Competitive binding of antagonistic peptides fine-tunes stomatal patterning

Jin Suk Lee1,2, Marketa Hnilova3, Michal Maes3, Ya-Chen Lisa Lin1,2, Aarthi Putarjunan2, Soon-Ki Han1,2, Julian Avila1,2 & Keiko U. Torii1,2

During development, cells interpret complex and often conflicting signals to make optimal decisions. Plant stomata, the cellular interface between a plant and the atmosphere, develop according to positional cues, which include a family of secreted peptides called epidermal patterning factors (EPFs). How these signalling peptides orchestrate pattern formation at a molecular level remains unclear. Here we report in Arabidopsis that Stomagen (also called EPF-LIKE9) peptide, which promotes stomatal development, requires ERECTA (ER)-family receptor kinases and interferes with the inhibition of stomatal development by the EPIDERMAL PATTERNING FACTOR 2 (EPF2)–ER module. Both EPF2 and Stomagen directly bind to ER and its co–receptor TOO MANY MOUTHS. Stomagen peptide competitively replaced EPF2 binding to ER. Furthermore, application of EPF2, but not Stomagen, elicited rapid phosphorylation of downstream signalling components in vivo. Our findings demonstrate how a plant receptor agonist and antagonist define inhibitory and inductive cues to fine–tune tissue patterning on the plant epidermis.

Development and pattern formation of multicellular organisms rely on diffusible signals that instruct cells to adopt a specific fate for optimal function, and hence organisal fitness. Often such signals are encoded by multiple gene families, which raises the question of how a given cell orchestrates the decision-making process. For instance, a family of secreted signals, such as FGFs, are used in an iterative manner to specify multiple, diverse developmental processes in animals1. While peptide signalling has recently emerged as a critical regulator of plant development2, how specific members of plant peptide families share signalling components in vivo is epistatic to Stomagen and the ER family act in the same pathway.

As reported, STOMAGEN-ami lines markedly reduced stomatal development in wild-type cotyledons (Fig. 1a, c, j and Extended Data Fig. 4)13. In contrast, STOMAGEN-ami had no effect on stomatal density, stomatal index, or stomatal clusters in er erl1 erl2 cotyledons, just like in tmm (Fig. 1 and Extended Data Fig. 3)13,14, suggesting that STOMAGEN and the ER family act in the same pathway.

To dissect the role of Stomagen on the TMM and ER module, we comprehensively investigated the effects of iSTOMAGEN on stomatal differentiation in tmm hypocotyls with additional er-family mutations (Fig. 2 and Extended Data Fig. 5). In hypocotyls, TMM and ER family have opposite functions: tmm hypocotyls lack stomata19, whereas er erl1 erl2 hypocotyls produce stomatal clusters19. While tmm is epistatic to er single mutation in hypocotyls, consecutive loss of ER-family genes reverts stomatal development in a dosage-dependent manner, with er erl1 erl2 being epistatic to tmm (ref. 7). iSTOMAGEN does not confer stomatal differentiation in tmm...
hypoctloty\textsuperscript{13}. However, in some instances arrested stomatal precursor cells (stomatal-lineage ground cells (SLGCs)) were observed, indicating that, in the absence of \textit{TMM}, \textit{iSTOMAGEN} could initiate stomatal development in hypocotyls\textsuperscript{13}. In addition, \textit{iSTOMAGEN} overexpression on stomatal development in \textit{tmm} hypocotyl epidermis with combinatorial loss-of-function in \textit{ER-family} genes. \textbf{a–h}, Representative confocal microscopy images of hypocotyl epidermis from 10-day-old light-grown transgenic \textit{Est::STOMAGEN} (oestradiol-induced \textit{STOMAGEN}) seedlings of \textit{tmm (a, b); tmm er (c, d); tmm erl1 erl2 (e, f); and tmm er erl1 erl2 (g, h)}. A control, uninduced phenotype (a, c, e, g) and \textit{iSTOMAGEN} phenotype (b, d, f, h) is shown. \textit{iSTOMAGEN} results in arrested stomatal precursor cells (asterisk) and stomatal-lineage ground cells (SLGCs (bracket)) in \textit{tmm} hypocotyls (b). Additional \textit{er} mutation exaggerated this effect (d), while additional \textit{erl1 erl2} mutations increased stomata (f). Images were taken under the same magnification. Scale bar, 30 μm.

Extended Data Figs 2-4. Among the \textit{ER} family, \textit{ER} primarily perceives \textit{EPF2} to restrict \textit{ER-family} act antagonistically, \textit{Stomagen} primarily acts via all three \textit{ER-family} receptor kinases. We delineated the role of \textit{Stomagen} in each of these steps. We first examined whether \textit{EPF1}, \textit{EPF2} and \textit{STOMAGEN} transcripts are under feedback regulation, which may complicate the genetic analyses. \textit{EPF1} and \textit{EPF2} transcript levels were slightly upregulated by \textit{iSTOMAGEN}, and conversely, slightly downregulated by \textit{STOMAGEN-ami} (Extended Data Fig. 2c, d). However, the endogenous \textit{STOMAGEN} transcript levels are unaffected by \textit{epf1}, \textit{epf2}, or \textit{epf1 epf2} (Extended Data Fig. 2d). Thus, altered expression of \textit{EPF1} and \textit{EPF2} by \textit{STOMAGEN} misregulation probably reflects the numbers of stomatal-lineage cells\textsuperscript{13,14}.

\textit{EPF2}–\textit{ER} or \textit{EPF1}–\textit{ERL1} signalling pathways with \textit{iSTOMAGEN} resulted in severe stomatal clusters, indicating that excessive \textit{Stomagen} promotes stomatal differentiation when either pathway is compromised (Extended Data Fig. 3). These genetic data support the notion that \textit{Stomagen}, when ectopically overexpressed, can bind to all \textit{ER-family} receptor kinases and inhibit signal transduction. Indeed, co-immunoprecipitation experiments using \textit{Nicotiana benthamiana} microsomal fraction expressing green fluorescent protein (GFP)-fused ectodomains of \textit{ER}, \textit{ERL1}, \textit{ERL2} or \textit{TMM} incubated in stomatal development\textsuperscript{16}. Finally, \textit{iSTOMAGEN} failed to enhance the severe stomatal clustering phenotype in \textit{tmm erl1 erl2} mutants (Fig. 2g, h and Extended Data Fig. 5q, r). Quantitative analysis of stomatal index and SLGC index (the percentage of SLGCs in total epidermal cells) supports these findings (Extended Data Fig. 5s, t). Together, the results suggest that in the hypocotyls, where \textit{TMM} and \textit{ER-family} act antagonistically, \textit{Stomagen} primarily acts via all three \textit{ER-family} receptor kinases.
Competitive binding of EPF2 and Stomagen

A series of genetic analyses leads to the possibility that Stomagen associates with all ER-family receptor kinases and TMM (Extended Data Fig. 9). Briefly, we immobilized GFP-fused receptors or control GFP from N. benthamiana on gold surfaces of QCM chips via anti-GFP antibody and then introduced the bioactive Stomagen or mEPF2 peptide solutions. The peptide–receptor binding was recorded as a function of frequency change (see Methods). Both Stomagen and mEPF2 exhibited saturable binding to the ER ectodomain fused to GFP (ER(ΔK)–GFP) with similar dissociation constants at a nanomolar range (Fig. 3a, b). Competitive binding. Microsomal fractions expressing ER(ΔK)–GFP were incubated with 1 μM of bioactive mEPF2 with increasing concentrations of bioactive Stomagen and subjected to immunoprecipitation. The mEPF2–MYC–His blot was re-probed with anti-Stomagen antibody. Asterisk indicates most likely isomer. c. Quantitative analysis of competition from four biological replicates. Error bars, s.e.m. The IC_{50} value is substantially higher than the K_{d} values for Stomagen–ER or mEPF2–ER, presumably owing to the immunoblot-based quantification. f. Wild-type cotyledon epidermis treated with mEPF2 alone or simultaneously co-treated with mEPF2 and increasing concentrations of Stomagen for 5 days, n = 3 for each treatment. Images were taken under the same magnification. Scale bar, 50 μM.

Figure 3 | Direct and competitive binding of Stomagen and EPF2 peptides to ER. a–c, QCM analysis for direct binding. a, b. The averages of experimental frequency shift values recorded from two to four independent experiments for Stomagen (a) or mEPF2 (b) onto biosensor chips functionalized with ER(ΔK)–GFP (red), TMM–GFP (blue) and GFP alone (grey) and fitted to the Langmuir adsorption model using least square regression. Error bars indicate s.d. Stomagen–ER, mEPF2–ER, n = 3; Stomagen–TMM, mEPF2–TMM, n = 3; mEPF2–GFP, n = 2. c. The average experimental frequency shift values recorded for LURE2 (dark grey) and mutant Stomagen (light grey) on ER(ΔK)–GFP. To calculate the dissociation constant (K_{d}) values, the ligand concentrations were increased to 1 μM to obtain fitted curves. See Extended Data Fig. 9 for raw recording data. Inactive mutant Stomagen, in which six cysteines were substituted with serines (Stomagen_{6C}–S)–ER, n = 3; LURE2–ER, n = 2. Error bars indicate s.d. Right insets: wild-type cotyledon epidermis treated with 2.5 μM mutant or bioactive Stomagen. Scale bars, 30 μm. n = 8 for each treatment. For a–c, each experimental point in independent binding experiment (referred to as ‘n = 1’) is generated from multi-point raw data (10–20 points) with average and s.d. values. d. Competitive binding. Microsomal fractions expressing ER(ΔK)–GFP were incubated with 1 μM of bioactive mEPF2 with increasing concentrations of bioactive Stomagen and subjected to immunoprecipitation. The mEPF2–MYC–His blot was re-probed with anti-Stomagen antibody. Asterisk indicates most likely isomer. e. Quantitative analysis of competition from four biological replicates. Error bars, s.e.m. The IC_{50} value is substantially higher than the K_{d} values for Stomagen–ER or mEPF2–ER, presumably owing to the immunoblot-based quantification. f. Wild-type cotyledon epidermis treated with mEPF2 alone or simultaneously co-treated with mEPF2 and increasing concentrations of Stomagen for 5 days, n = 3 for each treatment. Images were taken under the same magnification. Scale bar, 50 μM.

with synthetic Stomagen peptides demonstrated that Stomagen associates with all ER-family receptor kinases and TMM (Extended Data Fig. 6a).

Unlike overexpression, Stomagen co-suppression imposed different effects on EPF2–ER and EPF1–ERL1 signalling pathways. StOMAGEN-ami suppressed the stomatal-pairing phenotype of epf1 and dominant-negative, kinases-deleted ERL1 (ERL1(ΔK)) erl1 (Extended Data Fig. 4g–j). In contrast, StOMAGEN-ami exhibited complex interactions with epf2 and dominant-negative ER (ER(ΔK)) er, reducing numbers of stomata but not that of SLGCS (Extended Data Fig. 4c–f, k–n). This supports the idea that Stomagen counteracts EPF2 for ER-mediated stomatal initiation.13,14,16 This also suggests that, in the absence of both EPF2 and STOMAGEN, the default ER pathway is not activated while the later ERL1-mediated pathway remains capable of repressing the differentiation of mature stomata.

Competitive binding of EPF2 and Stomagen
Figure 4 | EPF2, but not Stomagen, triggers downstream MAPK activation in Arabidopsis seedlings. a, b, Differential MAPK activation in Arabidopsis wild-type seedlings treated with buffer only (a, mock), mEPF2 (a, b), Stomagen (a), and heat-denatured mEPF2 (b) for respective time intervals (min). The plots were probed with anti-phospho-ERK antibody (anti-pERK) to detect phosphorylated MPK6 (pMPK6) and pMPK3 upon peptide treatment. Asterisk indicates non-specific band. CBB, total proteins stained. Four and two biological replicates were performed for a and b, respectively. c, Confocal microscopy of Arabidopsis wild-type cotyledon abaxial epidermis treated with heat-denatured mEPF2 (top) and control, non-denatured mEPF2 (bottom). Scale bar, 40 μm. n = 3 for each treatment.

The figure exemplifies the specificity of Stomagen–ERECTA and Stomagen–TMM interactions.

Next, we performed ligand competition assays between Stomagen and EPF2. Microsomal fractions from N. benthamiana expressing ER ectodomain (ER(ΔK)–GFP) were incubated with bioactive epitope-tagged mEPF2 (mEPF2–MYC–HIS; 1 μM) and increasing concentrations of bioactive Stomagen peptides (0–23.4 μM) followed by immunoprecipitation of ER. Co-immunoprecipitated epitope-tagged mEPF2 was detected first. Then, the same blot was re-probed with anti-Stomagen antibody to detect co-immunoprecipitated Stomagen. Increasing concentrations of Stomagen peptide replaced mEPF2 for ER binding (Fig. 3d). Quantitative analysis confirmed the competitive binding of Stomagen and mEPF2 to ER, with a half-maximum inhibitory concentration (IC₅₀) value of 454 nM (Fig. 3e). Our results demonstrate that Stomagen and EPF2 peptides directly compete for binding to the same receptor, ER. Application of mEPF2 to wild-type seedlings inhibited stomatal development, while simultaneous treatment of mEPF2 with increasing concentration of Stomagen in a similar concentration range used in the competition experiments resulted in increased stomatal differentiation (Fig. 3f). The results are consistent with a previous report and further emphasize the in vivo biological relevance of peptide competition.

**Activation of downstream signalling**

To unravel the mechanism of Stomagen as a competitive antagonist of EPF2, we examined the activation of downstream signalling, specifically, using phosphorylation of MPK3 and MPK6 as readout. Genetic studies suggest that EPF2–ER ligand–receptor signalling acts via a MAPK cascade. However, a recent report of co-expressed stomatal signalling components in N. benthamiana failed to detect MPK6 activation by EPF2 (ref. 17), probably due to a limitation of the heterologous co-expression system for capturing fast and transient response. We therefore tested MAPK activation in vivo using Arabidopsis seedlings. Application of mEPF2 peptide to Arabidopsis wild-type seedlings rapidly elicited phosphorylation of MPK3 and MPK6 in 10 min, a characteristic signature of MAPK activation, which declined after 2 h (Fig. 4a, b). Heat-induced denaturation of mEPF2 greatly diminished MAPK phosphorylation, correlating with its loss of bioactivity (Fig. 4b, c). By contrast, Stomagen peptide treatment failed to trigger MAPK phosphorylation (Fig. 4a). We conclude that EPF2 activates ER signalling, leading to subsequent MAPK activation to inhibit stomatal development, while Stomagen prevents the signal transduction.

**Discussion**

Our work elucidates the competitive binding of Stomagen and EPF2 to ER as a molecular mechanism optimizing stomatal patterning. Plant genomes possess large numbers of peptide gene families, the functions of which remain largely unknown. The concept of fine-tuning signal transduction by related endogenous peptides that assume opposing functions may extend to other peptide families. EPF2 is expressed in a subset of protodermal cells, while Stomagen is secreted from an underlying internal tissue. Thus, it seems plausible that a protodermal cell might respond to differences in intrinsic concentrations of EPF2 and Stomagen on each neighbouring side. It remains to be tested whether local concentrations of Stomagen in the apoplast reflect the IC₅₀ values we have determined biochemically (Fig. 3e). The complex effects of STOMAGEN overexpression on a series of er-family mutants in the tmm background (Fig. 2) resemble that of challah (chal) higher-order mutants, which lack EPFL4 and EPFL6 peptides, another set of ER ligands promoting stem growth. This raises the possibility that complex fine-tuning of multiple EPF-family peptides may occur at developmental contexts far beyond stomatal patterning. Quantitative visualization of each peptide in vivo during epidermal development, as well as precise documentation of the dose–response effects of simultaneous mixed peptide applications at wide concentration gradients, may reveal the signalling complexity at the level of ligand–receptor associations. EPF2 and Stomagen bind to ER and TMM with a similar affinity (Fig. 3), suggesting the formation of co-receptor complexes, a hallmark of receptor activation and signal transduction in plant LRR-RKs in development and innate immunity response. Future structural and cell biological studies may reveal the intricacy behind how a cell interprets conflicting signals to make decisions during developmental patterning.

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**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions J.S.L and K.U.T. conceived the project. J.S.L., M.H., M.M., J.A. and Y.-C.L.L. purified peptides and performed ligand–receptor binding and bioassays. J.S.L and S.-K.H. performed RT–PCR. J.S.L. and A.P. performed MAPK assays. J.S.L. and Y.-C.L.L performed quantitative analysis of stomatal phenotypes. K.U.T. constructed STOMAGEN cDNA plasmid. K.U.T., J.S.L., M.H., M.M., Y.-C.L.L., A.P. and S.-K.H. analysed the data. K.U.T. wrote the manuscript with inputs from all co-authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.U.T. (ktorii@u.washington.edu).
METHODS

No statistical methods were used to predetermine sample size.

Plant materials and growth conditions. The Arabidopsis accession Columbia (Col) was used as wild type. All plants used in this study are in the Col background. For other genotypes, mutant and transgenic plant lines were generated and described previously 

Plasmid construction and transgenic plants generation. pKUT608 (PENTR-STOMAGEN) and pKUM127 (EST-STOMAGEN) were generated. See Extended Data Table 1 for plasmid and primer sequence information. Transgenic Arabidopsis plants were generated by the floral dip method. Multiple transgenic lines per construct were subjected to phenotypic characterization and representative lines (three 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 Agrobacterium tumefaciens. 

Chemical induction of transgene. Transgenic Arabidopsis seedlings carrying Est:STOMAGEN were germinated on 0.5 MS medium supplemented with 10 μM oestradiol (Sigma). Induction of STOMAGEN gene expression (istSTOMAGEN) 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. 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 or Leica SP5-WL 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 coloured using Photoshop CS6 (Adobe). Clearing of seedlings by chloral hydrate and observation using differential interference contrast (DIC) microscope was performed as described previously. For histological analysis, seedlings were stained with toluidine blue-O (TBO: Sigma) as follows. 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 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 were stained with TBO to reveal the cell outlines. For each genotype, type, sample size of 14–16 was used and over 1,000 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 analysed. For each seedling, representive image was taken at the exact location to minimize the variance. Specific numbers of stomata are listed for each genotype in corresponding figure legends. 

Transient protein expression in Nicotiana benthamiana. Agrobacterium tumefaciens strain GV3101 was transformed with expression clones and grown in yeast extract and beef medium supplemented with relevant antibiotics. Bacterial cultures were precultured and resuspended in infiltration medium (10 mM MgCl₂, 10 mM MES (pH 5.6) and 150 μM acetylsyringone). Culture densities were adjusted to an OD₀₆₀₀ of 1.0, and the cells were incubated at room temperature for 4 h before infiltration. Equal volumes of cultures carrying different constructs were mixed. To enhance transient expression in tobacco, the silencing suppressor p19 (a gift from D. Baulcombe) was co-infiltrated. The bacterial suspensions were infiltrated into young but fully expanded leaves of N. benthamiana plants. After infiltration, plants were incubated at 25°C and collected for further biochemical analyses after 45–72 h. 

Peptide expression, purification and refolding. Recombinant mEPF2 peptide was prepared as reported previously and the mature Stomagen peptide was 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, pH 8.8, 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 dialysed three times against 50 mM Tris–HCl, pH 8.0 for 1.5 d to remove glutathione. For non-folding mutant Stomagen, chemically synthesized peptides were dissolved in 50 mM Tris–HCl, pH 8.0. 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. 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 was determined using NanoDrop8000 (Thermo Scientific) using the following molar extinction coefficients: Stomagen, 5,360; EPF2, 6,460; LURE2, 23,950 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. Recombinant mEPF2 and Stomagen peptides in buffer were applied to 1–day-old Arabidopsis plants that had germinated on 0.5 MS medium. After 5 d of further incubation in 0.5 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. 

Immunoprecipitation, protein gel electrophoresis and immunoblots. For immunoprecipitation and co-immunoprecipitation assays, N. benthamiana leaves expressing CaMV35S::ERK–GFP, CaMV35S::FLS2–GFP, CaMV35S::TM–GFP, CaMV35S::GFP, or empty vector were subjected to protein preparation (mesoscopic 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-Flag (a gift from I. Hara-Nishimura) antibodies were performed as described previously. 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. The quartz 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 ER(–)–GFP and TM–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 by measuring the frequency shifts, Δf, at several peptide concentrations. To determine the dissociation constant (K₅) of each peptide–receptor pair, the experimental frequency shift values were fitted to the Langmuir adsorption model: −Δf = Δf₉₅₀(C/C₉₅₀ + K₅), where Δf₉₅₀ 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::ER(–)–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 mM NaF, 20 mM NaVa, 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 pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM PMSF and 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 pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM PMSF and 1:1,000 Complete protease inhibitor cocktail (Roche Applied Science)).

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PMSF, 1:1,000 Complete protease inhibitor cocktail) to release membrane proteins. The solution was sonicated on ice and centrifuged again at 100,000 g 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 2 h 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 unlabelled bioactive Stomagen peptide and then incubated at 4 °C for 1 h 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 2× SDS sample buffer at 80 °C for 5 min. 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 I. Hara-Nishimura)13 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 immunoprecipitates by monoclonal anti-GFP antibody. The intensity values shown in the paper are the ratios relative to the references, and values were analysed by nonlinear regression analysis using OriginLab version 6 (OriginLab) to calculate the IC₅₀ value.

**MAPK phosphorylation assays.** 12-day-old *Arabidopsis* seedlings were grown for 5 days on 0.5 MS media plates and then transferred to 0.5 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 peptide solution was treated at 95 °C for 2 h before 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, 10 mM Na₃VO₄, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM PMSF, 1 tablet per 50 ml extraction buffer of protease inhibitor mixture, 10% glycerol, 7.5% (w/v) PVPP). After centrifugation at 13,000 r.p.m. 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.

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Extended Data Figure 1 | Stomatal clustering phenotype of induced STOMAGEN overexpression in multiple independent transgenic lines. Shown are confocal microscopy images of abaxial cotyledon epidermis from 10-day-old light-grown seedlings of four independent transgenic lines carrying an oestradiol-inducible STOMAGEN overexpression construct (iSTOMAGEN). Left panels, no induction (control); right panels, oestradiol induction; each row shows representative images from individual lines. Yellow brackets indicate stomatal clusters. Images are taken under the same magnification. Scale bar, 40 μm. n = 3 for each panel.
Extended Data Figure 2 | RT–PCR analysis of STOMAGEN transcripts in transgenic lines used in this study. a, Expression of oestradiol-inducible STOMAGEN transgene (iSTOMAGEN) in transgenic lines expressing oestradiol-inducible STOMAGEN overexpression (Est::STOMAGEN) lines from wild-type (wt), tmm and er erl1 erl2 triple mutant background with or without oestradiol induction. b, Expression of the endogenous STOMAGEN transcripts in each genotype carrying STOMAGEN-ami construct. tmm or er erl1 erl2 mutation does not seem to affect STOMAGEN transcript levels. c, Expression of EPF1, EPF2, total STOMAGEN and STOMAGEN transgene (iSTOMAGEN) transcripts in transgenic Est::STOMAGEN lines (in two different T1 populations (s1 and s2) and a representative T3 line (s3)) with or without oestradiol induction. STOMAGEN overexpression by oestradiol causes modest increase in EPF1 and EPF2 transcripts, which accords with increased stomatal differentiation by iSTOMAGEN. d, EPF1, EPF2 and STOMAGEN transcript accumulation in wild-type (wt) and single- and higher-order loss-of-function mutants of epf1, epf2 and stomagen (STOMAGEN-ami). For epf1 STOMAGEN-ami and epf2 STOMAGEN-ami lines, two different F3 populations derived from the same genetic crosses were used to test the reproducibility. STOMAGEN expression is not influenced by epf1 and epf2 mutations, consistent with the proto-mesophyll expression of STOMAGEN. However, EPF2 expression is reduced by STOMAGEN-ami, consistent with reduced stomatal cell lineages by STOMAGEN co-suppression. As reported, epf1 has a T-DNA insertion within the 5’ UTR, which results in accumulation of aberrant transcripts. For all experiments, elF4A was used as a control. For primer sequences see Extended Data Table 1.
Extended Data Figure 3 | STOMAGEN overexpression promotes stomatal differentiation in genetic backgrounds missing/blocking EPF2–ER and EPF1–ERL1 signalling components. a–j, Representative confocal images of cotyledon abaxial epidermis from 10-day-old light-grown transgenic seedlings of the following genotypes, each carrying Est::STOMAGEN construct: epf2 (a, b); dominant-negative ER (ER(DK)) in er (c, d); epf1 (e, f); dominant-negative ERL1 (ERL1(DK)) in erl1 (g, h); er erl2 (i, j). For each genotype, a control uninduced phenotype (a, c, e, g, i) and induced STOMAGEN overexpression (iSTOMAGEN) (b, d, f, h, j) are shown. Blocking ER or lacking EPF2 produces small stomatal-lineage cells due to excessive entry divisions (a, c; yellow brackets). iSTOMAGEN confers stomatal clusters and small stomatal-lineage cells are no longer present (b and d). Blocking ERL1 or lacking EPF1 causes a stomatal pairing due to a violation of one-cell-spacing rule (e, g, dots). iSTOMAGEN enhances stomatal cluster phenotype in these genotypes (f, h). iSTOMAGEN does not enhance stomatal clustering defects in er erl1 erl2 (i, j). Images were taken under the same magnification. Scale bars, 30 μm. n = 29 (a); n = 24 (b); n = 16 (c); n = 17 (d); n = 22 (e); n = 23 (f); n = 17 (g); n = 20 (h); n = 24 (i); n = 24 (j).
k–m, Stomatal density (number of stomata per mm²) (k); stomatal index (% of number of stomata per stomata + non-stomatal epidermal cells) (l); and stomatal cluster distribution (in %) (m) from 10-day-old abaxial cotyledons of transgenic lines of each genotype carrying Est::STOMAGEN construct. –, no induction; +, induced by 10 μM oestradiol. Stomagen overexpression significantly increases stomatal density in all genotypes except for er erl1 erl2 and tmm. Error bars indicate s.e.m. ***P < 0.001; **P < 0.01; NS, not significant; Welch 2-sample t-test. Number of seedlings subjected to analysis, n = 14–16. Total numbers of stomata counted: wt, no induction 1,277, induction 2,639; epf1, no induction 1,390, induction 3,485; ERL1(DK) erl1, no induction 1,573, induction 3,991; epf2, no induction 2,502, induction 3,317; ER(DK) er, no induction 2,899, induction 4,397; tmm, no induction 2,948, induction 3,212; er erl1 erl2, no induction 4,454, induction 4,464. All genotypes carry Est::STOMAGEN. wt, no induction n = 16, induction n = 14; epf1, no induction n = 16, induction n = 17; ERL1(DK) erl1, no induction n = 15, induction n = 15; epf2, no induction n = 15, induction n = 15; ER(DK) er, no induction n = 15, induction n = 15; tmm, no induction n = 15, induction n = 15; er erl1 erl2, no induction n = 15, induction n = 15.
Extended Data Figure 4 | STOMAGEN co-suppression results in reduced stomatal development in genetic backgrounds missing or blocked in EPF2–ER and EPF1–ERL1 signalling pathways. a–j, Representative confocal images of cotyledon abaxial epidermis from 10-day-old light-grown transgenic seedlings of the following genotypes: wild type (a); STOMAGEN-ami (b); epf2 (c); epf2 STOMAGEN-ami (d); dominant-negative ER (ER(DK)) in er (e); ER(DK) er STOMAGEN-ami (f); epf1 (g); epf1 STOMAGEN-ami (h); dominant-negative ERL1 (ERL1(DK)) in erl1 (i); ERL1(DK) erl1 STOMAGEN-ami (j). STOMAGEN-ami markedly reduces stomatal differentiation in wild type (a, b). Blocking ER or lacking EPF2 produces small stomatal-lineage cells due to excessive entry divisions (c, e; yellow brackets). STOMAGEN-ami exaggerates the small stomatal-lineage cells of epf2 (d; yellow brackets). STOMAGEN-ami ER(DK) er shows excessive asymmetric entry as well as amplifying divisions (f; yellow and pink brackets, respectively). Blocking ERL1 or lacking EPF1 causes a stomatal pairing due to a violation of one-cell-spacing rule (g, i; dots). STOMAGEN-ami suppresses these mild stomatal pairing phenotypes and reduces stomatal differentiation (h, j). Images were taken under the same magnification. Scale bars, 30 μm. n = 13 (a); n = 26 (b); n = 15 (c); n = 23 (d); n = 11 (e), n = 17 (f); n = 12 (g), n = 22 (h); n = 18 (i), n = 13 (j).

k–n, Stomatal density (k), stomatal index (l), stomatal cluster distribution (in %; m), and non-stomatal epidermal cell density (n) from 10-day-old abaxial cotyledons of each genotype with or without carrying STOMAGEN-ami construct. Error bars, s.e.m. ***P < 0.001; *P ≤ 0.05; NS, not significant; Welch 2-sample t-test. n = 9–16. Total numbers of stomata counted: wt, 719; STOMAGEN-ami, 2,048; epf1, 1,004; epf1 STOMAGEN-ami, 383; ERL1(DK) erl1, 1,558; ERL1(DK) erl1 STOMAGEN-ami, 504; epf2, 1,505; epf2 STOMAGEN-ami, 1,165; ER(DK) er, 1,361; ER(DK) er STOMAGEN-ami, 782; tmm, 2,495; tmm STOMAGEN-ami, 2,688; er erl1 erl2, 1,853; er erl1 erl2 STOMAGEN-ami, 2,028. Total numbers of non-stomatal epidermal cells counted: wt, 1,494; STOMAGEN-ami, 1,299; epf1, 1,584; epf1 STOMAGEN-ami, 2,711; ERL1(DK) erl1, 871; ERL1(DK) erl1 STOMAGEN-ami, 1,348; epf2, 3,980; epf2 STOMAGEN-ami, 8,808; ER(DK) er, 5,739; ER(DK) er STOMAGEN-ami, 6,939; tmm, 790; tmm STOMAGEN-ami, 962; er erl1 erl2, 479; er erl1 erl2 STOMAGEN-ami, 391. wt, n = 8; STOMAGEN-ami, n = 8; epf1, n = 9, epf1 STOMAGEN-ami, n = 17; ERL1(DK) erl1, n = 13; ERL1(DK) erl1 STOMAGEN-ami, n = 9; epf2, n = 11; epf2 STOMAGEN-ami, n = 15; ER(DK) er, n = 9; ER(DK) er STOMAGEN-ami, n = 11; tmm, n = 8; tmm STOMAGEN-ami, n = 8; er erl1 erl2, n = 8; er erl1 erl2 STOMAGEN-ami, n = 8.
Extended Data Figure 5 | STOMAGEN overexpression on stomatal development in tmm hypocotyl epidermis with combinatorial loss-of-function in ER-family genes: a complete set. a–r, Representative confocal microscopy images of hypocotyl epidermis from 10-day-old light-grown transgenic seedlings of the following genotypes, each carrying Est::STOMAGEN: wild-type (wt) (a, b); tmm (c, d); tmm er (e, f); tmm erl2 (g, h); tmm erl1 (i, j); tmm er erl2 (k, l); tmm erl1 erl2 (m, n); tmm erl1 (o, p); and tmm er erl1 erl2 (q, r). A control, uninduced phenotype (a, c, e, g, i, k, m, o, q); iSTOMAGEN (b, d, f, h, j, l, n, p, r). iSTOMAGEN results in arrested stomatal precursor cells (asterisks) and stomatal-lineage ground cells (SLGCs; brackets) in tmm hypocotyls (d). iSTOMAGEN triggers entry divisions in tmm er and tmm erl2 (f, h; brackets), and exaggerate the SLGC clusters in tmm er erl2 (k, l; brackets). Images were taken under the same magnification. Scale bar, 30 μm. n = 19 (a); n = 19 (b); n = 20 (c); n = 19 (e); n = 22 (f); n = 20 (g); n = 17 (h); n = 18 (i); n = 19 (j); n = 19 (k); n = 21 (l); n = 17 (m); n = 20 (n); n = 19 (o); n = 21 (p); n = 20 (q); n = 20 (r). s, t, Stomatal index and SLGC index. s, ***P < 0.0001; **P < 0.01; *P < 0.05 (Wilcoxon rank sum test). NS, not significant. 0, no stomata or SLGCs observed, n = 15. Total number of stomata and SLGCs counted; tmm non-induced, 0 and 0; induced, 0 and 211; tmm er non-induced, 0 and 0; induced, 0 and 308; tmm erl2 non-induced, 0 and 32; induced, 0 and 171; tmm erl1 non-induced, 58 and 116; induced, 142 and 138; tmm er erl2 non-induced, 0 and 270; induced, 10 and 676; tmm er erl1 non-induced, 1,229 and 295; induced, 1,068 and 222. n = 15 for all genotypes (s, t).
Extended Data Figure 6 | Association of Stomagen with ER-family receptors and TMM. a, Shown are co-immunoprecipitation assays of ligand–receptor pairs expressed in N. benthamiana leaves. The ectodomains and membrane-spanning domains of ER, ERL1 and ERL2 fused with GFP were separately expressed in N. benthamiana, and microsomal fractions were incubated with 1 μM Stomagen peptides followed by immunoprecipitation using anti-GFP (anti-GFP) antibody. Inputs and immunoprecipitates were immunoblotted using anti-GFP (anti-GFP) or anti-Stomagen (anti-Stomagen) antibodies. Experiments were repeated three times (three biological replicates). b, Co-immunoprecipitation of LURE2 peptide fused with hexa-histidine tag (LURE2–His) with N. benthamiana microsomal fractions expressing the ectodomains and membrane-spanning domains of ER and FLS2 fused with GFP, a full-length TMM fused with GFP, or a control, uninoculated leaf sample. Immunoprecipitation was performed using anti-GFP and immunoblotted using anti-GFP (for detection of receptors) or anti-His (for detection of LURE2–His) antibodies. Experiments were repeated twice (two biological replicates). c, Co-immunoprecipitation of Stomagen peptide with N. benthamiana microsomal fractions expressing the ectodomains and membrane-spanning domains of ER and FLS2 fused with GFP or a control, uninoculated leaf sample. Immunoprecipitation was performed using anti-GFP and immunoblotted using anti-GFP (for detection of receptors) or anti-Stomagen antibodies. Experiments were repeated four times (four biological replicates).
Extended Data Figure 7 | Purified mEPF2 and Stomagen recombinant peptides and separation of bioactive mEPF2 by reverse-phase chromatography. 
a, SDS–PAGE gel of purified and refolded mEPF2-MYC-HIS and Stomagen recombinant peptides (asterisks). Left: molecular mass markers. 
b, HPLC chromatogram of purified, refolded mEPF2. Peaks 1 and 2 in UV chromatogram were collected and subjected to bioassays. 
c, Confocal image of cotyledon epidermis from wild-type seedling grown a solution with peak 1 for 5 days. No stoma is visible, indicating that peak 1 contains bioactive mEPF2. Scale bar, 20 µm. n = 19. 
d, Confocal image of cotyledon epidermis from wild-type seedling grown in a solution with peak 2 for 5 days, with normal stomatal differentiation, indicating that the peptide is not bioactive. Scale bar, 20 µm. n = 9.
Extended Data Figure 8 | Separation of properly folded, bioactive Stomagen and mutant Stomagen peptides by reverse-phase chromatography followed by mass spectrometry and bioassays. 

a, HPLC chromatogram of purified, refolded Stomagen. Peaks 1 and 2 in UV chromatogram were collected and subjected to MALDI-TOF mass spectrometry (b, d) as well as for bioassays (c, e). b, MALDI-TOF spectrum of peak 1 from a. A single-charged peptide corresponding to synthetic Stomagen peptide was observed at m/z = 5,185.4 ([M+H]+) and a double charged peptide at m/z = 2,599.8 ([M+2H]+). c, Confocal image of cotyledon epidermis from wild-type seedling grown a solution with peak 1. Severe stomatal clustering and overproduction of stomata are observed. Scale bar, 20 μm. n = 8. d, MALDI-TOF spectrum of peak 2 from a. e, Confocal image of cotyledon epidermis from wild-type seedling grown a solution with peak 2 from a, with no stomatal clustering, indicating that the fraction is not bioactive. Scale bar, 20 μm. n = 6. f, HPLC chromatogram and bioassays of an independent batch of Stomagen peptides used for QCM analysis in direct comparison with non-folding mutant Stomagen peptides in Fig. 3c. Peaks 1 and 2 in UV chromatogram were collected and subjected for bioassays. Insets: confocal microscopy images of cotyledon epidermis from wild-type seedling grown a solution with peak 1 (bioactive) and peak 2 (non-active) for 5 days. Scale bars, 50 μm. n = 8 (peak 1); n = 6 (peak 2). g, HPLC chromatogram of purified, mutant Stomagen peptide in which all cysteine residues were substituted to serine residues (Stomagen_6C→S). The mutant Stomagen peptide yielded a single peak, which was subjected for bioassays followed by confocal microscopy (inset). No stomatal clustering was observed, indicating that non-folding Stomagen peptide is not bioactive, confirming the previous results18. Scale bar, 50 μm. n = 8 for each peptide treatment.
Extended Data Figure 9 | Raw QCM recording data. Shown are raw recording data of frequency shifts for representative QCM analysis using biosensor chips immobilized with ER(DK)–GFP and GFP (a, b, inset) after sequential injection of active Stomagen (a, c), mEPF2 (b), non-folding, inactive mutant Stomagen (c, inset), or LURE2 (d) in increasing concentrations. Bioactive Stomagen and inactive Stomagen experiments in c were performed side by side. Arrows indicate time of additional peptide application. Numbers of experiments performed for each analysis: Stomagen–ER, n = 3; Stomagen–TMM, n = 2; Stomagen–GFP, n = 3; mEPF2–ER, n = 2; mEPF2–TMM, n = 3; mEPF2–GFP, n = 2; inactive Stomagen C6→S–ER, n = 3; and LURE2–ER, n = 2.
## Extended Data Table 1 | List of plasmids and primers used in this study

| Plasmid ID | Description | Insert | Vector | Bac R | Plant R |
|------------|-------------|--------|--------|-------|---------|
| pKUT608    | STOMAGEN in pENTR | STOMAGEN cDNA | pENTR | KAN | NA     |
| pKMP127    | proEst::STOMAGEN in pER8 | STOMAGEN cDNA | pER8 | SPEC/STREP | HYG |
| pJSL92     | ERL2 genomic γKinase in pENTR | ERL2 genomic γKinase | pENTR | KAN | NA     |
| pJSL93     | 35S::gERL2-γKinase-GFP in pGWBS | ERL2 genomic γKinase | pGWBS | KAN/HYG | KAN/HYG |
| pJSL73     | FLS2γK in pENTR | FLS2γK cDNA | pENTR | KAN | NA     |
| pJSL75     | 35S::FLS2γK-GFP in pGWBS | FLS2γK cDNA | pGWBS | KAN/HYG | KAN/HYG |

| Primer names | Sequences (5’ to 3’) | Purpose |
|--------------|----------------------|---------|
| EPFL9F       | CACCTCGAGATGAAGCATGAA | molecular cloning (pKUT608) |
| EPFL9Rf      | ACTAGTTATCTATGACAAACAC | molecular cloning (pKUT608) |
| FLS2 F       | CACCATGAAGTTACTCTCAAAGCTTTTTG | molecular cloning (pJSL73) |
| FLS2 F       | GATGTTGGCACTGTTGAATGAATCTGTTGC | molecular cloning (pJSL73) |
| FLS2 F       | TGTAGCAGCTGGTAACCAT | Sequencing |
| eIF4A F      | AGCCAGTGAAATCTTGGTGAAAGC | RT-PCR |
| eIF4A R      | CTAGTACGGAGAAGCACCAAGAGC | RT-PCR |
| STOMAGEN F   | TGATATCCAAGCTCAAGACCTTC | RT-PCR |
| STOMAGEN R   | ACTCGTTGACCTGACAAAGTTGGT | RT-PCR |
| pER8 Term R  | TCGAAACCGAGTACGGGACG | RT-PCR |
| EPF1+207F    | ATGCCGTCTTTGTAGGTTG | RT-PCR |
| EPF1+315rc   | TCAAGGGACAGGGTAGGACTT | RT-PCR |
| EPF2.1.cDNA.xhoI | CACCTCGAGATGAAATCTTGGTGAAAGC | RT-PCR |
| EPF2.360.cDNA.xhoI | CACCTCGAGATGAAATCTTGGTGAAAGC | RT-PCR |