S1 Text

Supplementary Methods

Model framework

I. Overview

This supplementary method provides a basic framework for modeling regulatory circuits initiating stomatal precursor cells. Here, each protodermal cell is represented by the hexagons, and each cell has an equal potential to differentiate into a stomatal initial cell. Based on the experimental observations reported in this manuscript and those by others (REF), we make a series of assumptions: (a) SPCH and SCRM form heterodimers; (b) SPCH is constitutively expressed and degraded by MAPK signaling; (c) SCRM is produced by SPCH•SCRM heterodimers and degraded by MAPK signaling; (d) EPF2 expression is regulated by SPCH•SCRM; (e) EPF2 diffuse at substantially faster rate than SPCH•SCRM; (e) TMM expression is regulated by SPCH•SCRM; (e) MAPK cascade is activated by ER and TMM, and ER forms homodimers or heterodimers with TMM; (f) this MAPK cascade leads to SPCH•SCRM degradation; and (g) an EPF2-independent MAPK cascade also leads to SPCH•SCRM degradation. See Fig 4 for the diagrams of regulatory circuits and simulated outcomes. The following are step-by-step descriptions of our modeling assumptions and actual mathematical equations used.

II. Regulatory relationships among SPCH, SCRM, EPF2, and TMM

We make the following assumptions based on experimental observations to describe the concentration changes of molecules in the i-th cell.

(i) SPCH ($u_1$) and SCRM ($u_2$) associate to form the heterodimer SPCH•SCRM ($u_3$) according to a reversible reaction:
SPCH + SCRM ⇌ SPCH•SCRM. \hspace{1cm} (1)

In this reaction, the increasing rate of $u_3$ concentration (equivalent to the decreasing rate of $u_1$ or $u_2$ concentration) is described by

$$\Delta = k^+ u_1 u_2 - k^- u_3,$$ \hspace{1cm} (2)

where $k^+$ and $k^-$ are the association and dissociation rate constants, respectively.

(ii) SPCH ($u_1$) is constitutively synthesized, reacts according to reaction (1), is degraded depending on MAPK signaling ($m$), and diffuses between cell $i$ and neighboring cell $j$:

$$\frac{du_{1i}}{dt} = A_1 - \Delta - \left( B_1 + B_{1m} \frac{m^q}{K_m^q + m^q} \right) u_{1i} + d_{u1} \sum_j (u_{1j} - u_{1i}), \hspace{1cm} (3)$$

where $A_1$ is the synthesis rate, $B_1$ is the background decay rate, $B_{1m}$ is the coefficient of MAPK-dependent degradation, $K_m$ is the half-saturation coefficient of $m$, and $d_{u1}$ is the coupling rate between neighboring cells.

(iii) SCRM ($u_2$) is synthesized in response to SPCH•SCRM ($u_3$), reacts according to reaction (1), is degraded depending on the MAPK signaling ($m$), and diffuses between cell $i$ and neighboring cell $j$:

$$\frac{du_{2i}}{dt} = A_0 + A_2 \frac{u_{3i}^p}{K_0^p + u_{3i}^p} - \Delta - \left( B_2 + B_{2m} \frac{m^q}{K_m^q + m^q} \right) u_{2i} + d_{u2} \sum_j (u_{2j} - u_{2i}), \hspace{1cm} (4)$$

where $A_0$ is the background synthesis rate, $A_2$ is the synthesis coefficient, $K_0$ is the half-saturation coefficient of $u_3$, $B_2$ is the background decay rate, $B_{2m}$ is the coefficient of MAPK-dependent degradation, $K_m$ is the half-saturation coefficient of $m$, and $d_{u2}$ is the coupling rate between neighboring cells.

In comparison to secreted peptide, EPF2, the nuclear localized SPCH and SCRM are likely have limited diffusion. We introduced 1/100 of the coupling rates to $u_1$ and $u_2$. It has been shown experimentally that enhanced diffusion in SPCH affect stomatal patterning[55], while the underlying mechanism is beyond the scope of this study.
(iv) SPCH•SCRM (u₃) reacts according to reaction (1) and is degraded at a constant rate:

$$\frac{du_{3i}}{dt} = \Delta - B_3 u_{3i},$$

where $B_3$ is the decay rate.

(v) EPF2 ($v_1$) is synthesized in response to SPCH•SCRM (u₃), is degraded at a constant rate, and diffuses between cell $i$ and neighboring cell $j$:

$$\frac{dv_{1i}}{dt} = C_0 + G \left[ \frac{u_{3i}^p}{K_1^p + u_{3i}^p} - D_1 v_{1i} \right] + d_{v1} \sum_j (v_{1j} - v_{1i}),$$

where $G$ is the reaction rate coefficient of negative feedback loop, $C_1$ is the synthesis coefficient, $K_1$ is the half-saturation coefficient of $u_3$, $D_1$ is the decay rate, and $d_{v1}$ is the coupling rate between neighboring cells. $C_0$ indicates the concentration of exogenously applied EPF2 peptide (S3 Fig D and S5 Fig A). For simplicity our model does not include peptide-processing steps, such CO₂-induced upregulation of EPF2 peptide cleavage reported very recently [37], as a function of activity.

(vi) TMM (w) is synthesized in response to SPCH•SCRM (u₃) and is degraded at a constant rate:

$$\frac{dw_i}{dt} = G \left[ \frac{u_{3i}^p}{K_3^p + u_{3i}^p} - D_3 w_i \right],$$

where $C_3$ is the synthesis coefficient, $K_3$ is the half-saturation coefficient of $u_3$, and $D_3$ is the decay rate.

We found that EPF2 peptide application triggered loss of SPCH and SCRM protein accumulation and hence the epidermis lacking stomatal-lineage cells (Fig 2D). Together with previous reports [11,12,56], the following can be proposed. The regulatory framework described in the above includes two feedback loops: a positive one by SPCH/SCRM and negative one by EPF2, which are expected to play central roles in patterning of stomatal differentiation. In fact, this regulatory relationship is equivalent to that of the activator–inhibitor system, one of well-known theoretical
models for pattern formation, which can generate stable patterns from a homogeneous state. In this framework, SPCH/SCRM and EPF2 correspond to the activator and inhibitor, respectively.

Thus, if this negative feedback regulation involving EPF2 is completely eliminated, every epidermal cell will highly express SPCH/SCRM and be differentiated into the stomatal lineage. This is the case for scrm-D mutation (Figs 2 and 4)[4]. However, despite the complete lack of this signaling pathway, er erl1 erl2 triple mutant shows clustered stomata, a phenotype weaker than that of scrm-D. This strongly indicates the presence of another negative feedback loop not involving EPF2. We accordingly introduce another diffusible molecule with a function similar to EPF2 (v1).

(vii) An assumed molecule (v2) is synthesized in response to SPCH•SCRM (u3), is degraded at a constant rate, and diffuses between cell i and neighboring cell j:

$$\frac{dv_{2i}}{dt} = G \left( C_2 \frac{u_{3i}^p}{K_2^p + u_{3i}^p} - D_2 v_{2i} \right) + d_{v2} \sum_j (v_{2j} - v_{2i}),$$

where $C_2$ is the synthesis coefficient, $K_2$ is the half-saturation coefficient of $u_3$, $D_2$ is the decay rate, and $d_{v2}$ is the coupling rate between neighboring cells.

(viii) Expression level of reporter $SPCHpro::GFP (g_i)$ is activated in the same manner as SPCH ($u_1$, Eq. (3)) and is degraded at a constant rate:

$$\frac{dg_{ui}}{dt} = A_1 - B g_{ui},$$

where $B_g$ is the decay rate.

(ix) Expression level of reporter $EPF2pro::GFP (g_i)$ is activated in the same manner as EPF2 ($v_1$, Eq. (6)) and is degraded at a constant rate:

$$\frac{dg_{ui}}{dt} = C_1 \frac{u_{3i}^p}{K_1^p + u_{3i}^p} - D_g g_{ui},$$

where $D_g$ is the decay rate.
III. Regulation of MAPK signaling activity

We now consider the MAPK signaling activity ($m$) that is induced by two pathways, EPF2 ($v_1$)-dependent and $v_2$-dependent:

$$m = m_1 + v_2,$$

where $m_1$ indicates the effect of the EPF2-dependent pathway, which involves two ligands (EPF2 and $S$, which is most likely Stomagen) and two receptor dimers constituted by ERECTA (ER) receptor kinase and TOO MANY MOUTHS (TMM) receptor-like protein (ER•ER homodimer and ER•TMM heterodimer). ER has been shown to both homodimerize with itself and heterodimerize with TMM[9]. In contrast, TMM does not form homodimers[9]. Thus, four ligand-receptor combinations exist: ER•ER•EPF2, ER•ER•Stomagen, ER•TMM•EPF2, and ER•TMM•Stomagen. Therefore, $m_1$ corresponds to the sum of effects of these ligand-receptor complexes:

$$m_1(v_1,w) = c_1[ER\cdot ER\cdot EPF2] + c_2[ER\cdot ER\cdot Stomagen] + c_3[ER\cdot TMM\cdot EPF2] + c_4[ER\cdot TMM\cdot Stomagen]$$

where $c_1$, $c_2$, $c_3$, and $c_4$ are constants that correspond to the signaling efficiency of each ligand-receptor complex. Because expressions of ERECTA and Stomagen are not directly influenced by the SPCH•SCRM-EPF2 module (Fig 3) we describe here that ER and Stomagen are expressed at a constant level, $m_1$ depends on EPF2 ($v_1$) and TMM ($w$). Note that here we do not discriminate ER from ERL1 and ERL2, because a mathematical description of their unequal redundancy complicates the simulation without benefit to our effort of identifying minimal regulatory circuits.

In order to determine the concentrations of the ligand-receptor complexes of Eq. (12), we consider the process of each reaction. ER molecules associate with each other to form the homodimer ER•ER, and also with TMM to the heterodimer ER•TMM:

$$ER + ER \rightleftharpoons ER\cdot ER$$

$$ER + TMM \rightleftharpoons ER\cdot TMM$$

These receptor dimers associate with their ligand EPF2 or Stomagen to form ligand-receptor complexes:
\[
\begin{align*}
\text{ER} \cdot \text{ER} + \text{EPF2} & \rightleftharpoons \text{ER} \cdot \text{ER} \cdot \text{EPF2} \quad (15) \\
\text{ER} \cdot \text{ER} + \text{Stomagen} & \rightleftharpoons \text{ER} \cdot \text{ER} \cdot \text{Stomagen} \quad (16) \\
\text{ER} \cdot \text{TMM} + \text{EPF2} & \rightleftharpoons \text{ER} \cdot \text{TMM} \cdot \text{EPF2} \quad (17) \\
\text{ER} \cdot \text{TMM} + \text{Stomagen} & \rightleftharpoons \text{ER} \cdot \text{TMM} \cdot \text{Stomagen} \quad (18)
\end{align*}
\]

If these reactions (13)–(18) are in equilibrium states, then we have

\[
\begin{align*}
[\text{ER} \cdot \text{ER}] &= k_1[\text{ER}]^2 \\
[\text{ER} \cdot \text{TMM}] &= k_2[\text{ER}][\text{TMM}] \\
[\text{ER} \cdot \text{ER} \cdot \text{EPF2}] &= k_3[\text{ER} \cdot \text{ER}][\text{EPF2}] \\
[\text{ER} \cdot \text{ER} \cdot \text{Stomagen}] &= k_4[\text{ER} \cdot \text{ER}][\text{Stomagen}] \\
[\text{ER} \cdot \text{TMM} \cdot \text{EPF2}] &= k_5[\text{ER} \cdot \text{TMM}][\text{EPF2}] \\
[\text{ER} \cdot \text{TMM} \cdot \text{Stomagen}] &= k_6[\text{ER} \cdot \text{TMM}][\text{Stomagen}]
\end{align*}
\]

where \(k_1, k_2, k_3, k_4, k_5, \) and \(k_6\) are equilibrium constants.

ER and Stomagen are constitutively expressed at a constant level. This conservation requirement can be written as

\[
E_0 = [\text{ER}] + 2[\text{ER} \cdot \text{ER}] + 2[\text{ER} \cdot \text{ER} \cdot \text{EPF2}] + 2[\text{ER} \cdot \text{ER} \cdot \text{Stomagen}] \\
+ [\text{ER} \cdot \text{TMM}] + [\text{ER} \cdot \text{TMM} \cdot \text{EPF2}] + [\text{ER} \cdot \text{TMM} \cdot \text{Stomagen}], \quad (25)
\]

\[
S_0 = [\text{Stomagen}] + [\text{ER} \cdot \text{ER} \cdot \text{Stomagen}] + [\text{ER} \cdot \text{TMM} \cdot \text{Stomagen}], \quad (26)
\]

where \(E_0\) and \(S_0\) are constants of the total concentration of ER and Stomagen molecules, respectively. In addition, \(v_1\) and \(w\) correspond to the total concentrations of EPF2 and TMM molecules, respectively:

\[
\begin{align*}
v_1 &= [\text{EPF2}] + [\text{ER} \cdot \text{ER} \cdot \text{EPF2}] + [\text{ER} \cdot \text{TMM} \cdot \text{EPF2}] \\
w &= [\text{TMM}] + [\text{ER} \cdot \text{TMM}] + [\text{ER} \cdot \text{TMM} \cdot \text{EPF2}] + [\text{ER} \cdot \text{TMM} \cdot \text{Stomagen}].
\end{align*}
\]

Thereby, concentrations of ligand-receptor complexes can be calculated by numerically solving Eqs. (19)–(28) (S7 Fig D). As EPF2 \((v_1)\) increases, \([\text{ER} \cdot \text{ER} \cdot \text{EPF2}]\) and \([\text{ER} \cdot \text{TMM} \cdot \text{EPF2}]\) increase and \([\text{ER} \cdot \text{ER} \cdot \text{Stomagen}]\) and \([\text{ER} \cdot \text{TMM} \cdot \text{Stomagen}]\) decrease. On the other hand, \([\text{ER} \cdot \text{TMM} \cdot \text{EPF2}]\) and \([\text{ER} \cdot \text{TMM} \cdot \text{Stomagen}]\) increase by increasing TMM \((w)\), but under such a condition
[ER•ER•EPF2] and [ER•ER•Stomagen] decrease. According to these numerical results, we use simplified forms for these concentrations in our model:

\[
[\text{ER•ER•EPF2}] \propto \frac{K_w v_1}{K_w + w K_v + v_1} \tag{29}
\]

\[
[\text{ER•ER•Stomagen}] \propto \frac{K_v}{K_w + w K_v + v_1} \tag{30}
\]

\[
[\text{ER•TMM•EPF2}] \propto \frac{w}{K_w + w K_v + v_1} \tag{31}
\]

\[
[\text{ER•TMM•Stomagen}] \propto \frac{w K_v}{K_w + w K_v + v_1}, \tag{32}
\]

where \(K_w\) and \(K_v\) are the half-saturation coefficients of \(w\) and \(v_1\), respectively (S7 Fig E). Using Eqs. (29)–(32), Eq. (12) can be described by

\[
m_1(v_1, w) = \frac{C_{EE} K_w v_1 + C_{ES} K_w v_1 + C_{TE} w v_1 + C_{TS} w K_v}{(K_w + w)(K_v + v_1)}, \tag{33}
\]

where \(C_{EE}, C_{ES}, C_{TE},\) and \(C_{TS}\) are constants.

In \(tmm\) mutant, we have

\[
m_1(v_1, 0) = \frac{C_{ES} K_v + C_{EE} v_1}{K_v + v_1}. \tag{34}
\]

In \(er erl1 erl2\) triple mutant, because the receptor dimers are absent (i.e. \(C_{EE} = C_{ES} = C_{TE} = C_{TS} = 0\)), we have

\[
m_1(v_1, w) = 0. \tag{35}
\]

IV. Numerical calculation

Numerical simulations are calculated by Euler’s method with a time step \(\Delta t = 0.002\) using Eqs. (2)–(10) and (33)–(35), until total time reaches \(t = 2000.0\) where patterns no longer change. Hexagonal cells are two-dimensionally arranged with periodic boundary conditions. Initial values of variables are given as their equilibrium with random fluctuation of 1.0%. In the wild type of Fig 4 and S4 Fig, parameter values
are set to be $G = 1.0$, $A_0 = 0.01$, $A_1 = 2.0$, $A_2 = 10.0$, $B_1 = 10.0$, $B_2 = 0.1$, $B_3 = 0.01$, $B_{1m} = B_{2m} = 20.0$, $B_g = 1.0$, $C_0 = 0.0$, $C_1 = 1.0$, $C_2 = 1.2$, $C_3 = 1.0$, $D_1 = D_2 = D_3 = D_g = 1.0$, $K_0 = 1.0$, $K_1 = K_2 = 0.2$, $K_3 = 0.1$, $K_m = 1.0$, $k_+ = 1.0$, $k_- = 0.1$, $d_{u1} = d_{u2} = 0.02$, $d_{v1} = d_{v2} = 2.0$, $p = 2$, $q = 3$, $K_v = 1.0$, $K_w = 0.1$, $C_{EE} = 0.1$, $C_{ES} = 0.1$, $C_{TE} = 1.6$, and $C_{TS} = 0.0$. Parameter values of mutants are the same as those of the wild type, except for $A_1 = 0.0$ in $spch$, $A_0 = A_2 = 0.0$ in $scrm scrm2$, $B_{2m} = 2.0$ in $scrm-D$, $C_3 = 0.0$ in $tmm$, and $C_{EE} = C_{ES} = C_{TE} = C_{TE} = 0.0$ in $erecta-triple$. Parameter values in S2 Fig are the same as those in Fig 4 except for $C_2 = 0.0$, $C_{EE} = 2.1$, $C_{ES} = 0.0$, $C_{TE} = 3.2$, and $C_{TS} = 0.0$. Parameter values in S8, S9, S12 and S13 Figs are the same as those in Fig 4 except for $d_v = d_{v1} = d_{v2} = 0.04-2.0$ and $G = 0.05-5.0$ in S8 Fig, $p = 1.0-3.0$ and $q = 1.0-5.0$ in S9 Fig, $A_1 = 0.2-20.0$ and $C_3 = 0.0-10.0$ in S12 Fig, and initial fluctuation of 1.0-100.0 % in S13 Fig.

References for Supplementary Methods

55. Guseman JM, Lee JS, Bogenschutz NL, Peterson KM, Virata RE, et al. (2010) Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in Arabidopsis CHORUS (GLUCAN SYNTHASE-LIKE 8). Development 137: 1731-1741.

56. Jewaria PK, Hara T, Tanaka H, Kondo T, Betsuyaku S, et al. (2013) Differential effects of the peptides Stomagen, EPF1 and EPF2 on activation of MAP kinase MPK6 and the SPCH protein level. Plant Cell Physiol 54: 1253-1262.