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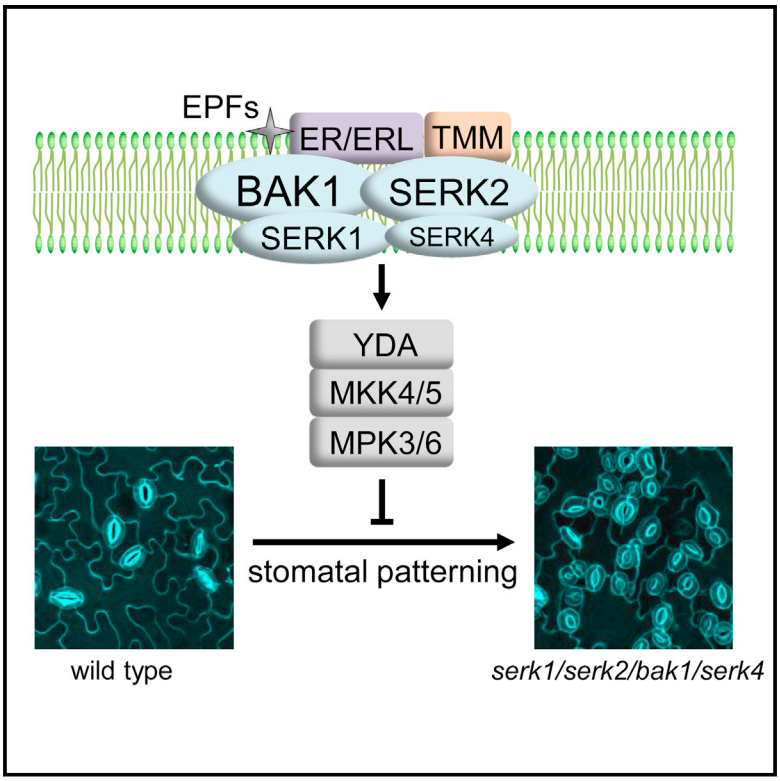
Differential Function of *Arabidopsis* SERK Family Receptor-like Kinases in Stomatal Patterning

Graphical Abstract



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In Brief

Meng et al. show that *Arabidopsis* SERK family receptor-like kinases regulate stomatal patterning via ligand-induced dimerization with ERECTA family receptors and ligand-independent association with the TMM receptor-like protein. The study reveals that ERECTA, SERKs, and TMM form a multiprotein receptorsome regulating stomatal patterning.

Highlights

- SERK family receptor-like kinases redundantly regulate stomatal patterning
- SERKs act downstream of EPF ligands and upstream of the YDA MAPKKK
- SERKs associate with ERECTA family receptors in a ligand-induced manner
- SERKs regulate stomatal patterning independent of brassinosteroid signaling

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Differential Function of *Arabidopsis* SERK Family Receptor-like Kinases in Stomatal Patterning

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SUMMARY

Plants use cell-surface-resident receptor-like kinases (RLKs) to sense diverse extrinsic and intrinsic cues and elicit distinct biological responses. In *Arabidopsis*, ERECTA family RLKs recognize EPIDERMAL PATTERNING FACTORS (EPFs) to specify stomatal patterning. However, little is known about the molecular link between ERECTA activation and intracellular signaling. We report here that the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family RLKs regulate stomatal patterning downstream of EPF ligands and upstream of a MAP kinase cascade. EPF ligands induce the heteromerization of ERECTA and SERK family RLKs. SERK and ERECTA family RLKs transphosphorylate each other. In addition, SERKs associate with the receptor-like protein (RLP) TMM, a signal modulator of stomata development, in a ligand-independent manner, suggesting that ERECTA, SERKs, and TMM form a multiprotein receptorsome consisting of different RLKs and RLP perceiving peptide ligands to regulate stomatal patterning. In contrast to the differential requirement of individual SERK members in plant immunity, cell-death control, and brassinosteroid (BR) signaling, all four functional SERKs are essential but have unequal genetic contributions to stomatal patterning, with descending order of importance from SERK3/BAK1 to SERK2 to SERK1 to SERK4. Although BR signaling connects stomatal development via multiple components, the function of SERKs in stomatal patterning is uncoupled from their involvement in BR signaling. Our results reveal that the SERK family is a shared key module in diverse *Arabidopsis* signaling receptorsomes and that different combinatorial codes of individual SERK members regulate distinct functions.

INTRODUCTION

Plants possess a largely expanded number of receptor-like kinases (RLKs) that are potentially involved in sensing intrinsic and extrinsic cues and lead to complex cellular networks with distinct signaling outputs [1, 2]. RLKs regulate a wide range of biological processes including plant growth, development, symbiosis, and immunity via perception of diverse signals, likely through different extracellular domains. The *Arabidopsis* genome contains more than 200 RLKs with extracellular leucine-rich repeat (LRR) domains [1]. An LRR-RLK typically contains an extracellular domain with different numbers of LRRs, a single transmembrane domain, and an intracellular kinase domain. Some well-known examples of LRR-RLKs include the BRI1 receptor for brassinosteroids (BRs), a class of plant hormones with essential roles in growth and development [3]; FLS2, which recognizes bacterial flagellin or flg22 (the 22-amino acid peptide of flagellin) and initiates plant immune signaling [4]; and the ERECTA (ER) family LRR-RLKs, which recognize the endogenous peptides EPIDERMAL PATTERNING FACTOR 1 (EPF1) and EPF2 to control stomatal patterning [5, 6].

Stomata are epidermal pores that control water vapor and gas exchange between plants and the atmosphere and consist of two highly specialized guard cells (GCs) that surround each stomatal pore. In *Arabidopsis*, the stomatal lineage is initiated from a subset of protodermal cells that undergo a cellular transition to become meristemoid mother cells (MMCs) [7, 8]. An asymmetric entry division of the MMC generates a smaller, triangular cell called a meristemoid and a larger cell called the stomatal lineage ground cell (SLGC). The meristemoid either differentiates into a round-shaped guard mother cell that further divides once into two GCs, or undergoes several amplifying divisions to produce more SLGCs. The SLGC either directly expands and differentiates into a pavement cell, or undergoes an asymmetric cell division to produce a satellite meristemoid that is oriented away from existing meristemoids or stomata [7, 8]. The ''spacing'' division of SLGCs ensures that stomata are always separated by at least one pavement cell, the so-called one-cell-spacing rule.

The signaling pathway controlling stomatal patterning is initiated by the secreted peptide ligands EPF1 and EPF2, which act as negative regulators with distinct functions. EPF1 functions mainly in the orientation of the cell spacing division, whereas

EPF2 primarily controls asymmetric entry cell division [5, 9, 10]. The ER family LRR-RLKs ER, ER-LIKE1 (ERL1), and ERL2 possess overlapping and distinct functions in the control of stomatal patterning [6]. EPF2-ER and EPF1-ERL1 function as ligand-receptor pairs to specify asymmetric entry division and spacing division, respectively [5]. TOO MANY MOUTHS (TMM), an LRR receptor-like protein (LRR-RLP), associates with ER family RLKs and differentially modulates stomatal development in different organs, with a negative role in cotyledons and positive role in hypocotyls and stems [5, 11]. A MAP kinase (MAPK) cascade composed of YDA (MAPKKK), MKK4/MKK5 (MAPKKs), and MPK3/MPK6 (MAPKs) functions downstream of ER family RLKs and negatively regulates stomatal development [12–14]. Potential targets of the MAPK cascade include the transcription factors SPEECHLESS (SPCH), MUTE, FAMA, SCRM1, and SCRM2 [15–18]. SPCH directly targets key regulators of cell-lineage specification and asymmetric cell division [19]. However, little is known about the molecular link between ER family receptor activation and intracellular signaling in stomatal development.

Receptor dimerization often constitutes the first step in the activation of downstream intracellular modules in RLK signaling [1]. BAK1, originally identified as a BRI1-associated receptor kinase mediating BR signaling [20, 21], is an important player in plant immunity via association with FLS2 and other immune sensors [22–25]. BAK1 is also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3), belonging to a subfamily of LRR-RLKs with five members [26]. SERK5 is likely a nonfunctional kinase [27]; SERK1–SERK4 possess diverse functions in male gametophyte development, BR-mediated growth, plant defense, and cell-death control [28, 29]. In this study, we report that SERK family RLKs regulate stomatal development and patterning through ligand-induced heteromerization and transphosphorylation with ER and ERL1. Successive mutation of four *SERK* genes causes excessive stomatal clustering, reminiscent of the loss-of-function mutant for the entire *ER* family. Importantly, each SERK member makes an unequal contribution to stomatal patterning, with descending order of importance from SERK3/BAK1 to SERK2 to SERK1 to SERK4. Our study indicates that SERK family RLKs act as coreceptors for ER family RLKs in regulating stomatal patterning and suggests that the combinatorial codes of individual SERK members control distinct cellular functions in cell-fate determination, growth, and immunity.

RESULTS

Ectopic Expression of Bacterial Effector AvrPto or AvrPtoB Impairs Stomatal Patterning Upstream of YDA

Pathogenic bacteria inject a repertoire of effector proteins into host cells to modulate diverse host cellular activities and physiology [30, 31]. Interestingly, ectopic expression of the bacteria *Pseudomonas syringae* pv *tomato* (*Pst*) effector AvrPto in *Arabidopsis* transgenic plants under the control of a dexamethasone (Dex)-inducible promoter led to excessively clustered stomata in the cotyledon epidermis, which violated the one-cell-spacing rule in stomatal development (Figure 1A; Figure S1A). The stomatal density indicated by the stomatal index was also much higher in the *Dex::AvrPto* transgenic plants after Dex treatment than that without Dex treatment (Figure S1B). Similarly, expression of AvrPtoB, another *Pst* effector sharing certain overlapping host targets with AvrPto [32], also caused a strong stomatal clustering phenotype (Figure 1A). However, transgenic plants expressing AvrRpt2 or AvrRpm1, which have distinct virulence mechanisms from AvrPto and AvrPtoB [33], exhibited a similar stomatal patterning as wild-type (WT) Columbia (Col-0) plants (Figure 1A). The MAPK cascade YDA-MKK4/MKK5-MPK3/MPK6 functions downstream of ER family RLKs in regulating stomatal development [12, 13]. AvrPto-mediated interference of stomatal development likely occurs upstream of YDA, because expression of a constitutively active form of YDA (YDAac) rescued the AvrPto-induced stomatal patterning defects (Figure 1B). In addition, overexpression of AvrPto in *Arabidopsis* protoplasts did not interfere with the YDAac-mediated activation of MPK3 and MPK6 (Figure 1C), which is consistent with its suppression function in plant immune signaling [34]. These results suggest that AvrPto and AvrPtoB target a common signaling component(s) upstream of YDA to interfere with stomatal development in *Arabidopsis*. Because the stomatal pore is a natural entry point for pathogen invasion [31], specific bacterial effectors may modulate stomatal density and patterning to promote pathogenicity.

SERK Family RLKs Redundantly Regulate Stomatal Patterning

BAK1 is one of the physiological targets of AvrPto and AvrPtoB, as supported by structural analysis of the BAK1-AvrPtoB complex and reduced virulence function of AvrPto/AvrPtoB in the *bak1* mutant [32, 35, 36]. In addition, AvrPto and AvrPtoB also interact with other SERKs, including SERK1, SERK2, and SERK4 (Figures S1C and S1D) [32]. Therefore, we tested whether the stomatal patterning defects in the *AvrPto* and *AvrPtoB* transgenic plants were caused by the dysfunction of BAK1 and other SERKs. However, neither the *serk1-1*, *serk2-1*, *bak1-4*, nor *serk4-1* single null mutants displayed abnormal stomatal patterning compared to WT Col-0 plants (Figure 2A). To reveal the potential functional redundancy, we systematically generated different combinations of *serk* higher-order mutants. The stomatal patterning is normal in the cotyledon of all double mutants, including *serk1-1/serk2-1*, *serk1-1/bak1-4*, *serk1-1/serk4-1*, *serk2-1/bak1-4*, *serk2-1/serk4-1*, and *bak1-4/serk4-1* (Figure 2B). Remarkably, clustered stomata were observed in the cotyledon epidermis of the *serk1-1/serk2-1/bak1-4* triple mutant but not in the other triple mutants, including *serk1-1/serk2-1/serk4-1*, *serk1-1/bak1-4/serk4-1*, or *serk2-1/bak1-4/serk4-1* (Figure 2C). The stomatal clusters in the *serk1-1/serk2-1/bak1-4* mutant often consisted of more than two stomata, similar to that of the *er105/erl1-2/erl2-1* triple mutant, which harbors loss-of-function mutations in all three *ER* family genes *ER*, *ERL1*, and *ERL2* [6] (Figure 2C). In addition, the *serk1-1/serk2-1/bak1-4* mutant, but not other mutants, exhibited similar growth morphology as the *er105/erl1-2/erl2-1* mutant (Figure 2D; Figure S2). Consistently, the stomatal index is also much higher in the cotyledon of the *serk1-1/serk2-1/bak1-4* mutant than that in WT and other mutant plants (Figure 2E). The clustered stomata were also observed in the true leaves of the *serk1-1/serk2-1/bak1-4* triple mutant but not in any other single, double, or triple mutants (Figure S3). These results indicate that BAK1, SERK1,

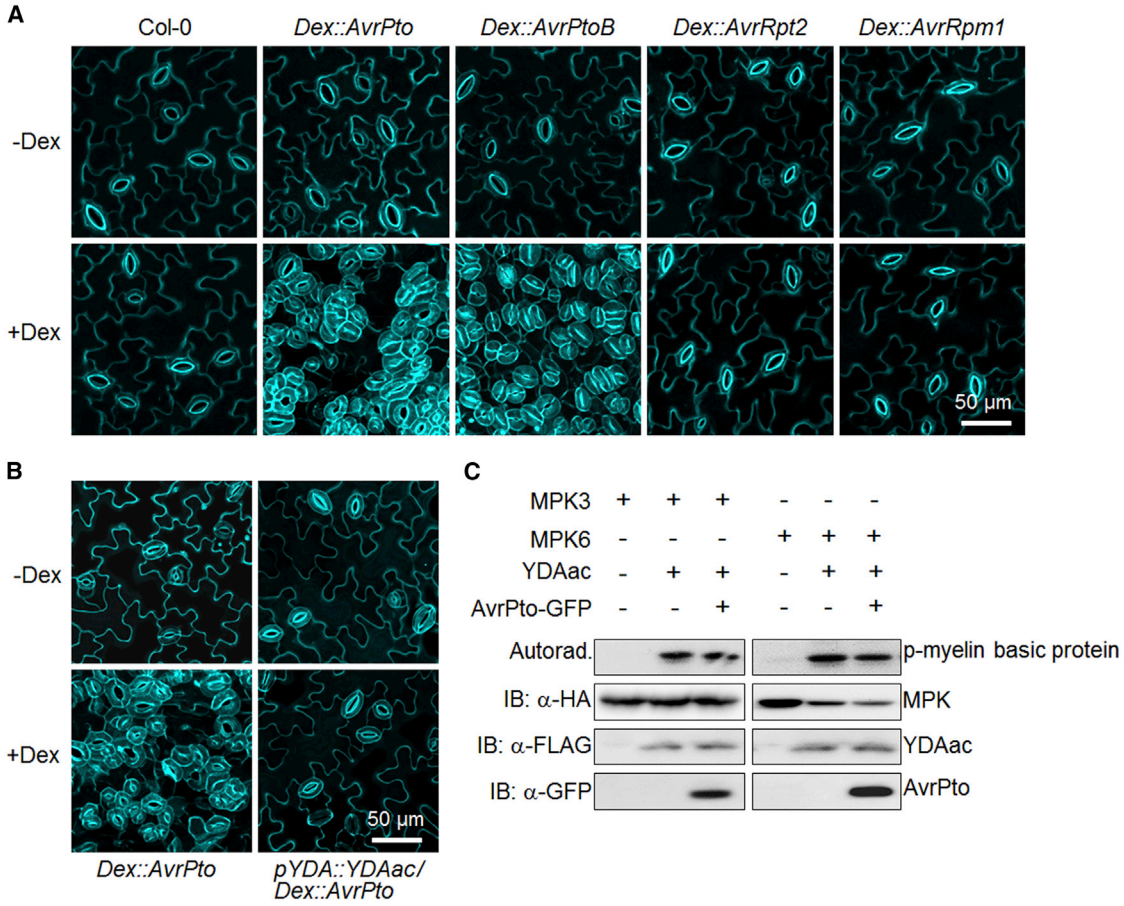

Figure 1. Ectopic Expression of Effector Protein AvrPto or AvrPtoB Impairs Stomatal Patterning

(A) Dex-induced expression of AvrPto or AvrPtoB but not AvrRpt2 or AvrRpm1 in *Arabidopsis* transgenic plants leads to severe stomatal clustering phenotypes.

(B) Expression of *YDAac* rescues the AvrPto-induced stomatal patterning defects.

Confocal images were taken on the abaxial cotyledon epidermis of 10-day-old seedlings grown on 1/2 MS medium with (A and B, bottom panels) or without (A and B, top panels) 100 μM Dex. Cell outlines were visualized with propidium iodide staining. The representative images in (A) and (B) were selected from at least five replicates.

(C) Expression of AvrPto does not affect YDAac-mediated activation of MPK3 and MPK6 in *Arabidopsis* protoplasts. The HA-tagged MPK3/MPK6 and FLAG-tagged YDAac were coexpressed with or without AvrPto in protoplasts. The MPK3/MPK6 proteins were immunoprecipitated with α-HA agarose beads for an in vitro kinase assay using myelin basic protein as the substrate. The phosphorylation of myelin basic protein by MPK3/MPK6 is shown with an autoradiograph (top panel), and protein expression is shown with immunoblotting (IB) (bottom three panels). The experiments were repeated three times with similar results.

See also Figure S1.

and SERK2 redundantly regulate stomatal development. The data are consistent with both AvrPto and AvrPtoB targeting multiple SERK family members in *Arabidopsis* (Figures S1C and S1D) [32]. Notably, the extent of stomatal clustering in the *serk1-1/serk2-1/bak1-4* mutant is weaker than that in *AvrPto* transgenic plants or the *er105/erl1-2/erl2-1* mutant (Figures 1A, 2C, and 2E). It is possible that SERK4 may also play certain roles in this process. However, the *serk1-1/serk2-1/bak1-4/serk4-1* quadruple null mutant is embryo lethal [27], which precludes the possibility of examining its stomatal development.

Unequal Redundancy of Individual SERK Members in Stomatal Patterning

In contrast to the null mutant *bak1-4*, the *bak1-5* mutant, a semi-dominant allele with a missense mutation in the kinase domain, is not impaired in cell-death control or BR signaling, yet is severely compromised in immune responses [37]. To circumvent the embryonic lethality and further explore the roles of different SERK members in stomatal development, we generated higher-order *serk* mutants in the *bak1-5* background. Although the *bak1-5* single mutant exhibited normal stomatal patterning, the *serk1-1/bak1-5*, *serk2-1/bak1-5*, and *bak1-5/serk4-1* double mutants displayed moderate stomatal clustering in the cotyledon compared to WT plants (Figure 3A). BAK1 is likely the most important SERK member in stomatal development, because stomatal patterning defects were only observed in the cotyledon of *serk* double and triple mutants harboring the *bak1* mutation but not in any other combinations (Figures 2B, 2C, 3A, and 3B). Apparently, the stomatal clustering was more pronounced in the cotyledon of *serk2-1/bak1-5* than those in the *serk1-1/bak1-5* and *bak1-5/serk4-1* mutants (Figures 3A and 3C), suggesting that SERK2 plays a more prominent role than SERK1

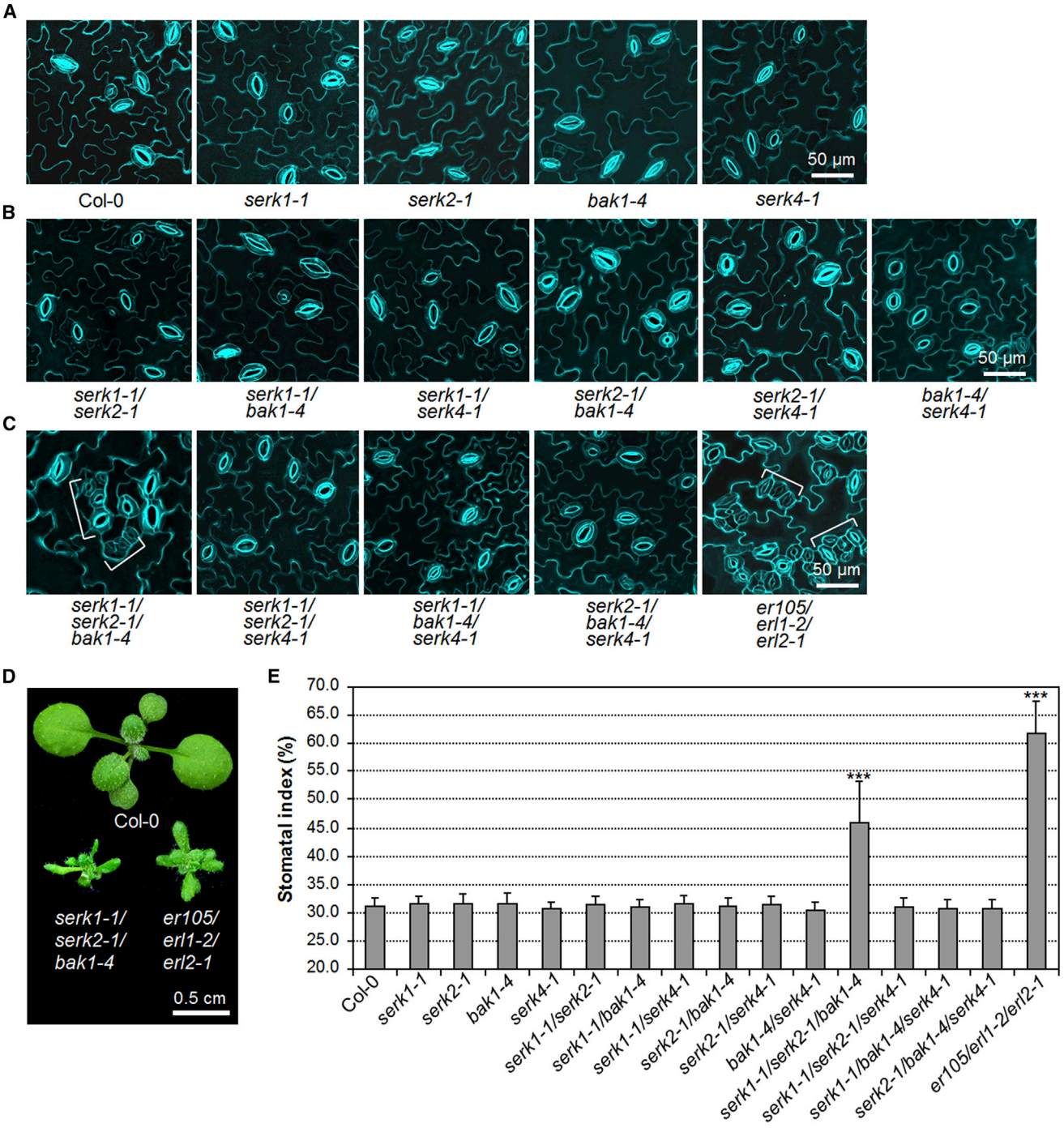

Figure 2. Redundant Function of SERK Family RLKs in Stomatal Patterning

(A–C) The *serk1-1/serk2-1/bak1-4* mutant, but not other *serk* mutants, shows stomatal patterning defects. Confocal images of the indicated genotypes were taken on the abaxial cotyledon epidermis of 10-day-old seedlings grown on 1/2 MS plates. The representative images were selected from at least five replicates. Brackets indicate clustered stomata (C).

(D) The seedling phenotypes of 2-week-old *serk1-1/serk2-1/bak1-4* and *er105/erl1-2/erl2-1* mutants grown on soil.

(E) Abaxial cotyledon stomatal index of 10-day-old seedlings, expressed as the percentage of the number of stomata to the total number of epidermal cells. The data are shown as mean ± SD (n = 8). Asterisks above the columns indicate significant difference compared with data from WT plants (***$p < 0.0001$, Student's t test). The experiments were repeated three times with similar results.

See also Figures S2 and S3.

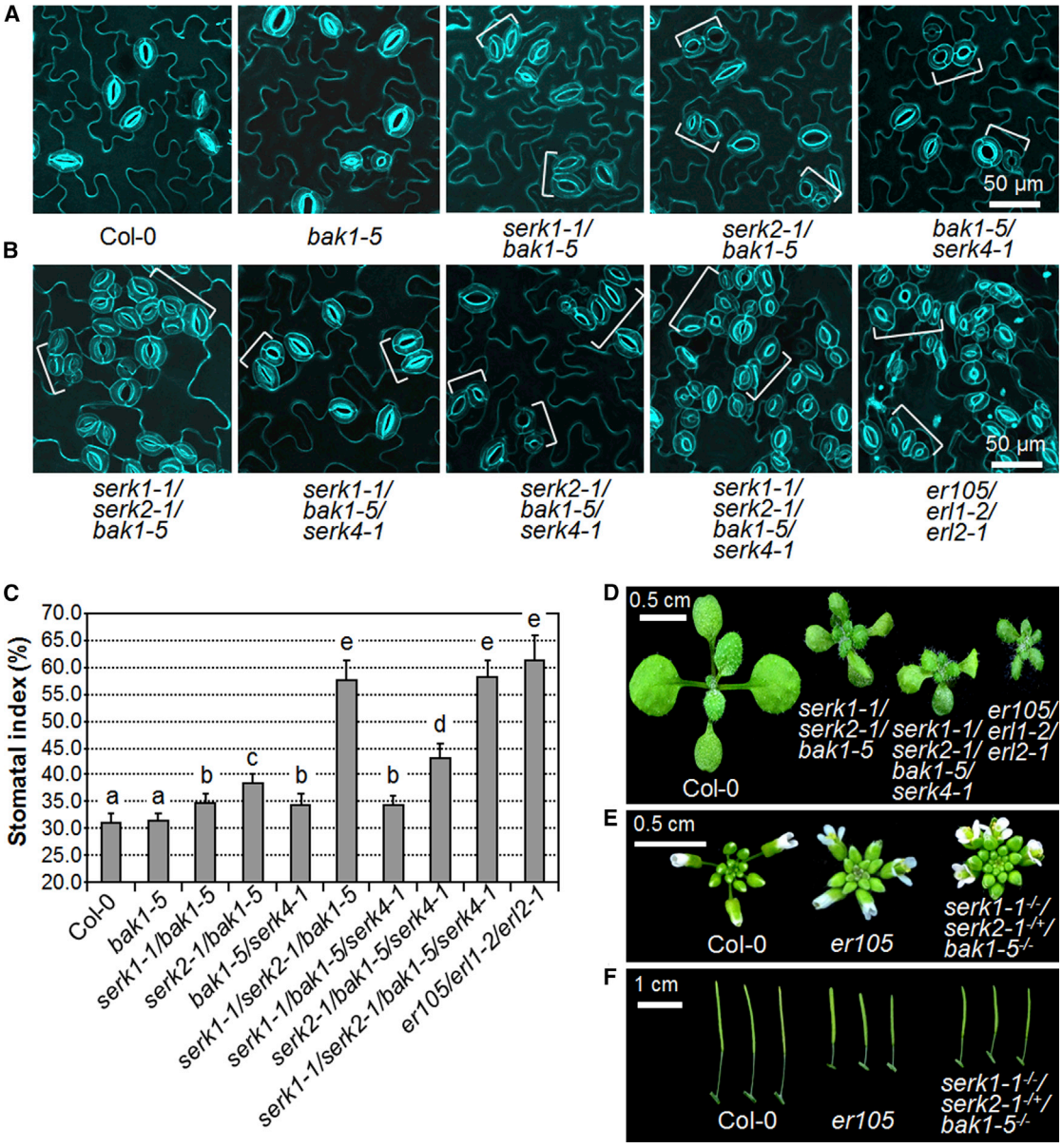

Figure 3. Differential Contributions of SERK Family RLKs to Stomatal Patterning

(A and B) The stomatal clustering phenotypes of *serk* higher-order mutants in the *bak1-5* background. Confocal images were taken on the abaxial cotyledon epidermis 10 days after germination on 1/2 MS medium. Brackets indicate clustered stomata.

(C) Abaxial cotyledon stomatal indexes of the indicated genotypes. The data are shown as mean ± SD (n = 8). The mean values marked with different letters are significantly different from each other ($p < 0.05$, Student's t test). The experiments were repeated three times with similar results.

(D) The phenotypes of 2-week-old seedlings grown on soil.

(E and F) *serk1-1*$^{-/-}$/*serk2-1*$^{-/+}$/*bak1-5*$^{-/-}$ plants phenocopy the *er105* mutant in inflorescence architecture (E) and pedicel length (F).

See also Figures S4 and S5.

and SERK4 in stomatal patterning. The stomatal clustering and index of *serk1-1/bak1-5/serk4-1* were similar to those of *serk1-1/bak1-5* and *bak1-5/serk4-1* (Figures 3A–3C), reinforcing the importance of *SERK2* in stomatal patterning. Introduction of the *serk4* mutation in *bak1-5* or *serk2-1/bak1-5* slightly but significantly increased stomatal clustering and index (Figures 3A–3C), indicating that SERK4 also plays an important but relatively minor role in stomatal development compared to BAK1 and SERK2. The stomatal clustering was much more severe in the cotyledon of *serk1-1/serk2-1/bak1-5* than that in *serk2-1/bak1-5/serk4-1* (Figures 3B and 3C), suggesting that SERK1 likely contributes more than SERK4 to stomatal development. Notably, the stomatal clustering and index in the cotyledon of *serk1-1/serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5/serk4-1* mutants were comparable to that in the *er105/erl1-2/erl2-1* mutant (Figures 3B and 3C). Similarly, the stomatal clustering in descending

order of severity was observed in the true leaves of *serk1-1/serk2-1/bak1-5*, *serk2-1/bak1-5/serk4-1*, and *serk2-1/bak1-5* (Figure S4). The extent of stomatal clustering in the true leaves of *serk1-1/serk2-1/bak1-5* was also comparable to that in *er105/erl1-2/erl2-1* (Figure S4). However, we did not observe the stomatal clustering in the true leaves of *serk1-1/bak1-5*, *bak1-5/serk4-1*, and *serk1-1/bak1-5/serk4-1* plants (Figure S4). Taken together, based on the extent of stomatal clustering in different *serk* double, triple, and quadruple mutants, it appears that each SERK member contributes differentially to stomatal development, with descending order of importance from BAK1 to SERK2 to SERK1 to SERK4. In contrast to stomatal lineage cell-specific genes such as *EPF1* and *EPF2* [9, 10], the expression of BAK1-GFP under the control of the *BAK1* native promoter was observed ubiquitously in the epidermal cells, including stomatal lineage cells in *pBAK1-BAK1-GFP* transgenic plants (Figure S5A), which is consistent with the multifunctionality of SERK family RLKs in diverse signaling pathways [28, 29].

In addition, the *serk1-1/serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5/serk4-1* mutants morphologically mimic the *er105/erl1-2/erl2-1* mutant in seedling stage (Figure 3D), whereas the morphologies of *serk1-1/bak1-5/serk4-1* and *serk2-1/bak1-5/serk4-1* are relatively normal compared to WT plants (Figure S5B). Moreover, the *serk1-1*$^{-/-}$*/serk2-1*$^{-/+}$*/bak1-5*$^{-/-}$ mutant also phenocopies the *er105* mutant in inflorescence architecture (Figure 3E) and pedicel length (Figure 3F) [38]. Compared with WT plants, both the *serk1-1*$^{-/-}$*/serk2-1*$^{-/+}$*/bak1-5*$^{-/-}$ and *er105* mutants exhibited clustered inflorescences (Figure 3E), which were associated with the shortened pedicels of these mutants (Figure 3F). The *serk1-1*$^{-/-}$*/serk2-1*$^{-/+}$*/bak1-5*$^{-/-}$ mutant was used here because the *serk1-1/serk2-1* homozygous mutant is male sterile and does not produce any seeds [39, 40]. The similar stomatal clustering and growth phenotypes in *serk* and *er* mutants suggest genetic interaction between SERK and ER family RLKs.

Uncoupled Functions of SERKs in Stomatal Patterning and BR Signaling

Members of the SERK family are also essential regulators of BR perception and signaling via complexing with the BR receptor BRI1 [20, 21, 27, 41]. It has been shown that BR regulates stomatal development through phosphorylation of YDA, MKK4/MKK5, and/or SPCH by the GSK3-like kinase BIN2 downstream of the BRI1-BAK1 complex [42–44]. To address whether the stomatal patterning defects in the *serk* mutants are caused by altered BR signaling, we examined the BR responses of the *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants, which displayed moderate and severe stomatal clustering, respectively. In contrast to the *bri1-119* mutant, which no longer exhibited hypocotyl elongation in response to exogenous brassinolide (BL) treatment, both *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants showed elongated hypocotyls upon BL treatment, similar to that observed in WT plants (Figures 4A and 4B). In addition, exogenous BL treatment induced the dephosphorylation of BES1 in both *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants, comparable to that in WT plants (Figure 4C). Apparently, the BR sensitivity of *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants is similar to that of WT plants. These data support that the stomatal patterning defects in *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants are not due to impaired BR signaling. In addition, the *serk1-8/bak1-4/serk4-1* triple null mutant, in which BR signaling is completely abolished (Figures 4A–4C) [27], exhibited normal stomatal patterning and index (Figures 4D and 4E), reinforcing the uncoupled functions of SERK family RLKs in BR signaling and stomatal patterning. Notably, SERK2 is not required for BR signaling [27] whereas it is essential in stomatal development (Figures 2 and 3), suggesting the functional specificity of individual SERK family members.

Interaction and Transphosphorylation between SERK and ER Family RLKs

We next tested whether BAK1 and other SERKs associate with ER or ERL1 to regulate stomatal development. A coimmunoprecipitation (coIP) assay using coexpressed FLAG-tagged SERKs and HA-tagged ER or ERL1 in *Arabidopsis* protoplasts indicated that SERK1, SERK2, BAK1, and SERK4 were able to coimmunoprecipitate both ER and ERL1 (Figure S6A). We further crossed *pBAK1::BAK1-GFP* transgenic *Arabidopsis* plants with *pER::ER-FLAG* or *pERL1::ERL1-FLAG* transgenic plants for the coIP assay. BAK1 could coimmunoprecipitate both ER and ERL1 when expressed under the control of their native promoters in transgenic *Arabidopsis* plants, indicating that they associate in vivo (Figure 5A). We further examined whether the EPF1 or EPF2 ligand could regulate the ER/ERL1-BAK1/SERK association dynamics. EPF1-ERL1 and EPF2-ER have been shown to function as ligand-receptor pairs specifying different steps of stomatal development [5]. Thus, we tested the ER-BAK1/SERK association in the presence of bioactive EPF2 peptide and the ERL1-BAK1/SERK association in the presence of EPF1 peptide. Importantly, EPF2 induced the association of ER with SERK1, SERK2, BAK1, and SERK4 (Figure 5B), and EPF1 induced the association of ERL1 with different SERKs (Figure 5C). The LRR-RLP TMM associates with ER and ERL1 and functions as a signal modulator in regulating stomatal patterning [5, 11]. TMM also shows binding ability to EPF2 but not EPF1 [5]. Interestingly, TMM also associated with SERK1, SERK2, BAK1, and SERK4 in coIP assays (Figure 5D). Apparently, the ligand EPF2 did not affect the association dynamics of TMM-BAK1/SERKs (Figure 5D). Taken together, these results suggest that ER/ERL1, BAK1/SERKs, and TMM form a multiprotein receptor complex consisting of different RLKs and RLP to perceive and transduce EPF peptide signals and regulate stomatal development.

To test whether BAK1 directly interacts with ER through their cytosolic kinase domains (CDs), we performed an in vitro pull-down assay. The maltose-binding protein (MBP)-tagged $BAK1_{CD}$ (MBP-$BAK1_{CD}$) could be pulled down by the glutathione S-transferase (GST)-tagged ER_{CD} (GST-ER_{CD}) but not by GST itself (Figure 6A). Moreover, in vitro kinase assays showed that MBP-$BAK1_{CD}$ phosphorylated GST-ER_{CD} (Figure 6B) and GST-ER_{CD} phosphorylated a kinase-inactive mutant of $BAK1_{CD}$ (MBP-$BAK1_{CD}$Km) (Figure 6C), indicating the transphosphorylation of the ER-BAK1 receptor complex. Notably, although both BAK1 and ER are RD-type RLKs (Figure S6B), the kinase activity of ER is very weak compared with that of BAK1. This allowed us to demonstrate the in vitro phosphorylation of ER by BAK1 using WT ER_{CD} (Figure 6B). Taken together, these data support that SERK family RLKs transduce stomatal development signaling through transphosphorylation with ER family RLKs.

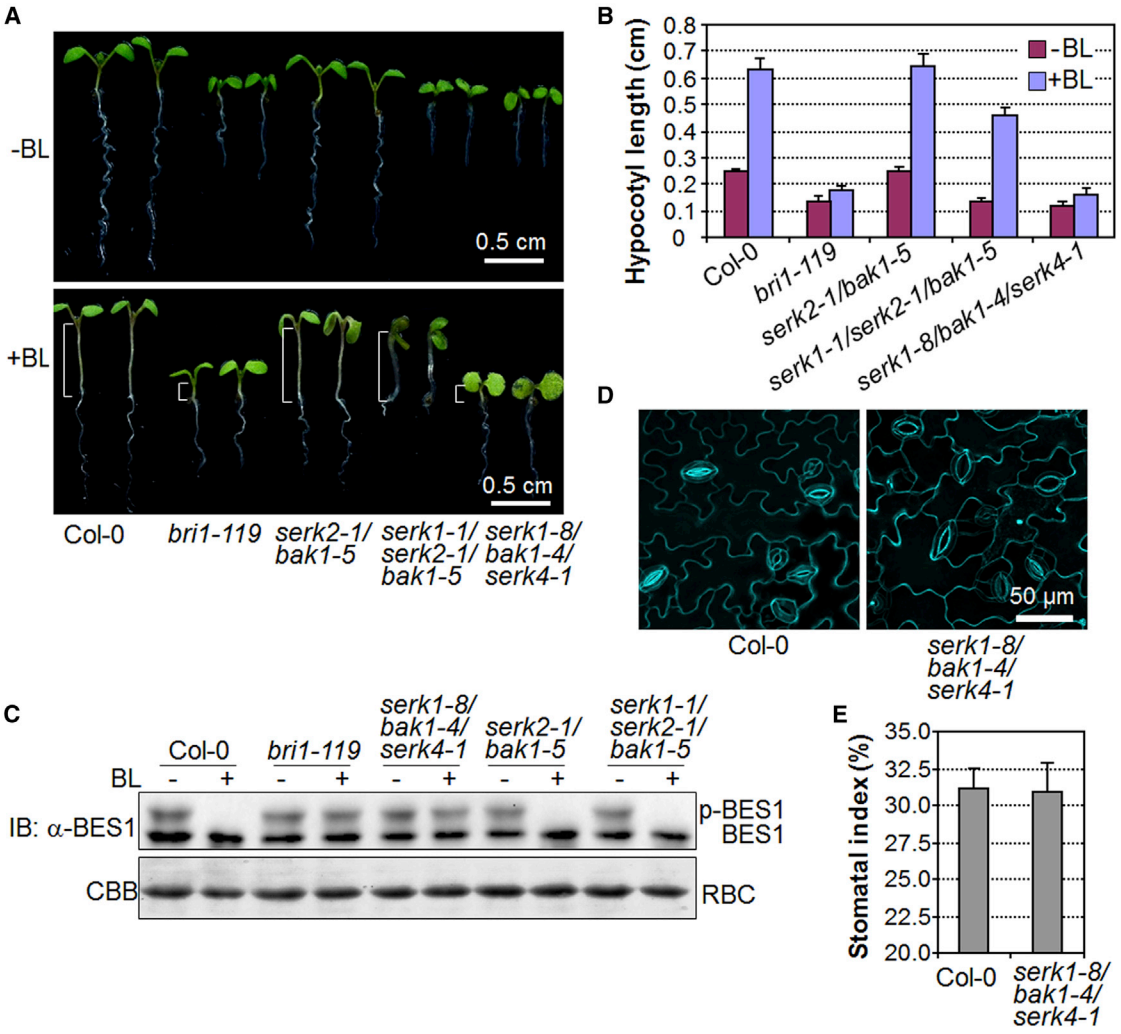

Figure 4. Uncoupled Functions of SERK Family RLKs in Stomatal Patterning and BR Signaling

(A and B) *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants show normal hypocotyl elongation in response to brassinolide treatment. The seedlings were grown under light for 10 days on 1/2 MS plates with or without 100 nM BL (A), and hypocotyl lengths were quantified (B). Brackets indicate hypocotyl (A). The data are shown as mean ± SD (n = 15) (B).

(C) BL treatment induces the dephosphorylation of BES1 in *serk2-1/bak1-5* and *serk1-1/serk2-1/bak1-5* mutants. Ten-day-old seedlings grown in liquid 1/2 MS medium were treated with 0 or 1 μM BL for 2 hr, and the total proteins were analyzed by immunoblotting with α-BES1 antibody (top panel). The protein loading is shown by Coomassie brilliant blue (CBB) staining for RuBisCO (RBC) (bottom panel).

(D and E) The *serk1-8/bak1-4/serk4-1* mutant exhibits normal stomatal patterning and index. Confocal images were taken on the abaxial cotyledon epidermis of 10-day-old seedlings (D), and the stomatal indexes were quantified (E). The data are shown as mean ± SD (n = 8). The experiments were repeated twice with similar results.

SERKs Function Downstream of EPFs and Upstream of YDA in Regulating Stomatal Development

To examine whether SERKs are required for EPF1- and EPF2-mediated stomatal development, we treated the *serk1-1/serk2-1/bak1-5* seedlings with bioactive EPF1 or EPF2 peptides and introduced the estradiol (Est)-inducible *EPF1* or *EPF2* transgene into the *serk1-1/serk2-1/bak1-5* mutant (Figure S7A). Similar to a previous report [5], application of EPF1 peptide or Est-induced overexpression of *EPF1* in WT seedlings rendered the epidermis devoid of stomata with arrested meristemoids (Figures 7A and 7B). In contrast, seedlings of *serk1-1/serk2-1/bak1-5* still exhibited excessively clustered stomata upon exogenous EPF1 treatment (Figure 7A) or induction of *EPF1* overexpression (Figure 7B). In addition, application of EPF2 peptide or overexpression of *EPF2* resulted in the epidermis with only pavement cells in WT seedlings, whereas the *serk1-1/serk2-1/bak1-5* seedlings were insensitive to EPF2 application or overexpression and still exhibited severe stomatal clustering (Figures 7A and 7B). These results demonstrate that EPF1- and EPF2-mediated stomatal development requires SERK family RLKs, and provide genetic evidence that SERKs function together with ER and ERL1 in regulating EPF2- and EPF1-mediated stomatal patterning.

To determine the genetic relationship between SERK family RLKs and the YDA-MKK4/MKK5-MPK3/MPK6 cascade, we

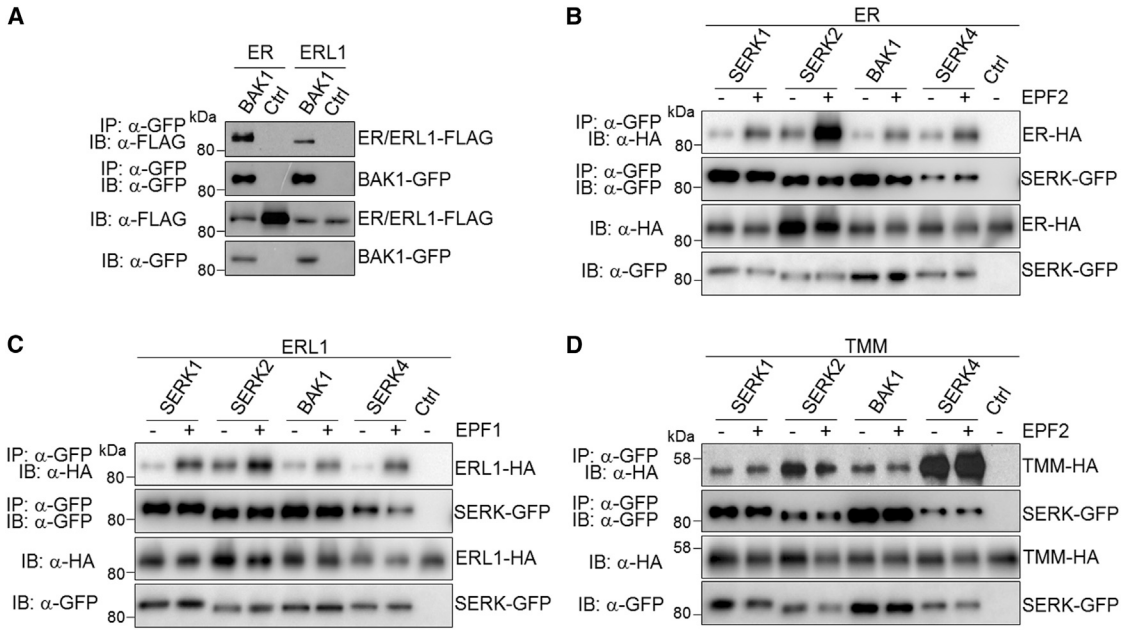

Figure 5. Interactions between SERK and ER Family RLKs

(A) BAK1 associates with ER and ERL1 in *pBAK1::BAK1-GFP/pER::ER-FLAG* and *pBAK1::BAK1-GFP/pERL1::ERL1-FLAG* transgenic plants. Protein extracts from transgenic plants were immunoprecipitated with α-GFP antibody (IP: α-GFP) and immunoblotted with α-FLAG (IB: α-FLAG) or α-GFP antibody (IB: α-GFP) (top two panels). The protein inputs are shown with immunoblotting before immunoprecipitation (bottom two panels). The *pER::ER-FLAG* and *pERL1::ERL1-FLAG* plants were used as controls.

(B) EPF2 induces the association of ER with SERKs in *Arabidopsis* protoplasts. SERK-GFP and ER-HA were transiently coexpressed in *Arabidopsis* protoplasts. After protoplasts were treated with or without 1 μM EPF2 for 5 min, protein extracts were immunoprecipitated with α-GFP antibody (IP: α-GFP) and immunoblotted with α-HA (IB: α-HA) or α-GFP antibody (IB: α-GFP) (top two panels). The protein inputs are shown with immunoblotting before immunoprecipitation (bottom two panels).

(C) EPF1 induces the association of ERL1 with SERKs in *Arabidopsis* protoplasts.

(D) SERKs associate with TMM in *Arabidopsis* protoplasts. Protoplasts were cotransfected with SERK-GFP and TMM-HA, and then treated with or without 1 μM EPF2 for 5 min. The experiments were repeated three times with similar results.

See also Figure S6A.

transformed a constitutively active form of *YDA* (*YDAac*) driven by its native promoter into the *serk1-1/serk2-1/bak1-5* mutant. As shown in Figure 7C, heterozygous *YDAac* was capable of fully rescuing the stomatal clustering defects in the *serk1-1/serk2-1/bak1-5* mutant. Notably, heterozygous *YDAac* was also able to rescue the growth defects of *serk1-1/serk2-1/bak1-5* plants (Figure 7C). Furthermore, a constitutively active *MKK5* variant (*MKK5*^{DD}) under the control of a Dex-inducible promoter was able to completely reverse the stomatal clustering phenotype in the *serk1-1/serk2-1/bak1-5* mutant and resulted in an epidermis solely composed of pavement cells (Figure 7D; Figure S7B). Collectively, these data further demonstrate that SERKs function in the same pathway with ER/ERL receptors upstream of the YDA-MKK4/MKK5-MPK3/MPK6 cascade in regulating stomatal development.

DISCUSSION

SERK family RLKs connect complex signaling networks via association with various RLK receptors and modulate distinct cellular responses [26, 29]. From the observation that ectopic expression of pathogen effectors targeting SERK family members led to clustered stomata in *Arabidopsis*, our study provides novel insights into host cellular signaling that BAK1, SERK1, SERK2, and SERK4 negatively regulate stomatal development via ligand-induced heteromerization and transphosphorylation with the ER and ERL1 receptors downstream of the EPF1 and EPF2 ligands and upstream of the YDA-MKK4/MKK5-MPK3/MPK6 cascade. Our study elucidates that SERK family RLKs function as a shared signaling node that modulates the interconnected architecture of complex cellular signaling networks yet disseminates diverse biological outcomes, including cell differentiation, growth, and immunity. Identification of SERK family RLKs as important regulators in stomatal development via association with ER family receptors substantiates the similarity of signaling pathways downstream of multiple RLK receptors.

Apparently, a diverse combinatorial code of individual SERK family RLKs contributes to their functional specificity. BAK1 and SERK4, but not SERK1 or SERK2, are important regulators in plant innate immunity and cell-death control [25, 45, 46]. In contrast, SERK1 and SERK2, but not BAK1 or SERK4, have a crucial and redundant function in anther development [39, 40]. BAK1, SERK1, and SERK4, but not SERK2, play an essential role in BR signaling [27]. We show here that all four functional SERKs (SERK1, SERK2, SERK3/BAK1, and SERK4) are involved in stomatal patterning (Figures 2 and 3). By comparison of

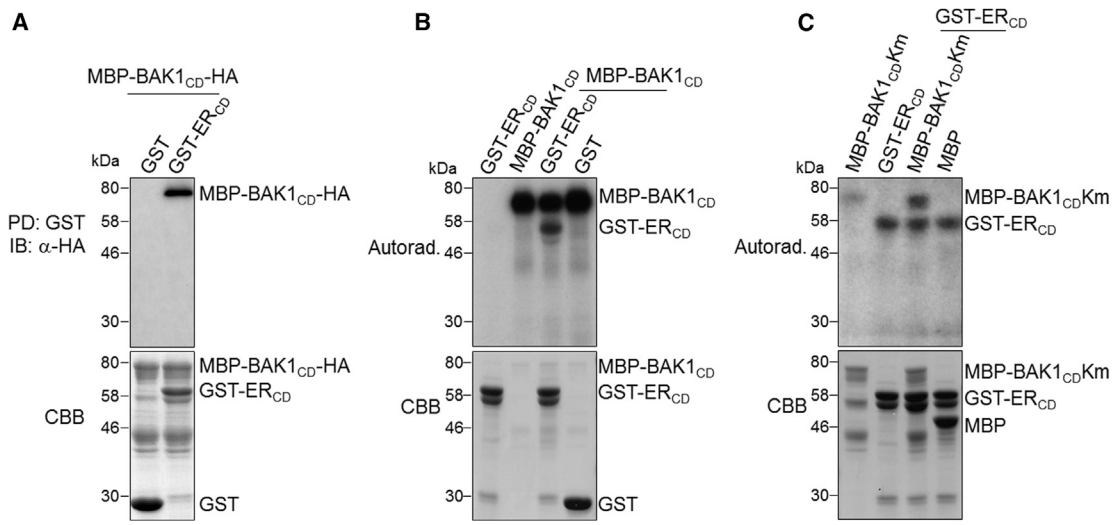

Figure 6. Transphosphorylation between the Cytosolic Kinase Domains of BAK1 and ER

(A) $BAK1_{CD}$ interacts with ER_{CD} in vitro. MBP-$BAK1_{CD}$-HA proteins were incubated with GST or GST-ER_{CD} glutathione beads, and the pulled-down (PD) proteins were immunoblotted with α-HA antibody (top panel). The CBB staining of input proteins is shown (bottom panel).

(B) The phosphorylation of ER_{CD} by $BAK1_{CD}$ (top panel).

(C) The phosphorylation of $BAK1_{CD}$ by ER_{CD} (top panel).

The kinase assays were performed using ER_{CD} and $BAK1_{CD}$ kinase mutant ($BAK1_{CD}$Km) proteins as substrates in (B) and (C), respectively. The CBB staining of input proteins is shown (bottom panels). The experiments were repeated three times with similar results.

See also Figure S6B.

stomatal clustering phenotypes in different combinations of *serk* higher-order mutants in the *bak1-5* background (Figures 3A–3C; Figure S4), we reveal the differential contributions of SERK family RLKs to stomatal patterning, with descending order of importance from BAK1 to SERK2 to SERK1 to SERK4. This unequal functional redundancy of different SERKs was also observed in plant immunity and BR signaling pathways [25, 27].

Accumulating evidence indicates that the diverse functions of SERK family RLKs are uncoupled. For instance, the involvement of BAK1 and SERK4 in cell-death control is independent of their function in BR signaling [45, 46]. The function of BAK1 in innate immunity can be separated from its involvement in cell-death control and BR signaling [37]. Similarly, several lines of evidence suggest that SERKs regulate stomatal patterning independent of BR signaling: (1) despite showing normal BR responses, the *serk1-1/serk2-1/bak1-5* mutant displayed severe defects in stomatal patterning (Figures 3B and 4A–4C); (2) the *serk1-8/bak1-4/serk4-1* mutant, in which the BR signaling is completely abolished [27], exhibited normal stomatal patterning (Figures 4D and 4E); (3) the *serk2* mutation in either *serk* single or higher-order mutants had an undetectable effect on BR signaling [27], whereas the introduction of the *serk2* mutation in the *serk1/bak1* double mutant dramatically exacerbated the stomatal clustering phenotype (Figure 3); and (4) the BR receptor mutant *bri1* and biosynthesis mutant *det2* showed much weaker stomatal clustering phenotypes than the *serk1/serk2/bak1* mutants [42, 44] (Figures 2 and 3). Thus, it appears that SERK family RLKs function independently in different signaling pathways.

With a relatively short extracellular LRR domain, SERK family RLKs appear not to be directly involved in the binding of ligands such as BL or flg22. Recent crystal structure analyses of the BL-BRI1-BAK1 and flg22-FLS2-BAK1 complexes indicate that BAK1 is involved in ligand sensing through contacting the BL- BRI1- or flg22-FLS2-binding interface [47–49]. Thus, although BAK1 itself does not confer BL- or flg22-binding activity, these structural studies support that SERK family RLKs function as coreceptors to interact directly with the ligand-receptor complexes. Consistent with this model, BL and flg22 induce the heterodimerization of SERKs with BRI1 and FLS2, respectively [22, 23, 25, 50]. Similarly, we observed that EPF2 and EPF1 peptides induce the heterodimerization of SERKs with their corresponding receptors ER and ERL1, respectively (Figures 5B and 5C). Therefore, it is likely that SERK family RLKs also serve as the coreceptors for ER and ERL in sensing EPF peptide signals. However, unlike FLS2, which does not oligomerize [49], both ER and ERL1 form receptor homomers and associate with the LRR-RLP TMM [5]. Lacking an obvious intracellular domain, TMM may not be directly involved in signal transduction. Genetic and biochemical studies indicate complex interactions with both antagonistic and cooperative roles between ER family receptors and TMM in regulating stomatal patterning [5, 6, 11]. Interestingly, SERK family RLKs also associate with TMM (Figure 5D), suggesting that ER/ERL1, SERKs, and TMM form a multiprotein receptor complex that perceives and transduces EPF peptide signals to regulate stomatal patterning. EPF ligands induce association of the cognate receptors ER and ERL1 but not the signal modulator TMM with SERKs (Figures 5B–5D), indicating the signaling role of ER/ERL1-SERK heterodimerization. It is likely that TMM may be involved in modulating the dimerization and/or activation of the ER/ERL1-SERK complexes. Future structural study of the EPF receptorsome consisting of multiple LRR-RLKs (ER/ERL and SERKs) and LRR-RLP (TMM) will

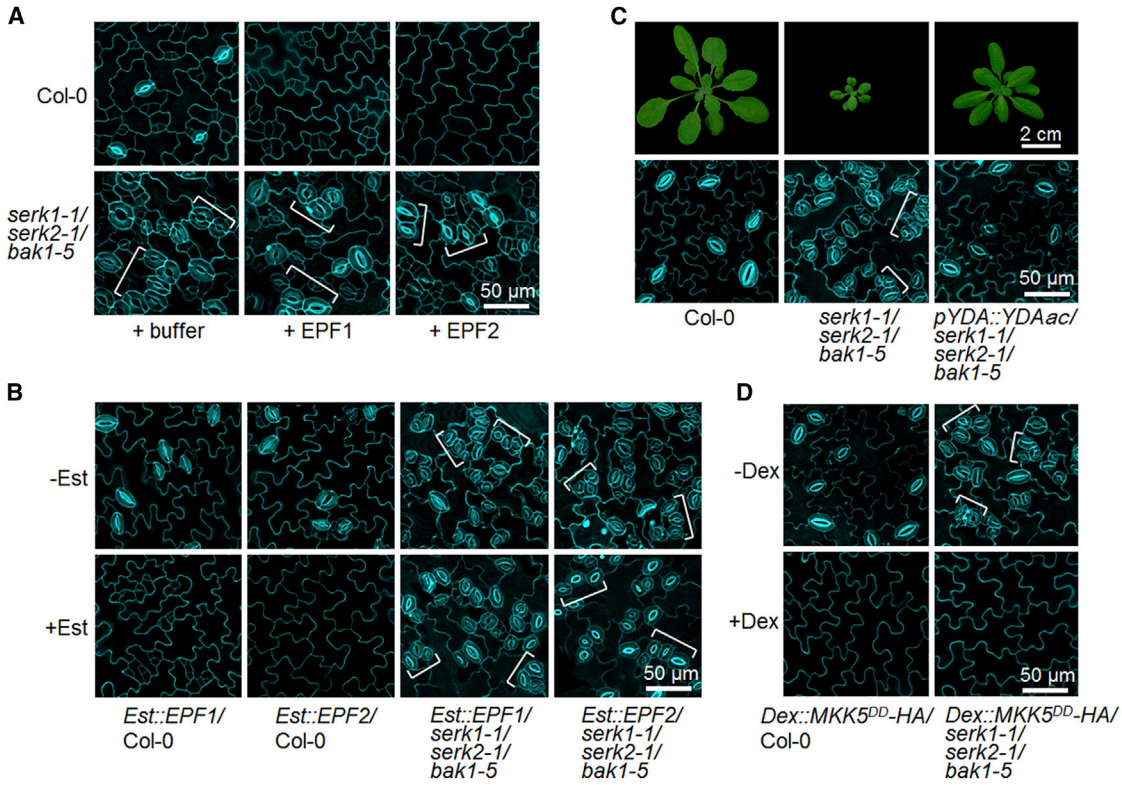

Figure 7. SERKs Function Downstream of EPFs and Upstream of YDA in Regulating Stomatal Development

(A and B) SERKs are required for EPF1- and EPF2-mediated stomatal development. Confocal images were taken on the abaxial cotyledon epidermis of 6-day-old Col-0 and *serk1-1/serk2-1/bak1-5* seedlings grown in 1/2 MS liquid medium containing 2.5 μM EPF1 or EPF2 (A) and 10-day-old transgenic seedlings of *Est::EPF1* or *Est::EPF2* grown on 1/2 MS plates with or without 10 μM estradiol (B).

(C) Expression of *YDAac* driven by its native promoter rescues the growth and stomatal patterning defects of *serk1-1/serk2-1/bak1-5*. The images were taken on 4-week-old plants (top panels) or 10-day-old cotyledon epidermis (bottom panels).

(D) Ectopic expression of $MKK5^{DD}$ eliminates stomata in the *serk1-1/serk2-1/bak1-5* mutant. Confocal images were taken on the abaxial cotyledon epidermis of 10-day-old transgenic seedlings of $Dex::MKK5^{DD}$ with or without 0.02 μM Dex treatment.

Brackets indicate clustered stomata. At least two transgenic lines for each construct in (B)–(D) were used, and similar results were obtained. The representative images were selected from at least five replicates.

See also Figure S7.

provide insights into the activation mechanism of the ligand-receptor-coreceptor complex.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Arabidopsis thaliana Columbia accession was used as wild-type. The mutants *bri1-119*, *bak1-4*, and *er105/erl1-2/erl2-1* and the transgenic plants of *pBAK1::BAK1-GFP*, *Dex::AvrPto*, *Est::EPF1*, and *Est::EPF2* in the Col-0 background, *pER::ER-FLAG* in *er105*, and *pERL1::ERL1-FLAG* in *erl1-2* were reported previously [5, 6, 32, 34]. The other *serk* T-DNA insertional mutants were obtained from the *Arabidopsis* Biological Resource Center (*serk1-1*, SALK_044330; *serk2-1*, SALK_058020; *serk4-1*, SALK_057955). The *Dex::AvrRpt2* (in the *rps2-101C* mutant background) and the *Dex::AvrRpm1* (in the *rpm1* mutant background) transgenic plants were obtained from Frederick Ausubel. The *Dex::AvrPtoB* transgenic plants in Nd-0 background were obtained from John Mansfield and Murray Grant [51]. The *bak1-5* mutant was obtained from Cyril Zipfel [37], and the *serk1-8/bak1-4/serk4-1* mutant was from Jia Li [27]. The *serk* double, triple, and quadruple mutants and *pBAK1::BAK1-GFP/pER::ER-FLAG* and *pBAK1::BAK1-GFP/pERL1::ERL1-FLAG* transgenic plants were generated by genetic crosses. *Arabidopsis* seeds were surface sterilized with 50% bleach and grown on half-strength Murashige and Skoog (1/2 MS) medium or on soil in a growth room at 23°C, 45% humidity, and 75 $\mu E\ m^{-2}\ s^{-1}$ light with a 12-hr light/12-hr dark photoperiod.

Plasmid Construction, Protoplast Transient Assay, and Generation of Transgenic Plants

The *Est::EPF1* and *Est::EPF2* constructs were reported previously [5]. The *pYDA::YDAac* construct was obtained from Wolfgang Lukowitz [52]. To make the $Dex::MKK5^{DD}$ construct, the PCR product of an *MKK5* variant containing constitutively active Ser-to-Asp mutations ($MKK5^{DD}$) was introduced into a modified pTA7002 vector and fused with an HA epitope tag at its C terminus. *ER*, *ERL1*, *SERK1*, *SERK2*, and *SERK4* genes were amplified by PCR from Col-0 cDNA and cloned into the plant expression vector for transient protein expression in protoplasts. The ER cytosolic domain was subcloned into a modified pGEX4T-1 vector (Pharmacia) for GST fusion protein expression in *Escherichia coli*, and the MBP fusion constructs of $BAK1_{CD}$ and $BAK1_{CD}$Km were generated previously [53]. The protoplast transient assay was carried out as described previously [54]. *Arabidopsis* transgenic plants were generated using *Agrobacterium*-mediated transformation by the floral-dip method. For all transgenic plants, >20 T1 plants per construct were screened for transgene expression using RT-PCR or immunoblotting, and two or three T2 lines with a single insertion and similar transgene expression levels were subjected to phenotypic characterization. The primers are listed in Table S1.

Histochemical Analysis and Microscopy

To visualize epidermal cell outlines, seedlings were stained with 0.2 mg/ml propidium iodide (PI) for 5 min and then washed twice with water. Confocal images were taken using a Zeiss LSM 700 microscope with a 20× objective lens. Histochemical staining of epidermis using toluidine blue O (TBO) (Sigma) was performed as described previously [5]. Stomatal index was quantified as the percentage of the number of stomata to the total number of epidermal cells using TBO-stained epidermal samples.

Chemical and Peptide Treatments

To characterize the BL-induced hypocotyl elongation, seeds were germinated on 1/2 MS plates containing 100 nM BL, and the hypocotyl length was measured 10 days after germination. To examine the BL-induced BES1 dephosphorylation, 10-day-old seedlings grown in 1/2 MS liquid medium were treated with 1 μM BL for 2 hr, and total proteins were analyzed by immunoblotting with an α-BES1 antibody (a generous gift from Yanhai Yin). Expression, purification, and refolding of recombinant bioactive EPF1 and EPF2 peptides were performed as described previously [5]. For peptide treatment, either buffer alone (50 mM Tris-HCl [pH 8.0]) or with 2.5 μM EPF peptides was applied to 1-day-old *Arabidopsis* plants germinated on 1/2 MS medium. After 5 days of further incubation in 1/2 MS liquid medium containing each peptide, stomatal phenotypes of abaxial cotyledons were analyzed with a confocal microscope. For chemical induction of transgenes, *Est::EPF1*, *Est::EPF2*, and *Dex::MKK5^{DD}* transgenic seeds were germinated on 1/2 MS plates containing 10 μM estradiol or 0.02 μM Dex, and stomatal phenotypes were examined 10 days after germination.

Coimmunoprecipitation, GST Pull-Down, and In Vitro Phosphorylation Assays

For the coIP assay, transfected protoplasts or leaf tissues of 4-week-old soil-grown transgenic plants were lysed with 0.5–1 ml extraction buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 1:200 complete protease inhibitor cocktail from Sigma). After vortexing vigorously for 30 s, the samples were centrifuged at 16,000 × *g* for 10 min at 4°C, and the supernatant was then incubated with α-FLAG (Sigma) or α-GFP agarose beads (ChromoTek) for 2 hr at 4°C with gentle shaking. The immunoprecipitated proteins were analyzed by immunoblotting with α-HA (Roche) or α-FLAG (Sigma) antibody. Expression and purification of the GST and MBP fusion proteins were performed using standard protocols. For the GST pull-down assay, 10 μg of MBP-BAK1$_{CD}$-HA proteins was incubated with prewashed GST or GST-ER$_{CD}$ glutathione beads in 0.5 ml pull-down buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Triton X-100) for 2 hr at 4°C with gentle shaking. The pulled-down proteins were analyzed by immunoblotting with α-HA antibody. For in vitro kinase assay, reactions were performed in 30 μl kinase buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 5 mM EGTA, 100 mM NaCl, and 1 mM DTT) containing 10 μg of fusion proteins with 0.1 mM cold ATP and 5 μCi [γ-^{32}P]ATP at room temperature for 2 hr with gentle shaking. The reactions were stopped by adding 4× SDS loading buffer, and the phosphorylation of fusion proteins was analyzed by autoradiography after separation with SDS-PAGE.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.068.

AUTHOR CONTRIBUTIONS

X.M., X.C., K.U.T., P.H., and L.S. designed the experiments; X.M., X.C., H.M., C.L., X.Y., and X.G. performed the experiments and analyzed the data; and X.M., P.H., and L.S. wrote the manuscript with input from all coauthors.

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Current Biology

Supplemental Information

Differential Function of *Arabidopsis* SERK Family Receptor-like Kinases in Stomatal Patterning

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SUPPLEMENTAL FIGURES

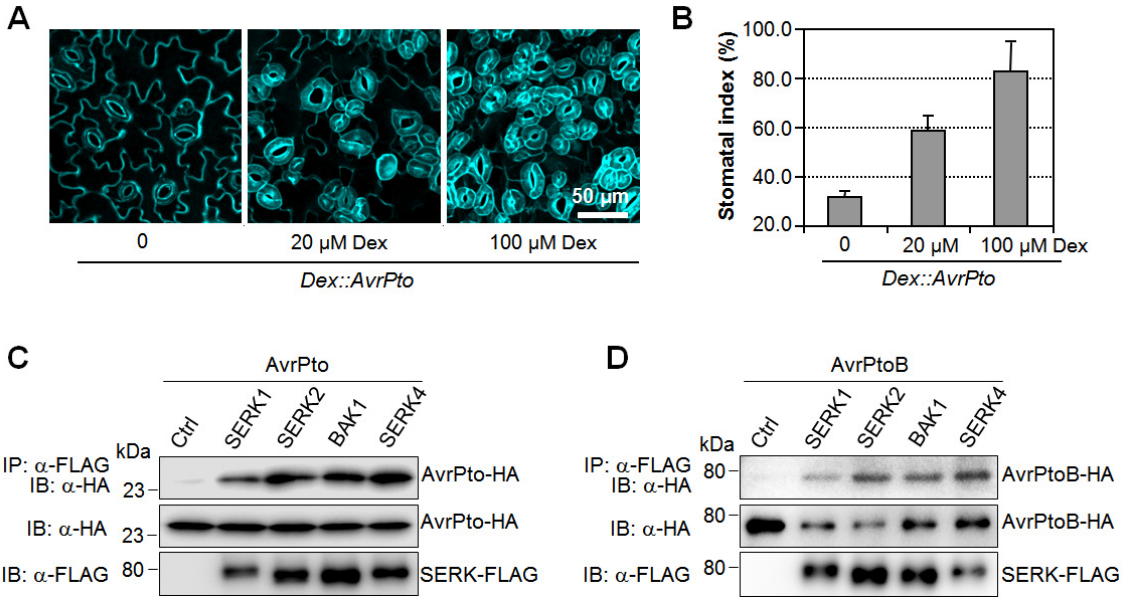

Figure S1, Related to Figure 1. Ectopic expression of AvrPto impairs stomatal patterning and AvrPto/AvrPtoB interact with the SERK family RLKs.

(A) Dex-induced expression of AvrPto in *Arabidopsis* transgenic plants caused severe stomatal clustering. Confocal images were taken on abaxial cotyledon epidermis of 10-day-old *Dex::AvrPto* transgenic seedlings grown on ½ MS medium with (20 or 100 μM) or without Dex. **(B)** Abaxial cotyledon stomatal index with the data shown as mean + SD (n=8). **(C, D)** AvrPto and AvrPtoB interact with SERK1, SERK2, BAK1 and SERK4 in *Arabidopsis* protoplasts. SERK-FLAG proteins were co-expressed with AvrPto-HA (C) or AvrPtoB-HA (D) in protoplasts. Protein extracts were immunoprecipitated with α-FLAG antibody (IP: α-FLAG), and immunoblotted with α-HA antibody (IB: α-HA) (top panel). The protein inputs are shown with immunoblotting before immunoprecipitation (bottom two panels). The experiments were repeated twice with similar results.

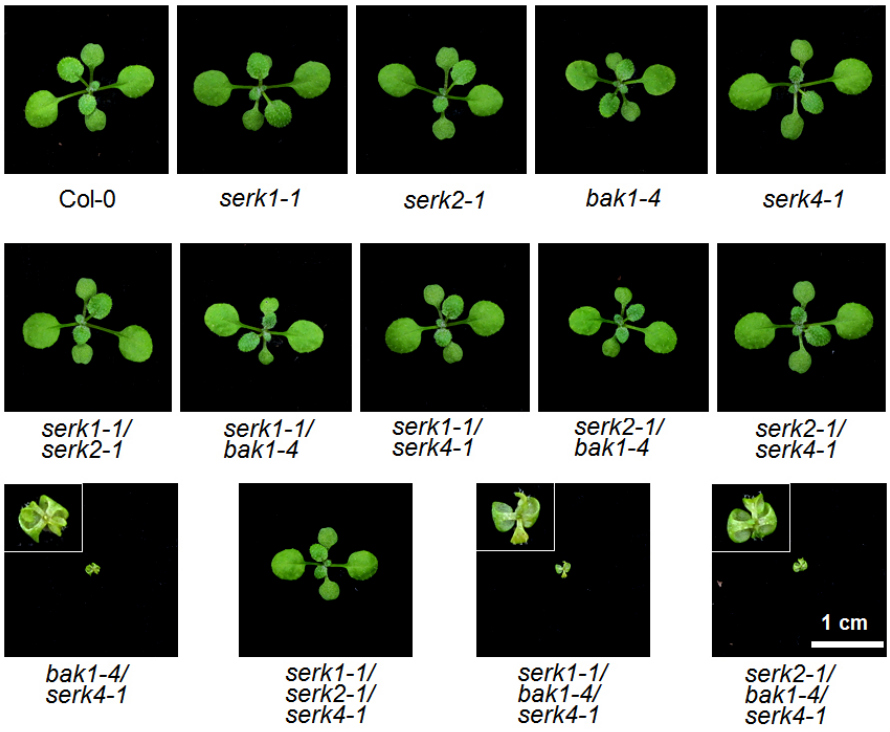

Figure S2, Related to Figure 2. The growth phenotypes of the *serk* single, double and triple mutants.

The *serk1-1/serk2-1/bak1-4* mutant shown in Figure 2D, but not other *serk* mutants shown here, exhibited similar growth morphology as the *er105/erl1-2/erl2-1* mutant (Figure 2D). The *bak1-4/serk4-1*, *serk1-1/bak1-4/serk4-1* and *serk2-1/bak1-4/serk4-1* mutants show seedling lethality. The images were taken on two-week-old seedlings grown on soil.

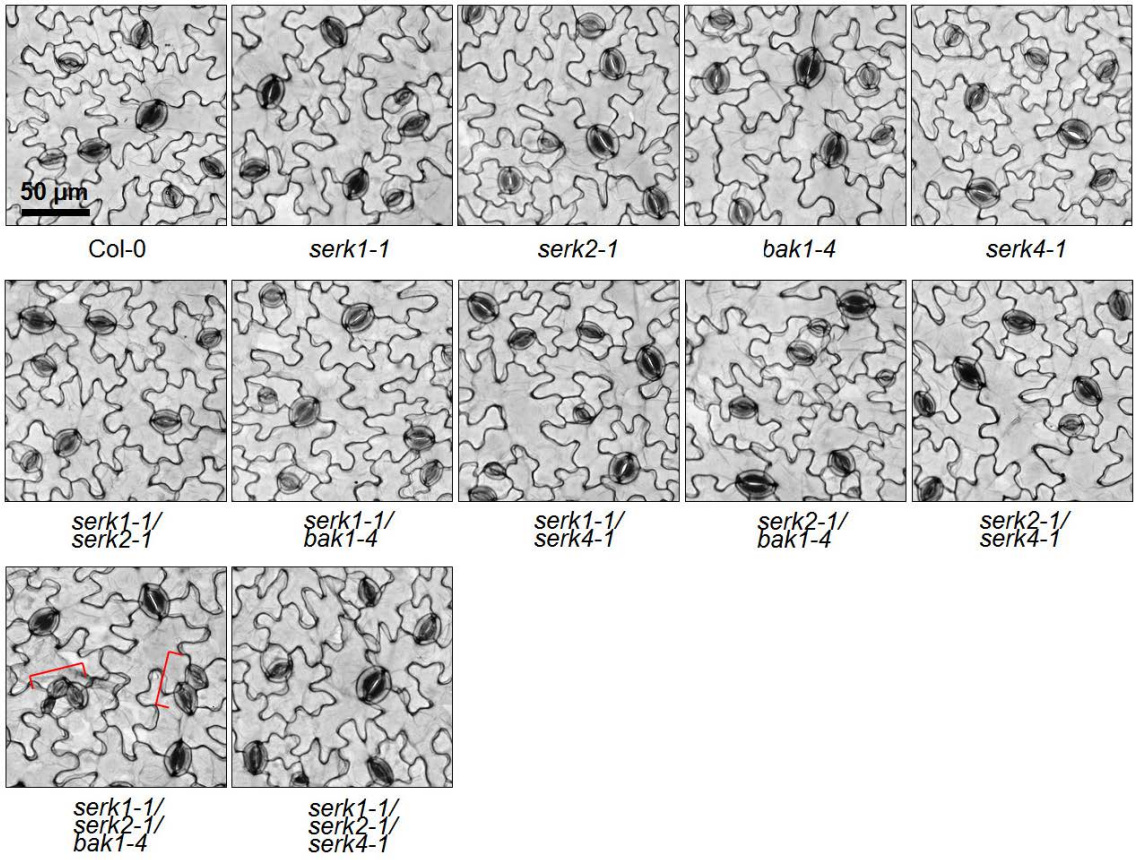

Figure S3, Related to Figure 2. The *serk1-1/serk2-1/bak1-4* mutant shows stomatal clusters in true leaves.

Optical microscopy images of abaxial epidermis of first true leaves from two-week-old seedlings of indicated genotypes grown on ½ MS medium. The *bak1-4/serk4-1*, *serk1-1/ bak1-4/serk4-1* and *serk2-1/bak1-4/serk4-1* mutants were not included in the assay because of seedling lethality and no true leaf developed. Cell outlines of peeled epidermal cell layers were visualized with toluidine blue staining. Brackets indicate clustered stomata. The representative images were selected from at least five replicates.

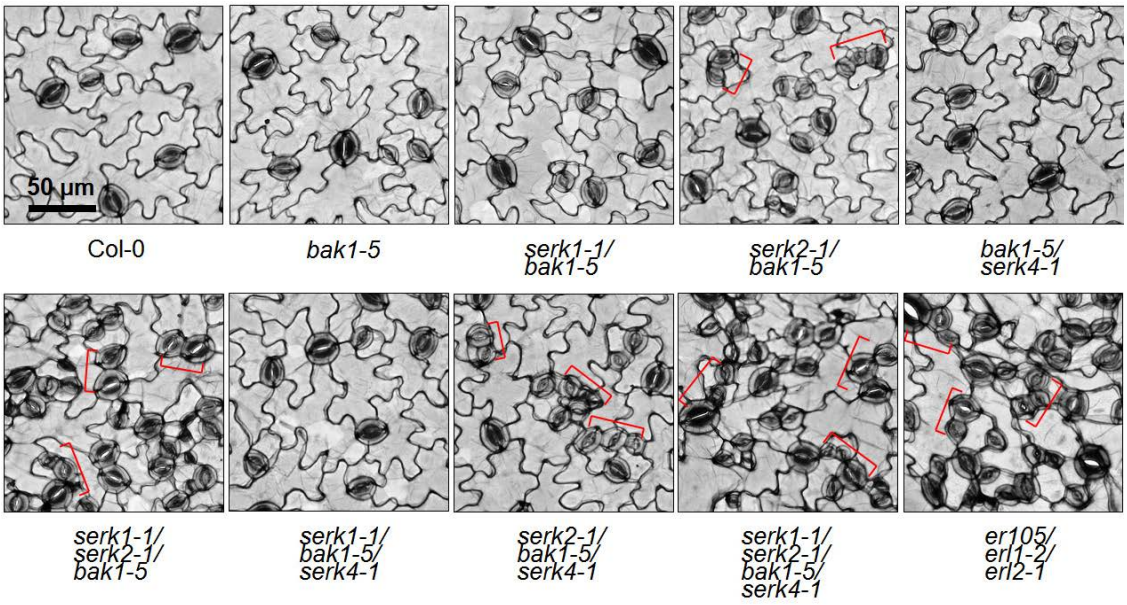

Figure S4, Related to Figure 3. The stomatal clustering phenotypes in true leaves of *serk* higher-order mutants in the *bak1-5* background.

Optical microscopy images of abaxial epidermis of first true leaves from two-week-old seedlings of indicated genotypes grown on ½ MS medium. Cell outlines of peeled epidermal cell layers were visualized with toluidine blue staining. Brackets indicate clustered stomata. The representative images were selected from at least five replicates.

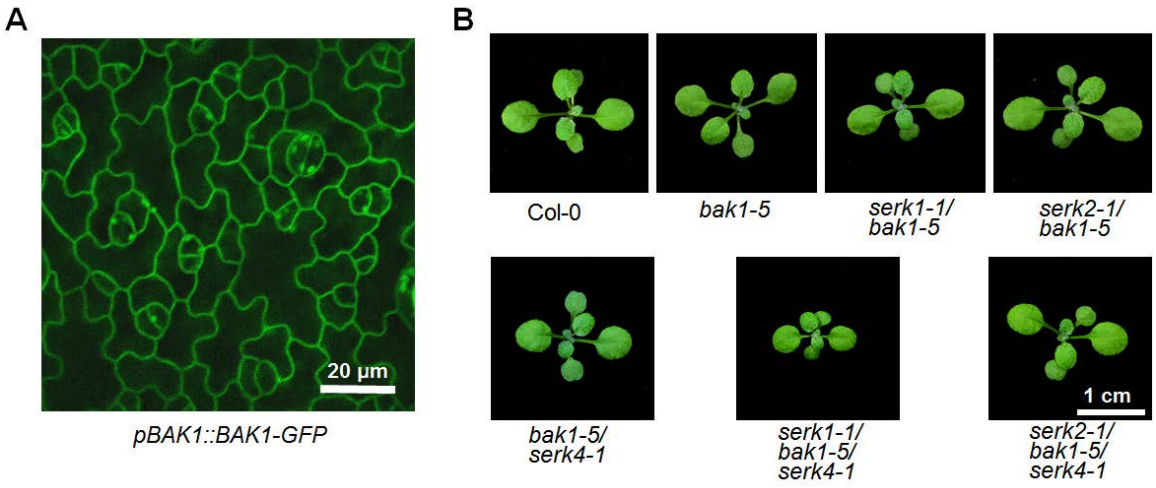

Figure S5, Related to Figure 3. Expression pattern of native promoter-driven BAK1-GFP and growth phenotypes of *serk* mutants in the *bak1-5* background.

(A) Native promoter-driven BAK1-GFP is ubiquitously expressed on the plasma membrane of epidermal cells. The expression of BAK1-GFP under the control of *BAK1* native promoter was observed using a confocal microscope in the abaxial epidermis of first true leaves of one-week-old *pBAK1::BAK1-GFP* transgenic plants. BAK1 is ubiquitously expressed in meristemoid cells, guard mother cells, guard cells and pavement cells. **(B)** The seedling phenotypes of *serk* single, double and triple mutants in the *bak1-5* background. Notably, the *bak1-5/serk4-1*, *serk1-1/bak1-5/serk4-1* and *serk2-1/bak1-5/serk4-1* mutants did not show seedling-lethal phenotype. The images were taken on two-week-old seedlings grown on soil.

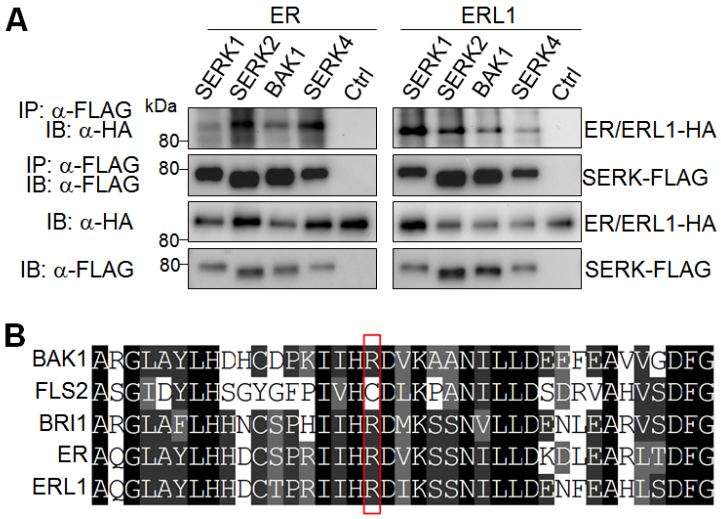

Figure S6, Related to Figure 5 (A) and Figure 6 (B). (A) SERKs associate with ER and ERL1 in *Arabidopsis* protoplasts. SERK-FLAG and ER/ERL1-HA proteins were co-expressed in protoplasts, immunoprecipitated with α-FLAG antibody (IP: α-FLAG), and immunoblotted with α-HA (IB: α-HA) or α-FLAG antibody (IB: α-FLAG) (top two panels). The protein inputs are shown with immunoblotting before immunoprecipitation (bottom two panels). **(B)** ER and ERL1 are RD-type RLKs. Amino acid sequence alignment of the kinase subdomain VI of BAK1, FLS2, BRI1, ER and ERL1. The dark shading represents identical amino acids among all five RLKs whereas the lightly shaded sequences represent similar amino acids. The red frame indicates presence or absence of the arginine (R) residue preceding the catalytic aspartate (D) in kinase subdomain VI that is used to differentiate RD kinases from non-RD kinases. In general, RD kinases are more active than non-RD kinases.

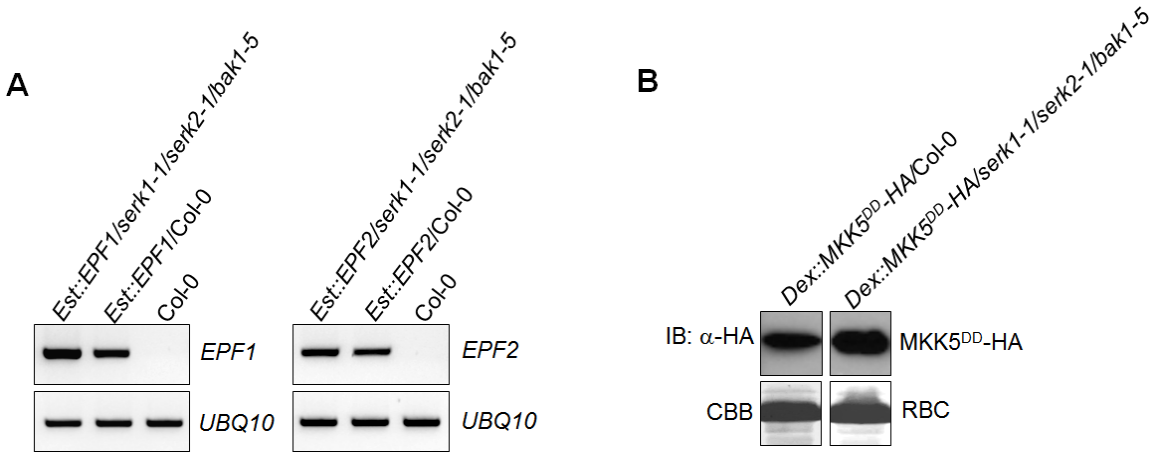

Figure S7, Related to Figure 7. (A) Estradiol-induced expression of *EPF1* and *EPF2* in transgenic plants. Ten-day-old seedlings of the indicated transgenic plants were treated with 10 μM estradiol for 24 hr. The expression of *EPF1* and *EPF2* genes was analyzed by RT-PCR with *UBQ10* as an internal control. **(B)** Dex-induced expression of *MKK5^{DD}* in transgenic plants. Ten-day-old seedlings of the indicated transgenic plants were treated with 5 μM Dex for 24 hr. The expression of MKK5^{DD}-HA was analyzed using immunoblotting with α-HA antibody and the protein loading is shown by CBB staining of RBC.

SUPPLEMENTAL TABLE

Table S1. Primers used for genotyping and plasmid construction

Primer Name	Sequences
serk1-1-LP	ATACACAAAAGTGAAACGGCG
serk1-1-RP	TTAGACGAAGAATTCGAAGCG
serk2-1-LP	GGAAAACTCAGGTGATCCATTAAG
serk2-1-RP	TTAACAGGTGATGCACTGCAC
bak1-4-LP	CAGGGGCTATATGACCAATTG
bak1-4-RP	TCCTATCTCTCCTACACCGCC
bak1-5-dCAPS-F	AAGAGGGCTTGCGTATTTACATGATCAGT
bak1-5-dCAPS-R	GAGGCGAGCAAGATCAAAAG
serk4-1-LP	TGGCTCAGAAGAAAACCACAG
serk4-1-RP	CTGCTCCACTTCTGTTTCCAC
SERK1-NcoI-F	CATGCCATGGAGTCGAGTTATGTGGTGTT
SERK1-StuI-R	GAAGGCCTCCTTGGACCAGATAACTCAACG
SERK2-NcoI-F	CATGCCATGGGGAGAAAAAAGTTTGAAGC
SERK2-StuI-R	GAAGGCCTTCTTGGACCAGACAACTCCATAG
BAK1-NcoI-F	CATGCCATGGAACGAAGATTAATGATC
BAK1-StuI-R	GAAGGCCTTCTTGGACCCGAGGGGTATTC
SERK4-BamHI-F	CGGGATCCATGACAAGTTCAAAAATGGA
SERK4-StuI-R	GAAGGCCTTCTTGGACCCGAGGGGTAAT
ER-NcoI-F	CATGCCATGGCTCTGTTTAGAGATAT
ER-StuI-R	GAAGGCCTCTCACTGTTCTGAGAAATAA
ER_{CD}-BamHI-F	CGGGATCCATGCCGCATAATCCTCCTCC
ERL1-BamHI-F	CGGGATCCATGAAGGAGAAGATGCAGCGA
ERL1-StuI-R	GAAGGCCTTATGCTACTTTTGGAGATGACTTCA
MKK5-XhoI-F	CCGCTCGAGATGAAACCGATTCAATCTCCTTC
MKK5-StuI-R	GAAGGCCTAGAGGCAGAAGGAAGAGGACGA
TMM-NcoI-F	CATGCCATGGCACGATATGAATTCTT
TMM-SmaI-R	TCCCCCGGGACTAGATATTAGCATAAAAATG