



Essential roles of SERKs in the ROOT MERISTEM GROWTH FACTOR-mediated signaling pathway

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J.L. supervised the project. Y.O. and B.T. performed all the experiments. Y.W. helped with the mbSUS analysis. Z.C. analyzed the expression patterns of *SHR*, *SCR*, and *WOX5* in Col-0 and *serk1-8 serk2-1 bak1-4*. H.L. and M.L. provided *SERK* mutants and some transgenic plants. X.G. and K.H. contributed to the discussions of the project. Y.O. and J.L. wrote the article.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is Jia Li (lijia@lzu.edu.cn).

Abstract

ROOT MERISTEM GROWTH FACTORS (RGFs), a group of peptide hormones, play key roles in root apical meristem development. In *Arabidopsis* (*Arabidopsis thaliana*), there are 11 members of RGFs, in which at least *RGF1*, *RGF2*, and *RGF3* are expressed at the root tip and are involved in root stem cell niche maintenance. RGFs are perceived by five functionally redundant receptor-like protein kinases, RGF1 INSENSITIVE 1 (RGI1) to RGI5, to maintain the expression of two downstream APETALA 2 (AP2) transcription factor genes, *PLETHORA 1* (*PLT1*) and *PLT2*, and to stabilize *PLT2*. RGI1 to RGI3 were also named RGF RECEPTOR 1 (RGFR1) to RGFR3, respectively. Although previous studies have suggested that BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and its paralogs, SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERKs), may act as coreceptors of RGIs, comprehensive genetic and biochemical analyses have not been well documented. Here, we report that single, double, and triple mutants of *SERKs* show various degrees of short root phenotypes and insensitivity to exogenously applied RGF1. The interaction between RGIs and BAK1 and their mutual phosphorylation are RGF1 dependent. We also found that RGF1-induced MAPK activation relies on both RGIs and SERKs. We demonstrate that RGIs play redundant roles in regulating root apical meristem development. Therefore, we genetically and biochemically substantiated that SERKs, as coreceptors, play essential roles in the RGF1-mediated signaling pathway.

Introduction

As an important vegetative organ, root plays key roles for the survival of land plants including supporting absorption, transportation, synthesis, and storage. The growth and development of root require a cell pool in root apical meristem to continuously produce new cells. This cell pool is consumed by cell differentiation and supplied by stem cell

division (Petricka et al., 2012). The quiescent center (QC) cells and their surrounding stem cells together compose a stem cell niche. The root apical meristem consists of a proximal root meristem and a distal root meristem, which are bounded by the QC cells. The maintenance of stem cell niche is critical for the growth and development of root apical meristem (Aichinger et al., 2012). There are a number of signaling pathways known to regulate the maintenance of

stem cell niche. One of these defined pathways is a CLV3/EMBRYO SURROUNDING REGION-related 40 (CLE40)-ARABIDOPSIS CRINKLY4 (ACR4)/CLAVATA1 (CLV1)-WUSCHEL-RELATED HOMEOBOX 5 (WOX5) pathway. WOX5 is specifically expressed in the QC cells, which are responsible for maintaining the division of the columella stem cells (van den Berg et al., 1997; Haecker et al., 2004). Shutting down the expression of WOX5 gives rise to the earlier differentiation of columella stem cells, similar to the results when the QC cells were laser ablated (Sarkar et al., 2007). In the distal root meristem, CLE40 expressed in columella cells restricts the expression of WOX5 through receptor-like protein kinases, ACR4 and CLV1, to maintain the stem cell niche (De Smet et al., 2008; Stahl et al., 2009, 2013). It was reported recently that CLAVATA3 INSENSITIVE RECEPTOR KINASEs (CIKs) act as coreceptors to mediate the signal transduction of CLE40 in regulating distal root meristem development (Zhu et al., 2021).

Another known pathway is a SHORT ROOT (SHR)-SCARECROW (SCR) pathway. SHR expressed in stele shuttles to adjacent cells including the QC cells, and activates the expression of SCR (Helariutta et al., 2000; Nakajima et al., 2001). SCR interacts with SHR to form a complex, disrupting the further intercellular movement of SHR and promoting the expression of specific genes including WOX5 in the QC cells (Sabatini et al., 2003; Levesque et al., 2006; Cui et al., 2007).

The third known pathway is an Auxin-TYROSYLPROTEIN SULFOTRANSFERASE/ACTIVE QUIESCENT CENTER 1 (TPST/AQC1)-ROOT MERISTEM GROWTH FACTORS (RGFs)-PLETHORAs (PLTs) pathway. The auxin concentration is maximized in the QC cells and gradually decreases in the surrounding cells, which is established under the action of various auxin transporters (Wisniewska et al., 2006; Grieneisen et al., 2007). The high concentrations of auxin are very important for the formation and maintenance of a functional stem cell niche. When QC cells are destroyed or root tip is removed, a new stem cell niche can be reconstructed under the action of high concentration of auxin (Sabatini et al., 1999; Xu et al., 2006; Sena et al., 2009). The role of auxin in the maintenance of stem cell niche depends on two transcription factors, PLT1 and PLT2 (Aida et al., 2004; Galinha et al., 2007). Similarly, the expression levels of PLT1 and PLT2 are enriched in the QC cells and gradually decrease in their surrounding cells (Aida et al., 2004; Galinha et al., 2007). It was found that auxin can up-regulate the expression of TPST and RGFs (Zhou et al., 2010). TPST is important for the maturation and activation of RGFs. The activated RGFs are able to maintain the expression of PLT1 and PLT2 (Matsuzaki et al., 2010).

RGFs were also designated as CLE-LIKEs and GOLVENs by two independent groups (Matsuzaki et al., 2010; Meng et al., 2012; Whitford et al., 2012). The mature forms of RGFs contain about 13 amino-acid residues; there are 11 RGF members in *Arabidopsis thaliana* (Fernandez et al., 2013). In root tips, RGF1 is expressed in QC and columella

stem cells, whereas RGF2 and RGF3 are expressed in columella cells adjacent to the stem cell niche (Matsuzaki et al., 2010). RGFs regulate many different processes during root growth and development, including maintenance of the stem cell niche, root gravitropism, and lateral root development in *Arabidopsis* (Fernandez et al., 2013).

The receptors of RGF1, RGF1 INSENSITIVEs (RGIs)/RGF RECEPTORS (RGFRs) were identified by three research groups independently (Ou et al., 2016; Shinohara et al., 2016; Song et al., 2016). Three leucine-rich repeat receptor-like kinases (LRR-RLKs) were found to interact with RGF1 through a photoaffinity labeling technology. They were named as RGFR1 to RGFR3 (Shinohara et al., 2016). Five phylogenetically related LRR-RLKs were found to interact with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) in a yeast two-hybrid analysis. The quintuple mutant of these LRR-RLKs showed an extreme short root phenotype and complete insensitivity to exogenously applied RGF1. These five LRR-RLKs were therefore named RGI1 to RGI5 (Ou et al., 2016). The same five LRR-RLKs were also identified via a structural signature approach (Song et al., 2016). Further analysis showed that RGIs/RGFRs can control the expression of downstream transcription factor genes PLT1 and PLT2 to promote the maintenance of stem cell niche in root tips, which is similar to the aforementioned function of RGFs (Ou et al., 2016; Shinohara et al., 2016; Song et al., 2016). Interestingly, the poly-ubiquitination and degradation of RGI1 are induced by RGF1 to turn off the signaling soon after the signaling pathway is initiated (Ou et al., 2016). Consistently, UBIQUITIN-SPECIFIC PROTEASE 12 (UBP12) and UBP13 are capable of stabilizing RGI1 in root tips (An et al., 2018).

The first SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK), DcSERK, was identified from *Daucus carota* (Schmidt et al., 1997). There are five orthologs of DcSERK in *Arabidopsis*, SERK1 to SERK5. Accumulated evidence indicated that SERKs, as coreceptors, are involved in regulating various aspects of plant growth, development, and immunity. BAK1, also named SERK3, was originally identified by two groups independently for its role as a coreceptor in the brassinosteroid (BR) signaling pathway (Li et al., 2002; Nam and Li, 2002). Genetic analysis showed that SERKs play an indispensable role in BR signaling (Gou et al., 2012). The crystal structures of the brassinolide (BL, the final product of the BR biosynthesis pathway)-BRI1 and BRI1-BL-BAK1 complexes were resolved which unfolded the early receptor-ligand-coreceptor formation mechanism (Hothorn et al., 2011; She et al., 2011; Santiago et al., 2013; Sun et al., 2013a). BAK1 and its functionally redundant SERKs were found to act as coreceptors in a number of different RLK-mediated signaling pathways to regulate different aspects of plant development, such as regulating root cell expansion in a phytosulfokine RECEPTOR 1 (PSKR)-sulfated peptide PSK pathway (Ladwig et al., 2015; Wang et al., 2015), controlling stomata development in an ERECTA (ER)/ERECTA-LIKE1 (ERL1)-EPIDERMAL PATTERNING FACTORS (EPFs)

pathway (Meng et al., 2015), mediating male gametophyte development in an EXCESS MICROSPOROCTES1 (EMS1)-TAPETUM DETERMINANT1 (TPD1) pathway (Li et al., 2017), and regulating floral organ abscission in a HAESA (HAE)/HAESA-LIKE2 (HSL2)-INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) pathway (Meng et al., 2016; Santiago et al., 2016). In addition, BAK1 and BKK1 (also named SERK4) were reported to mediate a light-dependent cell death pathway independent of the BR signaling pathway (He et al., 2007, 2008). It was recently found that loss of BAK1 and BKK1 triggers a nucleotide-binding leucine-rich repeat proteins (NLRs)-dependent autoimmune response and cell death (Wu et al., 2020). Thereafter, BAK1 and other SERKs act as coreceptors in various pattern-recognition receptors signaling pathways, such as in EF-TU RECEPTOR (EFR)-elongation factor thermo unstable (EF-Tu), FLAGELLIN-SENSITIVE 2 (FLS2)-flagellin, PEP1 RECEPTOR 1 (PEPR1)-plant elicitor peptides pathways to mediate plant innate immunity (Chinchilla et al., 2007; Postel et al., 2010; Roux et al., 2011; Sun et al., 2013b; Tang et al., 2015; Yamada et al., 2016).

Our previous studies showed that BAK1 and its paralogs are involved in regulating root development via BR dependent and independent pathways (Du et al., 2012). Subsequently, RGI1 to RGI5 were identified in a yeast two-hybrid screening with BAK1 as bait to regulate the maintenance of stem cell niche in the root apical meristem as the receptors of RGF1 (Ou et al., 2016). A different group reported that *serk1 serk2*, *serk2 bak1*, and *serk1 serk2 bak1* showed insensitivity to low concentration of RGF1. In addition, RGF1 can induce the interaction between the extracellular domains of SERKs and RGIs in a gel filtration chromatogram assay and a coimmunoprecipitation (Co-IP) analysis in *Nicotiana benthamiana* leaves (Song et al., 2016). Detailed genetic and biochemical data regarding the importance of BAK1 and its paralogs, SERKs, in the RGIs-RGFs pathways are yet to be elucidated.

Here, we report that a triple mutant of SERKs, *serk1-8 serk2-1 bak1-4*, exhibits an extreme short root phenotype with a smaller root apical meristem and insensitivity to RGF1, similar to the quintuple mutant of RGIs, *rgi12345*. The expression area of *CYCB1;1* is reduced and the expression levels of *PLT1* and *PLT2* are substantially downregulated in *serk1-8 serk2-1 bak1-4*. We further demonstrate that the interaction between RGIs and BAK1 *in vivo* is RGF1 dependent. We found that exogenous RGF1 treatment can induce the phosphorylation of RGIs and BAK1, and such a process requires the presence of both receptor and coreceptor. The cytoplasmic domains (CDs) of RGI1 and BAK1 can directly phosphorylate each other *in vitro*. The activation of downstream MAPK signaling components induced by RGF1 relies on both RGIs and SERKs, but not other RLKs such as BRL1, BRL2 and BRL3. We also genetically examined the contribution of each member of RGIs in root development. These results suggest that SERKs, as coreceptors, play key roles in the signal transduction of RGF1 to regulate the maintenance of stem cell niche in root apical meristem.

Results

SERK mutants display short root phenotypes and reduced sensitivity to exogenously applied RGF1

In order to comprehensively understand the importance of SERKs in regulating root meristem development in the RGIs-mediated signaling pathway, we first investigated the root phenotypes of a series of single, double, and triple mutants of SERKs. All these mutants exhibit various degrees of short root phenotypes except the single mutants, *serk1-8* and *serk2-1* (Figure 1, A and B). Particularly, the *serk1-8 serk2-1 bak1-4* triple mutant shows an extremely short root phenotype (Figure 1, A and B). An independent triple mutant, *serk1-1 serk2-2 bak1-6*, shows a similar root phenotype as that of *serk1-8 serk2-1 bak1-4* (Supplemental Figure S1, A and B). To examine whether SERKs are truly involved in the signal transduction of RGF1, we first tested the sensitivity of roots of *serk1-8 bak1-4* and *serk1-8 serk2-1 bak1-4* to exogenously applied RGF1. After the supplementation of RGF1, the root gravitropism of wild-type was substantially reduced, while the root gravitropism of *serk1-8 bak1-4* was only altered slightly (Figure 1, C and D). Microscopic analysis revealed that the meristematic cortex cell number of *serk1-8 serk2-1 bak1-4* is decreased significantly compared with that of wild type (Figure 1, E and F). After the treatment of RGF1, the meristematic cortex cell number was significantly increased in wild type, but not in *serk1-8 serk2-1 bak1-4*, indicating that *serk1-8 serk2-1 bak1-4* is less sensitive to the treatment of RGF1 (Figure 1, E and F). These results suggest that BAK1 and its paralogs are likely involved in the RGF1-mediated signal transduction to regulate the size of root apical meristem.

The expression domain of *CYCB1;1* is decreased and the expression levels of *PLT1* and *PLT2* are reduced in *serk1-8 serk2-1 bak1-4*

To examine whether the smaller root apical meristems in *rgi12345* and *serk1-8 serk2-1 bak1-4* are caused by the cell division defects, we introduced *pCYCB1;1-GUS* into *rgi12345* and *serk1-8 serk2-1 bak1-4* by genetic crossing. Our results showed that the expression domain of *CYCB1;1* in *rgi12345* and *serk1-8 serk2-1 bak1-4* are decreased substantially in comparison with that of wild-type (Figure 2, A–D), indicating that the cell division activities in the root apical meristems of *rgi12345* and *serk1-8 serk2-1 bak1-4* are reduced. Previous studies showed that the expression levels of *PLT1* and *PLT2* in *rgi12345* are decreased substantially compared to that in wild-type (Ou et al., 2016). We, therefore, introduced two reporter gene constructs, *pPLT1:PLT1-YFP* and *pPLT2:PLT2-YFP*, from wild type to *serk1-8 serk2-1 bak1-4* by genetic crossing. Similar to *rgi12345*, the expression levels of *PLT1* and *PLT2* in *serk1-8 serk2-1 bak1-4* are also substantially reduced (Figure 2, E–H). These results indicate that the genetic deficiency of RGIs and SERKs all lead to the decreased expression domain of *CYCB1;1* and the down-regulated expression of *PLT1* and *PLT2*, consistent with our hypothesis that they are all involved in RGF1-mediated

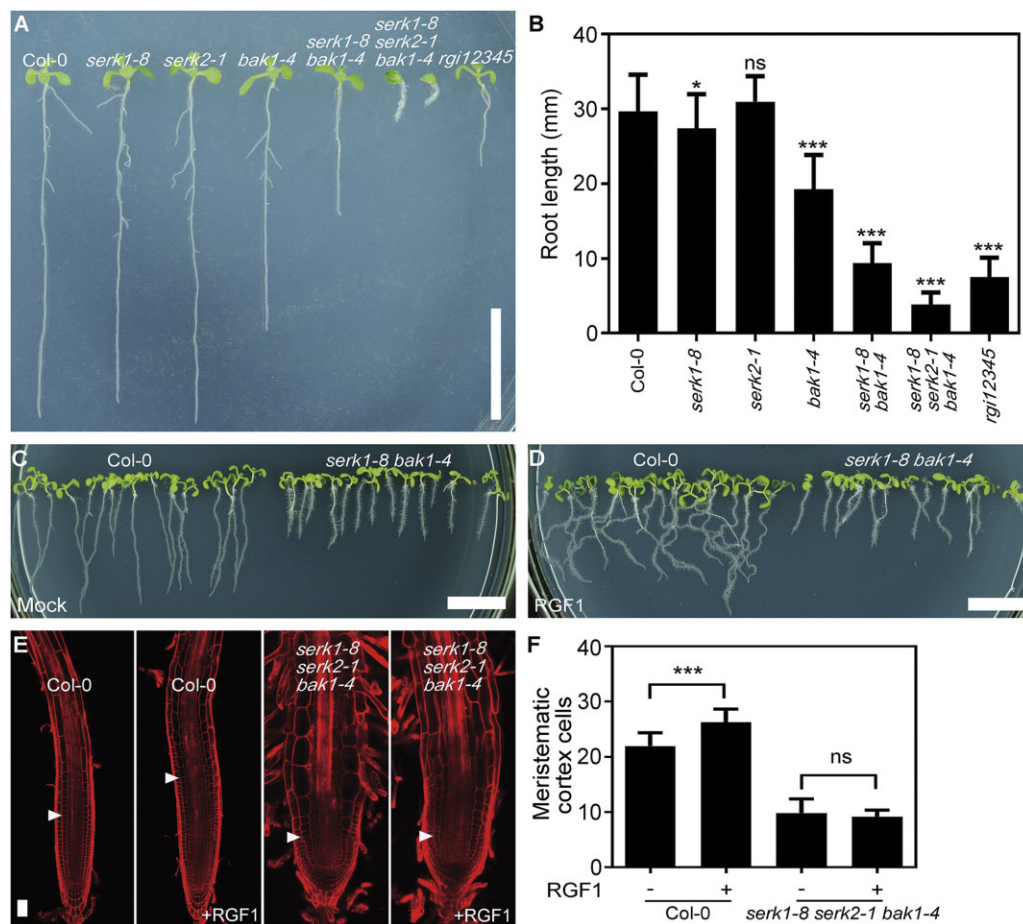


Figure 1 SERK mutants show reduced root length and decreased sensitivity to exogenously supplemented RGF1. A, Representative root phenotypes of 8-d-old Col-0, *serk1-8*, *serk2-1*, *bak1-4*, *serk1-8 bak1-4*, *serk1-8 serk2-1 bak1-4*, and *rgi12345*. The scale bar represents 10 mm. B, Measurements of the roots as shown in A. Each data represent the average and SD. $n > 30$. t test was carried out for the significance between *serk1-8*, or *serk2-1*, *bak1-4*, *serk1-8 bak1-4*, *serk1-8 serk2-1 bak1-4*, *rgi12345*, and wild type, respectively. “***” indicates $P < 0.001$. “*” indicates $P < 0.05$. “ns” means no significance. C and D, Root phenotypes of 6-d-old Col-0 and *serk1-8 bak1-4* on 0.5 MS medium without (C) and with (D) the treatment of 200 nM RGF1. The scale bar represents 10 mm. E, The responses of meristematic zone size in the root tips of wild type and *serk1-8 serk2-1 bak1-4* to 200 nM RGF1. The 4-d-old seedlings were used for PI staining and photography. The white arrow indicates the boundary between meristematic and transition zones. The scale bar represents 50 μ m. F, Measurements of meristematic cortex numbers for the roots as shown in E. Each data represent the average and SD. $n > 15$, “***” represents $P < 0.001$. “ns” means no significance.

signaling pathway. We also analyzed the expression patterns of *SHR*, *SCR*, and *WOX5* in Col-0 and *serk1-8 serk2-1 bak1-4*. Our data showed that the expression patterns of *SHR* and *SCR* in *serk1-8 serk2-1 bak1-4* are similar to that of Col-0 (Supplemental Figure S2, A–D). However, the expression pattern of *WOX5*, the QC specifically expressed gene, is expanded in *serk1-8 serk2-1 bak1-4* compared with that in wild type (Supplemental Figure S2, E and F). These results indicate that the genetic deficiency of *SERKs* affects the maintenance of stem cell niche.

In vivo interaction between RGLs and BAK1 is RGF1 dependent

Increasing evidence showed that BAK1 and its paralogs, *SERKs*, act as common coreceptors in many RLK-mediated signal transduction pathways (Li, 2010; Ma et al., 2016;

Gou and Li, 2020). RGLs were also previously identified through a yeast two-hybrid analysis utilizing BAK1 as bait (Ou et al., 2016). Therefore, we first confirmed the interaction between BAK1 and RGLs through a mating based Split Ubiquitin System (mbSUS). Our results showed that BAK1 interacts with different RGLs to various degrees in mbSUS (Figure 3A). The interactions between BAK1 and five different RGLs were verified through bimolecular fluorescence complementary (BiFC) technology in *B. Nicotiana* (Figure 3B). We also carried out Co-IP experiments to further confirm the interactions between BAK1 and all five RGLs. Native BAK1 can only associate with RGLs in the transgenic plants carrying 35S::RGL1-FLAG, pUBQ10::RGL2-FLAG, pUBQ10::RGL3-FLAG, 35S::RGL4-2HA, or 35S::RGL5-2HA upon the treatment of RGF1, but not in the same transgenic plants untreated with RGF1 or in wild-type (Figure 3, C–G). These results indicate that RGLs can

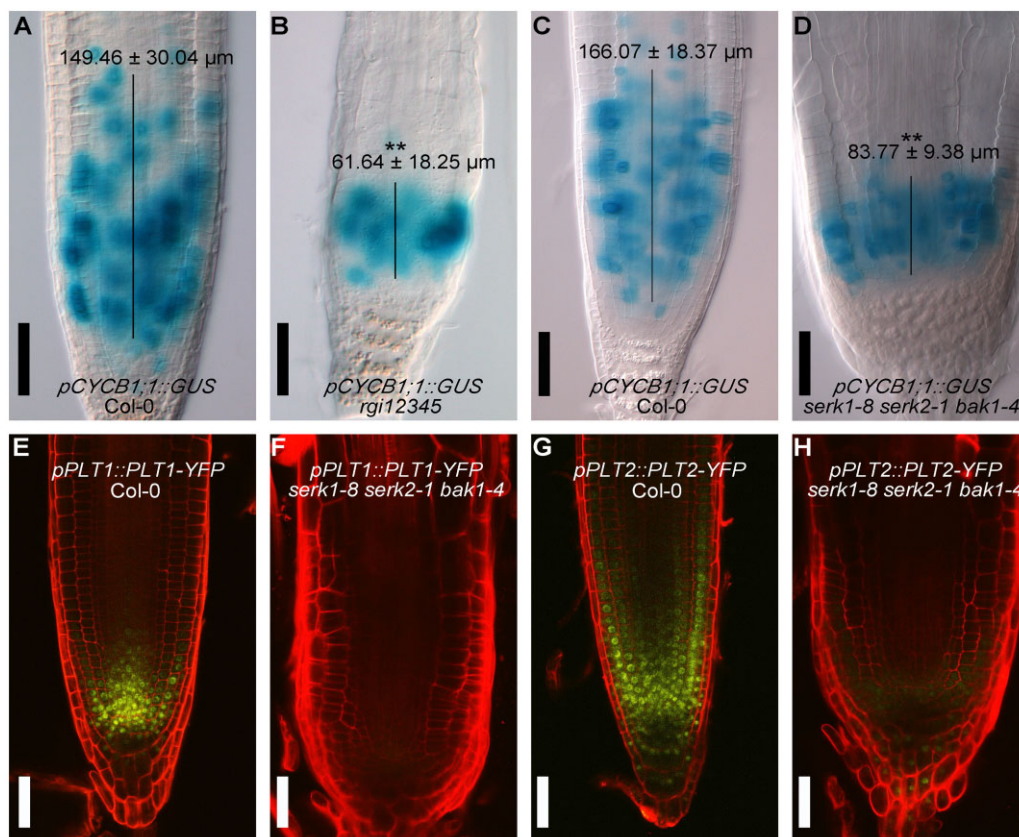


Figure 2 The expression patterns of *CYCB1;1*, *PLT1*, and *PLT2* in wild type, *rgi12345*, and *serk1-8 serk2-1 bak1-4*. A–D, The expression patterns of *pCYCB1;1::GUS* in 5-d-old Col-0 (A, C), *rgi12345* (B) and *serk1-8 serk2-1 bak1-4* (D). The scale bars represent 50 μm . Thin vertical black lines represent the length of the expression areas of *pCYCB1;1::GUS*. Each data represents the average and SD ($n \geq 10$). *t* test was carried out to show the significance between Col-0 and *rgi12345* or between Col-0 and *serk1-8 serk2-1 bak1-4*, respectively. “**” indicates $P < 0.01$. E–H, The expression patterns of *pPLT1::PLT1-YFP* or *pPLT2::PLT2-YFP* in 8-d-old Col-0 (E, G) and *serk1-8 serk2-1 bak1-4* (F, H). The scale bars represent 50 μm .

interact with BAK1 in vivo, and that the interaction between RGIs and BAK1 is RGF1 dependent.

The phosphorylation levels of RGIs and BAK1 are induced by RGF1, and the induced phosphorylation of RGIs and BAK1 is mutually dependent

A common activation mode for RLKs is mutual phosphorylation after hetero-dimerization induced by ligand (Wang et al., 2005, 2008). Therefore, we analyzed the phosphorylation levels of RGIs and SERKs with or without the treatment of RGF1. Our results showed that the phosphorylation levels of RGI1, RGI2, RGI3, and BAK1 are substantially increased upon the treatment of RGF1 (Figure 4, A–D). However, the phosphorylation levels of RGI4, RGI5, SERK1, and SERK2 after the treatment of RGF1 showed no detectable increase (Supplemental Figure S3, A–D).

In order to further investigate the activation mechanism of RGIs and BAK1, we generated transgenic plants overproducing a fusion protein RGI1-FLAG in wild-type or in *serk1-8 bak1-4*. After the treatment of RGF1, the phosphorylation level of RGI1-FLAG was substantially increased in wild-type but not in *serk1-8 bak1-4* background (Figure 5A). This result indicates that the phosphorylation of RGI1 induced by RGF1 is dependent on SERKs. Furthermore, we constructed

transgenic plants overproducing fusion protein BAK1-FLAG in wild type and in *rgi12345*. After the treatment of RGF1, the phosphorylation level of BAK1-FLAG was substantially induced in wild-type, but not in *rgi12345* (Figure 5B). This result suggests that the enhanced phosphorylation level of BAK1 by RGF1 relies on RGIs.

RGIs and BAK1 can phosphorylate each other

Our results indicated that RGF1 can induce the interaction between BAK1 and RGIs, and can promote the phosphorylation levels of BAK1 and RGIs. To examine whether RGI1 and BAK1 can transphosphorylate each other, we expressed fusion proteins GST-RGI1-CD (cytoplasmic domain), GST-RGI1-CD^m (K814E, a kinase dead mutant), His-BAK1-CD and His-BAK1-CD^m (D481N, a kinase dead mutant) in *Escherichia coli*, and performed the phosphorylation assays in vitro. We confirmed that GST-RGI1-CD and His-BAK1-CD are active kinases and can autophosphorylate themselves. At the meantime, GST-RGI1-CD^m and His-BAK1-CD^m are truly kinase inactive proteins. Our results showed that GST-RGI1-CD can substantially transphosphorylate His-BAK1-CD^m, while GST-RGI1-CD^m cannot transphosphorylate His-BAK1-CD^m (Figure 6A). In addition, His-BAK1-CD can substantially transphosphorylate GST-RGI1-CD^m, while the

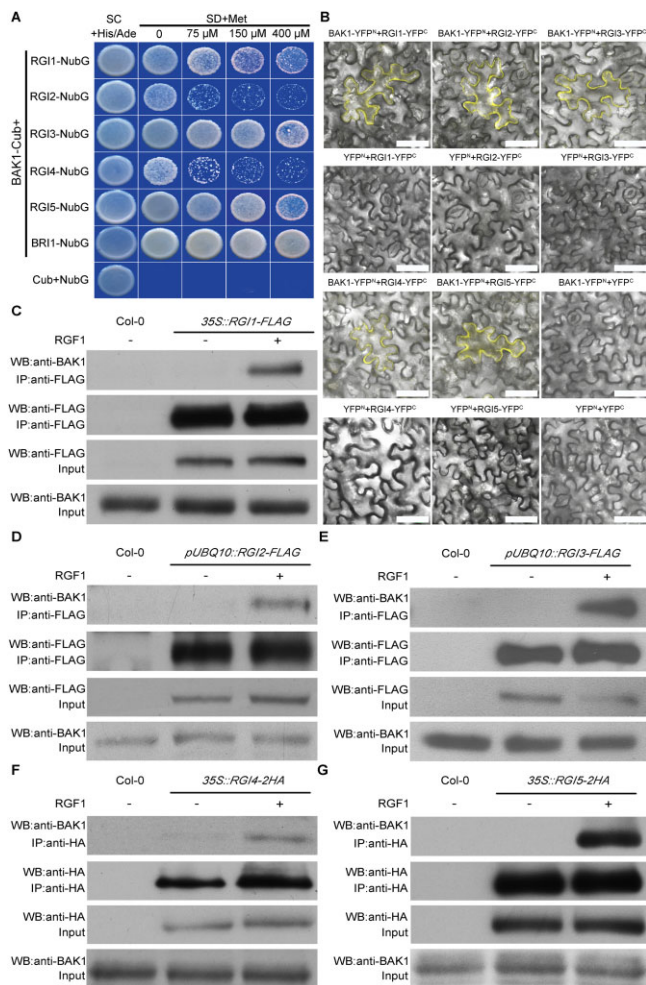


Figure 3 BAK1 and RGIs can physically interact. A, BAK1 interacts with RGIs in yeast using mbSUS. BRI1 was used as a positive control. B, BiFC analysis indicates that BAK1-YFP^N can interact with all five RGIs, including RGI1-YFP^C, RGI2-YFP^C, RGI3-YFP^C, RGI4-YFP^C, and RGI5-YFP^C in *N. benthamiana* leaves. The scale bars represent 50 μ m. C–G, Co-IP analysis also show that BAK1 can interact with RGI1 (C), RGI2 (D), RGI3 (E), RGI4 (F), and RGI5 (G) in planta upon the induction of 20 μ M RGF1. Total proteins were extracted from 10-d-old Col-0, 35S::RGI1-FLAG, *pUBQ10::RGI2-FLAG*, *pUBQ10::RGI3-FLAG*, 35S::RGI4-2HA, or 35S::RGI5-2HA transgenic seedlings after treated with or without 20 μ M RGF1 for 10 min for Co-IP. An anti-FLAG antibody or an anti-HA antibody was used for Co-IP. An anti-BAK1, or an anti-FLAG, or an anti-HA antibody was used for immunoblotting analysis. WB: Western Blot.

His-BAK1-CD^m cannot transphosphorylate GST-RGI1-CD^m (Figure 6B). These results indicate that the CDs of RGI1 and BAK1 can transphosphorylate each other directly in vitro.

The RGF1-induced phosphorylation of MPK3 and MPK6 depends on both RGIs and SERKs

It was reported that RGF1 can be sensed by RGI1 to regulate the size of root apical meristem via a YODA-MKK4/5-MPK3/6 signaling cascade (Lu et al., 2020; Shao et al., 2020). To investigate whether the activation of MPK3 and MPK6 induced by RGF1 depends on SERKs, the seedlings of wild

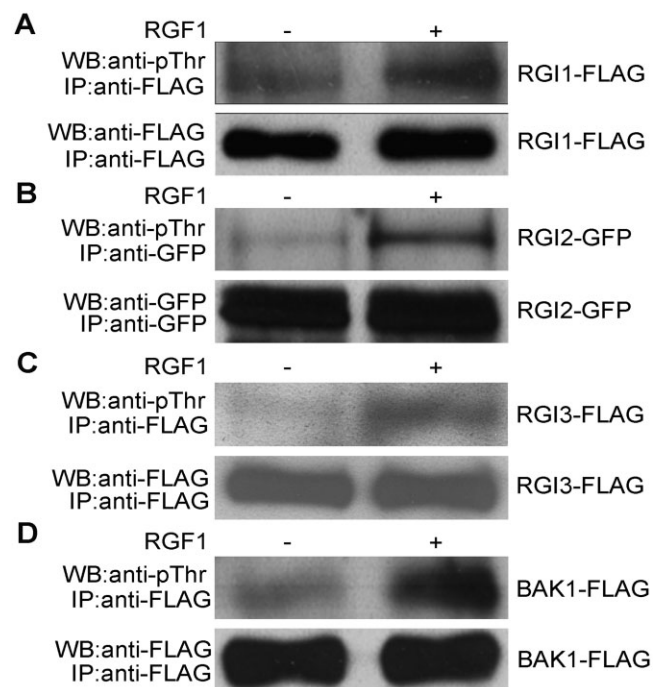


Figure 4 Exogenous treatment of RGF1 can induce the phosphorylation levels of RGI1, RGI2, RGI3, and BAK1. A and D, Membrane proteins were extracted from 10-d-old 35S::RGI1-FLAG (A) or 35S::BAK1-FLAG (D) transgenic seedlings treated or untreated with 20 μ M RGF1 for 5 min (A) or 10 min (D) for IP using an anti-FLAG antibody. B and C, Total proteins were extracted from 10-d-old 35S::RGI2-GFP (B) or *pUBQ10::RGI3-FLAG* (C) transgenic seedlings treated or untreated with 20 μ M RGF1 for 10 min were extracted for IP using an anti-GFP or an anti-FLAG antibody. The phosphorylation levels of RGI1-FLAG, RGI2-GFP, RGI3-FLAG and BAK1-FLAG were detected with an anti-pThr antibody. GFP: Green fluorescent protein.

type, *rgi12345*, *serk1-8* *serk2-1* *bak1-4*, and *bri1* *brl1* *brl3* were treated with RGF1, respectively, and the phosphorylation levels of MPK3 and MPK6 from various genotypes were analyzed. Our results showed that RGF1 can significantly enhance the phosphorylation levels of MPK3 and MPK6 in wild type and *bri1* *brl1* *brl3*, but not in *rgi12345* and *serk1-8* *serk2-1* *bak1-4* (Figure 7). Under the treatment of mock, the phosphorylation levels of MPK3 and MPK6 in *bri1* *brl1* *brl3* and *serk1-8* *serk2-1* *bak1-4* are higher than that in wild type (Figure 7). Interestingly, the induced phosphorylation of MPK3 and MPK6 in *bri1* *brl1* *brl3* is stronger than that in wild type (Figure 7). These results indicate that the activation of YODA-MKK4/5-MPK3/6 cascade induced by RGF1 depends on RGIs and SERKs, but not BRI1 and its paralogs.

Different RGI members contribute differently to the root development in Arabidopsis

In order to investigate the significance of each member of RGIs in regulating root development, we generated five different quadruple mutants including *rgi2345*, *rgi1345*, *rgi1245*, *rgi1235*, and *rgi1234*. These mutants exhibit SHR phenotypes in different degrees. The roots of *rgi2345*, *rgi1345*, *rgi1234*, and *rgi1235* are about 3/4, 2/3, 2/3, and 1/3 the length of

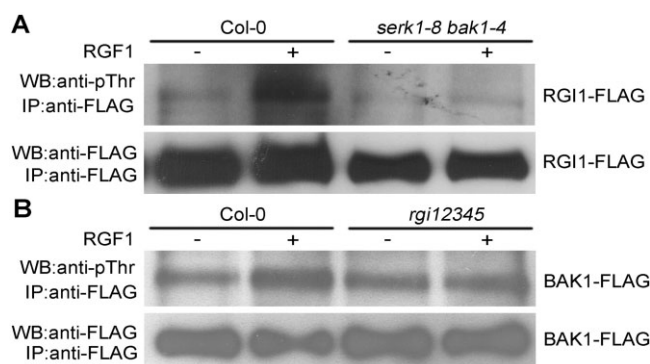


Figure 5 The RGF1-induced phosphorylation levels of RGI1 and BAK1 mutually depend on the presence of each other. **A**, The RGF1-induced phosphorylation of RGI1 depends on BAK1 and its paralog SERK1. Total proteins were extracted from 10-d-old *pUBQ10::RGI1-FLAG* transgenic seedlings in the background of Col-0 or *serk1-8 bak1-4* after treated with or without 20 μ M RGF1 for 10 min for IP using an anti-FLAG antibody. Phosphorylation levels of RGI1-FLAG in different backgrounds were analyzed with an anti-pThr antibody. **B**, Total proteins were extracted from 10-d-old *35S::BAK1-FLAG* transgenic seedlings in the background of Col-0 or *rgi12345* after treated or untreated with 20 μ M RGF1 for 10 min for IP using an anti-FLAG antibody. Phosphorylation levels of BAK1-FLAG in different backgrounds were analyzed with an anti-pThr antibody.

wild type, respectively. Interestingly, *rgi1245* showed SHR similar to *rgi12345*, possibly due to the undetectable expression level of *RGI3* at the root tip (Figure 8, A and B; Ou et al., 2016; Wu et al., 2016). We next counted the number of cortical cells in the meristematic zones of these mutants. On average, there were approximately 27.5 cells in wild-type, 23.1 cells in *rgi2345*, 20 cells in *rgi1345*, 5.2 cells in *rgi1245*, 9.5 cells in *rgi1235*, 14.8 cells in *rgi1234*, and 4.8 cells in *rgi12345* (Figure 8, C and D). These genetic data indicate that RGI1, RGI2 and RGI5 play more important roles in root meristem development in Arabidopsis. Furthermore, we found that the phosphorylation enhancement of MPK3 and MPK6 by RGF1 is substantial in wild type, but is essentially impaired in these mutants (Supplemental Figure S4). These results indicate that every member of RGIs plays a functionally redundant role in the RGF1-RGIs-SERKs-PLT1/2 signaling pathway.

Discussion

Our genetic and biochemical analyses demonstrate that BAK1 and its paralogs, SERKs, act as essential coreceptors of RGIs and play key roles in the RGF1-mediated signaling pathway. Without SERKs, the RGIs-RGF1-SERKs complex cannot be formed and signaling pathway cannot be initiated. Therefore, the YODA-MKK4/5-MPK3/6 downstream signal cascade cannot be activated. As a result, *PLT1* and *PLT2* expression remains low, and the cell division activity in the root apical meristem is greatly reduced (Figure 9A). In the presence of SERKs, on the other hand, RGF1 binds to RGIs. They then recruit SERKs to form a RGIs-RGF1-SERKs complex. The CDs of RGIs and SERKs can transphosphorylate each other,

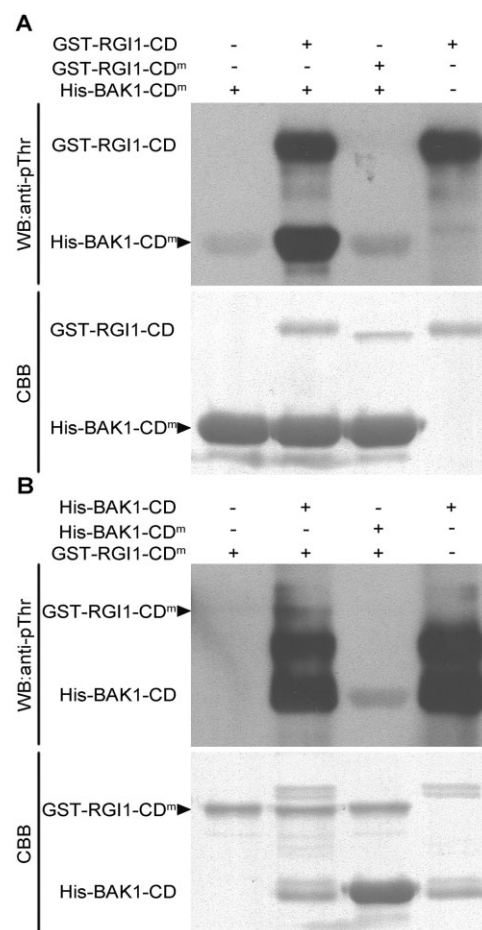


Figure 6 The CDs of RGI1 and BAK1 can transphosphorylate each other. **A**, The RGI1-CD is able to phosphorylate the kinase inactive BAK1-CD^m (D481N) whereas the kinase inactive RGI1-CD^m (K814E) cannot phosphorylate BAK1-CD^m. **B**, BAK1-CD is able to phosphorylate RGI1-CD^m, whereas the kinase BAK1-CD^m cannot phosphorylate RGI1-CD^m. CBB, Coomassie brilliant blue staining. GST, glutathione S-transferase.

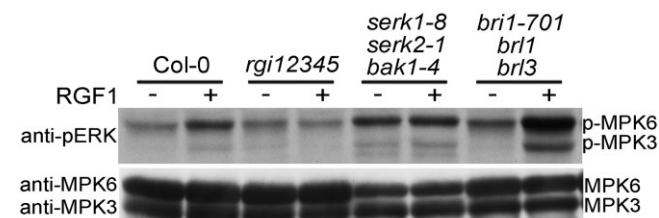


Figure 7 The RGF1-induced phosphorylation levels of MPK3 and MPK6 rely on both RGIs and SERKs. After pre-treated with 0.5 MS overnight, the seedlings of 9-d-old Col-0, *rgi12345*, *serk1-8 serk2-1 bak1-4*, and *bri1 bri1 bri3* were treated with 20 μ M RGF1 for 15 min. The phosphorylation levels of MPK3 and MPK6 were detected with an anti-pERK antibody.

triggering a series of downstream phosphorelay via the YODA-MKK4/5-MPK3/6 cascade. The expression levels of *PLT1* and *PLT2* are up-regulated and *PLT2* stability is increased to promote the cell division in the root apical meristem (Figure 9B).

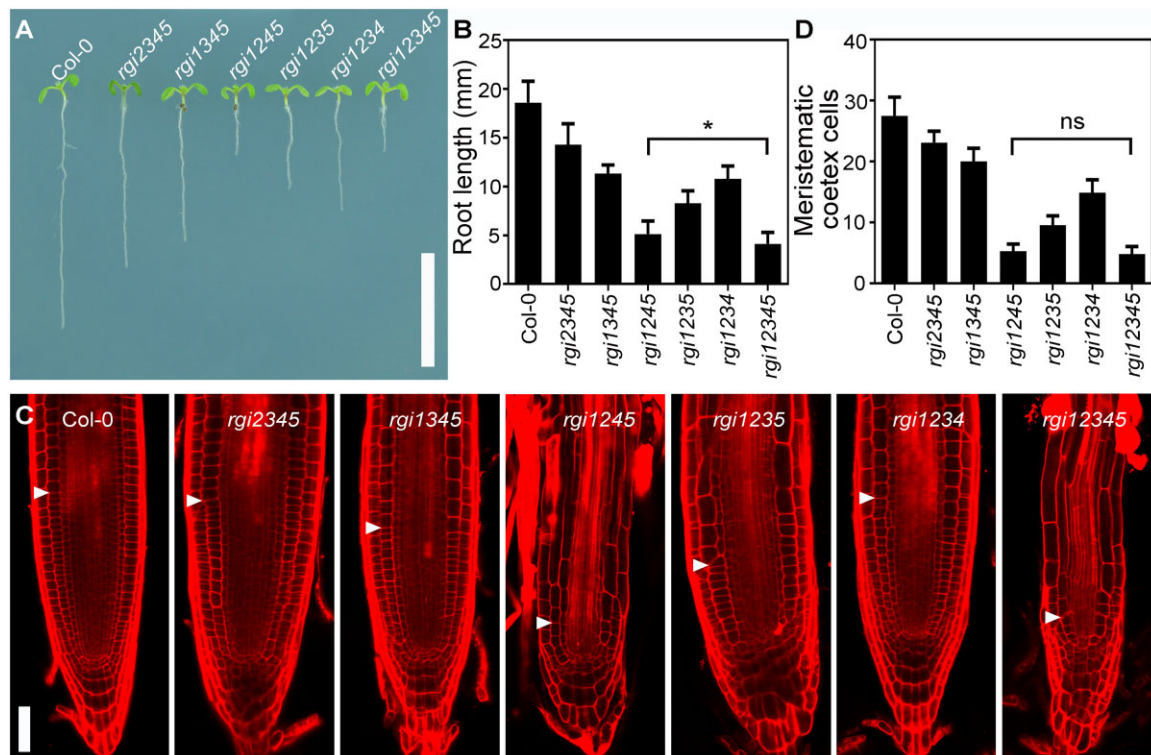


Figure 8 The root and root tip phenotypes of Col-0, different combinations of quadruple mutants, and the quintuple mutant of RGIs. A, Representative 6-d-old seedlings of Col-0, *rgi2345*, *rgi1345*, *rgi1245*, *rgi1235*, *rgi1234* and *rgi12345* grown on 0.5 MS medium. The scale bar represents 10 mm. B, The root length for the seedlings as shown in A. The data represent average and SD. $n > 20$. t test was carried out for the significance between *rgi1245* and *rgi12345*. “*” indicates $P < 0.05$. C, Confocal images of representative PI-stained 6-d-old root tips of Col-0, *rgi2345*, *rgi1345*, *rgi1245*, *rgi1235*, *rgi1234*, and *rgi12345*. The scale bar represents 50 μ m. The white arrows indicate the boundary between meristematic and transition zones. D, Measurements of the meristematic cortex cell numbers for the seedlings as shown in A. The data represent average and SD with $n > 15$. t test was carried out for the significance between *rgi1245* and *rgi12345*. “ns” means no significance.

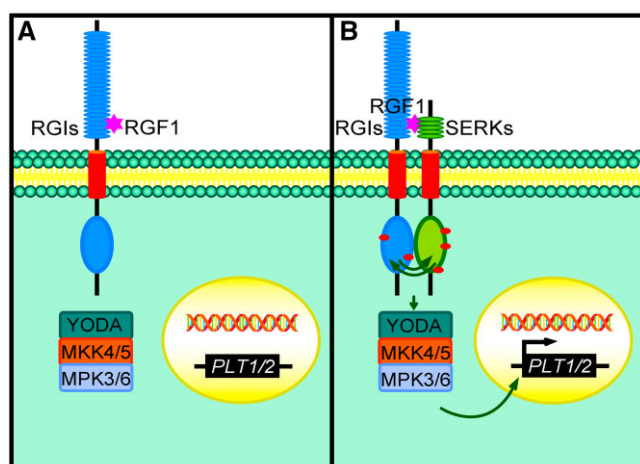


Figure 9 A model showing SERKs are essential coreceptors in the RGF1-RGI1-mediated signaling pathway to control root development. A, Without SERKs, RGF1-RGI1-mediated signaling pathway cannot be initiated. As a result, the transcriptions of *PLT1* and *PLT2* remain at a low level and the cell division activity in root tip is reduced. B, In the presence of SERKs, RGI1-BAK1 complex can be formed upon the induction of RGF1, the downstream signaling cascade can be activated. The expression levels of *PLT1* and *PLT2* are up-regulated and the protein stability of *PLT2* is enhanced which promotes the cell division in root tips.

The root of *serk1-8 serk2-1 bak1-4* appears to be shorter than that of *rgi12345*, but the number of meristematic cortex cells is slightly higher. There are a total of five members in the SERK subfamily and they are usually functionally redundant. Only when additional SERK members are knocked out, can the meristematic cortex cell numbers be further reduced. It is, however, impossible to generate a quadruple or a quintuple mutant for SERKs because they are embryo lethal (Li et al., 2019). In addition, BAK1 and SERKs are involved in a variety of developmental and immunity responses. The shorter root phenotype is likely the result of disrupting RGF1 and other signaling pathways. It is worthy to note that the root of *serk1-8 serk2-1 bak1-4* is much thicker than *rgi12345*. Previous studies indicated that SERKs also regulate the cell division orientation during zygotic embryogenesis (Li et al., 2019). But the ligand and receptor mediating such a process is not known.

The prolonged treatment of RGF1 can greatly alter root growth orientation in wild type, but only subtly in *rgi1234* and *serk1-8 bak1-4* mutants (Ou et al., 2016). Previous studies indicated that the treatment of BL can induce root “waving growth” (González-García et al., 2011). It was found that the inhibition of pectin methylesterase activity can trigger a similar phenotype, and BR signal is involved in the

dynamic maintenance of cell wall (Sebastian et al., 2012). Whether RGF1 signal is also related to plant cell wall remodeling needs to be investigated in the near future.

The phosphorylation levels of RGI1, RGI2, RGI3, and BAK1 are enhanced substantially upon the treatment of RGF1. However, the phosphorylation levels of RGI4, RGI5, SERK1, and SERK2 are not greatly increased under the similar research conditions. Genetic analysis indicated that they are also involved in regulating the root apical meristem development. It is possible that RGI4 and RGI5 are optimal receptors of other RGFs, such as RGF2 and RGF3, both of which were detected in root apical meristems (Matsuzaki et al., 2010). Likewise, SERK1 and SERK2 may also have their preference for RGF peptides.

Interestingly, the phosphorylation levels of MPK3 and MPK6 in *bri1 brl1 brl3* and *serk1-8 serk2-1 bak1-4* are much higher than that in wild type. RGF1 can enhance the phosphorylation levels of MPK3 and MPK6 in *bri1 brl1 brl3* but not in *serk1-8 serk2-1 bak1-4* (Figure 7). These results confirmed that MPK3 and MPK6 are the downstream components of the RGF1-mediated signaling pathway, in which SERKs, but not the BR signaling, play an essential role. Future investigation is needed to explain why the phosphorylation levels of MPK3 and MPK6 is substantially enhanced in *bri1 brl1 brl3* triple mutant.

Genetic evidence showed that RGI1, RGI2 and RGI5 play main roles in the regulation of cell division in root apical meristem, and RGI4 contributes less in this process. RGI3 has subtle function for root apical meristem development (Figure 8, A–D). These observations are consistent with the expression patterns of RGIs in root apical meristem. The expression levels of RGI1, RGI2 and RGI5 are much stronger than that of RGI4. The expression of RGI3 in root tips is undetectable in *pRGI3::GUS* transgenic plants (Ou et al., 2016; Wu et al., 2016).

Recently, it was reported that RGF1 can induce the expression of a transcription factor *RGF1-INDUCIBLE TRANSCRIPTION FACTOR 1 (RITF1)*. RITF1 mediates the distribution of ROS in root tips to maintain the protein stability of PLT2 (Yamada et al., 2020). The relationship between RITF1 and the YODA-MKK4/5-MPK3/6 signaling cascade is not understood. It is not clear whether the expression of RITF1 is regulated by the MAPK signaling cascade. There are also many additional questions that need to be investigated in the future. For example, we know that RGI1-BAK1 complex cannot directly phosphorylate YODA (Lu et al., 2020); what, then, is the regulatory component between RGI1-BAK1 and YODA in this signal transduction pathway? In addition, we still do not know how *PLT1* and *PLT2* are activated at a transcription level. We propose there is an unknown transcription factor which should be the substrate of MPK3 and MPK6 and is responsible for the RGF1-induced

expression of *PLT1* and *PLT2*. There might be some negative regulators that also need to be identified.

Materials and methods

Plant materials and growth conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col-0 was used as the wild-type control. The T-DNA insertion mutants of RGIs including *rgi1-1*, *rgi2-1*, *rgi3-1*, *rgi4-1*, *rgi5-1*, *rgi1234*, and *rgi12345* were described previously (Ou et al., 2016). Various quadruple mutants of RGIs were generated by genetic crossing, and the genotypes were confirmed by PCR analyses. The T-DNA insertion mutants of SERKs including *serk1-8*, *serk2-1*, *bak1-4*, *serk1-8 bak1-4*, *serk1-8 serk2-1 bak1-4*, *serk1-1 serk2-2 bak1-6*, and *bri1 brl1 brl3* were reported previously (Du et al., 2012; Gou et al., 2012). The reporter genes of *pCYCB1;1::GUS*, *pPLT1::PLT1-YFP*, and *pPLT2::PLT2-YFP* were introduced into various mutants by genetic crossing, and the genotypes were confirmed by PCR analyses. The transgenic plants used for comparing the expression levels in different genotypes were confirmed as homozygous lines. The reporter genes of *pSHR::NLS-YFP*, *pSCR::NLS-YFP* and *pWOX5::NLS-YFP* in Col-0 and *serk1-8 serk2-1 bak1-4* were described previously (Li et al., 2019).

The coding sequences of RGI1, RGI2, RGI3, RGI4, RGI5, SERK1, SERK2, and BAK1 were amplified and constructed into an entry vector as reported previously (Gou et al., 2010; Ou et al., 2016). Home modified vector *pBIB-BASTA-35S-FLAG-GWR* was used to construct expression vectors for RGI1, SERK1 and BAK1. *pBIB-BASTA-35S-GFP-GWR* was used to construct expression vectors for RGI2, RGI4, and SERK2. The coding sequences of RGI1, RGI2, and RGI3 were constructed in *pBIB-BASTA-pUBQ10-GFP-GWR*. Vectors of *pBIB-BASTA-35S-FLAG-GWR*, *pBIB-BASTA-35S-GFP-GWR*, and *pBIB-BASTA-pUBQ10-GFP-GWR* were used and described previously (Gou et al., 2010; Hu et al., 2018). Seeds for 35S-RGI4-2HA and 35S-RGI5-2HA were described previously (Wang et al., 2021). These expression vectors were transformed into *Agrobacterium* GV3101, and then transformed into *Arabidopsis* Col-0 through floral dipping.

The seeds were sterilized in 75% (v/v) ethanol for 1 min and 1% (w/v) NaClO for 10 min, washed with sterilized water for five times. The sterilized seeds were planted on 0.5 MS medium containing 1% (w/v) sucrose and 0.8% (w/v) agar. After vernalization for 2 d in 4°C, the seeds were cultured at 22°C with a photoperiod of 16 h light and 8 h dark for indicated days. RGF1 treatment was performed in liquid 0.5 MS medium containing 1% (w/v) sucrose.

Staining and microscopy observation

For propidium iodide (PI) staining, the seedlings were stained with PI (0.02 mg/mL) for 1–2 min and then photographed under a confocal microscope (Olympus-FV1000 MPE, or Leica-TCS SP8). For GUS staining, the seedlings of

Col-0, *rgi12345* and *serk1-8 serk2 bak1-4* harboring *pCYCB1;1-GUS* were stained with a GUS solution buffer for 3 hours as described previously (Wu et al., 2016).

Yeast two-hybrid assays

The coding sequences were amplified from the entry vectors of *RGI1*, *RGI2*, *RGI3*, *RGI4*, *RGI5*, and *BAK1* for yeast two-hybrid analysis. The coding sequence of *BAK1* and the linearized pMetYCgate were transformed and recombined into a THY.AP4 yeast strain. The coding sequence of *RGIs* and the linearized pX-NubWTgate were transformed and recombined into a THY.AP5 yeast strain. The mating strains were screened on synthetic complete medium (SC) with histidine and adenine. The mating strains were inoculated on synthetic minimal medium (SD) containing different concentrations of methionine for verifying the interaction. The mbSUS yeast two-hybrid method was described previously (Obdlik et al., 2004).

Bimolecular fluorescence complementation

The coding sequences of *BAK1* and *RGIs* were cloned into *pEarleygate201-YN* and *pEarleygate202-YC*, respectively, and transformed into *Agrobacterium* GV3101. The agrobacteria were cultured overnight in LB liquid medium containing 20 μ M acetosyringone. After collected through centrifugation, the agrobacteria were resuspended in MS medium containing 1% (w/v) sucrose, 150 μ M acetosyringone, 10 mM $MgCl_2$, 10 mM MES. The OD₆₀₀ was adjusted to 0.2. After sitting at room temperature for 2–3 h, different agrobacteria were mixed and injected into the 4-week-old *N. benthamiana* leaves. After 24–36 h, a confocal laser microscopy (Nikon, A1R + Ti2-E) was used for analysis.

Membrane and total protein extraction

About 5–10 g seedlings were ground into fine powder in liquid N₂. After N₂ was evaporated, Membrane Protein Extraction Buffer (MPEB20 mM Tris–Cl, 150 mM NaCl, 1 mM EDTA, 20% glycerol, pH 8.8) containing a protease inhibitor (Roche, 04693132001) and a phosphatase inhibitor (Rohce, PhosStop, 4906845001) was added and mixed. The mixture was centrifuged at 6,000 g in 4°C for 20 min to remove debris. The supernatant was further centrifuged at 100,000 g in 4°C for 60 min. After supernatant was removed, the Membrane Protein Soluble Buffer (MPSB; 20 mM Tris–Cl, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% TritonX-100, pH 7.3) containing the protease inhibitor and phosphatase inhibitor was added. The precipitate was resuspended in a 1.5-mL tube. After centrifugation at 15,000 g in 4°C for 10 min, the supernatant contains the extracted membrane proteins.

For total protein extraction, ~3–5 g plants were ground into fine powder in liquid N₂. Total Protein Extraction Buffer (TPEB; 10 mM HEPES, 100 mM NaCl, 1 mM EDTA-2Na, 10% glycerol, 0.5% Triton X-100, pH 7.5) containing the protease inhibitor and the phosphatase inhibitor was added. The extraction was transferred into a centrifugation tube and centrifuged at 16,000 g in 4°C for 10 min. The supernatant was

then transferred into a new centrifugation tube and centrifuged again at 16,000 g in 4°C for 10 min. The supernatant was the extracted total protein.

IP, protein phosphorylation analysis, and co-IP

After the membrane protein or total protein was extracted, beads were washed three times with MPSB or TPEB and then added into the protein extracts. After incubation at 4°C for 2–3 h, the beads were collected through centrifugation at 1,200 g in 4°C for 1 min. After washed four times with MPSB or TPEB, the supernatant was removed, 2 $\times$ SDS loading buffer was added. The sample was boiled for 5 min. The resulted sample was analyzed by immunoblotting after SDS-PAGE. The beads used in immuno-precipitation included α -FLAG beads (Sigma, St. Louis, MO, USA, A2220), α -GFP beads (KT HEALTH, KTSM1301), and α -HA beads (KT HEALTH, KTSM1305). The antibodies used in immunoblotting analyses included α -GFP (Roche, Basel, Switzerland, 11814460001), α -FLAG (Abmart, M20008L), α -pThr (Cell Signaling, 9381S), and α -BAK1 (Agrisera, AS121858).

Protein purification from E. coli and in vitro kinase activity analysis

The CD coding sequences of *RGI1* and *BAK1* were PCR amplified and constructed into the entry vector *pDONR/Zeo*. Primers used for cloning the CD coding fragments of *RGI1* and *BAK1* are listed in Supplemental Table S1. Mutation constructs *RGI1* CD^m Entry (K814E) and *BAK1* CD^m Entry (D481N) were generated via directed mutagenesis. These DNA fragments were reconstructed into *pET-28* (Novagen) and *pDEST15* (Invitrogen, Carlsbad, CA, USA), respectively. These destination vectors were then transferred into *Rosetta* for protein expression. The protein was induced by 0.3 mM IPTG and incubated at 20°C for 20 h. The bacteria were collected by centrifugation and washed once with TBS (25 mM Tris, 137 mM NaCl).

For the purification of His-BAK1-CD and His-BAK1-CD^m, the bacteria were broken in the His tagged lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 15 mM imidazole pH 8.0, 1 mM PMSF) by ultrasonication. After centrifugation at 18,000 g in 4°C for 15 min, the prewashed Ni-IDA Sefinose TMR resin (Sangon, C600029) was added in the supernatant. After incubation at 4°C for 4 h, the beads were collected through centrifugation at 2,000 g for 1 min. The beads were washed five times using the His tagged wash buffer (50 mM Tris–HCl, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF, pH 8.0). The protein was eluted in the His tagged elution buffer (50 mM Tris–HCl, 150 mM NaCl, 200 mM imidazole, 10% glycerol, pH 8.0).

For the purification of GST-*RGI1* CD and GST-*RGI1* CD^m, the bacteria were broken in the GST tagged lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, pH 8.0) by ultrasonication. After centrifugation at 18,000 g in 4°C for 15 min, the prewashed glutathione agarose beads (Sangon Biotech, Shanghai, China, C600031) were added in the supernatant. After incubation

at 4°C for 4 h, the beads were collected through centrifugation at 2,000g for 1 min. The beads were washed five times with the GST tagged wash buffer (25 mM Tris–HCl, 500 mM NaCl, 0.1% Triton X-100, pH 8.0). The protein was eluted in the GST tagged elution buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 20 mM GSH, pH 8.0).

The purified protein was added in the kinase reaction buffer (25 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 300 μM ATP). After incubation at 25°C for 1 h, 2 × SDS loading buffer was added. The samples were boiled for 5 min, and then analyzed by an immunoblotting analysis and Coomassie Blue staining after SDS-PAGE.

MAPK activation analysis

Seedlings were pre-treated in 0.5 MS medium overnight. Then RGF1 was added into the medium to make the final concentration of 20 μM. After 15 min, the seedlings were ground and solubilized in 2 × SDS loading buffer. After boiled for 5 min, the samples were analyzed with an SDS-PAGE. Coomassie Blue staining and immunoblotting analyses were carried out. The antibodies used in MAPK activation analysis included α-pERK (Cell Signaling Technology, 4370), α-MPK6 (Sigma-Aldrich, A7104) and α-MPK3 (Sigma-Aldrich, M8318).

Accession numbers

Sequence data from this article can be found in the Arabidopsis Information Resource (<http://www.arabidopsis.org/>) under the following accession numbers: *SERK1* (AT1G71830), *SERK2* (AT1G34210), *BAK1/SERK3* (At4g33430), *RGI1* (AT3G24240), *RGI2* (AT5G48940), *RGI3* (AT4G26540), *RGI4* (AT5G56040), *RGI5* (AT1G34110), *BRI1* (At4g39400), *BRL1* (At1g55610), *BRL3* (At3g13380), *SHR* (AT4G37650), *SCR* (AT3G54220), *WOX5* (AT3G11260), *CYCB1;1* (AT4G37490), *MPK3* (AT3G45640), *MPK6* (AT2G43790), *PLT1* (AT3G20840), and *PLT2* (AT1G51190).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Two independent sets of *SERK* triple mutants show similar short root phenotypes.

Supplemental Figure S2. The expression patterns of *SHR*, *SCR*, and *WOX5* in transgenic plants carrying *pSHR::NLS-YFP*, *pSCR::NLS-YFP*, or *pWOX5::NLS-YFP* in the wild type or *serk1-8 serk2-1 bak1-4*.

Supplemental Figure S3. RGF1 cannot substantially induce the phosphorylation levels of *RGI4*, *RGI5*, *SERK1*, and *SERK2* in vivo.

Supplemental Figure S4. The phosphorylation levels of *MPK3* and *MPK6* induced by RGF1 partially depend on each member of *RGI*s.

Supplemental Table S1. Primers used in this work.

Acknowledgments

We are grateful to Chuanyou Li (Institute of genetics and developmental biology, Chinese Academy of Sciences) for sharing the seeds of *pCYCB1;1-GUS*, *pPLT1:PLT1-YFP*, and *pPLT2:PLT2-YFP*. We thank Xiangzong Meng (College of Life Sciences, Shanghai Normal University) for providing the seeds of 35:RGI4-2HA and 35:RGI5-2HA.

Funding

This study was supported by the National Natural Science Foundation of China (31800236 to Y.O., 31720103902 and 31530005 to J.L.).

Conflict of interest statement. None declared.

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