Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE Proteins Serve Brassinosteroid-Dependent and -Independent Signaling Pathways[1][C][W]

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The Arabidopsis (*Arabidopsis thaliana*) *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) genes belong to a small family of five plant receptor kinases that are involved in at least five different signaling pathways. One member of this family, BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE1 (BAK1), also known as SERK3, is the coreceptor of the brassinolide (BR)-perceiving receptor BRI1, a function that is BR dependent and partially redundant with SERK1. BAK1 (SERK3) alone controls plant innate immunity, is also the coreceptor of the flagellin receptor FLS2, and, together with SERK4, can mediate cell death control, all three in a BR-independent fashion. SERK1 and SERK2 are essential for male microsporogenesis, again independent from BR. SERK5 does not appear to have any function under the conditions tested. Here, we show that the different *SERK* members are only redundant in pairs, whereas higher order mutant combinations only show additive phenotypes. Surprisingly, *SERK* members that are redundant within one are not redundant in another pathway. We also show that this evolution of functional pairs occurred by a change in protein function and not by differences in spatial expression. We propose that, in plants, closely related receptor kinases have a minimal homo- or heterodimeric configuration to achieve specificity.

In Arabidopsis (*Arabidopsis thaliana*), there are over 600 genes coding for receptor-like kinases (RLKs; Arabidopsis Genome Initiative, 2000; Shiu and Bleecker, 2001). Functional information is restricted to a relatively small number of these RLKs.

The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family consists of five Leu-rich repeat (LRR)-RLKs belonging to subgroup II (Hecht et al., 2001) that contain five LRRs in their extracellular domain and display similarity to the previously described DcSERK protein that marks embryogenic competence in carrot (*Daucus carota*) tissue cultures (Schmidt et al., 1997). The main feature distinguishing SERK proteins from other RLKs is the Pro-rich domain containing the SPP motif located between the LRRs and the transmembrane domain. The presence of the SPP domain together with precisely five LRRs was used as a criterion for the identification of the four other *SERK* genes (*SERK2*–*SERK5*) among the numerous LRR-RLK encoding genes in the Arabidopsis database (Hecht et al., 2001). Sequence analysis of the different SERK proteins indicates that they arose through gene duplication events that generated two ancestral precursors, SERK1-SERK2 and SERK3-SERK4-SERK5. Those precursors further duplicated and mutated to generate the five current SERK members (Hecht et al., 2001; He et al., 2007). The *SERK3* gene was identified as the *BRASSINOSTEROID INSENSITIVE1* (*BRI1*)-*ASSOCIATED KINASE1* (*BAK1*) through interaction with BRI1 in a yeast two-hybrid screen (Nam and Li, 2002) and in a genetic screen for suppressors of a weak *bri1* phenotype (Li et al., 2002). It has also been shown that BRI1 forms heterodimers with SERK3/BAK1 in living cells (Russinova et al., 2004; Hink et al., 2008) and that the interaction is dependent on the presence of brassinosteroids (BRs; Wang et al., 2005). Two other members of the family, SERK1 (Karlova et al., 2006) and SERK4/BKK1 (He et al., 2007), have also been reported to be involved in BR signaling. Mutant analysis within the SERK family suggested that SERK signaling pathways exist that cannot be directly linked to BRI1-mediated signaling. SERK1 and SERK2 proteins are functionally redundant and essential for tapetum specification and pollen

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development during male sporogenesis in Arabidopsis (Albrecht et al., 2005; Colcombet et al., 2005). Null or strong *bri1* mutants, although male sterile, are not reported to be altered in male sporogenesis. Recent studies have revealed that, independent from its function in BR signaling, SERK3 alone also controls innate immunity (Kemmerling et al., 2007) and is involved in flagellin perception (Chinchilla et al., 2007; Heese et al., 2007). In combination with SERK4/BKK1, the same SERK3 RLK controls plant cell death (He et al., 2007; Kemmerling et al., 2007). In cases where the main ligand-binding receptors are known, such as BRI1 or FLS2, the *serk3* null mutant allele only displays a subtle phenotype as compared to null mutant alleles of the main receptor (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007). These studies suggested genetic redundancy with other members of the *SERK* family.

To determine the level of redundancy within the *SERK* family, here we report the different roles of the SERK proteins, alone or in combination with other members, using a genetic and molecular approach. The results show that only SERK1 functions together with SERK3 in BRI1-mediated BR signaling. No other SERK combinations serve the BR-dependent pathway in our assays. In contrast, only the *serk4* mutant allele enhances the susceptibility to bacterial pathogens of *serk3* plants. Furthermore, we also provide evidence that the specificity of the SERK-mediated pathways can be largely ascribed to their biochemical function rather than the corresponding gene expression pattern. Hence, the SERK proteins have evolved to serve as coreceptors in multiple signaling pathways through hetero-oligomerization with different receptors.

In animals, signaling pathways exist as networks of interacting receptors of the same or different type (Citri and Yarden, 2006). The existence of similar receptor networks is proposed for plant RLKs as well (Dievart et al., 2003; Godiard et al., 2003; Shpak et al., 2003). We propose that, like in animal systems, specialization and redundancy of the *SERK* gene family members is at the core of a signaling network that provides signaling diversity together with robustness.

RESULTS

SERK1 and SERK3 (BAK1) Mediate BR Responses

Except for *serk3* (*bak1*), none of the *serk* single knockout mutants shows a morphological phenotype. The mutant alleles used are presented in Supplemental Figure S1 for the *serk1* alleles and Supplemental Figure S2 for the *serk2*, *serk3*, *serk4*, and *serk5* alleles.

serk3 displays some of the characteristic *bri1* mutant phenotypes, such as semidwarfism, reduction in hypocotyl and root length, and reduced BR sensitivity (Li et al., 2002; Nam and Li, 2002). However, all these phenotypes are weaker than those reported for *bri1* mutants. This suggests that other members of the SERK family have an overlapping function with SERK3 in BR signaling. Therefore, double mutants between *serk3* and the other *serk* mutants were generated and scored for enhancement of the three *serk3-1* BR-related phenotypes, rosette growth, reduced hypocotyl length, and reduced sensitivity of the roots to brassinolide (BL) application. Only for the *serk3-1 serk4-1* double mutant, we recorded additional reduction of the rosette size and increased dwarfism as compared to *serk3-1* (Supplemental Fig. S3). In double-mutant combinations of *serk3-1* with the *serk1-1* or *serk1-3* alleles, significant modification of the hypocotyl length of the *serk3-1* mutant was observed (Fig. 1B; χ^2: $P \leq 1.27e^{-08}$ and χ^2: $P \leq 1.17e^{-15}$, respectively; Supplemental Table S2A). In none of the other double-mutant combinations, a similar effect on hypocotyl length was observed (Fig. 1B; Supplemental Table S2A). In the root growth inhibition assay, only the single mutant *serk3-1* shows reduced sensitivity to BL (Supplemental Table S1). In double-mutant combinations, only the *serk1* alleles *serk1-1* and *serk1-3* enhance the *serk3-1* BR insensitivity in roots (Fig. 1A).

To further confirm that only SERK1 and SERK3 are involved in BR signaling, the *CPD* molecular marker was used. It was previously shown that the expression of *CPD*, involved in BR biosynthesis, is downregulated by BL treatment (Tanaka et al., 2005). Hence, the expression level of *CPD* can be used as an indicator of BL perception. A significant decrease of expression of *CPD* can be observed in wild-type plants treated with 100 nM BL as well as in untreated *bes1-D* plants. *bes1-D* is a constitutive inducer of the BRI1-dependent pathway. As expected, a decrease in expression of *CPD* is not detected in *serk3* mutants after treatment with 10 or 100 nM of BL (Fig. 1E). In double mutants of *serk3-1* with either *serk1-1* allele or *serk1-3* allele, this effect is further enhanced, suggesting an increased insensitivity to BL. No further increase of *CPD* expression is detected in double mutants between *serk3-1* and other *serk* alleles after BL treatment (Fig. 1E).

Given the high sequence similarity between the different SERK members, we cannot discard the hypothesis that SERK2, SERK4, and SERK5 might be functionally redundant, thereby masking a synergistic effect with SERK3. Therefore, higher order mutants were generated. Triple and quadruple mutants were tested in the hypocotyl and the root growth inhibition assay as described above. Our data show that introduction of additional mutant alleles of *serk2*, *serk4*, and/or *serk5* in the *serk1-1 serk3-1* background does not confer higher BR insensitivity in the root (Fig. 1C) or in the hypocotyl (Fig. 1D; i.e. *serk1 serk3* double mutants show the highest BR insensitivity).

To summarize, of all the mutant combinations tested, only *serk1* and *serk3* show synergy in the decrease of sensitivity to endogenous or exogenously applied BRs. Introduction of additional *serk2*, *serk4*, and/or *serk5* mutant alleles in the *serk1-1 serk3-1* background does not further enhance the phenotype of the *serk1-1 serk3-1* double mutant. These data suggest that, from the five closely related SERK family members,

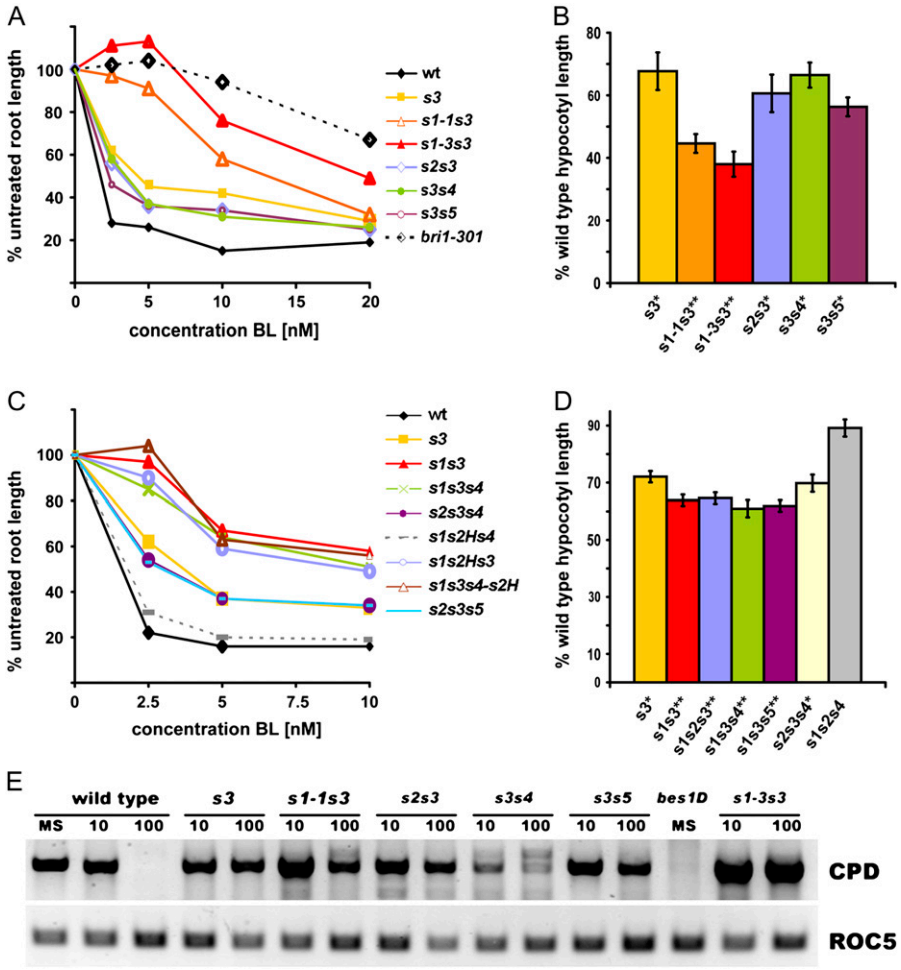

Figure 1. Phenotypic analyses of the multiple *serk* mutants. A and C, Root growth measurements of seedlings grown on medium containing different BL concentrations using various double-mutant combinations (A) or multiple mutant combinations (C). Each measurement is represented as a percentage of the root elongation of the control plants grown on medium containing the same volume of 80% (v/v) ethanol used to dilute BL. B and D, Quantitative analysis of the hypocotyl length of various double-mutant combinations (B) and multiple *serk* mutant combinations (D) grown in the dark for 5 d. Each measurement represents an average of hypocotyl lengths of 20 seedlings. Error bars indicate SD. E, RT-PCR analysis to evaluate the feedback regulation of the *CPD* gene in the different double-mutant backgrounds after treatment with different concentrations of BL. The constitutively expressed cyclophilin gene *ROC5* (Chou and Gasser, 1997) was used as control. *, Significant differences from Col-0 wild type ($P \leq 0.05$); **, significant differences from the *serk3-1* mutant ($P \leq 0.05$); *s1-1*, *serk1-1* allele; *s1-3*, *serk1-3*; *s2*, *serk2-2*; *s3*, *serk3-1*; *s4*, *serk4-1*; *s5*, *serk5-1*; H, heterozygote. [See online article for color version of this figure.]

only SERK1 and SERK3 have an overlapping function in BR signaling in roots and hypocotyls.

serk3 serk4 Dwarf Stature and *serk1 serk2* Male Sterility Are Not BR Related

BR biosynthetic or perception mutants share morphological and physiological changes that include reduced stature, rounded leaves, shortened hypocotyls and roots, and decreased sensitivity to BRs. The double mutant *serk3-1 serk4-1* displays severely altered architecture, loss of apical dominance, stunted and dwarfed stature, reduced organ size, and early flowering (Supplemental Fig. S4A). The phenotype of the double mutant could be rescued by either *SERK3* or *SERK4* under control of their own respective promoter (data not shown), thus confirming that the observed phenotype is caused by the T-DNA insertions in the *SERK3* and *SERK4* genes. Although showing a severe defect in growth and architecture, the observed phenotype does not resemble any of the known and described *bri1* mutant alleles (Supplemental Fig. S4B). Neither a reduction of the hypocotyl length nor an increase in root BR insensitivity was noted in the *serk3-1 serk4-1* double mutant as compared to *serk3-1* (Fig. 1, A and B). To further confirm that the observed phenotype does not relate to the BRI1-dependent pathway, we attempted to rescue the *serk3-1 serk4-1* by overexpressing the gain-of-function mutant gene *bes1-D*. *BES1* encodes a nuclear-localized protein and is essential for the transcription of BRI1 target genes (Yin et al., 2002). The *bes1-D* mutant allele was identified in a suppressor screen aimed at rescuing the weak mutant *bri1-119*. The mutant *bes1-D* gene fused to GFP and driven by the *35S* promoter was shown to rescue *bri1* mutant phenotypes (Yin et al., 2002). This construct was introduced in the *serk3 serk4* double mutant and the *serk1 serk2 serk3* triple mutant. The transgenic plants show curled leaves, a typical feature of *bes1-D*-overexpressing plants (Fig. 2A). Expression of the transgene was further confirmed by confocal microscopy and western-blot analysis (Fig. 2, C and D). The *serk3* BR-related phenotypes are successfully rescued in the transgenic lines generated in the *serk3 serk4* double mutant and the *serk1 serk2 serk3* triple mutant, as shown based on the root and hypocotyl assay (Fig. 2, E and F). However, in these same transgenic lines, showing rescue of the *serk3* mutant phenotype and the typical curly leaves of *bes1-D*

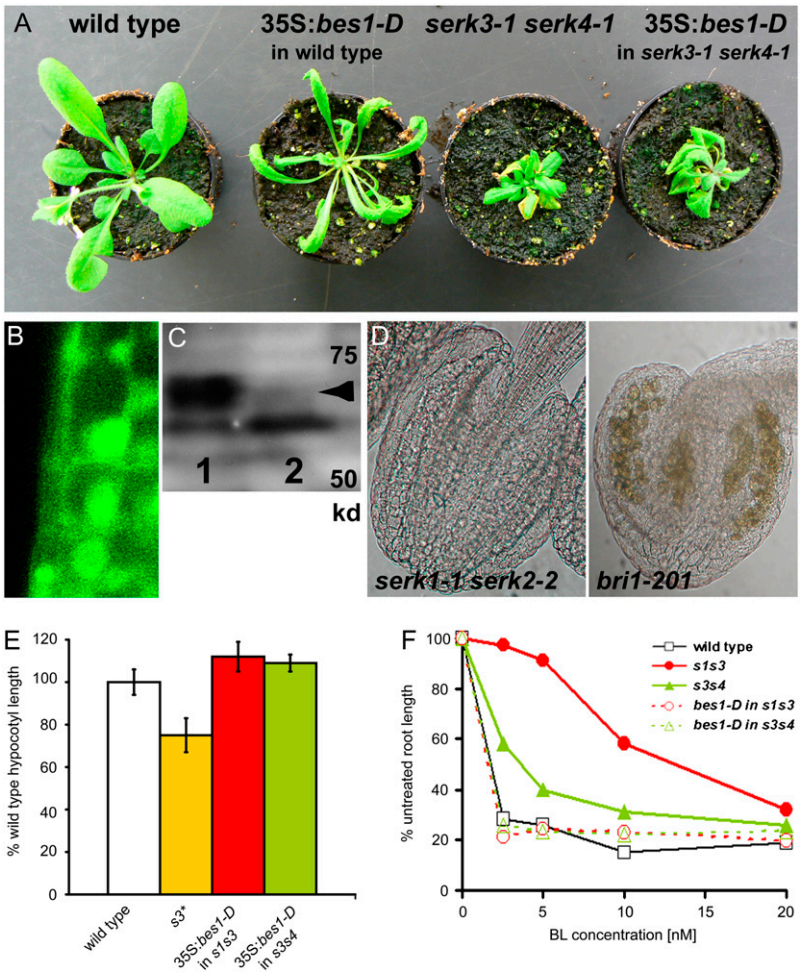

Figure 2. 35S:*bes1-D* rescues the *serk1-1 serk3-1* phenotype, but fails to rescue the *serk1-1 serk2-2* and *serk3-1 serk4-1* phenotypes. A, The *serk3-1 serk4-1* phenotype is not rescued by the 35S:*bes1-D* construct. B, Roots of transgenic plants transformed with the 35S:*bes1-D*-GFP fusion are visualized by confocal microscopy. C, Western analysis of the *serk3-1 serk4-1* double mutant transformed with the 35S:*bes1-D*-GFP constructs (1) as compared to the *serk3-1 serk4-1* double mutant (2). D, *serk1-1 serk2-2* anther showing no pollen grain and *bri1-201* anther showing pollen grain. E, Quantitative analysis of the hypocotyl length of *serk3-1 serk4-1* double mutants transformed with the 35S:*bes1-D*-GFP, *serk3-1*, and Col-0 wild-type plants grown in the dark for 5 d. Each measurement represents an average of hypocotyl lengths of 20 seedlings. Error bars indicate SD. F, Root growth measurements of seedlings grown on medium containing different BL concentrations. Each measurement is calculated as a percentage of the root elongation of the control plants grown on medium containing the same volume of 80% (v/v) ethanol used to dilute BL. *, Significant differences from Col-0 wild type ($P \leq 0.05$); *s1*, *serk1-1* allele; *s3*, *serk3-1*; *s4*, *serk4-1*; kd, kilodalton. [See online article for color version of this figure.]

overexpressing lines, *bes1-D* does not rescue the *serk1 serk2* anther phenotype nor the *serk3 serk4* growth phenotype (data not shown; Fig. 2A).

In contrast to the *serk1 serk2* double mutant of which the anthers do not produce any pollen (Supplemental Fig. S1B; Albrecht et al., 2005), the strong *bri1-201* mutant, which is also male sterile, does produce pollen (Fig. 2D).

Taken together, we conclude that the *serk1 serk2* male sterility phenotype and the *serk3 serk4* dwarf phenotype are not dependent on BRI1-mediated BR signaling.

SERK3 and SERK4 Are Partially Redundant in Pathogen-Induced Cell Death Control

Plants lacking full-length transcripts of *SERK3* show a significantly enhanced cell death phenotype after pathogen treatment, which cannot be determined in *serk4-1* single knockout lines (Fig. 3; Kemmerling et al., 2007). In double mutants of both genes, the plants look dwarfed (Fig. 2A), show spontaneous cell death, and seedling lethality (He et al., 2007). A combination of weaker alleles of both genes as used here is not seedling lethal anymore, but growth is more reduced (Supplemental Fig. S4A) and pathogen-induced cell death is much more severe than in the *serk3-1* parental lines (Fig. 3). *serk4-1* single mutants do not show any effect on growth or cell death control nor do single *serk1*, *serk2*, and *serk5* mutants. None of the double or triple mutants of these genes show any additional effect on growth or cell death responses compared to the *serk3-1* single or *serk3-1 serk4-1* double mutants, respectively. Taking these results together, we therefore conclude that, in addition to SERK3, SERK4 also exhibits a previously unrecognized effect in plant cell death control after pathogen treatment. However, SERK3 and SERK4 are only partially redundant because *serk3-1* single mutants already show the pathogen-related phenotype (Fig. 3).

SERK Overexpression Only Rescues the Rosette Phenotype of *bri1* Mutants

To further evaluate the role of the *SERK* genes in BR signaling, we used an overexpression approach. Overexpression of SERK3/BAK1 was shown to suppress a weak *bri1* allele, *bri1-5* (Li et al., 2002). To independently test whether other *SERK* genes shared this

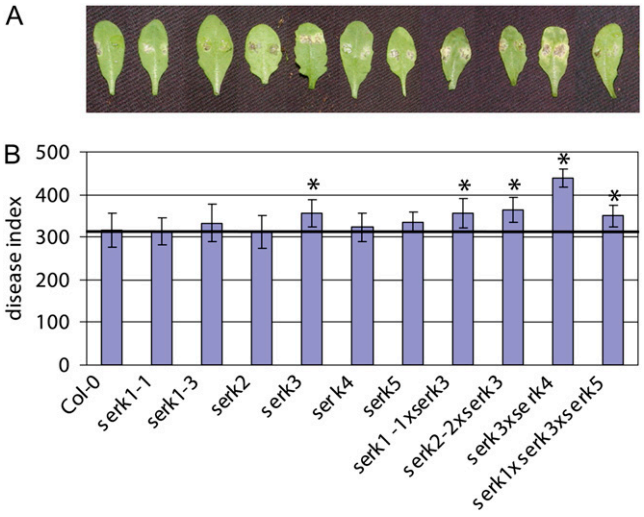

Figure 3. SERK3 and SERK4 are partially redundant in cell death control, but not SERK1, SERK2, and SERK5. A, Infection phenotypes of representative Col-0 wild type and various single and double *serk* mutants at 7 DAI with *A. brassicicola*. B, Quantitative analysis of the growth of *A. brassicicola* in wild-type Col-0 and various single and double *serk* mutants. Results represent means ± SD ($n \geq 8$). *, Significant differences from Col-0 wild type ($P \leq 0.05$). [See online article for color version of this figure.]

property, we tested whether SERK1, SERK2, and SERK4 overexpression could rescue the *bri1-301* phenotype. Overexpression of the transgenes was confirmed by western-blot analysis (Fig. 4B). The resulting transgenic lines were tested for rescue of the rosette, root, and hypocotyl phenotypes. In all the SERK overexpression lines, the *bri1-301* rosette phenotype is rescued (Fig. 4A). However, none of the transgenic lines is able to rescue the *bri1-301* hypocotyl (Fig. 4C) and root phenotypes (Fig. 4D; Supplemental Fig. S5, A and B). Because SERK5 was reported by He et al. (2007) to not rescue the rosette phenotype, we did not include the overexpression of SERK5 in that set of experiments. We could not discard the possibility that these results are linked to the different *bri1* alleles used here because previous results were obtained with the *bri1-5* allele (Li et al., 2002). Therefore, the *brs1-1D* mutant was analyzed to provide independent evidence. The *brs1-1D* locus was identified following activation tagging of the weak *bri1-5* allele (Li et al., 2001). Whereas rescue of the rosette phenotype was observed (Li et al., 2001), no rescue of the root phenotype was recorded in those lines (Supplemental Fig. S5C). These data confirm that a different requirement for BL signaling exists in the rosette when compared to hypocotyl and root.

SERK Genes Are Divergent Paralogs

The different double *serk* mutant combinations revealed specific pathways (*serk1 serk2*, *serk1 serk3*, and *serk3 serk4*) and suggest that the *SERK* genes are either differentially expressed or are not functional paralogs of one another. The expression pattern of the different *SERK* genes was therefore analyzed. Semiquantitative reverse transcription (RT)-PCR analysis and localization studies using fusion proteins of the different members indicate that the different SERK members are expressed in all tissues during development and share a largely overlapping pattern of expression (data not shown). To further confirm that the observed phenotypic differences within the double mutants are not due to subtle differences in expression pattern of the SERK members, we used a transgenic approach. We aimed at rescuing the *serk1 serk2* anther phenotype by expressing the *SERK1* and *SERK2* genes under control of the *SERK3* promoter. The *serk1 serk2* anther phenotype is rescued by the *SERK1* and *SERK2* genes driven by the *SERK3* promoter (Supplemental Fig. S6, A and C). However, *SERK3* under the control of the *SERK*3 promoter does not rescue the anther phenotype (Supplemental Fig. S6, B and D). Hence, subtle differences in the expression pattern of these *SERK* genes fail to explain the specific pathways observed within the double-mutant combinations. Similarly, the *SERK2* gene driven by the *SERK3* promoter, while rescuing the anther phenotype, cannot rescue the root insensitivity of the *serk1 serk3* double mutant (Supplemental Fig. S6E). This suggests that the SERK1 and SERK2 proteins are not interchangeable with the SERK3 or SERK4 proteins. Consequently, the respective genes are divergent paralogs.

DISCUSSION

Gene duplication is a common phenomenon and has been a key factor in the diversification of plants and animals. The high redundancy level detected in the Arabidopsis genome favors the evolution of new signaling pathways. Only a fraction of those genes have been assigned a function through the characterization of mutant alleles. Due to gene redundancy, this approach remains limited because most of the loss-of-function mutants do not show a phenotype. The *SERK* gene family appears to be a classic example of redundancy between the *SERK* gene members because no phenotypes were recorded for the single loss-of-function mutants, except for *serk3/bak1* (Li et al., 2002; Nam and Li, 2002; Kemmerling et al., 2007). We used a systematic genetic approach to analyze the function of the *SERK* genes by generating double, triple, and quadruple mutants. Because *serk3/bak1* is involved in BRI signaling and cell death control, the generated double, triple, and quadruple mutants were systematically analyzed for their BR-related phenotypes and their response to pathogen treatment. The phenotypes and the physiological responses observed do not support the hypothesis that all *SERK* genes are redundant and involved in BRI1 signaling and/or pathogen response. Our data suggest that, of the Arabidopsis SERK family, only SERK1 and SERK3 participate in BRI1-mediated signaling in Arabidopsis

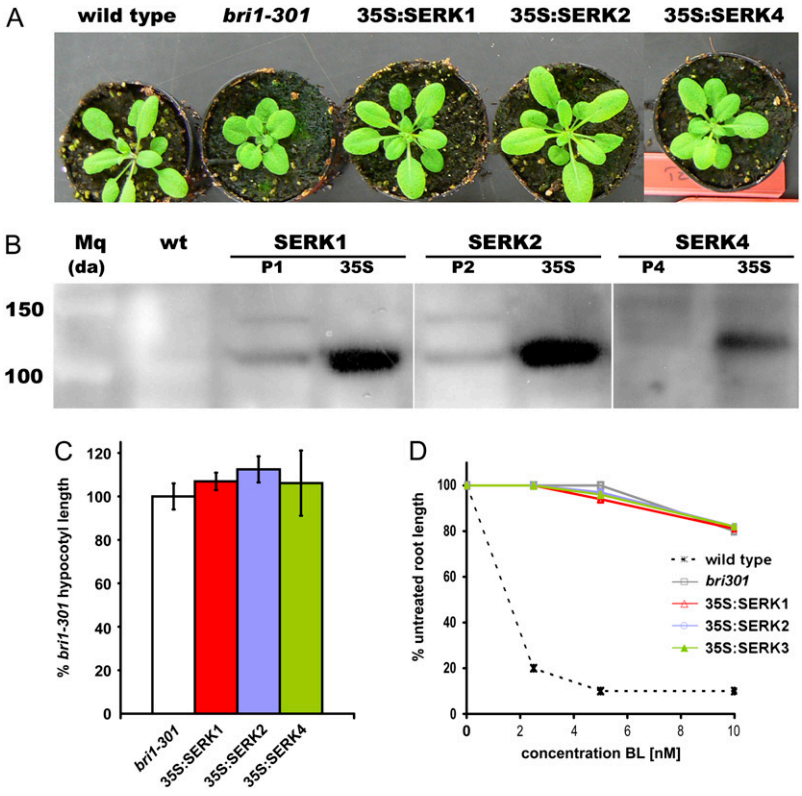

Figure 4. SERK overexpression only partially rescues the weak allele *bri1-301*. A, Overexpression of *SERK* genes partially suppresses the rosette phenotype of the weak *bri1-301* mutation. B, Western analysis to confirm the elevated expression of SERK proteins in the transgenic lines. C, Quantitative analysis of the hypocotyl length of plants overexpressing SERK proteins, *bri1-301* mutant, and Col-0 wild-type plants grown in the dark for 5 d. Each measurement represents an average of hypocotyl lengths of 20 seedlings. Error bars indicate SD. D, Root growth measurements of plants grown on medium containing different BL concentrations. Mq, Molecular size marker in daltons (da); P1, P2, and P4, *SERK1*, *SERK2*, and *SERK4* promoters, respectively; 35S, 35S promoter; wt, wild type. [See online article for color version of this figure.]

roots and hypocotyls. To further support those data, we also used an alternative approach. The gain-of-function construct *bes1-D* was used to complement the observed *serk* double-mutant phenotypes. In agreement with the phenotypic and physiological observations, only the hypocotyl and root BL sensitivity phenotype of the *serk1 serk3* double mutant could be rescued by this construct.

Triple and quadruple *serk* mutants only show additive phenotypes, suggesting that the different *SERK*s have no further redundancy besides the ones revealed by the double-mutant phenotypes. The strong *bri1* phenotypes cannot be phenocopied by creating a quadruple mutant, suggesting that genes other than *SERK*s play a role in BRI1-mediated BL signaling. *SERK5* is unlikely to perform this function because higher order mutants, containing the *serk5* mutation, do not show additional phenotypes. Furthermore, it was shown that *serk5* contains a mutation that inactivates SERK5 kinase activity (He et al., 2007). None of the double or triple *serk* mutants show any additional effect on cell death responses compared to the *serk*3 singles or *serk3-1 serk4-1* double mutants, respectively.

It was shown previously that SERK4 overexpression in weak *bri1-5* mutants rescued the rosette phenotype, suggesting that SERK4 mediates BR signaling (He et al., 2007). To confirm this, the *SERK* genes were overexpressed, using the 35S promoter, and introduced in weak *bri1-301* mutant plants. In all cases, SERK overexpression was able to rescue the rosette phenotype of the *bri1-301* mutant, but not the root and hypocotyl phenotypes, suggesting a difference in requirement for the different BR-related phenotypes. These data indicate that, although not redundant in the BRI1 pathway, *SERK* genes might have preserved a shared function that allows the rescue of some aspect of the *bri1* phenotypes. These data are in line with those reported by He et al. (2007), who further showed coimmunoprecipitation of SERK4/BKK1 with BRI1 in plants overexpressing both genes. However, the reported phenotypes and physiological observations are not related to any *bri1*-mediated aspect. Therefore, it remains to be determined whether this shared function operates in wild-type plants or whether it is caused by ectopic or overexpression of the *SERK* genes. The *serk3* allele used in this study is a weaker variant than the one used previously and this allowed us to demonstrate that the *SERK4* gene is partially redundant with *SERK3* in mediating a pathogen-induced response as reported by Kemmerling et al. (2007). However, we have not been able to observe the strong early seedling lethality phenotype reported previously for the same allele in the *serk3 serk4* double mutant (He et al., 2007).

In principle, gene duplication creates functionally identical copies that are fully redundant. Our data indicate that the *SERK* genes, although partially redundant, are involved in different signaling pathways (i.e. SERK1 and SERK3 act synergistically in BR signaling, SERK2 acts redundantly with SERK1 in male sporogenesis [Albrecht et al., 2005; Colcombet et al., 2005], and SERK4 redundantly with SERK3 in cell death control [He et al., 2007]). Hence, we observed

that the *SERK* genes have evolved to perform different functions. Acquisition of novel gene function can occur either by alteration of protein function, due to amino acid substitution, or gene expression pattern. SERK3 is unable to substitute for SERK1 or SERK2 activity in the anther, when driven by the *SERK1* or *SERK2* promoter, which indicates that the protein itself confers specificity. This suggests that the specificity for ligands may be altered or that different downstream targets are activated. So far, no ligand has been identified for the SERK receptors and they are thought to be non-ligand-binding coreceptors. Coreceptors are fully functional through the formation of hetero-oligomeric complexes and are capable of generating potent cellular signals. SERK3 heterodimerizes with at least two different main receptors, BRI1 and FLS2 (Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004; Chinchilla et al., 2007), whereas a function in cell death suggests the involvement of a third as-yet unidentified ligand-binding receptor (He et al., 2007; Kemmerling et al., 2007). SERK1 also heterodimerizes with BRI1 and SERK3. Independent of its role in BL signaling, SERK1, together with SERK2, activates targets involved in tapetum formation that cannot be activated by SERK3. Likewise, SERK3 is involved in cell death and innate immunity, whereas SERK1 and SERK2 are not involved in these processes. This situation is reminiscent of that described for the mammalian ERBB (erythroblastosis oncogene B) receptors. The system has evolved from a simple cascade with a single ortholog of ERBB in nematodes into a highly interconnected network in mammals through the duplication of genes encoding ligands and receptors (Citri and Yarden, 2006). Following gene duplication, partial inactivation due to mutations abolished the autonomy of two of the four receptors; ERBB2 lacks the capacity to bind ligands and ERBB3 is defective in kinase activity. This led to the transformation of a linear system of four receptors into a complex network where ERBB2 functions as the preferred heterodimeric partner of the other ERBB members and where ERBB3 needs to heterodimerize to be functional. At the core of such an interconnected network, autonomous dimer receptor modules function as essential signaling units that integrate diverse signals and activate a variety of downstream effectors. Along with its modular properties, the ERBB network displays redundancy that contributes to the robustness of that signaling system (Citri and Yarden, 2006).

Hetero-oligomerization of RLKs is essential in the activation of plant signaling cascades (e.g. Li et al., 2002; Wang et al., 2005). Our results support that hypothesis and show that the SERK receptors contribute to a network that controls the activation of multiple and partially independent pathways (Fig. 5). Based on our previously reported size estimate of the BRI1/SERK1/SERK3 receptor complex of 350 to 450 kD (Karlova et al., 2006), together with the observation that the BRI1 receptors can indeed homodimerize (Russinova et al., 2004), we proposed that a heterotetrameric receptor complex of two ligand-binding receptors and two coreceptors is a plausible configuration. This configuration would predict that no further enhancement of a particular phenotype would be possible upon removal of other members of the family of coreceptors than the ones present in either homodimeric or heterodimeric combination. Different specificities could then be attributed to a specific combination of main and coreceptors, in line with the results presented here. Understanding how the *SERK* genes are being recruited within the different receptor networks will help to understand how specificity of signaling using LRR-RLKs is being established in plant cells.

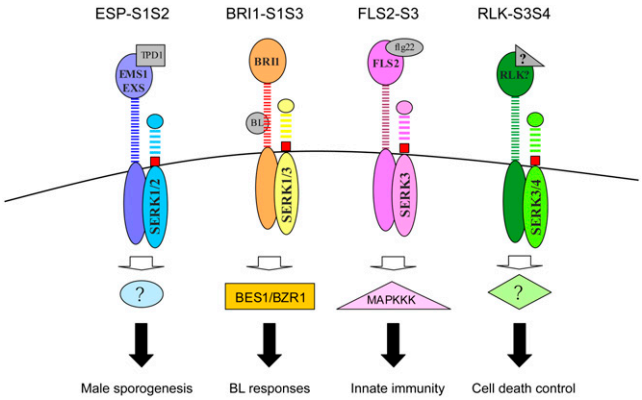

Figure 5. *SERK* genes are involved in several independent pathways. Model of pathways involving the five *SERK* genes (*SERK1–SERK5*) containing five LRRs (stripes) and one SPP (red square) domain as defined by Hecht et al. (2001). Shown are: BR pathway involving BRI1, SERK1, and SERK3 (Li et al., 2002; Nam and Li, 2002; Karlova et al., 2006); FLS2 pathway involving SERK3 essential to innate immunity (Chinchilla et al., 2007); pathway involving likely EMS1/EXS (Canales et al., 2002; Zhao et al., 2002), TPD1 (Yang et al., 2003), and SERK1 and SERK2 mediating male sporogenesis (Albrecht et al., 2005; Colcombet et al., 2005; C. Albrecht and S.C. de Vries, unpublished data); and pathway involving SERK3, SERK4, and probably an unidentified RLK leading to cell death control (He et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). [See online article for color version of this figure.]

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Columbia [Col-0]) were used as the wild type. Seeds were surface sterilized and germinated on 0.5× Murashige and Skoog medium (Duchefa) supplemented with 1% Suc. Plants were grown at 22°C under fluorescent light, with 16-h-light/8-h-dark photoperiods, unless otherwise specified. Transgenic seedlings were selected on 0.5× Murashige and Skoog medium containing either 50 mg/L kanamycin, 15 mg/L phosphinothricin, or 11.25 mg/L sulfadiazin. The *serk1-3* allele (line 448E10) was obtained from the GABI-KAT collection at the Max Planck Institute (Rosso et al., 2000). The *serk1-1* (SALK_044330), *serk3-1* (SALK_034523) or *bak1-3* (Russinova et al., 2004; Kemmerling et al., 2007), *serk4-1* (SALK_057955) or *bkk1-1* (He et al., 2007), and *serk5-1* (SALK_147275) alleles were obtained from the Signal Collection at the Salk Institute (Alonso et al., 2003). The *serk2-2* T-DNA-tagged allele was identified in the Syngenta Arabidopsis Insertion Library (SAIL) lines, nonredundant 119-G03. The genotyping for single and double mutants was performed by PCR reactions using primer combinations

for the *serk1-3* allele, GK_S1F (AGCAATTTTGTTTTGCAGAAAAGT)/GK_LB1 (CCCATTTGGACGTGAATGTAGACAC) and GK_S1F/S3 (AGAGATATTCTGGAGCGATGTGACCGATGG); the *serk1-1* allele, V3 (CGTGACAACAGCAGTCCGTGGCACCATCGG)/TgR1 (TGTTGCCGGTCTTGCGATGATTAT) and V3/KinR1 (TTTTTGCCATTCGTCCCATTTC); the *serk2-2* allele, F23M9_ZF (GTGTACTTGGTTTCACGTAACG)/LB1 (GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC) and F23M9_ZF/GSP1 (CGGCTAGTAACTGGGCCGCATAGATCC); and for the *serk3-1* allele, F17M5_ZF (GCACTGAAAAACAGTTTAGC)/LBb1 (GCGTGGACCGCTTGCTGCAACT) and F17M5_ZF/S3E6R (GATGCAGGAAGGGGAGTCAACTTGGTG) to amplify the T-DNA-tagged and the wild-type alleles, respectively. For the *serk4-1* allele, the following primers were used: IF (CTGAAGAAGACCCAGAGG)/sk4-R3 (GGAGTTGATATTCAAAAGTGCATGGG) and IF/LBb1 (GCGTGGACCGCTTGCTGCAACT), and for the *serk5-1* allele, IF-sk5R1 (GCTTAATGGAAGTGGAGAGA) and IF/LBb1 (GCGTGGACCGCTTGCTGCAACT) to amplify the wild-type and the T-DNA-tagged alleles, respectively. The *bri1-301* allele was obtained from Jianming Li (University of Michigan) and genotyped by PCR amplification of a product of 0.55 kb using primers Bri1-301_F (CATCGAAATCTTGTGCCTC)/Bri1-301_R (CCTTCATAAGCTCGGGGTC) followed by a restriction enzyme digest with *Mbo*I. The *bri1-301* mutant allele contained no restriction site, whereas the BRI1 wild-type allele generated two fragments of 0.16 and 0.39 kb, respectively.

Alternaria brassicicola infections were performed as described (Kemmerling et al., 2007). Bonitation of the symptom development was monitored at 7 d after infection.

Expression Analyses

RNA isolation, cDNA synthesis, and RT-PCR were performed as described by Hecht et al. (2001). For the detection of the *SERK1* transcript in the *serk1-3* mutant and wild-type background, cDNA synthesis was performed using random hexamer and oligo(dT) primers (Amersham Biosciences). PCR products were collected after 21, 23, 25, and 27 cycles for the constitutively expressed cyclophilin gene *ROC5* and 28, 30, 32, and 34 cycles for the *SERK1* gene. The PCR reaction was performed using primer combinations ROC5-5/ROC5-3 to amplify *ROC5* and either V1 (TTGGAAATCTGACAAACTTAGTGAGTTTGG)/S2 (TCGTCGCCACCAAGCAAAGGCTATTGCAGG) or V2 (GCTGCTCCTGCAATAGCCTTTGCTTGGTGG)/S3 (AGAGATATTCTGGAGCGATGTGACCGATGG) to amplify *SERK1* (Hecht et al., 2001).

For the detection of the *CPD* transcripts, cDNA synthesis was performed by using oligo(dT) primers and PCR amplification with CPD-f (ATGGCCTTCACCGCTTTTCTCCTC) and CPD-r (TCAAGTAGCAAAATCACGGCGCTT) primer combinations. PCR products were collected after 28 cycles. *ATA7* transcripts were analyzed as described by Albrecht et al. (2005).

Hypocotyl and Root Growth Assays

Freshly harvested seeds were surface sterilized and placed on either 0.5× Murashige and Skoog plates without hormones or 0.5× Murashige and Skoog plates containing different concentrations of BL (Sigma) or 2 μM brassinazole (Brz220; Tokyo Chemical Industry). The plates were kept at 4°C for 2 d and then placed at 22°C either in dark or grown under 16-h-light/8-h-dark photoperiods. The hypocotyl length with and without brassinazole was measured after 5-d incubation in dark and the root length with and without BRs was determined after 7 d of growth in light. Every experiment was performed in duplicate and repeated twice.

Microscopy and Histological Analysis

For the anther structure study, inflorescences of the *serk1-3* and *serk1-3 serk2-2* mutants were fixed in 5% (w/v) glutaraldehyde in 25 mM sodium phosphate, pH 7.4, dehydrated in ethanol series to 95% (v/v), and embedded in Technovit 7100 (EBSciences) according to the recommendations of the manufacturer. Seven-micrometer sections were stained with 0.25% (w/v) Toluidine Blue.

Gene Cloning and Arabidopsis Transformation

The entire open reading frames of *SERK1*, *SERK2*, *SERK3*, and *SERK4* cDNAs were amplified by RT-PCR from Col-0. The forward and reverse primers were engineered with an *Nco*I site to replace the *SERK1* and *SERK2* stop codons and allow an in-frame fusion with yellow fluorescent protein (YFP). The primers used were S1-*Nco*F and S1-*Nco*R for the *SERK1* cDNA, S2-*Nco*F and S2-*Nco*R for the *SERK2*, S3-*Nco*F and S3-*Nco*R for the *SERK3*, and S4-*Nco*F and S4-*Nco*R for the *SERK4* cDNA.

To prepare the *SERK1*, *SERK2*, *SERK3*, and *SERK4* promoter constructs, a 2-kb region upstream of the start codons of the *SERK1*, *SERK2*, *SERK3*, and *SERK4* genes was amplified from Col genomic DNA and cloned in the PGEM-T vector (Promega). The primers used were P1F and P1-*Nco*R for the *SERK1* promoter, P2F and P2-*Nco*R for the *SERK2* promoter, P3F and P3-*Nco*R for the *SERK3* promoter, and P4F and P4-*Nco*R for the *SERK4* promoter. The PGEM-T cloned promoters were inserted via *Sal*I-*Nco*I in a modified pBluescript vector containing the YFP gene inserted as *Nco*I-*Bam*H1 fragment in front of the Tnos terminator. The entire open reading frames of *SERK1* and *SERK2* as described above were then inserted as *Nco*I fragments. The resulting full cassettes were then subcloned into a modified pFluar vector via *Sal*I-*Sma*I (Stuitje et al., 2003). These constructs will be further referred to as P_{SERK1}:*SERK1-YFP*, P_{SERK2}:*SERK2-YFP*, P_{SERK3}:*SERK3-YFP*, and P_{SERK4}:*SERK4-YFP* for the *SERK1*, *SERK2*, *SERK3*, and *SERK4* transgenes, respectively.

The different *SERK* cDNAs were also cloned into the *Nco*I site of a modified pBluescript vector carrying the 35S promoter driving the YFP fluorophore. The resulting full cassettes were then subcloned into the binary vector as previously described. These constructs will be further referred to as *35S:SERK1-YFP*, *35S:SERK2-YFP*, and *35S:SERK4-YFP*.

These constructs were verified by sequencing and were electroporated in *Agrobacterium tumefaciens* strain C58C1 containing a disarmed C58 Ti plasmid (Koncz et al., 1989). The constructs were transformed into the different mutant backgrounds by the floral-dip method (Clough and Bent 1998).

Protein Extraction and Western Analysis

Protein extraction was performed as described by Karlova et al. (2006). The proteins were separated with 10% SDS-PAGE. GFP antibodies and western analysis procedures were as previously described (Karlova et al., 2006).

Fluorescence Microscopy

Anthers and root apices from transgenic plants harboring the 35S:*bes-1D*-GFP construct were used for confocal analyses. Transgenic roots were analyzed using a Zeiss confocal microscope (Zeiss Axiovert 100M equipped with a LSM510, argon laser with a 488-nm laser line). The settings for the GFP were as follows: 488-nm laser → HFT488/543 → sample → HFT488/543 → mirror → NFT545 → BP505-550 → detector. Autofluorescence spectral bleed-through was assessed by imaging at the same time with the YFP/GFP channel, a channel that detects red fluorescence: 514-nm laser → HFT458/514 → sample → NFT635vis → LP650 → detector. Pinhole was adjusted for each channel in such a way that Z resolution is equal (typically 2 μm). Amplifier gain for YFP/GFP and autofluorescence/spectral bleed-through channels is always the same between experiments.

Accession Numbers

The *serk1-3* allele (line 448E10) was obtained from the GABI-KAT collection at the Max Planck Institute (Rosso et al., 2000). The *serk1-1* (SALK_044330), *serk3-1* (SALK_034523) or *bak1-3* (Russinova et al., 2004; Kemmerling et al., 2007), *serk4-1* (SALK_057955) or *bkk1-1* (He et al., 2007), and *serk5-1* (SALK_147275) alleles were obtained from the Signal Collection at the Salk Institute (Alonso et al., 2003). The *serk2-2* T-DNA-tagged allele was identified in the SAIL lines, nonredundant 119-G03.

Supplemental Data

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

Supplemental Figure S1. Identification of the *serk1-3* allele.

Supplemental Figure S2. T-DNA insertion sites of single knockout lines.

Supplemental Figure S3. Growth phenotype of the different double and triple *serk* mutants.

Supplemental Figure S4. *serk3-1 serk4-1* double-mutant phenotype.

Supplemental Figure S5. Comparison of the root morphology of wild type, *bri1-5*, and *bri1-5 brs1-D* double mutant when grown on BL.

Supplemental Figure S6. *SERK* genes are divergent paralogs.

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