The Tomato Leucine-Rich Repeat Receptor-Like Kinases SlSERK3A and SlSERK3B Have Overlapping Functions in Bacterial and Nematode Innate Immunity

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Abstract

The Somatic Embryogenesis Receptor Kinase 3 (SERK3)/Brassinosteroid (BR) Insensitive 1-Associated Kinase 1 (BAK1) is required for pattern-triggered immunity (PTI) in Arabidopsis thaliana and Nicotiana benthamiana. Tomato (Solanum lycopersicum) has three SERK members. Two of them exhibit particularly high levels of sequence similarity to AtSERK3 and, therefore, were named SISERK3A and SISERK3B. To characterize a role for SISERK3A and SISERK3B in defense, we suppressed each gene individually or co-silenced both using virus-induced gene silencing (VIGS) in the tomato cv. Moneymaker. Co-silencing SISERK3A and SISERK3B resulted in spontaneous necrotic lesions and reduced sensitivity to exogenous BR treatment. Silencing either SISERK3A or SISERK3B resulted in enhanced susceptibility to root knot-nematode and to non-pathogenic Pseudomonas syringae pv. tomato (Pst) DC3000 hrcC indicating that both SISERK3s are positive regulators of defense. Interestingly, silencing SISERK3B, but not SISERK3A, resulted in enhanced susceptibility to the pathogenic strain Pst DC3000 indicating distinct roles for these two SISERK3 paralogs. SISERK3A and SISERK3B are active kinases, localized to the plasma membrane, and interact in vivo with the Flagellin Sensing 2 receptor in a flag22-dependent manner. Complementation of the Atserk3/bak1-1 mutant with either SISERK3A or SISERK3B partially rescued the mutant phenotype. Thus, SISERK3A and SISERK3B are likely to constitute tomato orthologs of BAK1.

Introduction

Innate immunity is the genetically determined and inheritable ability of any given host organisms to discriminate between self or non-self and activate defense responses against attempted microbial or pest/parasite infection. Plants utilize a multilayered immune system to protect themselves from invading pathogens or pests. One of the first layers of plant active defense is the ability of the host to sense microbes by perceiving microbe-associated molecular patterns (MAMPs). This type of recognition is mediated by pattern recognition receptors (PRRs) present at the cell surface and triggers a resistance response known pattern-triggered immunity (PTI) [1-3]. MAMP perception elicits a variety of defense responses including phosphorylation and dephosphorylation of proteins, production of reactive oxygen species (ROS), callose deposition and defense gene expression [4,5]. Microbial pathogens evolved effectors to suppress PTI. In return, plant sensed resistance (R) proteins that recognize effectors direct or indirect and activate effector-triggered immunity (ETI) [1]. Frequently, ETI responses are dependent on the defense hormone salicylic acid (SA).

Root-knot nematodes (RKN; Meloidogyne spp.) are sedentary endoparasites of great agricultural importance. RKN are obligate biotrophic, penetrate the host roots behind the root cap and move towards the vascular cylinder where they initiate feeding on the cytoplasm of live cells and develop an elaborate feeding site known as giant cells. Cells around the feeding site undergo hyperplasia and hypertrophy resulting in the formation of galls, root symptoms associated with this group of nematodes [6]. Nematode salivary secretions have been implicated in development and maintenance of the feeding site [7]. Once feeding is initiated, RKN become sedentary and mature females lay eggs in gelatinous sacs protruding on the root surface. Although no information exists about how nematodes induce PTI, host defense responses against RKN are similar to biotrophic microbial pathogens and resistance to this pest is mediated by classical R gene responses frequently associated with cell death [8,9].

Receptor like kinases (RLKs) are among the well characterized PRRs. Common features of the RLKs are the presence of an N-terminal signal sequence, an extracellular domain that varies in structure, a single membrane-spanning region, and a cytoplasmic protein kinase catalytic domain. RLKs with leucine-rich repeat (LRR)-containing extracellular domains comprise the largest subfamily of transmembrane RLKs in plants with over 200 members in Arabidopsis thaliana (Arabidopsis) [10,11].

The LRR-RLK FLS2 (FLAGELLIN SENSING 2 (FLS2), belonging to LRR-RLK subfamily XII, was first identified in Arabidopsis by its ability to perceive the bacterial flagellin including the minimal epitope flg22 [12]. Responsiveness to
Here, we describe the functional characterization of the remaining two SISERK3A, SISERK3A and SISERK3B, and their role in PTI to a bacterial pathogen and RKN. Using virus-induced gene silencing targeting SISERK3A and SISERK3B individually or combined revealed overlapping and unique roles for these SISERK3 paralogs in plant defense, cell death control and BR response. In addition, we show that both SISERK3A and SISERK3B co-immunoprecipitate with STLS2 and partially complement the bak1-4 null mutant.

Results

Molecular structure of SISERK3A and SISERK3B

The protein coding sequence (CDS) of SISERK3A (1,848 bp) and SISERK3B (1,854 bp) and their chromosome localization (chromosome 10 and 1, respectively) have been reported earlier [34]. The genomic sequences of both SISERK3A and SISERK3B were obtained from tomato cv. Moneymaker by amplifying overlapping regions based on cDNA sequences. Sequence analysis indicated that SISERK3A genomic (KC261564) sequence is 10,874 bp in length while the SISERK3B genomic (KC261565) sequences is 7,965 bp. As predicted, SISERK3A and SISERK3B contain 11 exons (Figure S1). The predicted proteins of SISERK3A (616 amino acids, 68.28 kD) and SISERK3B (618 amino acids, 68.27 kD) have domains characteristic of SERK proteins including a signal peptide (with a putative cleavage site between amino acids 24 and 25 for SISERK3A or amino acids 29 and 30 for SISERK3B), a LRR N-terminal domain followed by four successive LRR domains, a Pro-rich region including a SPP and the biotrophic oomycete pathogen Hyaloperonospora arabidopsidis [28].

BAK1 forms flg22-induced complexes with FLS2, directly interacts with FLS2 and recognizes the C-terminus of the FLS2-bound flg22 [30]. bak1 null mutants exhibit reduced flg22-responses including production of ROS, activation of mitogen-activated protein kinases (MAPK) and induction of defense genes indicating a role for this kinase in FLS2-mediated PTI [24,25,28]. BAK1 also forms complexes with additional PRRs and is required for responses triggered by a number of MAMPs from bacteria, fungi and oomycetes as well as signals generated from abiotic stresses such as cold shock and damage-associated molecular patterns indicating its role as a master regulator of stress responses [31].

In Solanaceae plants, SERK3 homologs have been characterized from N. benthamiana, N. attenuata and tomato. In N. benthamiana, two AtSERK3/BAK1 homologs, NbSERK3A and NbSERK3B were identified [32] while a single homolog NaBAK1 has been reported from N. attenuata [33]. The entire tomato SERK family members have been identified [34,35]. However, unlike Arabidopsis, tomato has only three SERKs (SERK) members. These were named based on their phylogenetic relationship to the Arabidopsis SERKs as SISERK1, SISERK3A and SISERK3B [34]. Interestingly, SISERK1 is required for potato aphid (Macrosiphum euphorbiae) resistance mediated by the presumed cytoplasmically localized nucleotide-binding (NB-LRR R protein Mi-1, indicating a role for LRR-RLK in NB-LRR-mediated ETI [34]. Surprisingly, SISERK1 is not required for Mi-1-mediated resistance to RKN suggesting distinct recognition processes or signaling responses for aphids and nematodes.
critical catalytic loop, which comprises a short stretch of residues in the kinase subdomain VI, is conserved. Recently, it has been shown that the cytoplasmic domains (CD) of SlSERK3B to have kinase activity with the ability to autophosphorylate and transphosphorylate kinase inactive SlBR1-CD [39]. To test whether SlSERK3A is also an active kinase, the CD of SlSERK3A (residues 263 to 615), including the juxtamembrane, kinase domain and C-terminal parts, was produced in a heterologous system as GST-fusion proteins (GST-SlSERK3A). As a control, the CD of SlSERK3B (residues 259 to 616) was also produced as a GST fusion protein (GST-SlSERK3B). We also developed the respective kinase-dead mutant variants SlSERK3A* CD (D418N) and SlSERK3B* CD (D420N), by introducing point mutations in the kinase catalytic loop based on a BAK1 kinase dead mutant [40], as GST- fusion proteins.

Purified proteins were subjected in vitro to an auto-phosphorylation assay as well as a trans-phosphorylation assay using the artificial substrate myelin basic protein (MBP). Analysis of the GST fusion proteins by SDS PAGE showed that both CD domains (66.32 and 67.64 kD) were soluble, and migrated as single bands at their predicted molecular masses (Figure 2, lower panel). A band corresponding to each of the auto-phosphorylated SlSERK3A and SlSERK3B proteins was observed when the kinase domains were used alone or in combination with MBP (Figure 2). In the presence of the wild type kinase domains, a phosphorylated MBP band was observed. As expected, both auto-phosphorylation and trans-phosphorylation of MBP were abolished by the kinase dead mutants of each SlSERK3* CD (Figure 2). Although kinase activity, both auto-phosphorylation and trans-phosphorylation, is stronger for SlSERK3A CD compared to SlSERK3B CD (Figure 2), this pattern of kinase activity was not consistently observed in replicated experiments. Taken together these results indicate that similar to SlSERK3B, SlSERK3A is also an active kinase catalyzing in vitro both auto- and trans-phosphorylation.

**SlSERK3A and SlSERK3B control cell death**

To assess the functional roles of SlSERK3A and SlSERK3B, we developed gene-specific silencing constructs able to suppress SlSERK3A or SlSERK3B transcripts using virus-induced gene silencing (VIGS). A third construct was developed to co-silence both SlSERK3A and SlSERK3B (Figure S3). The target specificities of the VIGS constructs in tomato were confirmed using quantitative RT-PCR (Figure 3A and Figure S4A). Co-silencing both SlSERK3A and SlSERK3B in tomato cultivar Moneymaker resulted in plants exhibiting reduced growth (Figure 3B) and spontaneous cell death in leaves (Figure 3C). Silencing SlSERK3A also reduced plant growth albeit to a lesser degree than the co-silenced plants, while silencing SlSERK3B did not have any obvious effect on plant growth (Figure 3B). Silencing either SlSERK3A or SlSERK3B individually did not result in spontaneous cell death (Figure S4B).

To investigate the molecular mechanism leading to the cell death phenotype in the co-silenced plants, we examined expression of the defense and senescence-related genes *SlPR5*, *SlPR1b1*, and *SlACS2* ([41]; Table S1). Expression of all four genes was upregulated in SlSERK3A SlSERK3B co-silenced leaves (Figure 3D). This overall expression is similar to transcript patterns for the respective Arabidopsis orthologs reported for bak1-1 bkk1-1 double mutant [29]. Strikingly, expression of none of these four genes was upregulated in plants individually silenced for SlSERK3A or SlSERK3B (Figure 3D). Tomato leaflets individually silenced for SlSERK3A or SlSERK3B or co-silenced, were further evaluated for callose deposition a known cell death-associated defense response. Aniline blue staining of individually silenced leaflets did not reveal callose deposition (Figure S4C), while callose deposits were detected in co-silenced leaflets in areas near tissues exhibiting cell death (Figure 3E). Co-silenced leaflets were also evaluated for cell-death associated H_2O_2 accumulation. In similar regions near dead tissues, H_2O_2 accumulation was detected as brown spots using 3,3’-diamino benzidine (DAB) staining (Figure S5A). Taken together, these results indicate that SlSERK3A and SlSERK3B have a redundant function in suppressing cell death. Because of the spontaneous nature of the cell death phenotype, co-silenced plants were not included in defense related experiments.

SlSERK3A and SlSERK3B co-silenced plants were smaller in overall stature compared to TRV-empty vector (TRV) control plants (Figure 3B). The semi-dwarf stature suggested BR-
deficiency or response in these plants. In Arabidopsis, expression of the CPD gene, involved in BR biosynthesis, is downregulated by BR treatment and this downregulation is compromised in the bri1 mutant as well as in most double and triple mutants of bak1 with other Atserks but not in any single Atserk mutant [22,42]. To assess whether SISERK3 silenced plants were affected in BR response, SISERK3 gene expression was evaluated in plants treated or untreated with BR. Basal SICPD transcript levels were similar in TRV control plants and plants individually silenced for SISERK3A or SISERK3B (Figure 3F). However, a reduction in SICPD transcript levels was observed in SISERK3A and SISERK3B co-silenced plants suggesting that SISERK3A or SISERK3B are required for the basal expression of SICPD (Figure 3F). Similar to Arabidopsis, exogenous application of BR downregulated SICPD transcript levels in tomato TRV control plants (Figure 3F). In addition, downregulation of SICPD transcript levels in response to BR was not affected in tomato plants individually silenced for SISERK3A or SISERK3B (Figure 3F and Figure S5B). In contrast, downregulation of SICPD transcript levels was greatly compromised in the SISERK3A and SISERK3B co-silenced plants treated with BR (Figure 3F and Figure S5B) suggesting a redundant function for these two paralogs in BR signaling.

Figure 3. SISERK3A and SISERK3B co-silenced plants are compromised in cell death control and BR sensitivity. (A) Transcript levels of VIGS-silenced genes were evaluated using qRT-PCR. Tomato cv. Moneymaker plants treated with TRV empty vector (TRV), TRV-SISERK3A, TRV-SISERK3B or TRV-SISERK3AB were evaluated. Expression was normalized against UBI3. Two independent samples were analyzed per construct. Values are average ± SE of three technical replicates. *P<0.05 significant difference from TRV (two-sample t-test). Experiment was repeated three times with similar results. (B) Phenotype of individually silenced SISERK3A, SISERK3B and co-silenced plants. (C) Cell death lesions in SISERK3A and SISERK3B co-silenced tomato leaflets. (D) Defense and senescence-related SIPR1a, SIPR2, SIPR5, and SIACS2 gene regulation in SISERK3A or SISERK3B silenced and co-silenced plants. Transcript levels were evaluated using qRT-PCR normalized against SLUB13. Values are average ± SE (n = 3). * indicates significant difference from TRV at P<0.05 (two-sample t-test). Experiment was repeated twice with similar results. (E) Aniline blue-stained tomato leaf discs. Callose accumulation was detected near the edges of leaf patches showing cell-death in co-silenced SISERK3A and SISERK3B plants and TRV control. Leaves treated with 1 μM flg22 for 24 h were used as control. (F) Leaflets of tomato plants silenced for SISERK3A, SISERK3B or co-silenced and TRV control were treated with 10 μM BL for 12 h for SICPD expression evaluation. Transcript levels were evaluated using qRT-PCR normalized against SLUB13. Values are average ± SE (n = 3). *P<0.05 and **P<0.01 indicate significant difference from the respective –BL control (two-sample t-test). This experiment was repeated twice with similar results.

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**SI**SERK3A and **SI**SERK3B are required for disease resistance

To investigate a possible role for a single **SI**SERK3 gene in disease resistance, we evaluated **SI**SERK3A or **SI**SERK3B silenced plants for resistance to the tomato pathogen *Pst* DC3000 and its nonpathogenic *hrcC* mutant derivative *Pst* DC3000 *hrcC*. To develop a control, we targeted the tomato flagellin receptor **SI**FLS2 (Figure S3) [16] for silencing in tomato and vacuum infiltrated the silenced plants (Figure S6) with *Pst* DC3000 *hrcC* and *Pst* DC3000. Silencing **SI**FLS2 enhanced the growth of both *Pst* DC3000 *hrcC* and *Pst* DC3000 relative to control plants (Figure 4A and 4B). Importantly, silencing either **SI**SERK3A or **SI**SERK3B (Figure S4A) also enhanced growth of *Pst* DC3000 *hrcC* indicating non-redundant roles for **SI**SERK3s in PTI against non-pathogenic *Pst* (Figure 4A). Interestingly, silencing **SI**SERK3B and not **SI**SERK3A resulted in enhanced *Pst* DC3000 growth suggesting an additional role for **SI**SERK3B in bacterial defense that may be distinct from its role in PTI against the non-pathogenic *Pst* strain (Figure 4B).

Root-knot nematodes are serious tomato pests and no information exists on PTI for this group of pests. We wondered whether resistance to nematodes might also involve PTI and the likely requirement for the master PTI regulator **SERK3**. To address this, we infected tomato plants silenced for **SI**SERK3A or **SI**SERK3B (Figure S7A) with *Meloxygone incognita* infective-stage juveniles and evaluated the roots for nematode infection and reproduction. Root weights of tomato plants silenced for either **SI**SERK3A or **SI**SERK3B compared to TRV control indicating a likely role for PTI in RKN resistance (Figure 4C). As reported earlier [43], VIGS in tomato roots was

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**Figure 4. ** **SI**SERK3A and **SI**SERK3B are involved in PTI in tomato.** Five-week-old tomato plants cv. Moneymaker silenced for **SI**FLS2, **SI**SERK3A or **SI**SERK3B and TRV control were used. (A) Plants were vacuum infiltrated with *Pst* DC3000 *hrcC* or *Pst* DC3000 and bacterial counts were performed at 0 and 3 days post infiltration (dpi). Results are average (±) SE (n=5). Letters above the graphs denote significance difference at *P*<0.01 (ANOVA Tukey HSD test). These experiments were repeated twice with similar results. (C) Plants were infected with 1,000 J2 each and evaluated 6 weeks later. No **FLS2** silenced plants were used in this assay. Results are average (±) SE (n=9). Letters above the graphs denote significance difference at *P*<0.05 (ANOVA Tukey HSD test). This experiment was repeated once with similar results. (D) Leaf samples were floated on water overnight. ROS burst was measured as relative light units (RLUs) emitted in a luminol-based assay within 15 min after 1 μM **flg22** treatment. Values are average ± SE (n=4). * indicates statistically significant difference from TRV at *P*<0.05 (two-sample t-test). This experiment was repeated once. (E) TRV-treated plants were vacuum infiltrated with *Pst* DC3000 *hrcC* and harvested 6 h later. Expression was evaluated using qRT-PCR normalized against **SI**UBI3. Values are average ± SE (n=3). *P*<0.05 and ** *P*<0.001 significant difference from TRV (two-sample t-test). This experiment was repeated twice with similar results.

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patchy (Figure S7A) suggesting the enhanced susceptibility values reported in this assay are likely an underestimate.

**SISERK3A and SISERK3B are required for flag22-triggered immunity**

To further characterize the role of SISERK3s in bacterial defense, we evaluated ROS production in SISERK3A-, SISERK3B- or SIFLS2-silenced plants. Tomato silenced for SISERK3A or SISERK3B were severely reduced in flag22-triggered ROS production, similar to SIFLS2 silenced plants, consistent with their role as positive regulators of bacterial PTI (Figure 4D). To confirm attenuation of PTI in SISERK3-silenced plants, expression of known PTI marker genes [15,44] was investigated. Transcripts of both SPTT5 and SIVWRT2B were upregulated within 6 h after *Pst Dc3000 hrcC* treatment in TRV-treated leaves (Figure 4E). In contrast, silencing SIFLS2, SISERK3A or SISERK3B severely reduced this up-regulation of both genes (Figure 4E). The observed attenuation of ROS production and reduction in defense marker gene induction further confirmed the role of SISERK3s in tomato PTI.

**SISERK3A and SISERK3B form a flag22-induced complex with SIFLS2 in N. benthamiana**

To test whether SIFLS2 heterodimerizes with SISERK3A or SISERK3B in *vivo*, we transiently co-expressed a SIFLS2-GFP fusion protein with either SISERK3A-HA or SISERK3B-HA fusion proteins in *N. benthamiana* for co-immunoprecipitation (Co-IP) experiments. Within 5 minutes after flag22-treatment, interactions between SIFLS2 and either SISERK3A or SISERK3B were detected by Co-IP with anti-GFP and immunoblotting with anti-HA, (Figure 5). Neither SISERK3A or SISERK3B were detected in the untreated anti-GFP immunoprecipitates (Figure 5). Reciprocal Co-IP using anti-HA to precipitate SISERK3A or SISERK3B and immunoblotting with anti-GFP, detected SIFLS2 only in flag22-treated samples (Figure 5). These results suggest flag22-induced complex formation between SIFLS2 and SISERK3A or SISERK3B consistent with the ligand dependency of the *Afls2-Bak1* association [24,25,45,46].

**Discussion**

In Solanaceae, SERK members have been identified in tomato, *N. benthamiana* and *N. attenuata*. However, it is not clear how many SERKs members *Nicotiana* species have. Only for tomato, all members of this family have been identified and unlike Arabidopsis, that has five members, tomato was found to have only three members [34]. In both tomato and *N. benthamiana*, two members have particularly high levels of sequence similarity to SISERK3/BAK1 suggesting recent duplication events in the lineage of these solanaceous species. Although a role for *NbSERK3* has been identified in microbial pathogen defense [25,32], it is not clear which of the two *NbSERK3* paralogs contribute to the resistance and whether the two members have redundant roles in defense. Similarly, *SISERK3* is required for the resistance to the vascular fungal pathogen *Verticillium* mediated by the receptor like protein (RLP) Vei1 and for defense responses induced by the fungal Ethylene-induced sylasase (Eix) mediated by RLP LeEix [47,48]. In these tomato and *N. benthamiana* studies, VIGS was used to evaluate the defense related roles of *SERK3* and because of the high level of sequence identity between the two *SERK3* paralogs from each plant species, the VIGS constructs used are capable to silence both members. However, the specificity of silencing was not evaluated in these experiments, consequently, the specific function of the individual paralog remains unclear. In this work, we were able to specifically silence individual *SISERK3A* and *SISERK3B* and co-silenced them by designing VIGS constructs partially targeting the respective untranslated gene regions. This allowed us to dissect the contributions of each of these gene paralogs and identify common and distinct roles for them.

*SISERK3A* silenced plants were smaller in size compared to TRV control or *SISERK3B* silenced plants which could be due to pleiotropic effect on BR signaling. However, molecular data indicate that individually silenced *SISERK3A* plants are not affected in BR signaling (Figure 3F). Although the reason for *SISERK3A* silenced plant short stature is unclear, our data indicate that BR signaling is not affected at a detectable level in either *SISERK3A* or *SISERK3B* silenced plants.

Interestingly, vacuum infiltration of *FLS2*-silenced tomato plants with *Pst* resulted in significant increase in bacterial growth a similar phenotype seen in *FLS2*-silenced *N. benthamiana* plants [14,15]. This is in contrast to Arabidopsis *fls2* mutant on which, compared to wild-type plants, no bacterial growth difference was observed after syringe infiltration [13]. Lower bacterial growth was seen on the *fls2* mutant only when bacteria were spray inoculated.
[13]. Our result with tomato, combined with that from N. benthamiana, indicates that flagellin perception by FLS2 in Solanaceae functions in the mesophyll cells while this perception in Arabidopsis is active in the guard cells. It remains to be determined whether FLS2 perception in Solanaceae functions also in the guard cells.

Our results showed that both SISERK3 paralogs contributed to resistance against the non-pathogenic Pst DC3000 hrcC strain and to RKN, while only SISERK3B promoted resistance against virulent Pst DC3000. This indicates that these two SERK3 members have evolved distinct immune related functions. The bacterial defense related role of SISERK3B is similar to that of BAK1, as shown with the bak1-5 mutant, suggesting that this tomato SERK member is an authentic BAK1 ortholog [28]. However, no clear SISERK3A Arabidopsis ortholog can be identified based on its defense function in tomato. Although a role in bacterial defense has been demonstrated for AtSERK3/BAK1, the closest BAK1 paralog, this role is only detectable in the bak1-5 bkk1 double mutant infected by a weakly virulent coronatine defective Pst strain [28]. Thus, BKK1 appears only to play a minor role in bacterial defense, unlike SISERK3A which strongly contributes to basal resistance against Pst DC3000 hrcC. Both BAK1 and BKK1 have non-redundant basal resistance functions against fungal and oomycete pathogens [28]. Although SERK3 paralogs have been implicated in fungal resistance in tomato it remains unclear which one of them contributes to this defense function because of the reasons stated above.

Recently the presence of PTI in roots was demonstrated using the well-known MAMPs chitin, flg22 and peptidoglycans and the immune responses to the latter two MAMPs were BAK1-dependent [49]. In addition, immunity function has been attributed to the bak1-4 Arabidopsis mutant to Verticillium

Figure 6. SISERK3A and SISERK3B partially complemented the Arabidopsis bak1-4 null mutant. (A) SISERK3A and SISERK3B transcript levels in transgenic bak1-4 plants expressing pBAK1-SISERK3A (bak1-4 SISERK3A) or pBAK1-SISERK3B (bak1-4 SISERK3B) were evaluated using qRT-PCR. Values are average ± SE (n = 3) normalized relative to AtActin and calibrated to expression of BAK1 in Col-0. *P<0.05 and ** P<0.001 significant difference from Col-0 (two-sample t-test). (B) Relative root growth of 9-day-old seedlings grown on medium with or without 1 mM BL. Root length is presented relative to untreated control for each genotype. Values are average ± SE (n = 30). Values were arcsine transformed for statistical analysis. Letters above the graphs denote significance difference at P<0.01 (ANOVA Tukey HSD test). This experiment was repeated twice with similar results. (C) Leaf discs were floated on water overnight. ROS burst was measured using a luminol-based assay within 25 min after 1 μM flg22 treatment. Values are average ± SE (n = 8). (D) A photo of representative short-day grown 4.5-week-old Arabidopsis plants of the indicated genotypes.

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indicating a role for BAK1 in basal defense to vascular pathogens [50]. Our results showing enhanced RKN susceptibility of SlSERK3A or SlSERK3B silenced plants indicate a role for SERK3 in resistance to RKN and the likely existence of PTI by nematode-associated molecular pattern(s). Silencing SlSERK3A resulted in reduced plant size and may be affected in BR signaling. However, it is unlikely that the enhanced RKN susceptibility is due to altered BR signaling as SlSERK3B silenced plants also exhibited enhanced RKN susceptibility but did not have altered plant size phenotype or are affected in BR signaling.

A number of nematode parasitism genes have been reported that play roles in virulence and suppression of host defenses [9,51,52]. However, no nematode-derived molecular patterns have been yet identified and it is difficult to speculate as to the nature of this pattern. Proteinaceous salivary secretions originating from esophageal gland cells have been implicated in nematode root invasion and migration as well as initiation and maintenance of their elaborate feeding sites [7]. Other sources of secretions from the nematode could be from sensory structures such as amphids or phasmids, or the excretory pore or the cuticle, none of which have been implicated in interactions with their hosts. Moreover, nematode penetration, feeding and secretion of cell wall degrading enzymes potentially produce damage-associated molecular patterns which could be the source of the nematode induced PTI. New research is needed to investigate nematode-induced PTI and to identify the nature of the nematode-associated molecular pattern(s) and its cognate PRR.

Our results showed that SlSERK3A and SlSERK3B have a redundant function in suppressing cell death. A similar function has been attributed to BAK1 and BAK1 [29], indicating that these SERK paralogs share similar cell death control functions in both Arabidopsis and tomato. However, co-silencing SERK3A and SERK3B in N. benthamiana does not result in cell death indicating this redundant cell death suppression function for two SERK members is not universal among plant species [32]. In Arabidopsis, it is speculated that these two SERK members suppress cell death through their interaction with the RLK, BIR1 (BAK1-Interacting Receptor Like Kinase 1) that possibly perceives an endogenous survival signal(s) [53]. As an alternative, it is discussed that SERK-associated PTI signaling complexes are guarded by R proteins [53,54]. In the latter case, the absence of both BAK1 and BKK1 may constitutively activate R protein-mediated defense responses, including cell death. Such a scenario is supported by the fact that the cell death phenotype in the baki bkk1 double mutant is dependent on the defense hormone SA which is required for many R protein-dependent immune responses [29]. The constitutive activation of the SA-regulated gene, NPR1 [55], we observed in SlSERK3A and SlSERK3B co-silenced plants suggests that the cell death phenotype in these tomato plants is also SA-regulated and could be triggered by an R protein. It remains to be seen whether an R protein guards SERK3-associated PTI signaling complexes in tomato.

SlSERK3A and SlSERK3B belong to the RD class of Ser/Thr kinases that share a conserved catalytic core. All AtSERK family members are active kinases and able to autophosphorylate in vitro [19,56]. Similarly, both SlSERK3A and SlSERK3B are active kinases able to auto-phosphorylate and trans-phosphorylate MPB in vitro. Multiple Ser and Thr residues are auto-phosphorylated in SlSERK3B-CD only a subset correspond to auto-phosphorylated Arabidopsis BAK1-CD residues [39]. It remains to be seen whether these additional conserved residues are also auto-phosphorylated in SlSERK3A-CD.

As the single amino acid mutation that eliminated the kinase activity of BAK1 also eliminated the kinase activities of both SlSERK3A and SlSERK3B [40], our data showed that the catalytic kinase core between BAK1 and the two SlSERK3 paralogs are structurally and functionally conserved. Although the substrates of most SERK members are not well defined, it is well documented that BAK1 trans-phosphorylates a number of RLKs [19,20,40,45,57]. Recently it has been shown that SlSERK3B can trans-phosphorylate SlBRI [39]. Based on the high sequence similarity between the SlSERK3A and SlSERK3B catalytic domains, and their redundant role in BR signaling, we hypothesize that SlSERK3A can also trans-phosphorylate SlBRI. Since SlSERK3 is required for signaling and immunity mediated by the tomato RLP LpEix2 and Vt1, respectively, it is likely that SlSERK3A or SlSERK3B individually or together are capable of trans-phosphorylating multiple receptors in a manner similar to BAK1.

Both SlSERK3A and SlSERK3B formed flg22-dependent complex with SlFLS2. Several AtSERK members are able to heterodimerize with FLS2, albeit at variable levels of association, in ligand dependent manner [25,26]. Further investigations should reveal whether SlSERK3A and SlSERK3B form a heterodimer in this interaction and whether SlFIL52 is also able to heterodimerize with SlSERK1.

The ability of SlSERK3A and SlSERK3B to form a ligand induced complex with SlFIL52 suggested a role for these two SERK paralogs in SlFIL52-dependent signaling. Indeed, both SlSERK3A and SlSERK3B have non-redundant functions in flg22-induced ROS production and activation of defense related genes. Among Arabidopsis SERK members, a similar function is only demonstrated for BAK1. Only bak1 single mutants are compromised in flg22-induced ROS and none of the remaining individual serk null mutants are impaired in ligand induced ROS production [24,28]. A minor role in flg22-induced ROS production was uncovered for bkk1 in the baki-5 bkk1 double mutant [29]. Taken together, this information indicates that SlSERK3A and SlSERK3B have BAK1-related functions and seem to be true orthologs of this Arabidopsis gene. Indeed, in complementation experiments either SlSERK3A or SlSERK3B partially rescued the baki1 mutant phenotype. The lack of full baki1 complementation is likely due to sequence divergence between these Arabidopsis and tomato orthologs.

Similar to Arabidopsis, the expression of SlCPD in tomato is down-regulated by exogenous application of BR via a presumed negative feedback mechanism [42]. The attenuation of SlCPD responsiveness to exogenous BR application in the SlSERK3A and SlSERK3B co-silenced plants, and not in the individual SlSERK3A or SlSERK3B silenced plants, indicates that SlSERK3A and SlSERK3B have redundant function in BR signaling. Since the SlSERK3A and SlSERK3B co-silenced plants had residual BR signaling competence, it suggests that the only other family member SlSERK1 also contributes to BR response. Based on CPD expression analysis in Arabidopsis, it is not clear the contribution of BAK1/SERK3 to BR signaling as BR effect on CPD expression have been analyzed in the double mutant baki1 serk1 or the triple mutant baki1 bkk1 serk1 and not in the double mutant baki1 bkk1 [22]. Nonetheless, our results show that SlSERK3A and SlSERK3B contribute to most of the BR effect on CPD expression in tomato.

In summary, our work provides functional characterization of SlSERK3A and SlSERK3B in an important crop and demonstrates differences and similarities in the role of BAK1 and SlSERK3A and SlSERK3B paralogs in immunity and BR signaling. This work also provides a foundation for future characterization of PTI against RKN in roots.
Materials and Methods

Plant material and growth conditions

One-week-old tomato (Solanum lycopersicum) cv. Moneymaker seedlings were transplanted into California mix II or sand. Plants were maintained in plant growth rooms at 24°C before VIGS treatment and then at 19°C until use in bioassays with a 16 h light and 8 h dark photoperiod. N. benthamiana plants were maintained in a plant growth room at 24°C at a similar photoperiod. Plants were fertilized biweekly with MiracleGro (The MiracleGro). Arabidopsis thaliana (Arabidopsis) Col-0 and T-DNA insertion mutant bak1-1 (SALK_116202) plants were grown in soil under fluorescent lights (10 h light and 14 h dark, 100 μEinsteins/m²/s) at 22°C.

Virus-induced gene silencing (VIGS)

The TRV-SlSERK3A (contains 152 bp of the SlSERK3A gene (+1837 to +1988)), TRV-SlSERK3B (contains 174 bp of the SlSERK3B gene (+1844 to +2017)), TRV-SlSERK3AB (contains 178 bp of the SlSERK3B gene (+1209 to +1466) and TRV-SlFLS2 (contains 111 bp of the SlFLS2 gene (+3460 to +1570)) were constructed by amplifying the desired fragments using gene-specific primers (Table S2) and tomato c. Moneymaker cdNA, and recombining into Gateway compatible pDONR207 vector (Invitrogen) and finally into pTRV2. After sequence verification, constructs were transformed into A. tumefaciens strain GV3101.

VIGS was performed using the bipartite TRV (pTRV1 and pTRV2; [50]) vector in Agrobacterium tumefaciens and syringe infiltration (agroinfiltration) of 2-week-old tomato leaflets. Equal volumes (OD600 = 1) of A. tumefaciens pTRV1 and suspensions containing pTRV2-derived constructs, pTRV2 empty vector or TRV-PDS were mixed before infiltration [50].

Constructs

The coding sequences (CDS) of SlSERK3A and SlSERK3B were PCR amplified from tomato cdNA using the primers given in Table S2. The BAK1 promoter was amplified from Arabidopsis genomic DNA using primers listed in Table S1 and fused with the CDS of either SlSERK3A or SlSERK3B and cloned into pDONR207 (pBak1-SlSERK3A and pBak1-SlSERK3B). The CDS without stop of SlSERK3A, SlSERK3B and SlFLS2 were PCR amplified from tomato cdNA using primers listed in Table S1 and cloned into pDONR207. All resulting constructs were sequence verified.

pENTR207 (pBak1-SlSERK3A) and pENTR207(pBak1-SlSERK3B) were recombined into pEarleyGate303. pENTR207-Sfls2, pENTR207-SlSERK3A and pENTR207-SlSERK3B were recombined into pEarleyGate103 generating C-terminal GFP- His-tag fusion constructs behind the 3SS promoter. pENTR207-SlSERK3A and pENTR207-SlSERK3B were also recombined into pGBW14 generating C-terminal HA-tag fusion constructs behind the 3SS promoter. All resulting constructs were sequence verified and transformed into A. tumefaciens strain GV3101.

The cytoplasmic domains (C) of SlSERK3A and SlSERK3B were amplified from tomato cdNA using gene-specific primers (Table S2). Single point mutation variants of SlSERK3A CD (D418N) and SlSERK3B CD (D420N) were generated by PCR-based site-directed mutagenesis [65]. The amplified products were cloned into the pGEX4T-1 vector (Pharmacia) using EcoRI and NotI (NEB) to generate N-terminal GST fusion constructs. The resulting constructs were sequence verified.

Recombinant protein purification and in vitro phosphorylation assays

Recombinant fusion proteins were produced in Escherichia coli strain BL21 (DE3). Bacteria were induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 30°C for 4 h and extracted with lysis buffer containing 1 × PBS, 1 M DTT, 0.1 M ATP and 1 tablet protease inhibitor cocktail (Roche) for 10 ml buffer. The soluble fraction was used to enrich for the fusion proteins. GST-tagged fusion proteins were enriched using glutathione-agarose beads (BD Biosciences) according to the manufactures protocol. The eluted fusion proteins were adjusted to the same concentration in 1 × PBS and 10% glycerol and incubated in the kinase buffer immediately. The in vitro phosphorylation of each kinase (1 μg) with [γ-32P] ATP was assayed as described earlier [34].

RNA extraction and quantitative RT-PCR

RNA from leaves was extracted using TRIzol (Invitrogen) and treated with DNase I (New England Biolabs), while RNA from roots was extracted using hot phenol [59]. Five μg RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo-dT primer. For quantitative PCR, transcripts were amplified from 1 μl of a 5x diluted cdNA in a 15 μl reaction using gene-specific primers (Table S1) and iQ SYBR Green Supermix (Bio-Rad). The PCR amplification consisted of 3 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, 15 min at 72°C, followed by the generation of a dissociation curve. The generated threshold cycle (CT) was used to calculate transcript abundance relative to tomato Ubi gene as described previously [60]. DNase-treated RNA was used as template for control.

Bacterial virulence assay

To prepare bacterial inoculum, a lawn of Pseudomonas syringae pv. tomato (Pst) DC3000 or Pst DC3000 hrcC was grown overnight at 30°C on King’s medium B plates with appropriate antibiotics. Cells were collected from plates with 10 mM MgCl2 and adjusted to the desired colony-forming units (CFU)/ml. Five-week-old tomato VIGS plants were vacuum infiltrated with bacterial suspension (Pst DC3000 104 CFU/ml and Pst DC3000 hrcC 5×104 CFU/ml). To assess bacterial titer, five 1 cm² leaf discs were harvested and ground in 1 ml 10 mM MgCl2, diluted and plated [61].

Nematode virulence assay

Meloidogyne incognita was maintained on tomato cv. UC82B. Nematode eggs were extracted from infected roots in 0.5% NaOCl and eggs were hatched as described in Martinez de Illarduya et al. (2001) [62]. Three weeks after agroinfiltration, tomato roots were infected with freshly hatched 1000 infective-stage juveniles and maintained at 24°C. Six weeks later, roots were washed from soil particles, weighed and stained in 0.001% erioglaucine (Sigma). Individual roots were chopped into small pieces, mixed and egg masses were counted in two 10 g subsamples and the average calculated.

Oxidative burst assay

For tomato, one leaf sample (two 2 mm² per sample) from four 5-week-old plants was dissected with a sharp blade. For Arabidopsis, one leaf disc (4 mm diameter) from eight 4-week-old plants was sampled. Samples were floated overnight in sterile water and water was replaced with a solution of 1.7 μg/ml luminol (Sigma) and 10 μg/ml horseradish peroxidase (Sigma) containing...
1 µM flg22. Luminescence was captured using a multiplate reader (BMG Lumistar Galaxy Luminometer or BertholdTech TriStar).

**BL assays**

For Arabidopsis root inhibition assays, sterilized seeds were vernalized at 4°C then sown on 1/2 MS medium supplemented with 1 mM epibrassinolide (BL) (Sigma) and 0.8% agar. Plates were incubated at 22°C, 16 h light and 8 h dark, 100 µEinstein/ m²/s, for 9 days. Root length was measured for 30 seedlings per genotype and plotted as inhibition percentage compared with untreated roots [46].

For *SICPD* expression analysis, tomato leaflets were syringe infiltrated with 10 µM BL 12 h before use.

**Transient expression in *N. benthamiana* for microscopy and immunoprecipitation**

*Agrobacterium tumefaciens* containing constructs pEARLEY-GATE103-SIPLS2, pEARLEYGATE103-SISERK3A, pEARLEY-GATE103-SISERK3B, pCAMBIA-AtBAK1-mCherry, pGWBI4-SISERK3A and pGWBI4-SISERK3B were grown overnight in LB medium supplemented with appropriate antibiotics. Cultures were resuspended in 10 mM MgCl₂, 10 mM MES, and 150 µM acetoxyeringone to a final OD₆₀₀ = 0.2 to 0.5. After 3 h induction, cultures were infiltrated into 3-week-old *N. benthamiana* leaves using a needleless syringe.

**Microscopy**

For localization, 35S-SISERK3A-GFP or 35S-SISERK3B-GFP (pEarleyGate103) and 35S-AtBAK1-mCherry (pCAMBIA) proteins were transiently co-expressed in *N. benthamiana* leaves by agroinfiltration. Fluorescence was monitored 48 h later using a Leica SP2 Confocal microscope, with laser set at 488- and 563-nm to excite the GFP and mCherry, respectively, and images were collected through band emission filters at 500-530 and 600-630 nm, respectively.

For callose visualization, leaf discs were cleared using hot 95% ethanol, stained with 150 mM K2P04 (pH 9.5), 0.01% aniline blue for 2 h, and examined for UV fluorescence using Olympus BX51 microscope.

For H₂O₂ accumulation, leaf discs were vacuum infiltrating with 3,3’-diaminobenzidine (DAB) as previously described [63]. Tissues were cleared with ethanol and examined under a bright-field microscope.

**Co-immunoprecipitation and immunoblot analysis**

Leaf samples were processed as described earlier [28]. Samples were centrifuged at 13000 g for 20 min at 4°C, adjusted to 2 mg/ml total protein concentration, and pretreated with Protein A-agarose (Roach) for 3 to 4 h. Immunoprecipitations were performed on 1.5 ml total protein by adding anti-HA (Santa Cruz; 1:100) or anti-GFP (Roach; 1:100) overnight at 4°C. After incubation with 20 µl protein A-agarose for 4°C for 3 to 4 h, beads were washed 4 times with Tris-buffered saline (TBS) containing 0.5% (v/v) ND-10, immunoprecipitates were analyzed by immunoblotting.

Samples were electrophoresed on 8% SDS-acrylamide gels, transferred onto nitrocellulose membranes (BIO-RAD), blocked, incubated overnight with primary antibody [anti-GFP (Roach) 1:5000; anti-HA-HRP (Santa Cruz) 1:2000], and washed in TBST (TBS with 0.1% (v/v) Tween-20). For anti-GFP, blots were incubated with a secondary antibody anti-mouse-HRP [Santa Cruz] 1:5000. Signals were visualized using chemiluminescent substrate (Thermo Scientific) before exposure to X-ray film.

**Arabidopsis transgenic plants**

*Agrobacterium tumefaciens* GV3101 containing pBAK1-SISERK3A or pBAK1-SISERK3B in pEARLEYGATE303 were transformed into the bak1-4 mutant by the floral-dip method [64].

**Supporting Information**

*Figure S1* The SISERK3s have conserved SERK gene structure. SISERK3A and SISERK3B gene structures with introns and exons shown as lines and boxes, respectively. Areas of the proteins coded by each exon are indicated beneath the boxes. SP, signal peptide; LRR, leucine-rich repeat; LRRNT, LRR N-terminal domain; SPP, proline-rich region; TM, transmembrane; & kinase subdomains (I–XI).

*Figure S2* The characteristic domains of SERK proteins are conserved in SISERK3s. The deduced amino acid sequence of tomato SISERK3s protein was aligned with the five Arabidopsis and two *Nicotiana benthamiana* SERK members. Conserved and most conserved amino acids residues are highlighted in black and grey, respectively. The protein domains are indicated below the sequences. Roman numerals indicate the position of the protein kinase catalytic subdomains. LRR, Leucine-rich repeat; LRRNT, LRR N-terminal domain. Double line in red indicate LRR C-terminal (LRRCT) domain. Single underline in black indicates the catalytic loop. Red star indicates the mutation to generate kinase dead mutants (D to N).

*Figure S3* Gene fragments used in VIGS. (A) Position of TRV-SFLS2 VIGS fragment used for silencing relative to the full-length open reading frame (ORF). (B) Upper panel, position of TRV-SISERK3A VIGS fragment used for silencing relative to the ORF. Lower panel, line-up of the TRV-SISERK3A fragment with the corresponding region in SISERK3B. (C) Upper panel, position of TRV-SISERK3B VIGS fragment used for silencing relative to the ORF. Lower panel, line-up of the TRV-SISERK3B fragment with the corresponding region in SISERK3A. (D) Upper panel, position of TRV-SISERK3AB VIGS fragment (originating from SISERK3B) used for co-silencing SISERK3A and SISERK3B relative to their ORF. Lower panel, line-up of the TRV-SISERK3AB fragment with the corresponding regions in SISERK3A and SISERK3B.

*Figure S4* Silencing individually SISERK3A or SISERK3B does not result in cell death. (A) Transcript levels of VIGS-silenced genes were evaluated using qRT-PCR. Additional samples (to those presented in Figure 3A) of tomato cv. Moneymaker plants (used in the bacterial screens) treated with TRV empty vector (TRV). TRV-SISERK3A, TRV-SISERK3B, and TRV-SISERK3AB were evaluated. Expression was normalized against *UBI3*. Values are average ± SE of three technical replicates. *P*<0.05 significant difference from TRV (two-sample *t*-test). (B) Tomato cv. Moneymaker leaflets from plants silenced with the indicated TRV constructs. Photos were taken 3 weeks after TRV treatment. (C) Aniline blue-stained tomato leaf discs. No callose deposits were detected in leaflets silenced for either SISERK3A or SISERK3B. Leaves treated with 1 mM flg22 for 24 h were used as control.

*Figure S5* Co-silencing SISERK3A and SISERK3B result in cell death and reduced BR sensitivity. (A) DAB-stained tomato leaf discs. Leaflets of tomato cv. Moneymaker plants co-
silenced for SlSERK3A and SlSERK3B showing cell death and TRV empty vector (TRV) control for H₂O₂ accumulation. (B) Leaves of tomato cv. Moneymaker plants silenced for SlSERK3A, SlSERK3B or co-silenced and TRV control were evaluated for BR-sensitivity. Leaves were infiltrated with 10 nM BL. 12 h before use. Transcript levels of VIGS-silenced genes and SlCPD were evaluated using qRT-PCR normalized against UBIs. Values represent the average ± SE of three biological replicates. *P<0.05 and **P<0.01 indicate significant difference from the respective − BL control (two-sample t-test). This experiment was repeated twice with similar results.

References