BSU1 family phosphatases mediate Flagellin-FLS2 signaling through a specific phosphocode.

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Abstract

Among the hundreds of receptor-like kinases (RLKs) in plants, the brassinosteroid (BR) hormone receptor BR-INSENSITIVE 1 (BRI1) and the immunity receptor FLAGELLIN SENSING 2 (FLS2) share a common co-receptor kinase, but lead to distinct growth and immunity responses, respectively. Here we show that the BSU1 family of phosphatases, known to mediate BR inactivation of GSK3-like kinases, also mediate flagellin-FLS2 signaling to the MAP kinases, through different phosphocodes. Flagellin treatment induced phosphorylation of BSU1 phosphatase at serine-251 (S251) located in the N-terminal kelch-repeat domain. The *bsu* quadruple mutant (*bsu-q*), with loss or compromised function of all four BSU family members, showed defects in flagellin-triggered MAP kinase activation. The Botrytis-induced kinase 1 (BIK1), a substrate of FLS2, phosphorylated BSU1 at S251 in a flagellin-dependent manner. Mutation of S251 to alanine in BSU1 reduced its phosphorylation by BIK1, interaction with MEKK1 and ability to restore flagellin-induced MAP kinase activation of the *bsu* quadruple mutant, without affecting its ability to activate BR-dependent growth. Our results demonstrate that BSU1 acts as a substrate of BIK1 downstream of FLS2 in the innate immunity pathway, in addition to its role in the BR pathway. Our results suggest that different RLKs sharing downstream components may maintain signaling specificity through phosphocodes in higher plants.

Text

The Arabidopsis genome encodes hundreds of receptor-like kinases (RLKs), which perceive diverse extracellular signals and trigger distinct cellular responses such as growth, differentiation, and immunity. The mechanisms that ensure specificity of RLK signal transduction are not fully understood. Among over two hundred leucine-rich repeat receptor-like kinases (LRR-RLK) in

Arabidopsis[1], the brassinosteroid (BR) receptor BR-INSENSITIVE1 (BRI1) and the flagellin receptor FLAGELLIN SENSING 2 (FLS2) have been most extensively studied[2, 3]. BR binding to BRI1 triggers its association with and activation by the co-receptor BRI1-ASSOCIATED KINASE1 (BAK1)[4, 5]. BRI1 in turn phosphorylates the BR-SIGNALING KINASE (BSK) and CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) family of receptor-like cytoplasmic kinases (RLCKs), to activate the BRI1-SUPRESSOR1 (BSU1) family of phosphatases[6, 7], which dephosphorylate and inactivate the GSK3-like kinases, leading to PP2A-mediated dephosphorylation of the BZR1 family of transcription factors and activation of growth responses[8, 9]. Similarly, flagellin, a pathogen associated molecular pattern (PAMP), activates FLS2 by inducing association with the BAK1 co-receptor[10], and downstream signal transduction also involves RLCKs[11, 12]. Members of the RLCK VII subfamily such as Botrytis-induced kinase 1 (BIK1) and PBS1-LIKE (PBL) kinases lead to activation of MAP kinases[13, 14]. There is evidence that BIK1 plays a role in BR signaling, and BSKs are also involved in FLS2 signaling[15-17]. As such, the BRI1 and FLS2 pathways share many similarities and common components. The molecular mechanisms underlying their signaling specificity and crosstalk are not fully understood, whereas interactions at multiple levels appear to contribute to the tradeoff between growth and immunity[18].

The Arabidopsis BSU1 family phosphatases includes 4 members named BSU1 and BSU1-LIKE 1 (BSL1) to BSL3 (together named BSLs, or PPKLs for protein phosphatases with kelch-like domains)[19]. The partial loss-of-function quadruple mutant (*bsu1;bsl1;amiBSL2/3, bsu-q*) displays pleiotropic developmental defects such as dwarfism and stomata clustering[20, 21]. The triple knockout mutant *bsl1;bsl2;bsl3* is embryo lethal[22], unlike the BR-insensitive mutants, suggesting that the BSU1 family phosphatases play additional roles beyond BR signaling.

To examine whether BSU1 family is involved in FLS2 signaling, we investigated flagellin-

induced posttranslational modification of BSU1 family proteins. We performed metabolic Stable Isotope Labeling In Arabidopsis followed by Immuno-Precipitation and Mass Spectrometry (SILIA IP-MS) analysis, with the isotopes switched in replicate experiments. We found that the serine-251 (S251) residue of BSU1 was phosphorylated in the samples treated with the 22-amino acid flagellin peptide (flg22) (Extended Data Fig. 1a, b). Targeted quantification using parallel reaction monitoring (PRM) analysis further showed that phosphorylation of BSU1 at S251 was increased by over 4 folds after flg22 treatment in both reciprocal treatment experiments (Fig. 1a, Extended Data Fig. 1c-e). The S251 residue of BSU1 is located in the N-terminal Kelch-repeat domain, and is conserved in BSLs and other plant species (Fig. 1b, Extended Data Fig. 2), suggesting that the S251 phosphorylation of BSU1 family members can be a general mechanism of pathogen-triggered immunity (PTI) signaling.

To determine whether BSLs play a role in flagellin signaling, we analyzed flagellin activation of MAP kinases in the mutants lacking various BSL members. In addition to *bsu-q*, we generated *bsl2;bsl3* double mutant, *bsu1;bsl1;bsl3* triple mutant, and an inducible quadruple mutant (*bsu-i*) by expressing the artificial microRNA targeting BSL2/3 (same as used in *bsu-q*) from the estradiol inducible promoter in the *bsu1;bsl1;bsl3* triple mutant background. We found that flg22 induced much weaker MAP kinase phosphorylation in the *bsu-q* mutant than in the wild-type plants (Fig. 1c). However, the *bsl2;bsl3* double mutant showed normal MAP kinase phosphorylation in response to flg22, (Extended Data Fig. 3a).Without estradiol treatment, the

bsu-i plants showed slightly reduced flg22-induced MAP kinase activation compared to the wild-type (Fig. 1d). Estradiol treatment significantly decreased the BSL2 protein level and the flg22-induced MAP kinase phosphorylation in the *bsu-i* plants compared to untreated plants, but had no

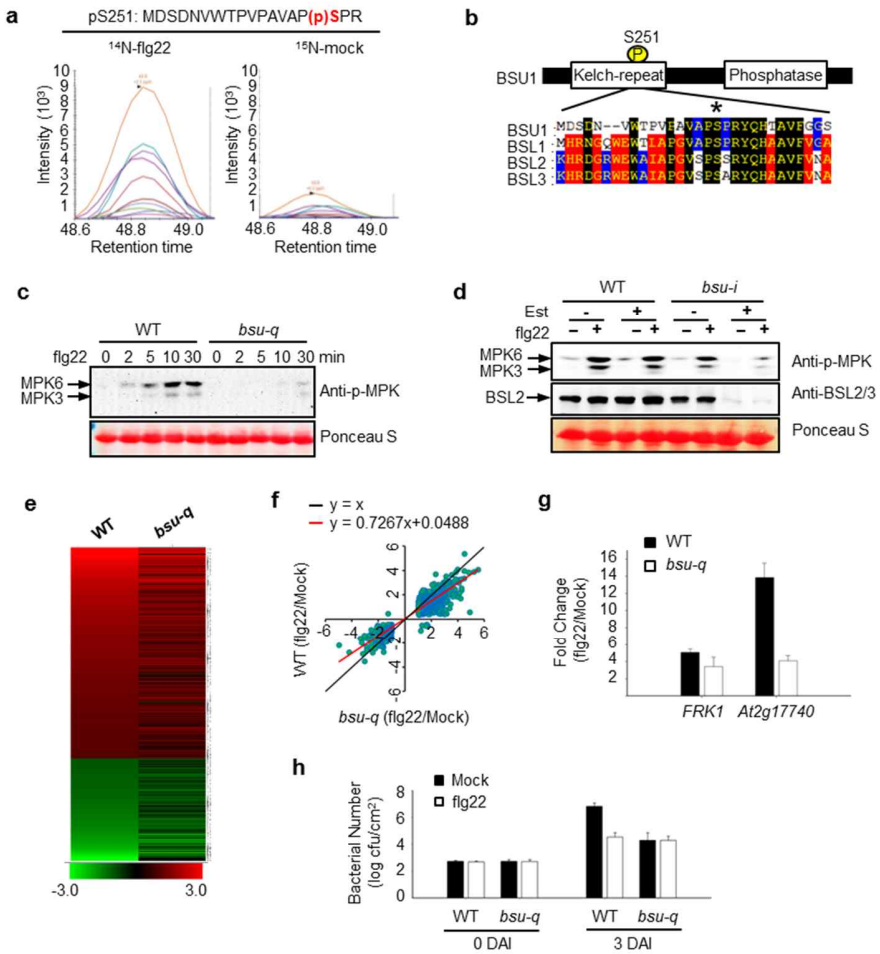

Fig. 1 BSU1 family phosphatases are required for flg22-induced immune responses.

a) Identification of flg22-induced BSU1 S251 phosphorylation by SILIA IP-MS using PRM analysis. b) The locus and conservation of BSU1 S251 residue. Asterisk denotes BSU1 S251. c) Flg22-induced MPK3/6 phosphorylation in wild-type (WT) and *bsu-q*. Eight-week short-day grown leaves were treated with 100 nM flg22 for 2, 5, 10 and 30 min. Fifty μg of protein extracts were loaded to 10% SDS-PAGE gel. d) Flg22-induced MPK3/6 phosphorylation of WT and *bsu-i*. Nine-day old seedlings grown on 20 uM estradiol or the same amount of DMSO were treated with 100 nM flg22 for 10 min. Immunoblot was performed using anti-phospho-p44/42 MAPK antibody. e) Heat map of $\log_2$ fold change values of flg22-responsive genes in WT and *bsu-q* from RNA-seq data. f) Scatter plot of $\log_2$ fold change values of flg22-regulated genes in WT vs. *bsu-q*. Total 1590 genes from flg22-regulated genes in WT($-2 \geq FC \geq 2$) were compared to those in *bsu-q*. g) qRT-PCR of flg22-induced *FRK1* and *At2g17740* expression in WT and *bsu-q*. h) Flg22-induced bacterial growth inhibition in WT and *bsu-q*. Each replicate includes four leaf discs and 12 biological replicates were used, and the experiment was repeated at least three times with similar results. The average bacterial titer ±SD is reported.

effect in the wild-type plants (Fig. 1d). Together these results demonstrate that the BSU1 family

members redundantly play an important role in flg22-induced MAP kinase activation. In addition, MAP kinase phosphorylation caused by another bacterial elicitor, elf18, was reduced in the *bsu-i* mutant, suggesting that BSU1 family members are involved in the MAP kinase activation by multiple PAMP signals (Extended Data Fig. 3b)[23].

Next, we examined whether mutations of BSU1 family members compromise immune responses. We performed RNA-Seq analysis of gene expression in the wild-type and *bsu-q* seedlings treated or untreated with flg22. The results show that the expression of 492 and 549 genes were increased or decreased in *bsu-q* compared to the wild-type (Extended Data Table 1). Interestingly, salicylic acid (SA)-responsive *PR1* and *BGL2* genes[24] and other defense-related genes were highly increased in *bsu-q*, suggesting that basal defense level including SA signaling was elevated in *bsu-q*. Similar elevated defense gene expression has been observed in other immune signaling mutants such as *bak1*, *bik1*, *mekk1*, and *mpk4,* and considered a result of the activation of plant's defense surveillance system[25-28]. In the wild-type plants, transcript levels of 1283 and 619 genes were increased and repressed, respectively, at least by 2 folds after flg22 treatment (Extended Data Table 2). In the *bsu-q* mutant, fewer numbers of genes were induced (977) and repressed (339) by the same flg22 treatment (fold change ≥ 2; Extended Data Table 3). Heatmap and scatter plot analysis showed that most of the flg22-responsive genes displayed lower magnitude of changes in *bsu-q* upon flg22 treatment than in the wild-type (Fig. 1e, f). Quantitative RT-PCR further confirmed that the *bsu-q* mutant showed high basal expression levels but weaker flg22 induction of the PTI marker genes, *FRK1* and *At2g17740*[29], suggesting BSU1 family phosphatases are required for flg22-induced PTI gene expression (Fig. 1g).

In a bacterial infection assay, *bsu-q* was more resistant to *Pseudomonas syringae* pathovar *tomato* (*Pst*) strain DC3000 than the wild-type without flg22 elicitation (Fig. 1h). Similar enhanced

basal defense responses were also observed in the *bik1* mutant, which was shown to be due to elevated level of SA[30]. Pretreatment with flg22 inhibited bacterial growth in wild-type plants, but had no significant effect in the *bsu-q* mutant, (Fig. 1h), indicating a role of BSU family in flg22-triggered defense against pathogen.

Given that BSU1 family members transduce the signal from the RLCKs (BSK1 and CDG1) to GSK3-like kinases in the BRI1 signal transduction pathway[6, 20], and BIK1 impacts both BR and flagellin signaling[15], we hypothesized that BSU1 family might mediate signaling from BIK1/PBLs RLCK proteins in FLS2 signaling. Thus, we tested the physical interaction of BSU1 family members with BIK1 and several PBLs. Yeast two-hybrid assays showed that BSU1 family members interacted with a subset of RLCK VII subfamily proteins with different specificity and affinity (Fig. 2a, Extended Data Fig. 4).

Consistent with the yeast two-hybrid assay result, *in vitro* pull-down assays showed that glutathione *S*-transferase (GST)-BIK1 interacted with maltose-binding protein (MBP)-BSU1 but the interactions with BSLs were weak or undetectable (Fig. 2b). Moreover, when BIK1-myc and BSU1-YFP were co-expressed in both *Nicotiana benthamiana* and *Arabidopsis* plants, BIK1-myc was co-immunoprecipitated with BSU1-YFP (Fig. 2c), indicating that BIK1 and BSU1 physically associate in plant cells. Further, flg22 treatment increased BIK1's ability to bind to BSU1 (Fig. 2d).

Consistent with their interaction, GST-BIK1 phosphorylated MBP-BSU1 *in vitro* (Fig. 2e). BIK1 more strongly phosphorylated the N-terminal Kelch domain than the C-terminal phosphatase domain *in vitro* (Fig. 2f). To test whether BIK1 phosphorylation of BSU1 is regulated by flg22 treatment, we performed kinase assays using immunopurified BIK1. Consistent with a previous

report[31], BIK1-HA immunoprecipitated from flg22-treated plants showed a slower mobility in SDS-PAGE gel and an enhanced autophosphorylation activity (Fig. 2g). Notably, flg22 treatment greatly increased BIK1-HA phosphorylation of MBP-BSU1 (Fig. 2g), indicating that the flg22-FLS2 signaling promotes BIK1 phosphorylation of BSU1.

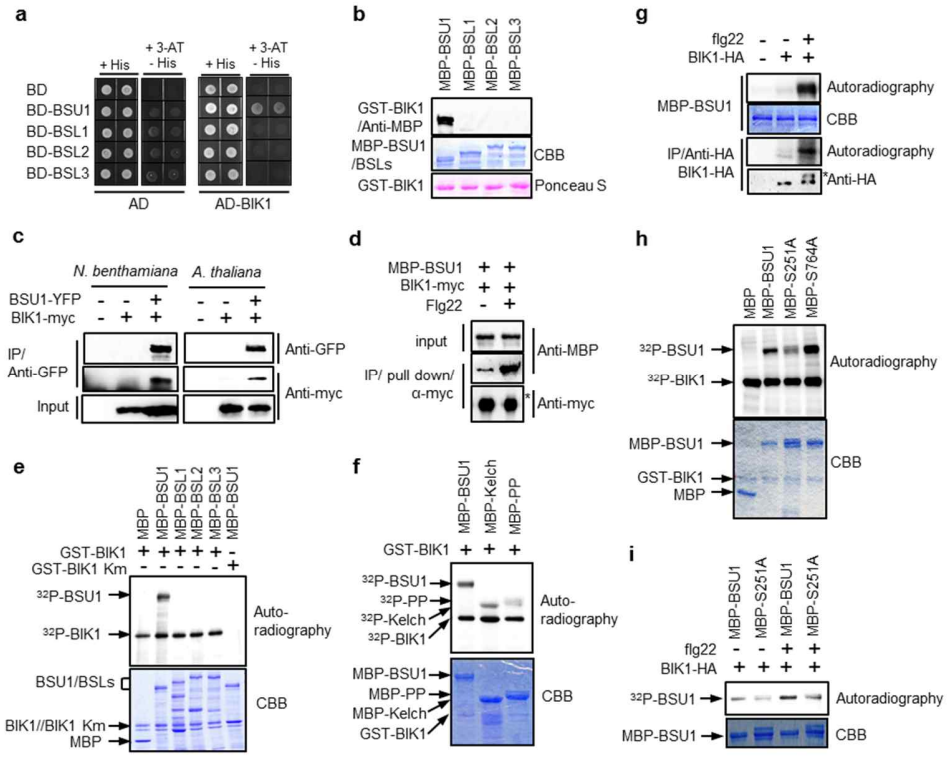

Fig. 2 BSU1 interacts with and is phosphorylated by BIK1 in a flg22-dependent manner.

a) Yeast-two-hybrid assay for interaction between BIK1 and BSU1 family members. Yeast cells co-expressing the indicated AD and BD proteins were grown on synthetic dropout media. b) *In vitro* pull-down of MBP-BSU1/BSLs by GST-BIK1, analyzed by anti-MBP immunoblotting. c) Co-immunoprecipitation assay of BSU1 with BIK1 protein expressed in *N. benthamiana* leaves and *Arabidopsis* transgenic plants. d) BIK1-myc proteins were immunopurified by anti-myc antibody from plants treated with 100 nM flg22 or mock for 30 min, and used to pulldown MBP-BSU1 *in vitro*. Asterisk indicates phosphorylated BIK1. e) Auto-radiograph of gel staining (CBB) *In vitro* kinase assay of BIK1 phosphorylation of the indicated MBP and MBP fusion proteins. f) *In vitro* kinase assay (as panel e) of BIK1 phosphorylation of BSU1 N-terminal (Kelch) and C-terminal (PP) domains. g) *In vitro* kinase assay of flg22-induced BIK1 phosphorylation of BSU1 using immunopurified BIK1-HA. BIK1-HA immunoprecipitated using anti-HA antibody from thirteen-day old *BIK1::BIK1-HA* seedlings treated with 1 uM flg22 or mock for 10 min was incubated with MBP-BSU1 and ^{32}P-γ-ATP. BIK1-HA proteins were analyzed by immunoblotting using anti-HA antibody. Asterisk indicates phosphorylated BIK1. h) *In vitro* kinase assay of BIK1 phosphorylation of wild-type and mutant (S251A and S764A) BSU1. i) *In vitro* kinase assay of flg22-induced BIK1 phosphorylation of wild-type and mutant (S251A) BSU1 using immunopurified BIK1-HA. BIK1-HA was immunoprecipitated using anti-HA antibody from twelve-day old *BIK1::BIK1-HA* seedlings treated with 1 uM flg22 or mock for 10 min, and was incubated with MBP-BSU1 or MBP-BSU1-S251A (MBP-S251A) and ^{32}P-γ-ATP.

To investigate whether BIK1 phosphorylates BSU1 at S251, which is phosphorylated upon flg22 treatment (Fig. 1a), *in vitro* kinase assays were performed using BIK1 and the wild-type or site-mutated BSU1. The results indicate that BIK1 phosphorylation of BSU1 was greatly reduced by mutation of S251 to alanine (S251A), but not by the mutation of S764 residue of which phosphorylation is required for BR-BRI1 signaling[6] (Fig. 2h). In addition, the flg22-induced BIK1 phosphorylation of BSU1 was also reduced by S251A mutation (Fig. 2i). These results indicate that BIK1 phosphorylates the S251 residue of BSU1 in response to bacterial flagellin.

To test if BSU1 directly regulates MAP kinase cascade activation, we analyzed BSU1 interaction with MEKK1, a MAP kinase kinase kinase (MAPKKK) involved in flg22-induced MAP kinase activation[32, 33]. We found that BSU1 can interact with MEKK1 *in vitro* and *in vivo* (Fig. 3a-d). Interestingly, S251A mutation decreased the BSU1 interaction with MEKK1 *in vivo* (Fig. 3d). To test whether phosphorylation of the S251 is required for BSU1-mediated MAP kinase activation, we generated transgenic *bsu-i* plants expressing BSU1-YFP or BSU1-S251A-YFP. Two independent *BSU1-YFP:bsu-i* transgenic lines, which expressed different levels of BSU1-YFP, showed normal flg22-induced MAP kinase activation as wild-type, whereas the *BSU1-S251A-YFP:bsu-i* plants showed lower flg22-induced MAP kinase activation, similar to the *bsu-i* mutant (Fig. 3e). In addition, the *BSU1-S251A-YFP:bsu-i* plant showed a weaker response to flg22 pre-treatment in bacterial growth inhibition assay than the *BSU1-YFP:bsu-i* plants (Fig. 3f). These results support that BIK1 phosphorylation of BSU1 at S251 contributes to flagellin induction of MAP kinase activation and immunity.

To evaluate the function of BSU1 S251 phosphorylation in BR signaling, we examined the BR-dependent growth in the transgenic plants expressing the wild-type BSU1-YFP or the mutant BSU1-S251A-YFP proteins. Overexpression of BSU1 reduced plant sensitivity to propiconazole

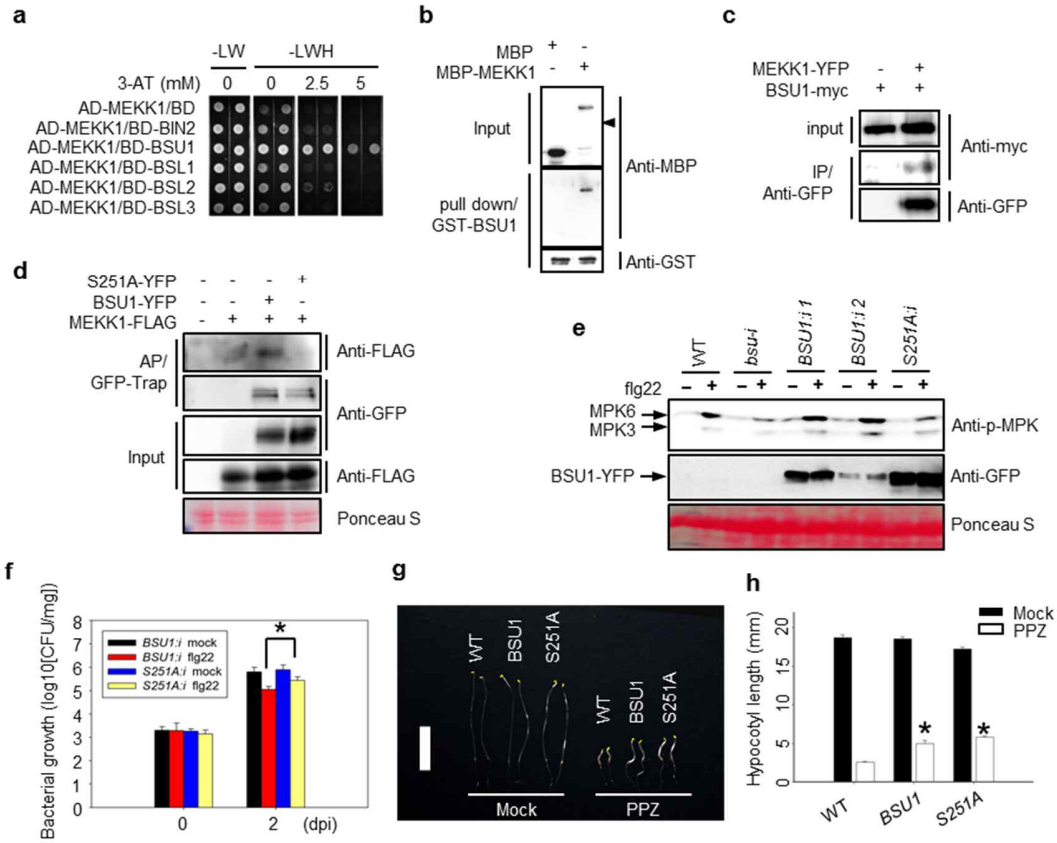

Fig. 3 BSU1 S251 is required for the MEKK1 interaction, MAP kinase activation and defense against bacteria but not growth response.

a) Yeast-two-hybrid assay for interaction between MEKK1 and BSU1 family members. Yeast cells co-expressing *AD-MEKK1* and *BD-BSU1/BSL*s were grown on synthetic dropout medium containing 2.5 and 5 mM 3-amino-1, 2, 4-triazole (3-AT). b) *In vitro* pull-down assay of GST-BSU1 and MBP-MEKK1. MBP-MEKK1 was pulled down by GST-BSU1 BP and the elutes were analyzed by immunoblotting using anti-MBP and anti-GST antibody. c) Co-immunoprecipitation assay of BSU1 with MEKK1 protein expressed in *N. benthamiana* leaves. d) Co-immunoprecipitation assay of BSU1 and BSU1-S251A with MEKK1 protein expressed in *Arabidopsis* transgenic plants. Seedling were grown for 9 days and treated with 1 uM flg22 for 10 min. BSU1-YFP and BSU1-S251A-YFP were pulled down by GFP-Trap beads and co-purified MEKK1-FLAG was detected by anti-FLAG antibody. e) Flg22-induced MPK phosphorylation in *35S::BSU1-YFP:bsu-i* (*BSU1:i*) and 35*S::BSU1-S251A-YFP:bsu-i* (*S251A:i*) plants. Eight-day old seedlings grown on 20 uM estradiol were treated with 10 nM flg22 for 15 min. Immunoblot was performed using anti-phospho-p44/42 MAPK and anti-GFP antibodies. Ponceau S staining image shows equal loading. f) Flg22-induced bacterial growth inhibition in *BSU1:i* and *S251A:i* seedlings. Each replicate includes 12 mg or more seedlings and 8 biological replicates were used. The average bacterial titer ±SD is reported. g, h) PPZ resistance of the wild-type, *35S::BSU1-YFP* (*BSU1*) and *35S::BSU1-S251A-YFP* (*S251A*) seedlings. g) Phenotype of 5-day-old etiolated seedlings grown in mock and PPZ media. Bar=10 mm. h) Hypocotyl length of etiolated seedlings. Asterisks denote significant difference from wild-type by t-test ($P<0.001$, n =13 for wild-type and n=14 for *BSU1* and *S251A*). Experiments performed 3 repeats and showed similar results.

(PPZ)[34], a BR biosynthesis inhibitor, in hypocotyl elongation of etiolated seedling (Fig. 3 e, f).

Similar to BSU1-overexpression plants, the BSU1-S251A-overexpression plants displayed considerable PPZ-resistance, indicating that S251 phosphorylation on BSU1 is not required for BR-mediated growth response (Fig. 3e, f, Extended Data Fig. 5). Given that the S764 residue in C-terminal region of BSU1 is required for BR signaling[6], these observations indicate that the signaling specificities for BSU1 family members in the BRI1 and FLS2 pathways are determined by phosphorylation of different residues, namely phosphocodes.

Collectively, our results show that BSU1 family members mediate FLS2 signaling downstream of RLCKs including BIK1 and upstream of the MAP kinases. Our data suggest that these protein phosphatases might be common signaling mediators of RLK-RLCKs pathways. Recent studies showed that RLCKs also directly interact with BIN2 and MAPKKKs to regulate growth and immune signaling, respectively[33, 35-37]. How BSU1 activates the MAP kinase cascade remains a subject for future study, but phosphocode-mediated direct interaction with downstream MAPKKKs seems to play a role. We showed that BSU1 family phosphatases play essential roles in BR and flagellin signaling pathways through different phosphocodes[20]. As genetic evidence supports broad functions of the BSU1 family phosphatases[20-22, 38], phosphocoding is likely a major mechanism that allows these cytoplasmic proteins to function in multiple RLK pathways. Our study further suggests that both expansion of the RLK gene family and addition of phosphocodes in shared downstream components contribute to the evolution of the large numbers of RLK pathways with specific cellular functions in plants.

Methods

Plant material and growth condition

All *Arabidopsis thaliana* plants used in this study are in Columbia ecotype background. *bsu1-4* (SALK_030721)[20], *bsl1-2* (SALK_051383)[20], *bsl2-3* (SALK_055335)[22], *bsl3-2* (SALK_072437)[22], *bsu-q*[20], *35S::BSU1-YFP*[9], *BSU1pro::BSU1-YFP*[39] and *BIK1::BIK1-HA*[11] were previously described. The *bsl2;bsl3* and *bsu1;bsl1;bsl3* were generated by crossing the single mutants. *Arabidopsis* seedlings were grown on 1/2 Murashige-Skoog (MS) agar medium containing 1% sucrose under continuous light in a growth chamber (Percival) at 22 °C. For estradiol treatment, seeds were planted on the MS medium treated with β-estradiol (Sigma E2758). For RNA-seq experiment, seedlings were grown in dark for 3 days and incubated in light for 12 days after removing *bsu-q* seedlings with weak phenotype (long hypocotyl). Plants were grown on soil in a greenhouse with a 16-h light/8-h dark cycle at 22–24 °C for general growth and seed harvesting.

Plasmid constructions

To generate yeast-two-hybrid, *35S::BIK1-myc*, *35S::MEKK1-YFP* and *MBP-MEKK1* constructs, full-length coding sequence (CDS) without stop codon of the genes were amplified by PCR from *Arabidopsis* cDNA using gene specific primers described in Extended Data Table 4. Each CDS was cloned into pENTR/SD/D-TOPO vector (Invitrogen) and subcloned into gateway-compatible destination vectors (pCAMBIA 1390-4myc-6His, pEarleyGate101[40], gcpMALc2x, pXDGATcy86, and pGADT7) by LR reaction. MBP-BSU1[20], MBP-BSU1-Kelch[6], MBP-BSU1-PP[6], MBP-BSL1~BSL3[6], GST-BIK1[12] and GST-BIK1Km[12] were previously described. Point mutations were generated by site-directed mutagenesis PCR according to the manufacturer's protocol (New England Biolab). BSU1 clone in pENTR/SD/D-TOPO vector[6] was used as a template for mutagenesis.

PAMP treatment

The flg22 peptide was synthesized by the Protein and Nucleic Acid Facility at Stanford University (http://pan.stanford.edu/). For immunoblot analysis, flg22 was treated to seedlings or rosette leaves in autoclaved distilled water containing 0.05% Silwet L-77. For RNA-seq, seedlings on plates were submerged in autoclaved distilled water containing 0.05% Silwet L-77 treated with 1 μM flg22 or water for 1 min. After decantation of the solution, the seedlings were incubated in growth chamber for 1 hr. For kinase assay using immunopurified BIK1, *BIK1::BIK1-HA* seedlings were treated with 1 μM flg22 in autoclaved distilled water for 10 min.

Immunoblot

Total proteins from plant materials were extracted with IP buffer (50 mM Tris at pH 7.5, 50 mM NaCl, 0.3 M sucrose, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail) or 2× SDS sample buffer (100 mM Tris at pH 6.8, 4% SDS, 20% glycerol and 4% β-mercaptoethanol). After boiling with 2× SDS sample buffer, proteins were loaded on SDS-PAGE gel. Separated proteins were transferred to a nitrocellulose membrane, and probed with anti-phosphop44/42 mitogen-activated protein kinase (Erk1/2) (Thr202/Tyr204) (Cell Signaling; 1:1,000 dilution), anti-MBP (New England Biolab; 1:2,000 dilution), anti-myc (Cell Signaling; 1:3,000 dilution), anti-HA (Roche; 1:1,000 dilution), anti-BSL2/3 (AbFrontier; 1:2,000 dilution) and anti-GFP (homemade or TRANS, M018) antibodies.

Bacterial growth assays

Bacterial growth assay was performed as described previously[41]. To monitor *Pst* DC3000 growth in plants, leaves of 6-week-old plants were hand-infiltrated with a 1×10^5 colony forming unit (CFU)/ml suspension of bacteria in 1 mM $MgCl_2$ using a needless syringe. For flg22 pretreatment, the leaves were hand-infiltrated with water or 1 μM flg22 one day before the bacterial inoculation. Leaf discs per treatment per time point were collected and ground in 1 mM $MgCl_2$ and then spotted on nutrient yeast glycerol agar plates in triplicate to determine the bacterial titer. Seedling flood inoculation assay was performed as described previously with minor modifications[42]. Briefly,

seedlings grown on media containing 10 μM estradiol were treated with 1 μM flg22 or distilled water one day prior to *Pseudomonas* inoculation. Bacteria inoculum (10^5 CFU/ml) containing 0.025% Silwet L-77 was dispensed into 7-day-old Arabidopsis seedlings grown on plates. Internal bacterial populations were evaluated from eight biological replicates and each replicate represented a pooled sample of at least 12 mg seedlings.

***In vitro* pull-down assay**.

Recombinant GST-tagged proteins were purified from *E*. coil using Glutathione Sepharose bead (GE healthcare). The GST-tagged protein-bead complexes were incubated with 2 μg of MBP-tagged proteins in 1 mL of pull-down buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% NP-40 and 0.05 mg/mL BSA) at 4 °C for 1 hr. Then, the protein-bead complex was washed twice with 1 ml of washing buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.1% NP-40). Associated proteins were eluted with 2× SDS sample buffer and analyzed by immunoblotting.

***In vitro* kinase assays**.

In vitro kinase assays using recombinant proteins were performed as described previously[6]. Briefly, one μg of GST–BIK1 or GST–BIK1-Km was incubated with MBP-BSU1, MBP-BSL1, MBP-BSL2 or MBP-BSL3 in the kinase buffer (20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT) containing 100 μM ATP and 10 μCi [^{32}P]-γ-ATP at 30 °C for 3 hr. For kinase assay using immunopurified BIK1, total protein was extracted from *BIK1::BIK1-HA* seedlings using immunoprecipitation (IP) buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 0.3 M sucrose, 1% Triton X-100, 1 mM PMSF and 1× protease inhibitor cocktail), centrifuged at 4,000 rpm for 5 min. Supernatants were centrifuged at 12,000 rpm for 10 min. The cleared supernatants were incubated with anti-HA antibody (Roche, 3F10) immobilized on Protein A/G agarose beads (Pierce) for 2 hr. The beads were then washed five times with 1 mL of washing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) followed by the kinase buffer (25 mM Tris, pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT). Aliquots of BIK1-HA-bead complex were incubated with 1 ug of MBP-BSU1 in the kinase buffer containing 10 μM ATP and 10 μCi [^{32}P]-γ-ATP at room temperature with

shaking at 1,000 rpm for 1.5 hr. Proteins were eluted with 2× SDS sample buffer and separated by SDS-PAGE. Autoradiograph images were obtained by Typhoon Trio imager (GE healthcare).

Co-immunoprecipitation assays

For transient protein co-expression in *N. benthamiana*, *Agrobacterium* cells containing *35S::BIK1-myc* or *35S::BSU1-YFP* expression vectors were washed and resuspended in the induction medium (10 mM MES buffer at pH 5.6, 10 mM $MgCl_2$ and 150 mM acetosyringone) and were infiltrated into young *N. benthamiana* leaves. Two days after infiltration, the infiltrated leaves were harvested. For the MEKK1-BSU1 interaction, *Agrobacterium* cells containing *35S::BSU1-myc* and *35S::MEKK1-YFP* expression constructs were co-infiltrated in *N. benthamiana* leaves in the same way. Total proteins were extracted with IP buffer. The proteins were immunoprecipitated using anti-GFP antibody-bead complex. After washing twice with 0.5 mL of washing buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 30 mM Sucrose, 0.2% Triton X-100, 1X protease inhibitor, 0.2 mM PMSF), the co-immunoprecipitated proteins were eluted by 2× SDS sample buffer.
For Arabidopsis, 12-day old wild-type, *35S::BIK1-myc*, and *35S::BIK1-myc;BSU1pro::BSU1-YFP* seedlings were crosslinked for 15 min in 1% formaldehyde under vacuum. Total protein was extracted with IP buffer. After centrifugation at 12,000 rpm for 10 min, the supernatant was incubated with anti-GFP (10 μg) immobilized on UNOsphere SUPrA beads (Biorad) at 4 °C for 2.5 hr. The beads were then washed five times with 1 ml of washing buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and eluted samples were analyzed by immunoblot. For the MEKK1- BSU1 interaction, 9-day old wild-type, *MEKK1-FLAG*, *35S::BSU1-YFP;MEKK1-FLAG* and *35S::BSU1-S251A-YFP;MEKK1-FLAG* seedlings were treated with 1 μM flg22 for 10 min. Total protein was extracted with IP buffer 2 (50 mM Tris, pH7.5, 50 mM NaF, 0.3 M sucrose, 0.2% Triton X-100) with 1× Protease Inhibitor (Pierce). After centrifugation at 12,000 rpm for 10 min, the supernatant was incubated with GFP-Trap magnetic agarose beads at 4 °C overnight. The beads were then washed three times with 1 mL of washing buffer 2 (50 mM Tris at pH 7.5, 100 mM NaCl, 50 mM NaF, 0.1% Triton X-100) and eluted samples were analyzed by immunoblot.

Stable Isotope Labeling In Arabidopsis followed by Immuno-Precipitation and Mass Spectrometry (SILIA IP-MS) analysis

Samples for initial screening for flg22-regulated phosphorylation sites on BSU1 were prepared as the following. Transgenic *35S::BSU1-YFP* seedlings were grown on ^{14}N (1/2 MS without nitrogen media (PhytoTechnology Laboratories), NH_4NO_3 [0.5 g/L, Sigma], KNO_3 [0.5 g/L, Sigma], pH 5.7) or ^{15}N media (1/2 MS without nitrogen media (PhytoTechnology Laboratories), $NH_4{}^{15}NO_3$ [0.5 g/L, Cambridge Isotope Laboratory], $K^{15}NO_3$ [0.5 g/L, Cambridge Isotope Laboratory], pH 5.7) for 10 days. ^{14}N- and ^{15}N-labeled samples were treated with 1 μM flg22 or mock solution for 10 min in reciprocal experiments. Equal amount of tissues ground in liquid nitrogen were mixed. Proteins were then extracted in IP buffer with a phosphatase inhibitor (Roche, PhosSTOP), centrifuged at 4,000 rpm for 5 min and filtered through miracloth, and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new tube, and the same volume of IP buffer without detergent was added to dilute the detergent to 0.5%, then was incubated with anti-GFP antibody with UNOsphere SUPrA bead (Biorad) at 4 °C for 1 hr, followed by 5 times washing with wash buffer. Samples for targeted quantification of S251 phosphorylation on BSU1 were prepared as the following. The BSU1-YFP seedlings were grown on ^{14}N or ^{15}N media for 13 days and treated with 1 μM flg22 or mock solution for 10 min. Total proteins from mixed samples (^{14}N-flg22/^{15}N-mock and ^{14}N-mock/^{15}N-flg22) were extracted with IP buffer 2 (50 mM Tris, pH7.5, 50 mM NaF, 0.3 M sucrose, 0.2% Triton X-100) with 1× Protease Inhibitor (Pierce) and phosphatase inhibitor (Roche, PhosSTOP), and centrifuged for 2,000 rpm for 5min. Resulting supernatant was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new tube and incubated with anti-GFP nanobody conjugated magnetic agarose bead (Allele) at 4 °C for 1 hr, followed by 5 times washing with wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Triton X-100). Samples were eluted with 2× SDS sample buffer, separated by SDS-PAGE, followed by Colloidal Blue staining (Invitrogen).

BSU1-YFP protein bands were excised and subjected to in-gel digestion with trypsin. Peptide mixtures were desalted using C18 ZipTips (Millipore). Data were acquired in the Data Dependent Acquisition (DDA) mode. Briefly, peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS) on a Nanoacquity ultraperformance liquid chromatography system (Waters) connected to Linear Trap Quadrupole (LTQ) Orbitrap Velos mass spectrometer

(Thermo). Peptides were separated using analytical column Easy-Spray C18 column (75 µm × 150 mm) (Thermo, ES800). The flow rate was 300 nL/min, peptides were eluted by a gradient from 2 to 30% solvent B (acetonitrile/0.1%formic acid) over 57 min, followed by a short wash at 50% solvent B. After a precursor scan was measured in the Orbitrap by scanning from mass-to-charge ratio 350 to 1400, the six most intense multiply charged precursors were selected for collision-induced dissociation (CID) in the linear ion trap.

MSMS data was converted to peaklist using an in-house script PAVA, and data were searched using Protein Prospector against the TAIR database *Arabidopsis thaliana* from December 2010 (https://www.arabidopsis.org/), concatenated with sequence randomized versions of each protein (a total of 35386 entries). A precursor mass tolerance of 20 ppm and a fragment mass error tolerance of 0.6 dalton were allowed. Carbamidomethylcysteine was searched as a constant modification. Variable modifications include protein N-terminal acetylation, peptide N-terminal Gln conversion to pyroglutamate, and Met oxidation. Subsequent searches were performed to find those peptides modified by phosphorylation. The search parameters were as above, but this time allowing for phosphorylation on Ser, Thr, and Tyr. Assignment of modified peptide was checked manually and is illustrated in Extended Data Fig. 1a. Quantification was done using Protein Prospector.

For targeted quantification, data was acquired in the Parallel Reaction Monitoring (PRM) mode. Peptides were separated using an EasyLC1200 system (Thermo) connected to high performance quadruopole Orbitrap mass spectrometer Q Exactive HF (Thermo). Peptides were separated using analytical column Easy-Spray C18 column (75 µm × 250 mm) (Thermo, ES802). The flow rate was 300 nL/min, peptides were eluted by a gradient from 3 to 28 % solvent B (80% acetonitrile/0.1%formic acid) over 45 min, 28 to 44% solvent B for 15 min, followed by a short wash at 90% solvent B. Samples were analyzed using PRM mode with an isolation window of 1.4 Th. PRM scans were done using 30000 resolutions (AGC target 2e5, 200 ms maximum injection time) triggered by an inclusion list (Extended Data Table 5). Normalized collision energy 27 was used in a higher-energy dissociation mode (HCD). PRM data were analyzed using skyline software[43].

Transcript analysis

Total RNA was extracted from seedlings using the Spectrum Plant Total RNA kit (Sigma). M-MLV reverse transcriptase (Fermentas) was used to synthesize complementary DNA (cDNA) from the RNA. Quantitative real-time PCR (qPCR) was carried out using LightCycler 480 (Roche) and the Bioline SYBR Green Master Mix. *FRK1* and *At2g17740* primers were previously described[29]. *PP2A* was used as an internal control[44].

RNA-seq analysis

Total RNA was extracted from the wild-type and *bsu-q* seedlings treated with flg22 or mock solution using the Spectrum Plant Total RNA kit (Sigma). cDNA libraries were constructed using a TruSeq RNA sample preparation kit (Illumina) according to the manufacturer's instruction. Samples of three replicates were sequenced on an Illumina HiSEQ2000 sequencing machine. Mapping of reads to the reference genome (TAIR10) and calling differentially expressed genes was conducted via the iPlant collaborative Discovery Environment (de.iplantcollaborative.org/de/)[45]. Expression data was clustered and visualized with the TIGR MeV program (http://mev.tm4.org/)[46]. Scatter plot of flg22-responsive genes were analyzed with Microsoft EXCEL.

References

1. Torii, K. U. in International Review of Cytology 1-46 (Academic Press, 2004).
2. Wang, Z.-Y., Seto, H., Fujioka, S., Yoshida, S. & Chory, J. BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410, 380-383 (2001).
3. Gómez-Gómez, L. & Boller, T. FLS2: An LRR Receptor-like Kinase Involved in the Perception of the Bacterial Elicitor Flagellin in Arabidopsis. Molecular Cell 5, 1003-1011 (2000).
4. Li, J. et al. BAK1, an Arabidopsis LRR Receptor-like Protein Kinase, Interacts with BRI1 and Modulates Brassinosteroid Signaling. Cell 110, 213-222 (2002).
5. Wang, X. et al. Identification and Functional Analysis of in Vivo Phosphorylation Sites of the Arabidopsis BRASSINOSTEROID-INSENSITIVE1 Receptor Kinase. The Plant Cell 17, 1685 (2005).

6. Kim, T.-W., Guan, S., Burlingame, Alma L. & Wang, Z.-Y. The CDG1 Kinase Mediates Brassinosteroid Signal Transduction from BRI1 Receptor Kinase to BSU1 Phosphatase and GSK3-like Kinase BIN2. Molecular Cell 43, 561-571 (2011).
7. Tang, W. et al. BSKs Mediate Signal Transduction from the Receptor Kinase BRI1 in Arabidopsis. Science 321, 557 (2008).
8. Tang, W. et al. PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating BZR1. Nature Cell Biology 13, 124 (2011).
9. Kim, T.-W. & Wang, Z.-Y. Brassinosteroid Signal Transduction from Receptor Kinases to Transcription Factors. Annual Review of Plant Biology 61, 681-704 (2010).
10. Chinchilla, D. et al. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defense. Nature 448, 497-500 (2007).
11. Zhang, J. et al. Receptor-like Cytoplasmic Kinases Integrate Signaling from Multiple Plant Immune Receptors and Are Targeted by a Pseudomonas syringae Effector. Cell Host & Microbe 7, 290-301 (2010).
12. Lu, D. et al. A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. Proceedings of the National Academy of Sciences 107, 496-501 (2010).
13. Feng, F. et al. A Xanthomonas uridine 5'-monophosphate transferase inhibits plant immune kinases. Nature 485, 114-118 (2012).
14. Rao, S. et al. Roles of receptor-like cytoplasmic kinase VII members in pattern-triggered immune signaling. Plant Physiology (2018).
15. Lin, W. et al. Inverse modulation of plant immune and brassinosteroid signaling pathways by the receptor-like cytoplasmic kinase BIK1. Proceedings of the National Academy of Sciences 110, 12114-12119 (2013).
16. Shi, H. et al. BR-SIGNALING KINASE1 Physically Associates with FLAGELLIN SENSING2 and Regulates Plant Innate Immunity in Arabidopsis. The Plant Cell Online 25, 1143-1157 (2013).
17. Majhi, B. B., Sreeramulu, S. & Sessa, G. BRASSINOSTEROID-SIGNALING KINASE5 associates with immune receptors and is required for immune responses. Plant Physiology, pp.01492.2018 (2019).
18. Wang, W. & Wang, Z.-Y. At the Intersection of Plant Growth and Immunity. Cell Host & Microbe 15, 400-402 (2014).
19. Mora-Garcia, S. et al. Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in Arabidopsis. Genes Dev 18, 448-60 (2004).
20. Kim, T.-W. et al. Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. Nat Cell Biol 11, 1254-1260 (2009).
21. Kim, T.-W., Michniewicz, M., Bergmann, D. C. & Wang, Z.-Y. Brassinosteroid regulates stomatal development by GSK3-mediated inhibition of a MAPK pathway. Nature 482, 419-422 (2012).
22. Maselli, G. A. et al. Revisiting the Evolutionary History and Roles of Protein Phosphatases with Kelch-Like Domains in Plants. Plant Physiology 164, 1527 (2014).
23. Zipfel, C. et al. Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts Agrobacterium-Mediated Transformation. Cell 125, 749-760 (2006).
24. Cabot, C., Gallego, B., Martos, S., Barceló, J. & Poschenrieder, C. Signal cross talk in Arabidopsis exposed to cadmium, silicon, and Botrytis cinerea. Planta 237, 337-349 (2013).

25. Chen, T. et al. Arabidopsis Mutant bik1 Exhibits Strong Resistance to Plasmodiophora brassicae. Frontiers in physiology 7, 402-402 (2016).
26. He, K. et al. BAK1 and BKK1 Regulate Brassinosteroid-Dependent Growth and Brassinosteroid-Independent Cell-Death Pathways. Current Biology 17, 1109-1115 (2007).
27. Ichimura, K., Casais, C., Peck, S. C., Shinozaki, K. & Shirasu, K. MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J Biol Chem 281 (2006).
28. Kong, Q. et al. The MEKK1-MKK1/MKK2-MPK4 Kinase Cascade Negatively Regulates Immunity Mediated by a Mitogen-Activated Protein Kinase Kinase Kinase in Arabidopsis. The Plant Cell 24, 2225 (2012).
29. He, P. et al. Specific Bacterial Suppressors of MAMP Signaling Upstream of MAPKKK in Arabidopsis Innate Immunity. Cell 125, 563-575 (2006).
30. Veronese, P. et al. The Membrane-Anchored BOTRYTIS-INDUCED KINASE1 Plays Distinct Roles in Arabidopsis Resistance to Necrotrophic and Biotrophic Pathogens. The Plant Cell 18, 257-273 (2006).
31. Kadota, Y. et al. Direct Regulation of the NADPH Oxidase RBOHD by the PRR-Associated Kinase BIK1 during Plant Immunity. Molecular Cell 54, 43-55 (2014).
32. Asai, T. et al. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415, 977-983 (2002).
33. Bi, G. et al. Receptor-like Cytoplasmic Kinases Directly Link Diverse Pattern Recognition Receptors to the Activation of Mitogen-activated Protein Kinase Cascades in Arabidopsis. The Plant Cell (2018).
34. Hartwig, T. et al. Propiconazole Is a Specific and Accessible Brassinosteroid (BR) Biosynthesis Inhibitor for Arabidopsis and Maize. PLOS ONE 7, e36625 (2012).
35. Ren, H. et al. BRASSINOSTEROID-SIGNALING KINASE 3, a plasma membrane-associated scaffold protein involved in early brassinosteroid signaling. PLOS Genetics 15, e1007904 (2019).
36. Yan, H. et al. BRASSINOSTEROID-SIGNALING KINASE1 Phosphorylates MAPKKK5 to Regulate Immunity in Arabidopsis. Plant Physiology 176, 2991 (2018).
37. Yamada, K. et al. The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation. The EMBO Journal 35, 2468–2483 (2016).
38. Saunders, D. G. O. et al. Host Protein BSL1 Associates with Phytophthora infestans RXLR Effector AVR2 and the Solanum demissum Immune Receptor R2 to Mediate Disease Resistance. The Plant Cell 24, 3420 (2012).
39. Kim, E.-J. et al. Oligomerization between BSU1 Family Members Potentiates Brassinosteroid Signaling in Arabidopsis. Molecular Plant 9, 178-181 (2016).
40. Earley, K. W. et al. Gateway-compatible vectors for plant functional genomics and proteomics. The Plant Journal 45, 616-629 (2006).
41. Fan, M. et al. The bHLH Transcription Factor HBI1 Mediates the Trade-Off between Growth and Pathogen-Associated Molecular Pattern-Triggered Immunity in Arabidopsis. The Plant Cell 26, 828-841 (2014).
42. Ishiga, Y., Ishiga, T., Uppalapati, S. R. & Mysore, K. S. Arabidopsis seedling flood-inoculation technique: a rapid and reliable assay for studying plant-bacterial interactions. Plant Methods 7, 32 (2011).

43. MacLean, B. et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 26, 966-968 (2010).
44. Oh, E., Zhu, J.-Y. & Wang, Z.-Y. Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. Nature Cell Biology 14, 802 (2012).
45. Goff, S. A. et al. The iPlant Collaborative: Cyberinfrastructure for Plant Biology. Frontiers in plant science 2, 34 (2011).
46. Saeed, A. I. et al. TM4: a free, open-source system for microarray data management and analysis. BioTechniques 34, 374-378 (2003).

Supplementary Information is available in the online version of the paper.

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Author Contributions

C.H.P., T-W.K. and Z-Y.W designed the research. C.H.P performed most of the experiments. C.H.P. performed RNA-seq. C.H.P. and B.Y. analyzed RNA-seq data. C.H.P., J-H.K., S-K.K. and T-W.K. performed and analyzed yeast-two-hybrid assay and in vitro pull-down assay. C.H.P. and S-L.X performed MS analysis. C.H.P., J-G.K. and M.B.M performed and analyzed bacterial

growth assay. C.H.P. and N.X performed immunoblot and seedling flood inoculation assay. C.H.P., S-K.K., T-W.K. and Z-Y.W wrote the manuscript.

Author Information

Competing interests

The authors declare no competing interests.

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Figure legends

Fig. 1 BSU1 family phosphatases are required for flg22-induced immune responses.

a) Identification of flg22-induced BSU1 S251 phosphorylation by SILIA IP-MS using PRM analysis. b) The locus and conservation of BSU1 S251 residue. Asterisk denotes BSU1 S251. c) Flg22-induced MPK3/6 phosphorylation in wild-type (WT) and *bsu-q*. Eight-week short-day grown leaves were treated with 100 nM flg22 for 2, 5, 10 and 30 min. Fifty µg of protein extracts were loaded to 10% SDS-PAGE gel. d) Flg22-induced MPK3/6 phosphorylation of WT and *bsu-i*. Nine-day old seedlings grown on 20 uM estradiol or the same amount of DMSO were treated with 100 nM flg22 for 10 min. Immunoblot was performed using anti-phospho-p44/42 MAPK antibody. e) Heat map of $\log_2$ fold change values of flg22-responsive genes in WT and *bsu-q*

from RNA-seq data. f) Scatter plot of log_2 fold change values of flg22-regulated genes in WT vs. *bsu-q*. Total 1590 genes from flg22-regulated genes in WT (-2≥ FC ≥2) were compared to those in *bsu-q*. g) qRT-PCR of flg22-induced *FRK1* and *At2g17740* expression in WT and *bsu-q*. h) Flg22-induced bacterial growth inhibition in WT and *bsu-q*. Each replicate includes four leaf discs and 12 biological replicates were used, and the experiment was repeated at least three times with similar results. The average bacterial titer ±SD is reported.

Fig. 2 BSU1 interacts with and is phosphorylated by BIK1 in a flg22-dependent manner.

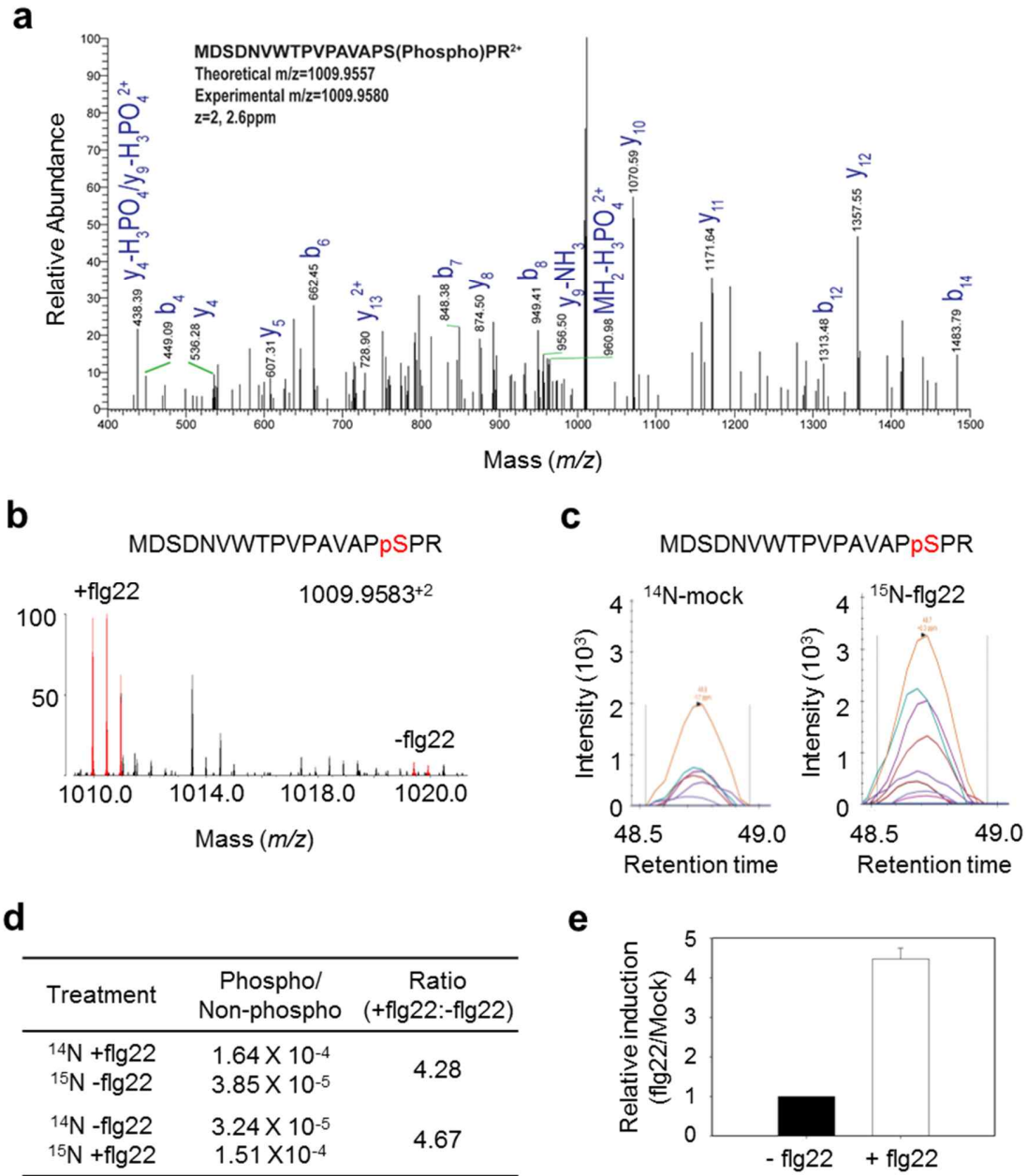
a) Yeast-two-hybrid assay for interaction between BIK1 and BSU1 family members. Yeast cells co-expressing the indicated AD and BD proteins were grown on synthetic dropout media. b) *In vitro* pull-down of MBP-BSU1/BSLs by GST-BIK1, analyzed by anti-MBP immunoblotting. c) Co-immunoprecipitation assay of BSU1 with BIK1 protein expressed in *N. benthamiana* leaves and *Arabidopsis* transgenic plants. d) BIK1-myc proteins were immunopurified by anti-myc antibody from plants treated with 100 nM flg22 or mock for 30 min, and used to pulldown MBP-BSU1 *in vitro*. Asterisk indicates phosphorylated BIK1. e) Auto-radiograph of gel staining (CBB) *In vitro* kinase assay of BIK1 phosphorylation of the indicated MBP and MBP fusion proteins. f) *In vitro* kinase assay (as panel e) of BIK1 phosphorylation of BSU1 N-terminal (Kelch) and C-terminal (PP) domains. g) *In vitro* kinase assay of flg22-induced BIK1 phosphorylation of BSU1 using immunopurified BIK1-HA. BIK1-HA immunoprecipitated using anti-HA antibody from thirteen-day old *BIK1::BIK1-HA* seedlings treated with 1 uM flg22 or mock for 10 min was incubated with MBP-BSU1 and ^{32}P-γ-ATP. BIK1-HA proteins were analyzed by immunoblotting using anti-HA antibody. Asterisk indicates phosphorylated BIK1. h) *In vitro* kinase assay of BIK1 phosphorylation of wild-type and mutant (S251A and S764A) BSU1. i) *In vitro* kinase assay of

flg22-induced BIK1 phosphorylation of wild-type and mutant (S251A) BSU1 using immunopurified BIK1-HA. BIK1-HA was immunoprecipitated using anti-HA antibody from twelve-day old *BIK1::BIK1-HA* seedlings treated with 1 uM flg22 or mock for 10 min, and was incubated with MBP-BSU1 or MBP-BSU1-S251A (MBP-S251A) and ^{32}P-γ-ATP.

Fig. 3 BSU1 S251 is required for the MEKK1 interaction, MAP kinase activation and defense against bacteria but not growth response.

a) Yeast-two-hybrid assay for interaction between MEKK1 and BSU1 family members. Yeast cells co-expressing *AD-MEKK1* and *BD-BSU1/BSL*s were grown on synthetic dropout medium containing 2.5 and 5 mM 3-amino-1, 2, 4-triazole (3-AT). b) *In vitro* pull-down assay of GST-BSU1 and MBP-MEKK1. MBP-MEKK1 was pulled down by GST-BSU1 BP and the elutes were analyzed by immunoblotting using anti-MBP and anti-GST antibody. c) Co-immunoprecipitation assay of BSU1 with MEKK1 protein expressed in *N. benthamiana* leaves. d) Co-immunoprecipitation assay of BSU1 and BSU1-S251A with MEKK1 protein expressed in *Arabidopsis* transgenic plants. Seedling were grown for 9 days and treated with 1 uM flg22 for 10 min. BSU1-YFP and BSU1-S251A-YFP were pulled down by GFP-Trap beads and co-purified MEKK1-FLAG was detected by anti-FLAG antibody. e) Flg22-induced MPK phosphorylation in *35S::BSU1-YFP:bsu-i* (*BSU1:i*) and 35*S::BSU1-S251A-YFP:bsu-i* (*S251A:i*) plants. Eight-day old seedlings grown on 20 uM estradiol were treated with 10 nM flg22 for 15 min. Immunoblot was performed using anti-phospho-p44/42 MAPK and anti-GFP antibodies. Ponceau S staining image shows equal loading. f) Flg22-induced bacterial growth inhibition in *BSU1:i* and *S251A:i* seedlings. Each replicate includes 12 mg or more seedlings and 8 biological replicates were used. The average

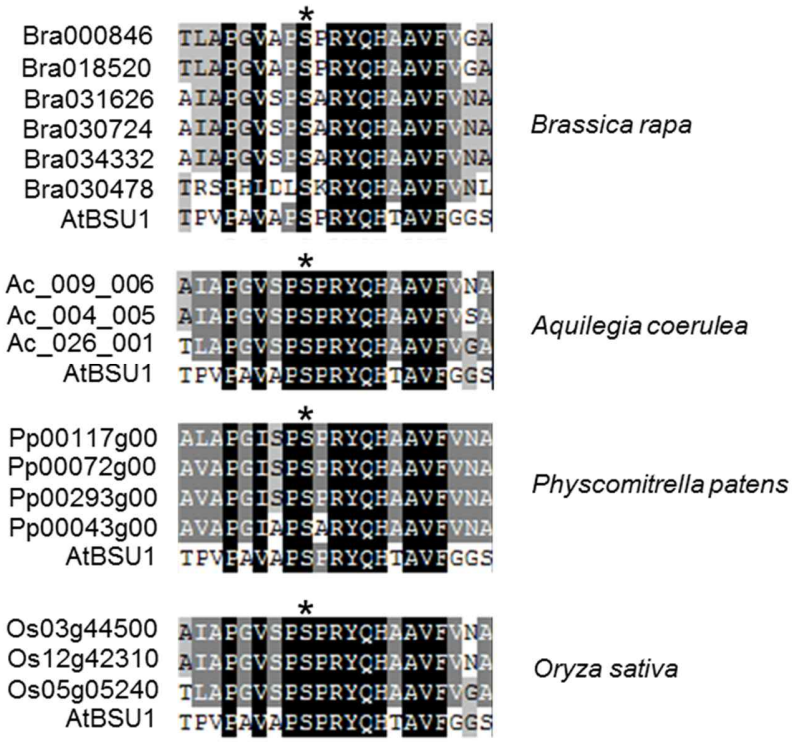
bacterial titer ±SD is reported. g, h) PPZ resistance of the wild-type, *35S::BSU1-YFP* (*BSU1*) and *35S::BSU1-S251A-YFP* (*S251A*) seedlings. g) Phenotype of 5-day-old etiolated seedlings grown in mock and PPZ media. Bar=10 mm. h) Hypocotyl length of etiolated seedlings. Asterisks denote significant difference from wild-type by t-test ($P<0.001$, n =13 for wild-type and n=14 for *BSU1* and *S251A*). Experiments performed 3 repeats and showed similar results.



Treatment	Phospho/ Non-phospho	Ratio (+flg22:-flg22)
^{14}N +flg22 ^{15}N -flg22	1.64×10^{-4} 3.85×10^{-5}	4.28
^{14}N -flg22 ^{15}N +flg22	3.24×10^{-5} 1.51×10^{-4}	4.67

EXTENDED DATA Fig. 1. SILIA IP-MS result of fl22-treated *BSU1-YFP*.
a) MSMS spectrum of phosphorylated S251 of BSU1. b) flg22-induced BSU1 S251 phosphorylation by SILIA IP-MS. The phosphorylated S251-containing peptide peaks and S251 residue are indicated in red. c) PRM analysis of S251 phosphorylation using reciprocal labeling. d) The ratio of phosphorylated and non-phosphorylated MDSDNVWTPVPAVAPSPR peptides. The values of peak area were used for quantification. The ratio of S251 phosphorylation between flg22 non-treated (-flg22) and treated (+flg22) samples is shown. e) Quantification of flg22-induced BSU1 S251 phosphorylation. Columns indicate the average of relative S251 phosphorylation induction of the two reciprocal analysis. Bars are S.D.

1



EXTENDED DATA Fig. 2. Sequence alignment of BSU1 paralogs. Amino acid sequences of BSU1 paralogs were compared with Arabidopsis BSU1. Amino acid sequences were obtained from Maselli et al., 2014. Asterisks indicated BSU1 S251.

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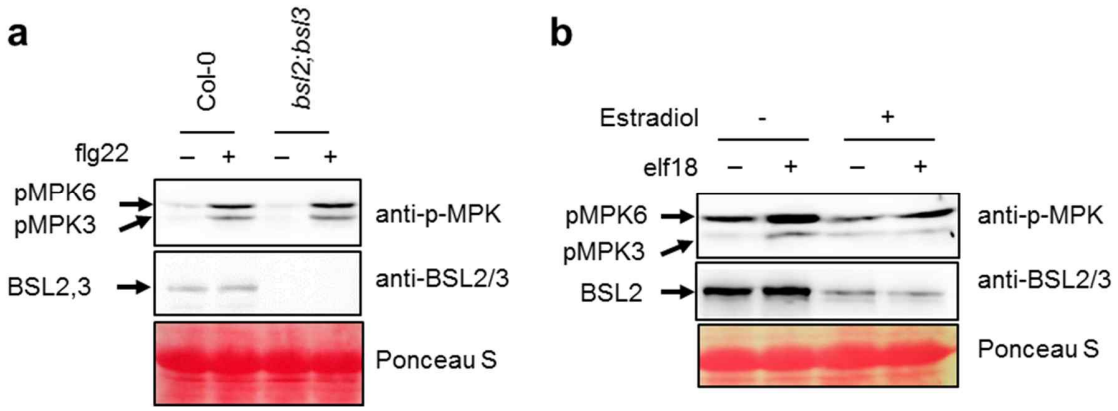

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EXTENDED DATA Fig. 3. The flg22 and elf18-induced MPK3/6 phosphorylation in BSU1 family mutants.
a) Flg22-induced MPK3/6 phosphorylation in *bsl2;bsl3*. Ten day-old seedlings were treated with 100 nM flg22 for 10 min. Immunoblot was performed using anti-phospho-p44/42 MAPK and anti-BSL2/3 antibodies. Ponceau S staining shows equal protein loading. b) Elf18-induced MPK3/6 phosphorylation in *bsu-i*. Twelve day-old seedlings grown on 20 uM estradiol containing media were treated with 1 μM elf18 for 10 min. Immunoblot was performed using anti-phospho-p44/42 MAPK and anti-BSL2/3 antibodies. Ponceau S staining shows equal protein loading.

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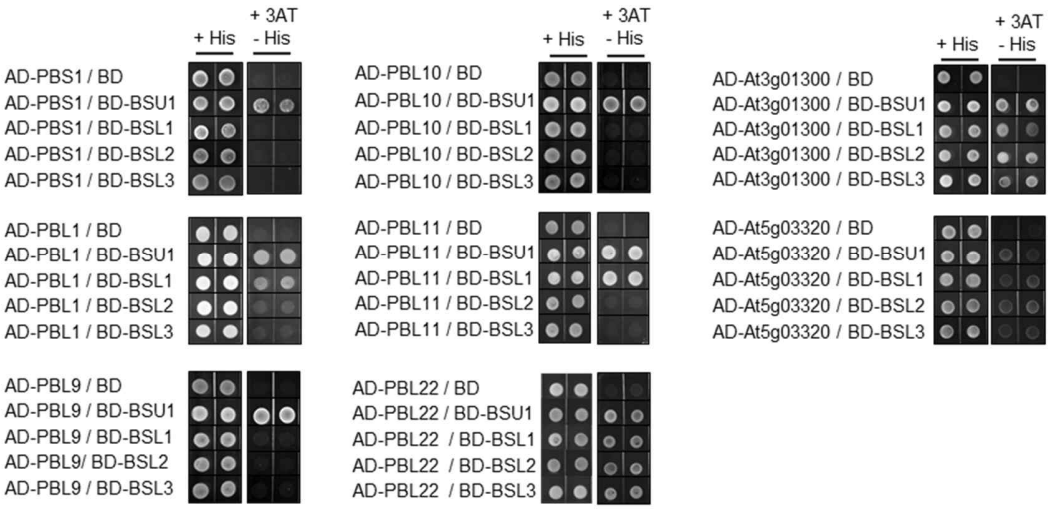
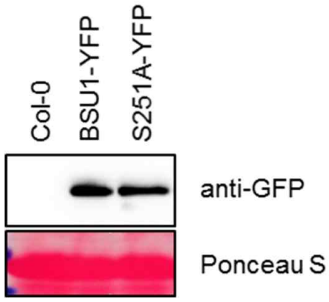


EXTENDED DATA Fig. 4. Yeast-two-hybrid assay for interaction between BIK1 family and BSU1/BSLs proteins.
Yeast-two-hybrid assay for interaction between BIK1/PBLs and BSU1/BSLs proteins. Yeast cells co-expressing AD-BIK1/PBLs and BD-BSU1/BSLs were grown on synthetic dropout medium containing 2.5 mM 3-amino-1, 2, 4-triazole (3AT).

1



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EXTENDED DATA Fig. 5. BSU1 protein level in *35S::BSU1-YFP* (*BSU1-YFP*) and *35S::BSU1-S251A-YFP* (*S251A-YFP*) seedlings. Total protein was extracted from 9-day old seedlings. BSU1-YFP protein level was analyzed by immunoblotting using anti-GFP antibody. Ponceau S staining image shows equal protein loading.