The MAP4 Kinase SIK1 Ensures Robust Extracellular ROS Burst and Antibacterial Immunity in Plants

Highlights
- The conserved MAP4K SIK1 is required for PAMP-induced ROS production in Arabidopsis
- SIK1 binds, phosphorylates, and stabilizes the central immune regulator BIK1
- sik1 mutants display resistance to P. syringae due to high salicylic acid accumulation
- SIK1 binds to and phosphorylates an NADPH oxidase to enhance ROS production for defense

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In Brief
Zhang et al. identify the conserved MAP4 kinase SIK1 as required for pattern-triggered immunity in plants. SIK1 associates with, phosphorylates, and stabilizes the central immune regulator BIK1. Upon perception of pathogens, SIK1 and activated BIK1 phosphorylate the NADPH oxidase RBOHD to enhance ROS production and promote defense.
The MAP4 Kinase SIK1 Ensures Robust Extracellular ROS Burst and Antibacterial Immunity in Plants

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SUMMARY
Microbial patterns are recognized by cell-surface receptors to initiate pattern-triggered immunity (PTI) in plants. Receptor-like cytoplasmic kinases (RLCKs), such as BIK1, and calcium-dependent protein kinases (CPKs) are engaged during PTI to activate the NADPH oxidase RBOHD for reactive oxygen species (ROS) production. It is unknown whether protein kinases besides CPKs and RLCKs participate in RBOHD regulation. We screened mutants in all ten Arabidopsis MAP4 kinases (MAP4Ks) and identified the conserved MAP4K SIK1 as a positive regulator of PTI. sik1 mutants were compromised in their ability to elicit the ROS burst in response to microbial features and exhibited compromised PTI to bacterial infection. SIK1 directly interacts with, phosphorylates, and stabilizes BIK1 in a kinase activity-dependent manner. Furthermore, SIK1 directly interacts with and phosphorylates RBOHD upon flagellin perception. Thus, SIK1 positively regulates immunity by stabilizing BIK1 and activating RBOHD to promote the extracellular ROS burst.

INTRODUCTION
Plants rely on their innate immune system to actively recognize and respond to pathogenic organisms. Immune receptors with extracellular domains act to perceive conserved microbial patterns, resulting in pattern-triggered immunity (PTI). These microbial features are referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). Well-characterized PAMPs include bacterial flagellin and fungal chitin (Thomma et al., 2011). PTI responses include the generation of reactive oxygen species (ROS), a transient influx of calcium from the apoplast, activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CPKs), as well as transcriptional reprogramming of immune-related genes (Couto and Zipfel, 2016).

The Arabidopsis receptor FLS2 is a well-characterized pattern recognition receptor (PRR) that perceives a conserved 22-amino-acid epitope from bacterial flagellin (flg22) (Chinchilla et al., 2006). The kinase BIK1, a member of the receptor-like cytoplasmic kinase (RLCK) family, is important for downstream immune signaling (Tang et al., 2017). BIK1 associates with the FLS2 complex and is rapidly phosphorylated upon flg22 perception. Activated BIK1 is released from the FLS2 complex and directly phosphorylates the NADPH oxidase (NOX) RBOHD to activate extracellular ROS production (Kadota et al., 2014; Li et al., 2014). The extracellular ROS burst is significantly compromised in the bik1 mutant, but MAPK activation is not affected (Feng et al., 2012; Zhang et al., 2010), indicating that BIK1’s primary role is to enhance the extracellular ROS burst. BIK1 also positively regulates immune responses triggered by other PAMPs, suggesting that BIK1 serves as a central and convergent regulator of distinct PRR-dependent pathways (Couto and Zipfel, 2016). For example, BIK1 associates with the CERK1 PRR and is required for chitin-induced immune responses (Zhang et al., 2010). Given the importance of BIK1 in immune signaling, its protein stability is tightly regulated during PTI (Liang et al., 2016; Monaghan et al., 2014; Wang et al., 2018).

The extracellular ROS burst can act as an antimicrobial molecule, strengthen the plant cell wall through oxidative crosslinking, and act as a local and systemic messenger to induce downstream immune responses (Kadota et al., 2015; Wang et al., 2018).
Ca²⁺ binding to EF-hand motifs (Kadota et al., 2015). BIK1 and activating sites as well as conformational changes induced by cations, including phosphorylation of RBOHD’s N terminus at NOX activity primarily occurs through post-translational modifications, including phosphorylation of RBOHD’s N terminus at activating sites as well as conformational changes induced by Ca²⁺ binding to EF-hand motifs (Kadota et al., 2015). BIK1 and CPKs have been shown to be important for activation of RBOHD by phosphorylating its N-terminal regions (Dubiella et al., 2013; Kadota et al., 2014; Li et al., 2014). However, it is unknown whether other protein kinases besides CPKs and RLCKs participate in the regulation of RBOHD to ensure its robust activation upon pathogen or PAMP perception.

The mitogen-activated protein kinase kinase kinase kinase (MAP4K) family is evolutionarily conserved and related to yeast STE20 (Dan et al., 2001). In yeast and humans, MAP4Ks can directly activate MAPK cascades, but can also phosphorylate diverse substrates (Brenner et al., 2009; Leberer et al., 1997). In humans, MAP4Ks are also involved in immunity and activate nuclear factor kB immune signaling (Brenner et al., 2009; Jiao et al., 2015). In Arabidopsis, the MAP4K family contains 10 members. The SIK1 MAP4K regulates cell proliferation and cell expansion (Xiong et al., 2016). The Arabidopsis BLUS1 MAP4K is phosphorylated by phototropins and serves as a primary regulator of stomatal opening (Takeuchi et al., 2013). However, the functions and mechanisms of MAP4Ks in plant immunity remain unexplored.

Here, we show that sik1 mutants were compromised in the ROS burst upon PAMP perception and displayed compromised immunity to infection with the bacterial pathogen Pseudomonas syringae, indicating that MAP4K family is involved in plant innate immunity. In addition, we show that SIK1 phosphorylates BIK1 and stabilizes it in a kinase activity-dependent manner. Interestingly, SIK1 also associates with RBOHD in Arabidopsis and phosphorylates RBOHD’s N terminus. Together, these results indicate SIK1 positively regulates PTI by stabilizing BIK1 and activating RBOHD to promote the extracellular ROS burst.

 results

sik1 Mutants Are Compromised in flg22-Induced ROS Production

To define the role of plant MAP4Ks, we first identified homologous transfer DNA insertion mutants in the ten Arabidopsis MAP4K members. Sequence analyses and PCR confirmed the transfer DNA insertions and RT-PCR analyses verified the absence of full-length transcripts in transfer DNA lines, indicating that these lines are null mutants (Figure S1). We were unable to amplify MAP4K8 and MAP4K9 transcripts in wild-type Columbia-0 (Col-0) (Figure S1B), likely due to the low expression levels of these genes in leaf tissue. Unlike the other Arabidopsis MAP4K members, SIK1 (MAP4K3, At1g69220) exhibits unique domain architecture with a central kinase domain and additional N- and C-terminal regions (Figure 1A). Interestingly, the sik1 mutants displayed a dwarf phenotype, which is consistent with previous findings (Xiong et al., 2016), whereas other mutant lines did not show obvious differences in plant growth compared with wild-type Col-0 (Figure 1B).

To analyze the role of MAP4Ks in plant immunity, we phenotyped each mutant for alterations in the flg22-induced ROS burst. ROS production was detected using a luminol-based assay with water used as a negative control. The ROS burst was significantly reduced in the sik1-1 mutant upon flg22 treatment compared with wild-type Col-0 (Figures 1C and S2). No alterations in the flg22-induced ROS burst were observed in the other nine transfer DNA mutants (Figures 1C and S2). These results indicate that SIK1 is involved in flg22-induced ROS production.

SIK1 Is a Functional and Conserved Kinase in Land Plants

SIK1’s kinase domain exhibits conserved features of active protein kinases (Figure S3). It contains a characteristic glycine-rich loop with the consensus GXGXXG motif (Figure 2A), which is a structural hallmark of protein kinases and nucleotide binding proteins (Taylor and Kornev, 2011). It also possesses an invariant lysine residue at position 278 and a conserved aspartate (D371) (Figure 2A), which are generally required for kinase activity. A conserved activation segment was identified in the SIK1 kinase domain (Figure 2A), and the DFG motif in this segment has been shown to be important for function in other kinases by coordinating Mg²⁺ at the active site (Taylor and Kornev, 2011). These features indicate that SIK1 is an active kinase.

We next experimentally examined the kinase activity of SIK1 using an in vitro phosphorylation assay. Since full-length SIK1 is toxic to Escherichia coli (Xiong et al., 2016), truncated SIK1 protein lacking its N-terminal region was utilized (SIK1ΔN, amino acids 249–836). We purified recombinant SIK1ΔN and its kinase-dead variant K278E (SIK1ΔNΔK) from E. coli, and evaluated their kinase activities by analyzing autophosphorylation using a radiolabeled assay. The SIK1ΔN protein can autoprophosphorylate itself, but the kinase-dead variant K278 cannot (Figure 2B), demonstrating that SIK1 is an active kinase.

To determine whether SIK1 is conserved in plants, we searched for homologs in other plant species by BLAST analyses. SIK1 homologs are ubiquitously in land plants, and they all possess conserved domain architecture with a central kinase domain (Figure 2C). The kinase domains of these homologs are highly conserved (92%–99% amino acid identity) and also share conserved features with SIK1 (Figure S3).

sik1 Mutants Exhibit Enhanced Resistance to P. syringae due to High Levels of SA

The compromised ROS production in sik1-1 led us to propose that SIK1 may positively regulate PTI. We inoculated two sik1 knockouts (sik1-1 and sik1-4) with the virulent bacterial pathogen P. syringae pv. tomato DC3000 (Pst). Unexpectedly, the sik1 mutants displayed significantly lower bacterial titers than Col-0 (Figure 3A). Since activation of plant immunity can restrict the delivery of type III effectors (Crabill et al., 2010), we evaluated the delivery of the Pst AvrRpm1 effector in sik1-1 using the adenylate cyclase assay. Pst is still able to deliver AvrRpm1 into sik1-1 (Figure S4A), indicating that effector delivery is not grossly altered in
Figure 1. The SIK1 MAP4K Is Involved in flg22-Induced ROS Production
(A) Domain architecture and phylogeny of the Arabidopsis MAP4K family. Red circles represent nodes with posterior probability greater than 0.5. MAP4K kinase domains are highlighted in blue.

(B) Top: SIK1 gene structure and transfer DNA insertion sites. Black lines indicate introns. Boxes indicate exons. Red boxes represent exons in the kinase domain. Bottom: growth phenotypes of 3-week-old map4k transfer DNA insertion mutants.

(C) The ROS burst in transfer DNA mutants of the MAP4K family after treatment with 100 nM flg22 or water. Total relative luminescent units (RLU) were detected over a 35-min period. Values are means ±SEM of RLU (n = 12). Asterisks indicate significant differences (Dunnett’s test, **p < 0.01). Experiments were repeated three times with similar results.

See also Figures S1 and S2.
The *sik1* mutants also exhibit a lesion-mimic phenotype under long-day conditions (Figure S4B). The presence of dwarf and lesion-mimic phenotypes is frequently associated with higher levels of salicylic acid (SA) and autoimmune responses (Rodriguez et al., 2016). The *sik1* mutants exhibited significantly higher levels of basal SA (Figure S4C), constitutive expression of the SA-response defense marker gene *PR1* (Figure S4D), and higher *PR1* protein accumulation compared with wild-type Col-0 (Figure S4E). Jasmonic acid (JA) levels were decreased in the *sik1* mutants (Figure S4F), consistent with the antagonism between SA and JA pathways (Fu and Dong, 2013). No significant alterations in the abundance of abscisic acid and cis-(+)-12-oxo-phytodienoic acid were detected in *sik1* mutants (Figure S4F).

To test whether high levels of SA lead to enhanced resistance to *Pst* in *sik1*, we generated transgenic plants constitutively expressing the salicylate hydroxylase NahG, which degrades SA (Gaffney et al., 1993), in both Col-0 and *sik1-1* backgrounds. Both Col-0 NahG and *sik1-1 NahG* transgenic lines exhibited comparable levels of *PR1* gene expression (Figure 3B) and NahG protein (Figure 3C), indicating similar SA levels. Therefore, we should be able to uncouple pleiotropic effects from high SA levels by using the *sik1-1 NahG* transgenic line. NahG expression did not completely restore the dwarf phenotype in *sik1* mutant (Figure S4B). However, NahG expression suppressed the *sik1-1* resistance phenotype to virulent *Pst* (Figure 3D). Together, these results demonstrate that the enhanced resistance in the *sik1* mutants is caused by high SA accumulation.

*sik1* NahG Plants Are Compromised in flg22-Triggered Immunity

To investigate whether SIK1 is required for PAMP-induced resistance to *Pst*, we carried out a flg22-protection assay. Pretreatment with PAMPs, such as flg22, results in enhanced resistance
to subsequent inoculation with virulent bacteria (Zipfel et al., 2004). Col-0 NahG and sik1 NahG leaves were infiltrated with water or flg22 1 day prior to inoculation of the same leaf with virulent Pst. After flg22 pretreatment, sik1 NahG carried approximately 14-fold more bacteria than Col-0 NahG (Figure 4A), indicating that sik1 NahG is substantially inhibited in flg22 responsiveness. Taken together, these results suggest that SIK1 positively regulates flg22-triggered immunity to Pst.

**sik1 Mutants Are Compromised in Different PAMP-Induced ROS Bursts but Not in MAPK Activation**

To confirm the role of SIK1 in ROS regulation, we analyzed a second independent homozygous transfer DNA mutant line (sik1-4) as well as a UBQ::SIK1-GFP complementation line (Xiong et al., 2016). flg22-induced ROS production was significantly reduced in both sik1-1 and sik1-4 compared with wild-type Col-0 (Figures 4B and S5A), ROS production in the SIK1 complementation line was not significantly different from wild-type Col-0, further confirming the role of SIK1 in flg22-induced extracellular ROS burst (Figure 4B). The knockout mutant of the PTI regulator BIK1 also displays high basal levels of SA accumulation and compromised extracellular ROS production (Veronese et al., 2006; Zhang et al., 2010). The bik1 mutant, which has been shown to be compromised in both elf18- and chitin-induced ROS burst, was used as a control (Zhang et al., 2010). Interestingly, the ROS production in sik1 mutants was even lower than that in bik1 after flg22 treatment (Figure 4B), highlighting the importance of SIK1 in ROS production. PBL1 is a close homolog of BIK1, and is also required for PTI signaling. Consistent with previous results (Zhang et al., 2010), ROS production was further reduced in the bik1 pbl1 double mutant compared with the bik1 single mutant after flg22 treatment (Figures S5B and S5C). ROS production in the sik1 mutants was still lower than that in the bik1 pbl1 mutant (Figures S5B and S5C). We also observed a reduced ROS burst in sik1 NahG compared with Col-0 NahG (Figures 4C and S5D), indicating that the compromised ROS production in sik1 mutants is not due to high SA accumulation. Interestingly, NahG expression significantly reduced ROS production after flg22 treatment in Col-0 and sik1 (Figure 4C), demonstrating that SA is involved in regulation of apoplastic ROS production. Chitin- and elf18-induced ROS production was also reduced in sik1 mutants compared with wild-type Col-0 (Figures 4D, 4E, S5E, and S5F), indicating that SIK1 regulates responsiveness to a broad range of PAMPs. In parallel with ROS production, PTI also induces MAPK activation (Couto and Zipfel, 2016; Yu et al., 2017). We did not observe compromised PAMP-triggered MAPK activation in sik1 and sik1 NahG compared with wild-type Col-0 and Col-0 NahG (Figure 4F). MAPK activation was slightly higher in the sik1 mutant, but not in sik1 NahG lines (Figure 4F). Together, these results indicate that SIK1 primarily regulates PAMP-induced ROS production.

**SIK1 Associates with and Phosphorylates BIK1**

To investigate how SIK1 modulates ROS production, we first analyzed the abundance of the FLS2 immune receptor and the central immune regulator BIK1 in sik1 mutant lines. The
compromised flg22-induced ROS burst in the sik1 mutant is not caused by reduced levels of the FLS2 immune receptor (Figure S6A) or reduced transcriptional expression of BIK1 (Figure S6B). The sik1 mutants phenocopied the bik1 mutant with respect to decreased ROS production after PAMP treatment and high basal levels of SA. Therefore, we then investigated whether SIK1 associates with BIK1. To test this hypothesis, we expressed BIK1-FLAG and HA-SIK1 in Arabidopsis and performed co-immunoprecipitation (co-IP). A strong association between SIK1 and BIK1 was observed in the presence and absence of flg22 (Figure 5A), supporting the hypothesis that SIK1 resides within the same protein complex as BIK1.

To test whether SIK1 directly interacts with BIK1, we purified GST-SIK1ΔN, MBP-BIK1 and MBP-BSK1 recombinant proteins from E. coli. BSK1 is an Arabidopsis RLCK that is involved in both brassinosteroid perception and innate immunity (Shi et al., 2013). The glutathione S-transferase (GST) pull-down assay showed that SIK1ΔN specifically interacts with BIK1 compared with BSK1 (Figure 5B). We next investigated SIK1’s ability to phosphorylate BIK1 in vitro. SIK1ΔN phosphorylated a kinase-dead variant BIK1KD (BIK1K105A/K106A), but the SIK1ΔNKD did not (Figure 5C), indicating that trans-phosphorylation of BIK1 was due to SIK1ΔN phosphorylation. To identify BIK1 sites phosphorylated by SIK1, we co-expressed BIK1KD with SIK1ΔN or SIK1ΔNKD in E. coli and then purified BIK1KD recombinant protein for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. Twelve serine (S) residues, twelve threonine (T) residues, and one tyrosine (Y) residue were identified as potential BIK1 phosphorylated sites (Table S1) and only detected after co-expression with SIK1ΔN. Among these

Figure 4. sik1 Mutants Exhibit Compromised PAMP-Triggered Immunity and ROS Burst
(A) Susceptibility of the sik1-1 NahG line #1 to Pst. Leaves were pretreated with either water or flg22 (1 μM) 24 hr before inoculation with Pst. Statistically significant differences (Fisher’s LSD; p < 0.01) between the Col-0 NahG and the sik1-1 NahG lines are indicated. Different letters indicate significant differences. Similar results were obtained in three independent experiments. (B and C) The ROS burst in the indicated lines after treatment with 100 nM flg22 or water. Total relative luminescent units (RLU) were detected over a 40-min period. (D) The ROS burst in the indicated lines after treatment with 100 nM elf18 or water. Total RLU were detected over a 40-min period. (E) The ROS burst in the indicated lines after treatment with 10 μM chitin or water. For chitin-induced ROS burst, the more sensitive probe L012 was used. Total RLU were detected over a 40-min period. (F) MAPK activation was detected after flg22 treatment by an immunological assay. The expected identities of the respective bands are marked on the right. This experiment was repeated twice with similar results. Values in (B)–(E) are means ± SEM of RLU (n ≥ 12). Different letters indicate significant differences (Fisher’s LSD, p < 0.01). Similar results were obtained in three independent experiments. See also Figure S5.
ROS burst in the lines. Expression of BIK1-HA can significantly enhance ROS production in transgenic lines of the sik1-1 BIK1-HA protein, indicating that SIK1 regulates extracellular ROS production through BIK1 stabilization.

Next, we investigated at what point SIK1 functions to stabilize BIK1. In the absence of PAMP perception, BIK1 exhibits reduced accumulation in the sik1-1 mutant (Figures 6A–6D). Treatment with fig22 did not rescue BIK1-HA accumulation in the sik1-1 mutant compared with that in Col-0 (Figure 6F), indicating that SIK1 mainly stabilizes BIK1 at a resting state. After fig22 treatment, we detected a partial shift of BIK1 protein by western blot, which has been previously demonstrated to be phosphorylated BIK1 (Lu et al., 2010). We detected a similar pattern of BIK1 mobility in Col-0 and the sik1-1 mutant after fig22 perception, indicating that sik1-1 is not compromised in BIK1 hyperphosphorylation (Figure 6F).

We next generated protoplasts from the bik1 complementation line pBIK1::BIK1-HA. Expression of T7-SIK1 in pBIK1::BIK1-HA enhanced BIK1 accumulation (Figures 6G and 6H). However, expression of a T7-SIK1 kinase-dead variant (SIK1KD) did not enhance BIK1 accumulation (Figures 6G and 6H), indicating that SIK1 kinase activity is required for BIK1 stability. Protein accumulation of PBL1, a close homolog of BIK1, was not affected in sik1-1 (Figure S6E). Interestingly, wild-type T7-SIK1 accumulated at a low level, whereas T7-SIK1KD robustly accumulated (Figure 6G), suggesting that SIK1 protein accumulation is also tightly regulated in a kinase activity-dependent manner. These results strongly support that SIK1 regulates extracellular ROS burst through BIK1 stabilization.

Similar to BIK1, SIK1 associates with the PRR FLS2 at a resting state (Figure S6F). It was reported recently that heterotrimeric G proteins are directly coupled with the FLS2-BIK1 immune complex to positively regulate FLS2-mediated immune responses by stabilizing BIK1 at a resting state (Liang et al., 2016, 2018). These previous findings prompted us to investigate the link between SIK1 and heterotrimeric G proteins. We expressed the extra-large G-protein subunit XLG2 and SIK1 in Arabidopsis, and performed coIP. Interestingly, XLG2 associates with SIK1 in planta (Figure 6I), indicating that SIK1 enhances BIK1 stability at a resting state by coupling with heterotrimeric G proteins.
Figure 6. SIK1 Regulates BIK1 Stability

(A) BIK1-HA accumulation in seedlings of the independent transgenic lines.

(B) Quantification of band intensity based on eight independent transgenic lines per genotype, including those shown in (A). Asterisks indicate significant differences (Student’s t test, **p < 0.01).

(C) BIK1-HA protein levels in mature plants of the independent transgenic lines.

(D) Quantification of band intensity based on eight transgenic lines per genotype, including those shown in (C). Asterisks indicate significant differences (Student’s t test, **p < 0.01).

(E) flg22-induced ROS burst in sik1-1 and sik1-1 BIK1-HA transgenic lines. Total relative luminescent units (RLU) were detected over a 40-min period. Similar results were obtained in two independent experiments. Different letters indicate significant differences (Fisher’s LSD, p < 0.01).

(F) Detection of BIK1-HA in transgenic lines after flg22 treatment. Protoplasts were isolated from Col-0 BIK1-HA and sik1-1 BIK1-HA transgenic lines, and treated with water or flg22 for 10 min. CBB, Coomassie brilliant blue staining.

(G) Enhanced BIK1 stability by SIK1 expression. The indicated constructs were expressed in pBIK1::BIK1-HA protoplasts. The accumulation of BIK1 and SIK1 was determined by immunoblot analyses after treatment for 6 hr with either 0.2% DMSO (-) or 50 μM MG132 (+). KD, kinase-dead variant; SE, short exposure; LE, long exposure. This experiment was repeated three times with similar results.

(H) Quantification of BIK1-HA band intensity based on three replicates. Different letters indicate significant differences (Fisher’s LSD, p < 0.01).

(I) Co-immunoprecipitation of HA-SIK1 and XLG2-FLAG after co-expression in Arabidopsis.

See also Figure S6.
SIK1 Associates with and Phosphorylates RBOHD

PAMP-induced ROS production was lower in the sik1 mutants than bik1 pbl1 after flg22 perception (Figures S5B and S5C), indicating that SIK1 may also regulate the extracellular ROS independently of RLCKs. To investigate whether SIK1 directly regulates RBOHD, we first expressed SIK1 and RBOHD in Arabidopsis and performed coIP to test their interaction. A specific signal for HA-SIK1 was clearly observed in the FLAG-RBOHD immunoprecipitate (Figure 7A), indicating that SIK1 can associate with RBOHD. SIK1 remains physically associated with RBOHD after flg22 treatment (Figure 7A), indicating that the SIK1-RBOHD association is not dynamic.

Figure 7. SIK1 Directly Interacts with RBOHD and Phosphorylates Its N Terminus

(A) SIK1 associates with RBOHD in Col-0. HA-SIK1 and FLAG-RBOHD were co-expressed in Col-0 protoplasts, and co-immunoprecipitation was performed. Protoplasts were treated with 1 μM flg22 or water, after which total proteins were subjected to anti-FLAG immunoprecipitations.

(B) SIK1 associates with RBOHD in bik1 pbl1. The indicated constructs were co-expressed and subjected to anti-FLAG immunoprecipitations.

(C) MBP pull-down assay demonstrates direct interaction between SIK1 and RBOHD N terminus in vitro. GST-RBOHD-N, MBP-SIK1ΔN, and MBP-BSK1 recombinant proteins were affinity purified from E. coli, and pull-down assays were performed using Amylose beads.

(D) SIK1 phosphorylates RBOHD’s N terminus. MBP-RBOHD-N was co-expressed with His-SIK1ΔN or His-SIK1ΔN D in E. coli and then MBP-RBOHD-N was purified. Phosphorylation was detected by anti-pThr immunoblot (top panel). Total MBP-RBOHD-N was detected by anti-MBP immunoblot (bottom panel).

(E) RBOHD phosphorylation sites identified in vitro by LC-MS/MS. The observed y and b ions are numbered.

(F) RBOHD phosphosites identified in vivo upon flg22 treatment. The observed y and b ions are numbered.

(G and H) Quantification of phosphorylation of S8 (G) and S347 (H) residues in vivo. FLAG-RBOHD was transiently expressed in Col-0 and sik1-1 protoplasts, and treated with 1 μM flg22 for 10 min. Total proteins were subjected to immunoprecipitation with anti-FLAG beads followed by on-bead digestion. Phosphorylated peptides were quantified by PRM. Data are means ± SE of four biological replicates. Asterisks indicate significant differences (Student’s t test, *p < 0.05). Similar results were obtained in two independent experiments.

See also Figure S7.

To investigate whether SIK1 associates with RBOHD through BIK1 or PBL1 RLCK proteins, we examined the SIK1-RBOHD association in the bik1 pbl1 genetic background. Interestingly, SIK1 still associates with RBOHD in the bik1 pbl1 double mutant (Figure 7B), indicating that SIK1 may directly interact with RBOHD. We purified GST-RBOHD-N, MBP-SIK1ΔN, and MBP-BSK1 recombinant proteins from E. coli and conducted a maltose binding protein (MBP) pull-down assay. The result clearly showed that SIK1ΔN specifically interacts with the N terminus of RBOHD (Figure 7C).

Positive regulation of RBOHD activity is mainly through phosphorylation of its N terminus (Kadota et al., 2015). Therefore, to
test whether SIK1 can directly phosphorylate the N terminus of RBOHD, we co-expressed MBP-RBOHD-N with SIK1ΔN or SIK1ΔN<sup>KD</sup> in E. coli, and purified MBP-RBOHD-N for detection of phosphorylation and LC-MS/MS analyses. SIK1ΔN phosphorylated the RBOHD N terminus (MBP-RBOHD-N) but the SIK1ΔN<sup>KD</sup> did not (Figure 7D), indicating that trans-phosphorylation of MBP-RBOHD-N was due to SIK1ΔN. LC-MS/MS identified six potential phosphorylation residues (S8, S9, T177, T179, S339, and S347) in RBOHD’s N terminus only after co-expression with SIK1ΔN (Figures 7E and S7). Among these residues, S339 and S347 are essential for RBOHD activity and are also phosphorylated by BIK1 or CPKs upon PAMP perception (Kadota et al., 2014; Li et al., 2014). Phosphorylation of S8, S9, T177, and T179 residues has not been previously reported. To test whether these phosphorylation sites occur after PAMP perception, we expressed FLAG-RBOHD in <i>Arabidopsis</i> protoplasts, and enriched FLAG-RBOHD protein using anti-FLAG agarose beads for mapping phosphorylation sites <i>in vivo</i> by MS. The spectra demonstrated phosphorylation of S8, S9, S339, and S347 residues after flg22 treatment <i>in vivo</i> (Figure 7F). To further determine whether the ratio of phosphorylation of these residues is altered in the siki-1 mutant, we conducted parallel reaction monitoring (PRM). Unmodified peptides for the S339 site were undetectable by MS and S9 phosphorylation was not detected by PRM, so we focused on the S8 and S347 residues. We did not observe a significant difference for phosphorylation of residue S8 between Col-0 and the siki-1 mutant (Figure 7G). However, phosphorylation of S347 was significantly reduced in siki-1 (Figure 7H), suggesting that SIK1 contributes to S347 phosphorylation <i>in vivo</i>. These results suggest that SIK1 positively regulates ROS by directly phosphorylating RBOHD at promoting sites in addition to enhancing BIK1 stability.

### DISCUSSION

Multiple MAP4Ks have been identified across eukaryotes based on their homology to the yeast kinase STE20 (Dan et al., 2001). Here, we characterized the <i>Arabidopsis</i> MAP4K SIK1 as an important component of plant immune responses that positively regulates extracellular ROS production and promotes PTI against the bacterial pathogen <i>Pst</i>. SIK1 associates with and stabilizes the central immune regulator BIK1 as well as directly phosphorylating RBOHD to promote the ROS burst. Importantly, we further demonstrated that, similar to yeast STE20, the <i>Arabidopsis</i> MAP4K SIK1 also associates with heterotrimeric G proteins. These results indicate that the STE20 MAP4K family exerts similar function to transduce signals from surface-localized receptors to downstream responses via heterotrimeric G proteins. Our results not only reveal regulatory mechanisms that ensure robust immune responses but also reveal the importance and functional conservation of the STE20 family across eukaryotes.

The post-translational modification and stability of PRRs and their associated members are tightly regulated to ensure appropriate and robust immune activation. Overexpression of BIK1 results in enhanced defense responses, indicating that BIK1 contributes to immune signaling in a dose-dependent manner (Monaghan et al., 2014). SIK1-mediated stabilization of BIK1 likely contributes to appropriate and robust immune signaling. Expression of BIK1-HA significantly enhances ROS production in the siki-1 mutant, further highlighting the importance of BIK1 accumulation for mediating PTI responses. Heterotrimeric G proteins also maintain BIK1 stability by attenuating the proteasome-dependent degradation of BIK1 (Liang et al., 2016; Wang et al., 2018). Interestingly, the yeast STE20 MAP4K associates with the β subunits of heterotrimeric G proteins to activate downstream signaling upon perception of pheromone by the G-protein-coupled receptor (Leberer et al., 1997). In this study, we demonstrated that SIK1 stabilizes BIK1 at a resting state and associates with XLG2, indicating that SIK1 may work together with the heterotrimeric G proteins to regulate BIK1 protein accumulation.

SIK1 promotes BIK1 stability in a kinase-dependent manner, indicating that phosphorylation by SIK1 may play a positive role in BIK1 protein stability. Previously, the CPK28 kinase was demonstrated to phosphorylate BIK1 and enhance BIK1 turnover by the proteasome (Monaghan et al., 2014). Protein turnover can also be inhibited by phosphorylation. For example, the transcriptional factor ERF6 is stabilized by MPK3/MPK6-mediated phosphorylation to ensure activation of defense-related genes (Meng et al., 2013). Phosphorylation of BIK1 S236 and T237 residues stabilizes BIK1 (Wang et al., 2018), and we show here that S236 is phosphorylated by SIK1 <i>in vitro</i>, indicating that SIK1 modulates BIK1 accumulation by direct phosphorylation. SIK1 associates with XLG2 to enhance BIK1 stability at a resting state, thus it is likely that BIK1 S236 phosphorylation occurs in the absence of pathogen perception. Given the opposing phenotypes of the cpk28 and siki knockouts, CPK28 likely phosphorylates different BIK1 residues, resulting in an opposite effect on BIK1 protein turnover. BIK1 activity and abundance is regulated by multiple mechanisms (Couto et al., 2016; Liang et al., 2016; Monaghan et al., 2014), further highlighting its importance in plant immune signaling.

The NADPH oxidase RBOHD is required for the extracellular ROS burst in response to PAMP perception (Kadota et al., 2015). This ROS burst is critical for mediating many PTI responses, including stomatal closure, callose deposition, systemic signaling, and inhibition of bacterial growth (Kadota et al., 2015). RBOHD is positively regulated by N-terminal phosphorylation mediated by multiple kinases to ensure robust immune responses (Kadota et al., 2015). BIK1 is able to phosphorylate RBOHD at four critical residues, S39, S339, S343, and S347, whose phosphorylation levels rapidly increase upon PAMP perception (Kadota et al., 2014; Li et al., 2014). RBOHD S347 was also shown to be phosphorylated by CPKs (Kadota et al., 2014). We show here that SIK1 directly interacts with and phosphorylates RBOHD’s N terminus. Among these residues, S8, S9, S339, and S347 are phosphorylated <i>in vivo</i> after flg22 treatment. Interestingly, phosphorylation of S347 is significantly reduced in siki-1, further confirming that SIK1 affects RBOHD’s phosphorylation status. S347 phosphorylation has been shown to be required for RBOHD activation (Kadota et al., 2014; Li et al., 2014; Nühse et al., 2007). These findings indicate that, in addition to regulating BIK1 protein accumulation, SIK1 directly interacts with and phosphorylates RBOHD to ensure a robust extracellular ROS burst.

Although siki and bik1 knockouts are clearly compromised in PTI responses, they also exhibit autoimmune phenotypes.
including high levels of basal SA and enhanced resistance to virulent Pst. Phosphorylated BIK1 was recently demonstrated to be directly involved in regulating levels of the defense hormones JA and SA (Lal et al., 2018). When autoimmune effects are removed, the corresponding genes of some mutants, such as saul1, have been shown to facilitate immune signaling (Tong et al., 2017). Similarly, when the high level of basal SA was removed by the expression of the SA dehydrogenase NahG, the sik1 knockout was compromised in flg22-induced bacterial growth inhibition and no longer exhibited enhanced resistance to virulent Pst. These results highlight the importance of careful phenotyping of autoimmune mutants. SIK1 homologs are present across land plants and display similar domain architecture, with a long N-terminal region and central kinase domain. The conservation of SIK1 in diverse plants indicates that homologs may use similar mechanisms to regulate RLCK stability and RBOHD phosphorylation. Thus, modification of SIK1 expression or stability could be a promising strategy to enhance crop disease resistance. Our study has uncovered a role of a conserved MAP4K family member in plant innate immunity, which motivates us to investigate the functions of other MAP4K members in the future.

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SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and four tables and can be found with this article online at https://doi.org/10.1016/j.chom.2018.08.007.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

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SUPPORTING CITATIONS
The following references appear in the supplemental Information: Laluk et al., 2011; Lin et al., 2014; Xu et al., 2013.

REFERENCES


### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to will be fulfilled by the Lead Contact, Gitta Coaker (glcoaker@ucdavis.edu).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Arabidopsis thaliana

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control. Arabidopsis T-DNA insertion lines for MAP4K members (Table S2) were obtained from the Arabidopsis Biological Resource Center (ABRC) and are in the Col-0 genetic background. T-DNA insertion mutants were genotyped by PCR using T-DNA specific and the gene-specific primers as listed in Table S3. Gene-specific primers (Table S3) were then used for RT-PCR to detect full-length transcripts. Seeds of bik1 (Lu et al., 2010), bik1 pbl1 (Zhang et al., 2010), the bik1 complementation line pBIK1::BIK1-HA (Lin et al., 2013), fls2 (SALK_062054) (Ranf et al., 2012), sik1-4 (Xiong et al., 2016) and the sik1-4 complementation line pUBQ::SIK1 (Xiong et al., 2016) used in this study were described previously.

Arabidopsis seeds were stratified for 3 days in the dark at 4 °C then sown on soil. To detect lesion mimic phenotypes, Arabidopsis plants were grown at 22 °C, with a 16-hr-light/8-hr-dark photoperiod (100 μmol m−2 s−1). For all other experiments, Arabidopsis plants were grown at 23 °C and 70% relative humidity with a 10-hr-light/14-hr-dark photoperiod (100 μmol m−2 s−1).

Nicotiana benthamiana

N. benthamiana was grown in growth chamber at 22 °C under a long-day photoperiod (16 hr light and 8 hr dark).

Bacterial Strains

P. syringae pv. tomato (Pst) DC3000 was grown at 28 °C on nutrient yeast-glycerol agar (NYGA) with appropriate antibiotics.

METHOD DETAILS

Phylogenetic Analyses and Prediction of Protein Domain Architecture

SIK1 orthologs were determined by bi-directional BLAST. Multiple sequence alignments were generated by T-Coffee (Tommaso et al., 2011). Trees were built using PhyML with default settings (Guindon et al., 2010), and displayed with FigTree (http://tree.bio.ed.ac.uk/software/figtree) and iTOL (http://itol.embl.de). The kinase domains in SIK1, SIK1 orthologs, and MAP4K family members were predicted using Pfam (Finn et al., 2016), and domain architecture was illustrated by DOG (Ren et al., 2009).
PAMP-Triggered ROS Burst

Leaf disks of four-week-old Arabidopsis plants were harvested with a cork borer (4.76 mm) and ROS burst was detected after addition of 100 nM flg22 or elf18 peptide as previously described (Lin et al., 2015). For the chitoheptaose-induced ROS assay, the L-012 chemiluminescent probe was used because it is more sensitive than luminol (Liang et al., 2013). For FLS2 protein detection, total protein extract from the indicated lines were separated on SDS-PAGE and immunoblot was performed using anti-FLS2 (1:5,000) (Agrisera) primary antibody followed by anti-rabbit-HRP (1:2,000) (BioRad) secondary antibody.

Phytohormone Measurement

Arabidopsis rosette leaves from five-week-old plants were harvested and ground in liquid nitrogen. Extraction and quantification of SA, JA, ABA, and OPDA were carried out by gas chromatography-mass spectrometry (GC-MS) as previously described (Chehab et al., 2008; Engelberth and Engelberth, 2009) using dihydro-JA, deuterated SA, and ABA as internal standards.

Quantitative RT-PCR

Total RNA from Arabidopsis leaves was extracted via the Trizol method. One microgram of total RNA was reverse transcribed with an oligo(dT) primer using M-MLV Reverse Transcriptase (Promega). The relative expression levels of the tested genes were normalized to the AtEF1α in Arabidopsis. PCR primers are provided in Table S3. Quantitative RT-PCR was performed on a CFX96 real-time System (Bio-Rad) under the following conditions: 95 °C for 30 s, then 40 cycles at 95 °C for 5 s and 60 °C for 15 s, followed by a melting curve analysis to validate specificity. The relative expression level of each gene was determined using the delta-delta Ct method (Livak and Schmittgen, 2001).

Transgenic Lines

The NahG gene was PCR amplified from pCIB200-NahG (Gaffney et al., 1993), cloned into pENTR/D-TOPO (Thermo Fisher Scientific), and recombined into pGWB14 (Nakagawa et al., 2007) by LR reaction (Thermo Fisher Scientific). pBiK1::BiK1-HA construct was described previously (Zhang et al., 2010). Binary vectors were then transformed into Agrobacterium tumefaciens strain C58C1, and transgenic lines were generated by floral dip (Clough and Bent, 1998). Transformants were selected for one-half-strength Murashige and Skoog medium supplemented with 15 μg mL\(^{-1}\) Hygromycin B. For NahG-trangenic lines, expression of NahG-3XHA was confirmed by immunoblot in T3 homozygous lines. Horseradish peroxidase (HRP)-conjugated anti-HA antibody (Sigma) was used at a 1:1,000 concentration in conjunction with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for detection. PR1 protein expression in NahG-transgenic lines was detected using anti-PR1 antibody (Wang et al., 2005). The PR1 mRNA abundance was measured by qPCR. PR1 protein expression, ROS, and bacterial growth assays were performed on sik1 NahG-HA line #1. MAPK activation was performed on sik1 NahG-HA lines #1 and #3. For BiK1-HA transgenic lines, expression of BiK1-HA protein was confirmed by western blot using an anti-HA antibody in independent T1 transgenic lines.

Bacterial Inoculation

P. syringae pv. tomato (Pst) DC3000 was grown at 28°C for 2 days on NYGA containing 100 mg mL\(^{-1}\) rifampicin and 25 mg mL\(^{-1}\) kanamycin. Bacterial cells were collected and diluted to the appropriate concentration with 5 mM MgCl\(_2\). To measure bacterial growth, Arabidopsis leaves were infiltrated with the Pst DC3000 at a concentration of 1 × 10\(^{8}\) colony-forming units mL\(^{-1}\) (CFU mL\(^{-1}\)). Bacterial titers were detected 3 days post-inoculation (dpi) as previously described (Liu et al., 2009). For the flg22 protection assay, five-week-old plants were first infiltrated with 1 μM flg22 or H\(_2\)O 24 hr before infiltration with 1 × 10\(^{5}\) CFU mL\(^{-1}\). Bacterial titers were determined 2 dpi.

Recombinant Protein Purification and Kinase Assays

SIK1 variants were cloned as maltose-binding protein (MBP) fusions for recombinant protein expression in E. coli. SIK1ΔN refers to amino acids 249-836. SIK1 cDNA fragments were PCR amplified and cloned into pENTR/D-TOPO (Invitrogen). SIK1ΔN\(^{KD}\) (K278E) variants were generated by PCR-based site-directed mutagenesis. The resulting pENTR SIK1 constructs were recombined into a modified pMAL-C4X vector (New England Biolabs) containing a Gateway cassette. The RBOHD N terminus was cloned into the pMAL-C4X vector by LR reaction. Primers used are listed in Table S3.

His-BiK1\(^{KD}\) was expressed and purified as previously described (Lal et al., 2016). The remaining expression constructs were transformed into E. coli BL21(DE3) and protein expression was induced by addition of 0.3 mM IPTG to a 250 mL culture at OD600 = 0.4, followed by incubation at 28°C for 4 hr. Cells were harvested and resuspended in column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100) for MBP-tagged proteins, and buffer A (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, and 0.1% Triton X-100) for His-tagged proteins, followed by lysis using a microfluidizer. Cell lysates were centrifuged at 20,000 g to separate insoluble cell debris, and soluble supernatant was applied to MBPTrap HP (GE Healthcare) or HisTrap HP (GE Healthcare) 5 mL column on an AKTA FPLC (GE Healthcare). MBP fusion proteins were eluted with 10 mM maltose in column buffer; His-BiK1\(^{KD}\) was eluted with a gradient of 20-500 mM imidazole in buffer A.

In vitro kinase assays were performed in kinase buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl\(_2\), 1 mM CaCl\(_2\), 100 μM ATP, 1 mM DTT). 0.5 μg of SIK1N or SIK1ΔN\(^{KD}\) and 3-5 μg of substrate were used per reaction. Reactions were initiated by incubating at 30°C for 30 min and stopped with the addition of 3 × Laemmli sample buffer. Samples were subjected to immunoblots with anti-pThr301n antibody (Cell Signaling) at a 1:2,000 concentration, followed by anti-rabbit-HRP secondary antibody (BioRad). Blots were visualized using chemiluminescent substrate.
were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for detection. For radiolabeled kinase assays, 10 μCi of γ-32P-ATP was added to each reaction and performed as described above. The proteins were separated on a 12% SDS-PAGE gel and phosphorylated proteins were visualized by X-ray film exposure.

**In Vitro Pull-Down Assays**

The coding region of BIK1, BSK1, RBOHD-N, and SIK1 was amplified using PCR. For GST-tagged proteins, GST vector (Addgene #29707) linearized with SspI was used. MBP-tagged proteins were cloned into the MBP vector (Addgene #29708) linearized with SspI. Cloning was performed using Gibson assembly (Gibson et al., 2009). Sequenced plasmids were transformed into the BL21 expression strain of *E. coli*.

Recombinant proteins were purified separately, and in vitro GST pull-downs were performed by incubating 1 μg of GST-SIK1ΔN with 4 μg of MBP-BIK1, or MBP-BSK1 with GST beads calibrated with pull-down buffer (30 mM HEPES, 300 mM NaCl, 0.2% Triton X-100, pH 7.5). Proteins were incubated with moderate shaking at 4°C for 2 hr. GST beads were washed 3 times with 100 column volumes of wash buffer (30 mM HEPES, 350 mM NaCl, 0.2% Triton X-100, pH 7.5) for 2 hr each. Protein bound to GST resin was eluted by boiling the resin in SDS sample buffer and analyzed by western blot. Similar protocol was followed for GST-RBOHD-N and MBP-SIK1ΔN pull-down assay.

**Phosphorylation Site Identification**

To identify BIK1 phosphorylation sites in vitro, the kinase inactive His-BIK1 recombinant protein was co-expressed with SIK1ΔN or SIK1ΔN<sup>CD</sup> in *E. coli* BL21(DE3) and purified using a HisTrap HP column. The purified recombinant protein was then digested with trypsin (Minkoff et al., 2014). Briefly, the recombinant protein was reduced with 5 mM DTT, alkylated with 15 mM iodoacetamide, and digested overnight with trypsin (Promega) at 37°C. To identify RBOHD phosphorylation sites, the RBOHD N terminus was co-expressed with SIK1ΔN or SIK1ΔN<sup>CD</sup> in Rosetta(DE3), and purified using a MBPTrap HP column. The RBOHD-N recombinant protein was separated on a 10% precast protein gel (BioRad), and stained with Coomassie brilliant blue. The band for MBP-RBOHD-N was excised and subjected to tryptic in-gel digestion (Elmore et al., 2012). Tryptic peptides were analyzed by LC-MS/MS using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). The Peptides were identified using X!Tandem (Craig and Beavis, 2004). Parameters were set for 20 ppm peptide tolerance. Phosphorylation of Ser, Tyr or Thr residues were allowed as variable modifications. X!Tandem results were combined in Scaffold version 4 (Searle, 2010) and exported to Excel (Microsoft Office).

To quantify phosphosites in vivo, FLAG-RBOHD (Li et al., 2014) was transiently expressed in Col-0 and sik1-1 protoplasts, and after 12 hr incubation protoplasts were treated with 1 μM flg22 for 10 min. Then total proteins were subjected to immunoprecipitation using anti-FLAG beads and followed by on bead digestion with trypsin. Isolation list for phosphopeptide and control peptides, as shown in Table S4, were acquired on the Orbitrap mass spectrometer using Skyline software (MacLean et al., 2010). The peptides were analyzed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). The resulted raw data were imported into Skyline and the peak areas were then exported into Excel (Microsoft) for further analysis.

**MAPK Activity Assay**

Four-week-old *Arabidopsis* plants were sprayed with 10 μM flg22 or water containing 0.025% Silwet L-77 for 10 min and frozen in liquid nitrogen. Tissue samples were ground in liquid nitrogen and resuspended in 50 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, 1× complete protease inhibitors (Thermo Fisher Scientific) and 1× phosphatase inhibitors (Thermo Fisher Scientific). The protein concentration was determined using the Pierce 660 nm protein assay supplemented with the Ionic Detergent Compatibility Reagent (Thermo Fisher Scientific). Equal amounts of total protein were loaded on a 12% SDS-PAGE gel, and an anti-phospho-p44/42 (Erk1/2) (Cell Signaling) monoclonal antibody was used to determine the phosphorylation state of MPK3, MPK4 and MPK6.

**RLCK Stability in Arabidopsis Protoplasts**

To generate gateway compatible vectors for transient expression in protoplasts, the 35S-T7-R1-CmR-ccdB-R2 fragment from pGW827 (Nakagawa et al., 2007) was PCR amplified and cloned into pUC19 vector (New England Biolabs). pENTR clone of SIK1 was recombined into the modified vector mentioned above by LR reaction. BAK1 cDNA fragment was cloned as an XhoI and BstBI restriction sites. All constructs were transiently expressed in *Arabidopsis* protoplasts as previously described (Yoo et al., 2007). Protein accumulation was analyzed by immunoblot with anti-HA-HRP (1:1,000) (Roche), anti-T7-HRP (1:3,000) (Millipore), and anti-FLAG-HRP (1:2,000) (Sigma) antibodies.

**Co-immunoprecipitation**

To test the association between SIK1 and BIK1, RBOHD or XLG2, the *Arabidopsis* protoplasts were transfected with the indicated constructs (Liang et al., 2016; Li et al., 2014). After 12 hr of incubation, protoplasts were treated with 1 μM flg22 for 10 min, and total protein was isolated with an extraction buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% glycerol, 5 mM DTT, 1 mM Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1% IGEPAL, 1× complete protease inhibitor and 1× phosphatase inhibitors). The extract was pre-cleared by centrifugation at 14,000 rpm for 10 min. Total protein was incubated with an agarose-conjugated anti-Flag antibody (Sigma) for 2 hr, and then washed
four times with a buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM DTT, 1 mM Na$_2$MoO$_4$·2H$_2$O and 1% IGEPAL. Immunoprecipitates were separated on a 10% SDS-PAGE gel and detected by anti-HA or anti-Flag immunoblot.

To test association between SIK1 ΔN and FLS2 in *N. benthamiana*, Agrobacterium suspensions containing p1776-SIK1 ΔN (35S: T7-SIK1 ΔN) or pEarlygate103-FLS2 (35S: FLS2-GFP-His) were co-infiltrated into *N. benthamiana* leaves. One gram of leaf tissue was collected at 48 hpi, and homogenized in IP buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM DTT, 1 mM Na$_2$MoO$_4$·2H$_2$O, 2% IGEPAL (Sigma), 1 × complete protease inhibitor (Thermo Fisher Scientific) and 1 × phosphatase inhibitors (Thermo Fisher Scientific). The homogenate was pre-cleared by centrifugation at 14,000 rpm for 20 min and further filtered using two layers of cheesecloth. Subsequently, 30 µL of GFP-Trap (Chromotek) was added to the homogenate and incubated at 4°C for 2 hr. Beads were washed 3 times with IP buffer and re-suspended in 3 × Laemmli buffer. Samples were separated on SDS-PAGE and immunoblot was performed using anti-T7-HRP (1: 3,000) (Millipore) antibody, or anti-GFP (1: 500) (Abcam) primary antibody followed by anti-rabbit-HRP (1: 2,000) (BioRad) secondary antibody.

**Effector Translocation Assays**
The adenylate cyclase (CyaA) delivery assay was performed as previously described, with slight modifications (Crabill et al., 2010). Five-week-old Col-0 and *sik1-1* leaves were infiltrated with *Pst* DC3000 containing *avrRpm1-CyaA* (Casper-Lindley et al., 2002; Mudgett and Staskawicz, 1999) at 3 × 10$^7$ CFU mL$^{-1}$. Eight hours post-infiltration, 0.56 cm$^2$ leaf discs from six individual plants were harvested as a biological replicate. Samples were flash frozen in liquid nitrogen and stored at -80°C. To extract cAMP, leaf discs were grounded to fine powder and re-suspended in 200 µL of 0.1 M HCl. Samples were diluted 300-fold with 0.1 M HCl. cAMP levels were quantified by using a direct cAMP ELISA kit (Enzo). Three biological replicates were performed.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Statistical analyses were performed using Prism 7 software (GraphPad). The data are presented as mean ± SEM. For luminal-based ROS burst and bacterial growth curve assays, n represents the number of individual plants. For quantification of BIK1-HA band intensity, PRM, and hormone measurement, n represents the number of experimental replicates. Student’s t test was used to compare means for two groups. One-way ANOVA with Dunnett’s multiple-comparison test was performed to compare means from several groups against a control group mean. Fisher’s LSD test was used to compare means between groups. Statistical analyses and the exact value of n are described in detail in the figures and figure legends.