Phosphorylation of BIK1 is critical for interaction of downstream signaling components

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Abstract

Botrytis-induced Kinase 1 (BIK1) is a receptor-like cytoplasmic kinase (RLCK) involved in the defense, growth, and development of higher plants. It interacts with various receptor-like kinases (RLKs) such as Brassinosteroid Insensitive 1 (BRI1), Flagellin Sensitive 2 (FLS2), and Perception of the Arabidopsis Danger Signal Peptide 1 (PEPR1), but little is known about signaling downstream of BIK1. Interestingly, *Arabidopsis thaliana* BIK1 (AtBIK1) displays strong autophosphorylation kinase activity on tyrosine and threonine residues, whereas *Brassica rapa* BIK1 (BrBIK1) does not exhibit autophosphorylation kinase activity *in vitro*. Herein, we demonstrated that four proteins (RGP2, PATL2, PP7, and SULTR4.1) interact with BrBIK1 but not AtBIK1 in a yeast two-hybrid (Y2H) system. We subsequently employed bimolecular fluorescence complementation (BiFC) to confirm interactions between BIK1 and candidates in *Nicotiana benthamiana*, and found that only BrBIK1 bound the three proteins tested. We selected three phosphosites, T90, T362, and T368, based on amino acid sequence alignment between AtBIK1 and BrBIK1, and performed site-directed mutagenesis (SDM) on AtBIK1 and BrBIK1. S90T, P362T, and A368T mutations in BrBIK1 restored autophosphorylation kinase activity on threonine residues comparable with AtBIK1. However, T90A, T362P, and T368A mutations in AtBIK1 did not alter autophosphorylation kinase activity on threonine residues compared with wild-type AtBIK1. Interestingly, BiFC results showed that BIK1 mutations restored kinase activity but not binding to RGP2, PATL2, or PP7 proteins. Our results suggest that phospho-BIK1 might be involved in plant innate immunity, while non-phospho BIK1 may regulate plant growth and development through interactions with RGP2, PATL2, and PP7.

Introduction

Plants must adapt to changing environmental factors in order to grow and develop. Receptor-like kinases (RLKs) are extremely important in transmembrane signaling-mediated regulation of plant growth, development, and adaptation to diverse environmental conditions, including pathogens, and they are believed to play critical roles in the perception and transmission of external signals¹. Therefore, transcriptional and post-translational regulation of their activity is pivotal for receptor signaling in plants. Recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) triggers the induction of various proteins such as FLS2 (flagellin) and Elongation Factor Tu (EFR), which is the first line of inducible defense against invading pathogens².

Among RLKs, Receptor-like Cytoplasmic Kinases (RLCKs) share homology with RLKs in their kinase domains, but lack a transmembrane domain, and they have emerged as a major class of signaling proteins that regulate plant cellular activities in response to biotic/abiotic stresses and endogenous extracellular signaling molecules as downstream components of RLKs³. Some functionally characterized RLCKs from plants play roles in development and stress responses. For example, 149 and 187 RLCK-encoding genes have been identified in *Arabidopsis thaliana* and *Oryza sativa*, respectively⁴. However, to date, little is known about the precise functions of RLCKs in higher plants.

As we know, the AtBIK1 gene is regulated by *Botrytis cinerea* infection, and inactivation of BIK1 causes severe susceptibility to necrotrophic fungal pathogens but enhances resistance to a virulent strain of the bacterial pathogen *Pseudomonas syringae* pv tomato⁵. Interestingly, AtBIK1 associates with the flagellin receptor complex to initiate plant innate immunity⁶, and BIK1 is rapidly phosphorylated by flg22 perception in an FLS2- and BAK1-dependent manner. As another aspect, BIK1 is monoubiquitinated following phosphorylation, then released from the FLS2-BAK1 complex, and internalized dynamically into endocytic compartments⁷.

BIK1 associates with multiple RLKs to regulate pathogen defense responses and brassinosteroid (BR) signaling. Since BR signaling regulates plant growth and development, BIK1 might be involved in both defense and growth through different post-translational modifications such as phosphorylation status. BIK1 and ERECTA (ER) play opposing roles in leaf morphogenesis and inflorescence architecture, and BIK1 is required to maintain appropriate auxin responses during leaf margin morphogenesis⁸. Based on genetic analysis, flg22-induced PTI against *Botrytis cinerea* requires BIK1, EIN2, and HUB1, and BIK1-mediated PTI against *P. syringae* is modulated by salicylic acid (SA), ethylene (ET), and jasmonate signaling⁹. An Asp residue (D²⁸⁷) of BAK1 is crucial for its proteolytic cleavage, and plays an essential role in BAK1-regulated plant immunity, growth hormone BR-mediated responses, and cell death containment. BAK1^{D287A} mutation impairs BAK1 phosphorylation of its substrate BIK1, and its plasma membrane localization¹⁰.

As a signaling molecule, Pep1 is an endogenous elicitor that potentiates PAMP-inducible plant responses and association with PEPR1, and acts as a negative regulator in BR signaling through direct interaction with the BR receptor BRI1 cytosolic kinase domain¹¹. The small peptide Pep1 trigger immunity through Perception of the Arabidopsis Danger Signal Peptide 1 and 2 (PEPR1 and PEPR2) in *A. thaliana*. Interestingly, PEPR1 specifically interacts with BIK1 to mediate defenses in the presence of Pep1 and PEPR1, and PEPR2 phosphorylates BIK1 with Pep1 treatment *in vitro* and *in vivo*¹². Generally, phosphorylation of proteins by protein kinases controls many cellular and physiological processes, including intracellular signal transduction. BIK1 also interacts with PBL1 in PTI defenses¹² and acts as a positive regulator of the PAMP flg22-induced increase in cytosolic calcium.

Although much is known about BIK1, the relationships between BIK1 and downstream components related to plant growth, development, and defense signaling remain poorly understood in higher plants. In the present study, we found that AtBIK1 displayed strong autophosphorylation kinase activity on tyrosine and threonine residues, unlike BrBIK1. We employed yeast two-hybrid (Y2H) assays to identify downstream components and explore BIK1 downstream signaling interactors with AtBIK1 and BrBIK1 as bait. We identified four proteins: UDP-arabinopyranose mutase 2 (RGP2), which is essential for cell wall and pollen development¹³; PATL2, a carrier protein that may be involved in membrane trafficking events associated with cell plate formation during cytokinesis¹⁴; serine/threonine-protein phosphatase 7 (PP7), which acts as a positive regulator of cryptochrome signaling involved in hypocotyl growth inhibition and cotyledon expansion under white and blue light conditions¹⁵, control of stomatal aperture¹⁶, and

thermotolerance in *Arabidopsis*¹⁷; and SULTR4.1, a sulfate transporter that interacts with BrBIK1 but not AtBIK1, and displays strong autophosphorylation kinase activity. The results suggest that interactions between BIK1 and downstream components are dependent on the BIK1 phosphorylation status.

Material And Methods

Yeast Two-hybrid Assay

To construct the BIK1 bait, we amplified full-length BIK1 (392 residues) using primers 5'-GC GAA TTC GGT TCT TGC TTC AGC TCT TG-3' (forward) and 5'-CG GGA TCC TTA ATG CTT CCC AAA AGG TCT T-3' (reverse). The BIK1 bait (1182 nt) was cloned into *EcoRI/BamHI* restriction sites of the pGBKT vector, which contains the DNA-binding domain of GAL4 (GAL4DB), the *TRP1* yeast selection marker, the Kan^r marker, and the replication origin sequence of the yeast 2μ plasmid. It contains a fully functional pADH1 promoter in front of the bait gene. The junction between the GAL4 DNA-binding domain and the BrBIK1 gene was confirmed by DNA sequencing. For Y2H screening, a *Brassica rapa* cDNA activation domain (AD) library and an *A. thaliana* cDNA library were used. The cDNA inserts were integrated into the pGADT7 vector in three different frames. The cDNA inserts were introduced into yeast strain PBN204 using a *SmaI*-linearized pGADT7-Rec vector in three different frames by yeast homologous recombination. For self-activity testing of the BIK1 bait, we used a Y2H system based on transcriptional activity of the reconstituted GAL4 transcriptional activator. Therefore, the bait by itself should not function as a transcriptional activator.

To test the transcriptional activity of the BIK1 bait, the bait plasmid was introduced alongside an empty prey vector into yeast strain PBN204. The negative control prey vector was pACT2, which contains a GAL4 transcriptional activation domain, the *LEU2* yeast selection marker, the Amp^r marker for *Escherichia coli* selection, and the replication origin of the 2μ plasmid for replication in yeast. PBN204 contains *ADE2*, *URA3*, and *lacZ* genes as reporters based on different *gal* promoters. Yeast transformants were selected on synthetic dextrose (SD) minimal medium lacking leucine and tryptophan (SD-LW). Transformants were replica plated onto various SD selection media, including medium lacking leucine, tryptophan, and uracil (SD-LWU), and medium lacking leucine, tryptophan, and adenine (SD-LWA). Yeast transformants containing bait did not grow on SD selection media and they did not show beta-galactosidase activity in X-gal filter assays. This indicates that the BIK1 bait does not have transcriptional activator function, making it suitable for YTH selection in the PBN204 yeast strain. We also carried out BIK1 bait self-activity tests in the AH109 strain, which has a different genetic background, and the results were the same as those in PBN204. As a positive control, yeast were transformed with the Tag bait plasmid and the p53 prey plasmid; pGBKT7-53 encodes the Gal4 DNA-binding domain fused with murine p53, and pGADT7-Tag encodes the Gal4 AD fused with the SV40 large T-antigen. Negative controls were transformed with parental bait vector (pGBKT7) and prey vector (pGADT7).

BiFC Assay

Reverse reaction (LR) recombinations of appropriate open reading frames (AtBIK1, BrBIK1, AtRGP2, AtPATL2, AtPP7) in either pDONR207 or pDONR/zeo were performed with the split-YFP destination vectors pDEST-GWVYNE and pDEST-GWVYCE to generate N- or C-terminal fusions with the N- and C-terminal yellow fluorescent protein (YFP) moieties, respectively ¹⁸. Recombined vectors were transformed into *Agrobacterium* strain GV3101. Six-week-old *N. benthamiana* leaves were agro-infiltrated as previously described ¹⁹. After 48 h, YFP fluorescence was visualized using a Super Resolution Confocal Laser Scanning Microscope (LSM 880 with Airyscan, Zeiss, Jena, Germany). For each experiment, five cells from each of three leaves were measured (n = 20).

Amino Acid Sequence Alignment

Amino acid sequences of AtBIK1 and BrBIK1 were obtained from TAIR (<https://www.arabidopsis.org>) and Brassica (<http://brassicadb.org>) databases, and sequence alignment was performed using genedoc(<https://genedoc.software.informer.com/2.7>). Ser, Thr, and Tyr phosphorylation sites were predicted by UniProt (<https://www.uniprot.org/>).

Recombinant Protein Production and Purification

To produce LRR-RLK recombinant proteins, *E. coli* BL21 (DE3) cells (Novagen, Temecula, CA, USA) were transformed with vectors containing the genes of interest. *E. coli* cells were grown in Luria-Bertani (LB) medium, and expression of receptor kinases was induced with 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA) when the optical density at 600 nm (OD₆₀₀) of the cell culture reached 0.6. For time series analysis of activity, *E. coli* cells were incubated at room temperature with shaking for the indicated times (up to 16 h) following IPTG induction. Cells were harvested by centrifugation and resuspended in buffer containing 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS; pH 7.5), 150 mM NaCl, and protease inhibitors before being lysed by sonication. Cell lysates were fractionated by centrifugation at 35,000 $\times$ g into soluble and pellet fractions. Recombinant FLAG-tagged protein kinases in the soluble fractions were immunopurified on an anti-FLAG M2 affinity gel (Sigma-Aldrich). After purification, protein solutions were dialyzed against a 1000 $\times$ volume of dialysis buffer containing 20 mM MOPS (pH 7.5) and 1 mM dithiothreitol (DTT; Sigma-Aldrich) as previously described ²⁰.

Electrophoresis and Immunoblotting

Recombinant protein preparations were mixed with pre-heated (95°C) 1 $\times$ sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 1 M urea, 0.7 M 2-mercaptoethanol, 5 mM NaF, 1 mM Na₂MoO₄, 1 mM Na₃VO₄, 1 mM aminoethylbenzenesulfonyl fluoride, and 2 mM EDTA. Protein concentrations were determined by dye-binding assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a protein standard. Proteins were separated on 12% polyacrylamide (0.1% SDS) gels and transferred to polyvinylidene difluoride (PVDF) fluorescence-specific membranes (Millipore, Bedford, MA, USA). Membranes were blocked in a 2% (v/v) fish gelatin solution in phosphate-buffered saline (PBS; 5 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) before being incubated with primary

antibodies, which were diluted as specified in PBS containing 0.1% (v/v) Tween-20 (PBST). Purified recombinant proteins or proteins in the soluble fraction were analyzed using SDS-PAGE and immunoblotting with anti-FLAG (1:5000) and anti-GST (1:5000), antiphosphothreonine (1:500), and antiphosphotyrosine (1:500) antibodies to monitor the overall pattern of phosphorylation and level of recombinant protein expression in *E. coli*. Immunoblots were scanned using an Odyssey C-Digit scanner (LI-COR Bioscience, Lincoln, NE, USA) for visualization ²⁰.

Site-directed Mutagenesis

The described GST-AtBIK1, Flag-AtBIK1, and Flag-BrBIK1 constructs were used as templates for site-directed mutagenesis with a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). Six individual constructs were generated with the substitutions T90S, T362P, and T368A for AtBIK1, and S90T, P362T, and A368T for BrBIK1. All constructs were sequenced in both directions to verify specific mutations and the absence of additional mutations.

Results And Discussion

Higher plants have evolved numerous RLKs and RLCKs that modulate many different biological processes, including innate immunity, growth and development, abiotic stresses, and stomatal aperture. Phosphorylation of the RLK/RLCK complex is an essential step in the initiation of diverse biological and physiological processes. Among RLCKs in Arabidopsis, BIK1 is a serine/threonine kinase that possesses tyrosine kinase activity, and mass spectrometry, immunoblot, and genetic analyses have shown that it is autophosphorylated at multiple Ser/Thr/Tyr residues. BAK1, a co-receptor of BRI1, is able to phosphorylate BIK1 at both tyrosine and serine/threonine residues ²². BIK1 (Y150) blocks both tyrosine and serine/threonine kinase activity, whereas BIK1 (Y243 and Y250) are more specifically involved in tyrosine phosphorylation ²². In the present study, AtBIK1 showed strong autophosphorylation kinase activity on tyrosine and threonine residues, in contrast to BrBIK1, despite high amino acid similarity between these two proteins (Fig. 1). To identify downstream signaling components of BIK1 related to growth and defense signaling in *A. thaliana*, we used an Arabidopsis library for Y2H screening, and identified four proteins that interact with BrBIK1 but not AtBIK1 (Fig. 2A); UDP-arabinopyranose mutase 2 (RGP2), Patellin-2 (PATL2), serine/threonine-protein phosphatase 7 (PP7), and sulfate transporter 4.1 (SULTR4.1). RGP2 is essential for proper cell wall and pollen development ¹³. PATL2 may be involved in membrane trafficking events associated with cell plate formation during cytokinesis ¹⁴. PP7 acts as a positive regulator of cryptochrome signaling involved in hypocotyl growth inhibition, cotyledon expansion under white and blue light conditions ¹⁵, and control of Arabidopsis stomatal aperture ¹⁶. SULTR4.1 is an H⁺/sulfate cotransporter that may play a role in the regulation of sulfate assimilation. Y2H results suggested that BrBIK1 interactors might be involved in plant growth and development processes, including sulfate assimilation, which indicates that non-phosphorylated BIK1 might not be involved in defense mechanisms, including PTI.

We also employed BiFC experiments to confirm interactions between BIK1 and RGP2, PP7, and PATL2 in *N. benthamiana* leaves. As a positive control we used SOS2, which encodes a novel Ser/Thr protein kinase that also functions in salt tolerance in Arabidopsis²², and SOS3 that encodes a novel EF-hand Ca^{2+} sensor²³. We confirmed that AtBIK1 does not interact with RGP2, PP7, and PATL2 (Fig. 2C), whereas BrBIK1 strongly interacts with these proteins (Fig. 2B). The results indicate that phosphorylation of BIK1 is not necessary for RGP2, PP7, PATL2, and BrBIK1 interactions, suggesting that BIK1 phosphorylation is essential for plant defense signaling, but not necessary for plant growth and development signaling.

Therefore, we performed amino acid sequence alignments and investigated known phosphorylation sites of AtBIK1 in *A. thaliana*. Protein phosphorylation is a key event in signal transduction pathways. When upstream signals are stimulated through receptor kinases, protein kinases are activated and phosphorylate their substrates on Ser/Thr and tyrosine residues, modulating their localization, conformation, and activity. In some cases, phosphorylated substrates become recognizable to other proteins, and such interactions transduce and propel the signal onward. Certain domains specifically recognize phosphorylated residues of proteins, regulating cell growth and differentiation²⁴. Analysis of recombinant RLCKs revealed distinct autophosphorylation activity at tyrosine and threonine residues in *E. coli*²⁵. Given that phosphorylation of RLCKs generally initiates and mediates signal transduction pathways, these two RLCKs might also be involved in sensing and responding to cellular needs in Arabidopsis²⁵.

In the present work, we focused on the status of BIK1 phosphorylation, which may be essential for interactions between BIK1 and downstream components to regulate diverse physiological processes. Therefore, we cloned BrBIK1 and AtBIK1 into the pDEST15B and pFlag recombinant expression vectors. The results of western blotting analysis with anti-pThr and anti-pY antibodies showed that GST-BrBIK1 and Flag-BrBIK1 did not undergo autophosphorylation at threonine and tyrosine residues. However, in contrast with BrBIK1, AtBIK1 exhibited strong autophosphorylation kinase activity at threonine and tyrosine residues (Fig. 3A and B). Thus, we performed site-directed mutagenesis (SDM) to manipulate autophosphorylation of AtBIK1 and BrBIK1 based on amino acid sequence alignment (Fig. 1), and generated three threonine phosphorylation site mutants in GST-AtBIK1 (T90S, T362P, and T368A). The three sites differ from those in BrBIK1. The results showed that GST-AtBIK1(T90S), GST-AtBIK1(T362P), GST-AtBIK1(T368A), and wild-type GST-AtBIK1 exhibited almost identical autophosphorylation kinase activities (Fig. 3B). We also used SDM to generate Flag-BrBIK1 mutants P362T and A368T. Interestingly, both mutations rescued autophosphorylation kinase activity at threonine residues to levels comparable with AtBIK1 *in vitro* (Fig. 3C). Thus, we tested protein interactions using BiFC experiments with BrBIK1 (P362T, A369T) and RGP2, PATL2, and PP7. Surprisingly, BrBIK1 (P362T, A369T) did not bind downstream components RGP2, PATL2, and PP7 (Fig. 4A and B). These results suggest that the phosphorylation status of BIK1 may play an important role in regulating between growth and defense in higher plants. The findings indicate that autophosphorylation of RLCKs such as BIK1 provides a convenient and powerful system for elucidating kinase specificity *in vitro* and *in vivo*.

Declarations

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, MH.O. and E.S.O.; Original draft preparation, JH.C., MH.O., and MH.O.; Reviewing and editing, all authors. All authors read and agreed to the published version of the manuscript.

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Figures

Figure 1

Figure 1

Amino acid sequence alignment of AtBIK1 and BrBIK1. Amino acid sequences of AtBIK1 and BrBIK1 were obtained from TAIR (<https://www.arabidopsis.org>) and Brassica (<http://brassicadb.org>) databases. Ser, Thr, and Tyr phosphorylation sites were predicted by UniProt (<https://www.uniprot.org/>).

Figure 2

Figure 2

Protein-protein interaction analysis of BIK1 and RGP2, PATL2, PP7, and SULTR4.1 using Y2H and BiFC assays. (A) For Y2H screening, a Brassica rapa cDNA AD library and an Arabidopsis thaliana cDNA library were used. Each insert DNA was integrated into the pGADT7-Rec vector by yeast homologous recombination. As a positive control, tagged bait and p53 prey plasmids were transformed into yeast cells; pGBKT7-53 encodes the Gal4 DNA-binding domain fused with murine p53; pGADT7-Tag encodes the Gal4 AD fused with SV40 large T-antigen. Cells were transformed with parental bait vector (pGBKT7) and prey vector (pGADT7) as negative controls. (B, C) Colocalization between BrBIK1-YFPn, AtBIK1-YFPn, and three different proteins fused with YFPc. The overlay picture shows transiently transformed N. benthamiana leaves co-infiltrated with BrBIK1-YFPn and AtRGP2, AtPP7, or AtPATL2. Fluorescence signals were detected using a super-resolution confocal laser scanning microscope. Scale bars = 100 μm.

Figure 3

Figure 3

Phosphorylation of recombinant BIK1 and mutant proteins. (A) Immunoblot analysis of recombinant BIK1 proteins autophosphorylated during production in *E. coli* using previously described anti-pThr and anti-pY antibodies. (B and C) Phosphorylation of recombinant proteins at engineered threonine residues on AtBIK1, and the effects of site-directed mutagenesis of threonine residues in AtBIK1 and BrBIK1 on autophosphorylation of recombinant proteins.

Figure 4

Interactions between AtBIK1 and BrBIK1 mutants and candidate interacting proteins. (A) BiFC assay analysis of interactions between AtBIK1 mutants (T362P, T368A) and truncated proteins (AtRGP2, atPATL2, AtPP7). (B) BiFC assay analysis of interactions between BrBIK1 mutants (P362T, A368T) and truncated proteins (AtRGP2, atPATL2, AtPP7). Interactions between SOS2-YFPn and SOS3-YFPc served as controls. Scale bars = 100 μ m.

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