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RESEARCH ARTICLE

Identification and functional analysis of phosphorylation residues of the *Arabidopsis* BOTRYTIS-INDUCED KINASE1

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ABSTRACT

***Arabidopsis* BOTRYTIS-INDUCED KINASE1 (BIK1) is a receptor-like cytoplasmic kinase acting early in multiple signaling pathways important for plant growth and innate immunity. It is known to form a signaling complex with a cell-surface receptor FLS2 and a co-receptor kinase BAK1 to transduce signals upon perception of pathogen-associated molecular patterns (PAMPs). Although site-specific phosphorylation is speculated to mediate the activation and function of BIK1, few studies have been devoted to complete profiling of BIK1 phosphorylation residues. Here, we identified nineteen *in vitro* autophosphorylation sites of BIK1 including three phosphotyrosine sites, thereby proving BIK1 is a dual-specificity kinase for the first time. The kinase activity of BIK1 substitution mutants were explicitly assessed using quantitative mass spectrometry (MS). Thr-237, Thr-242 and Tyr-250 were found to most significantly affect BIK1 activity in autophosphorylation and phosphorylation of BAK1 *in vitro*. A structural model of BIK1 was built to further illustrate the molecular functions of specific phosphorylation residues. We also mapped new sites of FLS2 phosphorylation by BIK1, which are different from those by BAK1. These *in vitro* results could provide new hypotheses for more in-depth *in vivo* studies leading to deeper understanding of how phosphorylation contributes to BIK1 activation and mediates downstream signaling specificity.**

KEYWORDS phosphorylation, BIK1, receptor-like cytoplasmic kinase, quantitative mass spectrometry

INTRODUCTION

In plants, innate immunity is triggered by cell-surface pattern-recognition receptors (PRRs) which usually perceive conserved pathogen- or microbe-associated molecular patterns (PAMP/MAMPs) (Boller and Felix, 2009; Boller and He, 2009; Zhang and Zhou, 2010). Receptor-like kinases (RLKs) constitute this major superfamily of PRRs in plant cells and they have been identified as critical early determinants of PAMP/MAMP-triggered immunity (PTI) (Antolin-Llovera et al., 2012; Greeff et al., 2012). Compared to RLKs, the biological functions of receptor-like cytoplasmic kinases (RLCKs) remain much less understood. Although lacking an apparent extracellular domain, RLCKs are classified within the RLK superclass due to their sequence homology. The *Arabidopsis* genome contains over 600 RLKs and RLCKs that are predicted to function in plant responses to microbial infection, hormones, and other endogenous and environmental cues (Shiu and Bleecker, 2001, 2003). Similar to RLK, RLCKs have been implicated to function in PTI and ETI (effector-triggered immunity) responses (Lu et al., 2010a; Zhang et al., 2010; Kim and Hwang, 2011; Laluk et al., 2011). They are speculated more likely to mediate intracellular signal transduction rather than ligand perception, particularly acting in concert with surface-localized RLKs or indirectly as intracellular receptors of microbial effectors (Zhang et al., 2010; Laluk et al., 2011).

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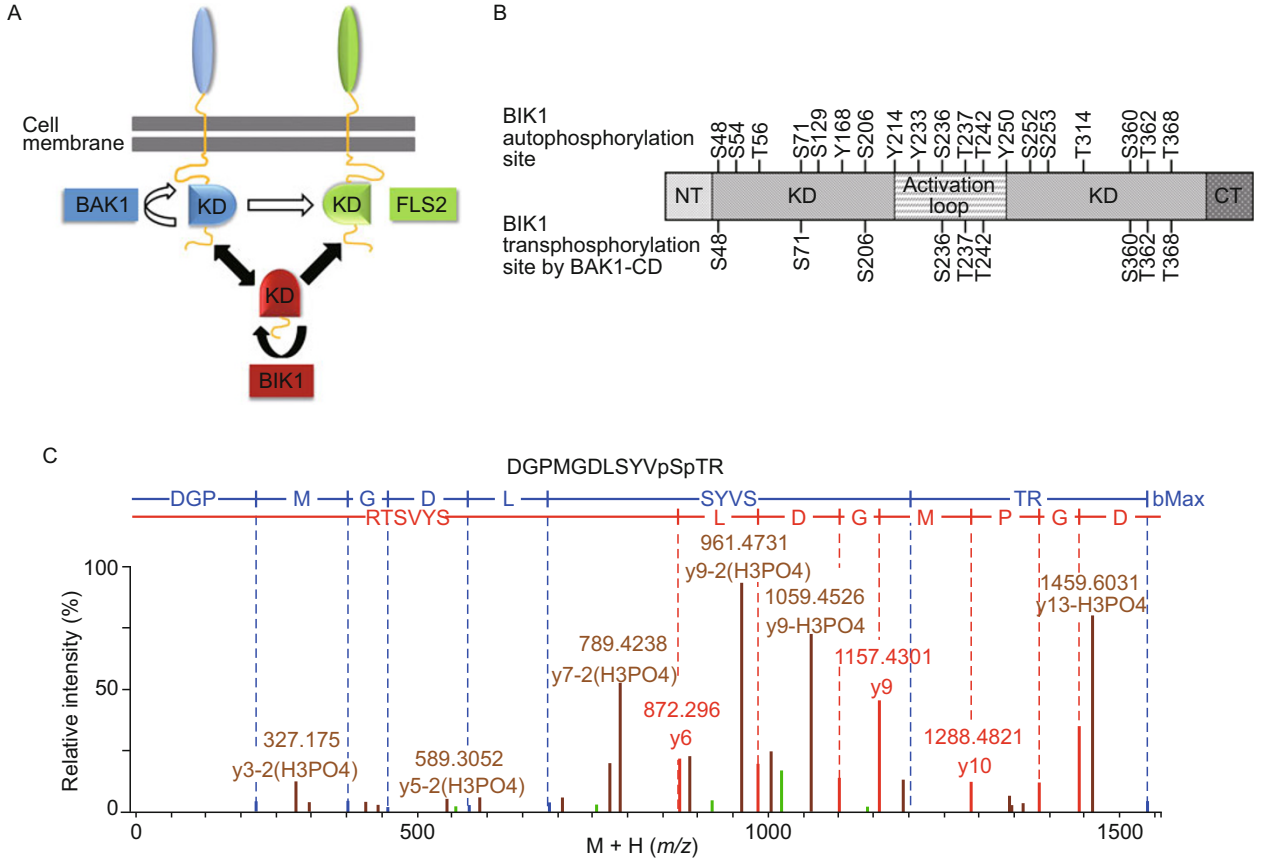

Figure 1. The recombinant BIK1 is highly phosphorylated. (A) A model depicts known phosphorylation events within the FLS2/BAK1/BIK1 complex. KD, kinase domain. A single arrow: phosphorylation of a substrate by its kinase; a double arrow: reciprocal phosphorylation between the two kinases; a curved arrow: autophosphorylation. Black arrows refer to phosphorylation events studied in this work while white arrows indicate those reported elsewhere. (B) Summary of phosphorylation sites identified in different domains of BIK1. KD, kinase domain; NT, N-terminus; CT, C-terminus. Shown upper are autophosphorylation sites whereas lower are sites of BIK1 KM phosphorylation by BAK1-CD. (C) MS/MS spectrum of a BIK1 di-phosphopeptide with the phosphosites assigned to be S236 and T237.

BIK1 is a typical RLCK localized to the plasma membrane that was found to act early in multiple signaling pathways important for plant growth, development as well as immune responses against pathogens (Lu et al., 2010a; Zhang et al., 2010; Laluk et al., 2011). It was originally identified as a component in plant defense against necrotrophic fungal pathogens (Veronese et al., 2006). More recently, elegant studies by several groups have showed BIK1 plays a central role in signal integration from multiple PAMP receptors including the flagellin receptor FLS2, the chitin receptor CERK1 and the receptor for bacterial EF-TU EFR (Lu et al., 2010a; Zhang et al., 2010). Specifically, BIK1 associated with these PAMP receptors and a signaling co-receptor BRI1-associated receptor kinase 1 (BAK1) *in vitro* and *in vivo*, and its phosphorylation was rapidly induced upon flagellin treatment in an FLS2- and BAK1-dependent manner.

Site-specific phosphorylation, either by itself or upstream kinases, is known to be essential for RLK/RLCK functions *in vitro* and *in vivo* (Wang et al., 2008; Lu et al., 2010b; Schwessinger et al., 2011). Auto- and trans-phosphorylation events between BIK1 and the receptor complex FLS2/BAK1 *in vitro* documented previous work (Lu et al., 2010a, 2010b; Zhang et al., 2010) are depicted in Fig. 1A. The kinase domain of both BIK1 and BAK1 can phosphorylate itself as well as the cytoplasmic domain of FLS2. In addition, reciprocal phosphorylation occurs between BIK1 and BAK1. In contrast to BAK1 on which distinctive phosphorylation sites have been clearly identified (Wang et al., 2005, 2008; Yan et al., 2012), few studies have been devoted to complete profiling of phosphorylation residues of BIK1. Biochemical analysis of site-directed mutants suggested Thr-237 was the major phosphorylation site of BIK1 in response to flagellin (Lu et al., 2010a). Another extensive study on BIK1 *in vivo* functions revealed several other putative phosphorylation sites (e.g. Ser-236, Thr-242) contributing to BIK1 kinase activity as well as its biological roles in PTI and ETI responses (Laluk et al., 2011). Little evidence was provided, however, to confirm any of them as a *bona fide* phosphorylation site except Ser-236 identified in a previous study (Zhang et al., 2010).

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In general, plant RLKs are classified as serine/threonine kinases whereas most of the animal cell-surface receptor kinases are tyrosine kinases (Oh et al., 2010). However, a couple of plant leucine-rich repeat RLKs such as the brassinosteroid-insensitive 1 (BRI1) and BAK1 were reported to phosphorylate on tyrosine residues in addition to serine/threonine residues (Oh et al., 2009, 2010, 2012). Importantly, previous studies implicated that tyrosine phosphorylation plays an important role in receptor kinase signaling essential for plant growth and innate immunity (Oh et al., 2009, 2010). Whether BIK1 is a dual-specificity kinase and how tyrosine phosphorylation mediates its activation remain largely unknown.

In the current study, we identified a number of autophosphorylation residues in the kinase domain of BIK1 including three tyrosine residues, thereby proving BIK1 is a dual-specificity kinase for the first time. The distinct impact of specific phosphorylated residues on BIK1 activation and further phosphorylation of a natural substrate BAK1 was explicitly defined by an established quantitative MS approach (Voolstra et al., 2010; Soderblom et al., 2011). A structural model was built to further illustrate the molecular functions of specific phosphorylated residues. Given that BIK1 serves as both a kinase and a substrate in the FLS2 receptor complex (Fig. 1A), we also mapped the sites of FLS2 phosphorylation in the kinase domain by BIK1, as well as the sites of BIK1 phosphorylation by BAK1.

RESULTS

Identification of BIK1 autophosphorylation residues and residues phosphorylated by BAK1-CD *in vivo*

We first sought to identify the autophosphorylation residues of the recombinant BIK1 by mass spectrometric analysis. Using a sensitive workflow combining phosphopeptide enrichment and nanoLC-MS/MS analysis (Yan et al., 2012), we were able to unambiguously map 16 Ser/Thr autophosphorylation sites on BIK1 (Fig. 1B, see details of MS analysis in Table S3 and Fig. S2). A representative fragmentation spectrum for the identification of phosphorylated Ser-236 and Thr-237 is shown in Fig. 1C. Among these phosphorylated residues, S233, S236, T237 and T242 are located in the activation loop (AL) of BIK1 (Fig. 1B). A BIK1 mutant D202A serves as a kinase-dead mutant (KM) as it disrupts the RD domain essential for the kinase function (Johnson et al., 1996; Laluk et al., 2011). Notably, no phosphopeptides were detected from BIK1 KM using the same approach, indicating all the identified phosphorylation events were dependent on the kinase activity.

BIK1 is known to be a substrate of another multi-functional plant RLK, BAK1, within the flagellin receptor complex initiating PTI response in plants (Lu et al., 2010a). We then determined the sites on BIK1 KM phosphorylated by BAK1 cytoplasmic domain (BAK1-CD), and found them all included in the autophosphorylation sites of BIK1 (Fig. 1B). Although BIK1 was associated with the cell-surface receptor FLS2 as well as BAK1, and both of them were required for BIK1 phosphorylation *in vivo* (Lu et al., 2010a), we didn't detect any direct phosphorylation of BIK1 KM by FLS2-CD. Therefore, we speculate that flg22-induced BIK1 activation might be enhanced through transphosphorylation by BAK1 rather than FLS2, with the latter possibly serving more as a scaffold protein contributing to the receptor complex assembly. Certainly this hypothesis awaits further investigation *in vivo*.

Phosphorylation residues of BIK1 have distinct impact on its autophosphorylation activity

To investigate a potential role of the identified phosphorylation sites in mediating BIK1 activity, we need to examine the site-specific impact on BIK1 activation using a quantitative and unbiased approach. Label-free quantitative mass spectrometry has emerged as a powerful tool for sensitive and accurate measurement of protein PTM dynamics in a site-specific manner (Voolstra et al., 2010; Gunawardena et al., 2011). Herein, we adapted this approach to the relative quantitation of phosphorylation status in BIK1 mutants vs. the wild-type.

Six phosphorylated Ser/Thr residues including four in AL that are highly conserved in BIK1-related kinases from *Arabidopsis* and other species (Laluk et al., 2011) were selected for site-directed mutagenesis (Fig. 2A). Only phosphopeptides that were detected in all experimental replicates of the mutant and the wild-type were subjected to quantitation. The four phosphorylation sites in AL and four other sites in the kinase domain met these criteria and were quantified (Fig. 2A and Table S5). Notably, for phosphorylated Ser-233, Ser-236 and Thr-237, they are all located in the same peptide sequence (226–238) and thus comprise multiple regioisomers of the singly-phosphorylated peptide that were inseparable by nano-UPLC. Therefore, these regioisomers had to be quantified together, though the fragmentation spectra revealed it was more likely to be modified on either Ser-233 or Ser-236 (Fig. S2). Phosphorylation of Thr-237 was predominantly detected on the di-phosphorylated peptide variant with pSer-236 and pThr-237, thereby it was quantified based on the signal of this di-phosphopeptide. Phosphorylation on Thr-242, Ser-252 or Ser-253 had to be quantified together based on the signal of the mono-phosphopeptide although MS/MS data suggested T242 was the major phosphorylation site (Fig. S2).

Distinct impact of mutating individual phosphorylation sites on the autophosphorylation patterns of BIK1 is illustrated in Fig. 2A. Detailed quantitation data reported in Table S5 showed excellent reproducibility, with all experimental variations below 15%. The six phosphorylation sites can be divided into three groups according to their relative importance for the kinase activation. Group 1 includes Thr-237 and Thr-242, of which substitution to Ala eliminated or substantially suppressed (<30% of the wild-type) phosphorylation at all quantified sites. Intriguingly, the phosphomimic mutant of Thr-237 (T237D) fully or partially restored phosphorylation levels at most sites except S233/S236, whereas T242D mutant remained almost completely dephosphoryated at all quantified sites (<5% of the

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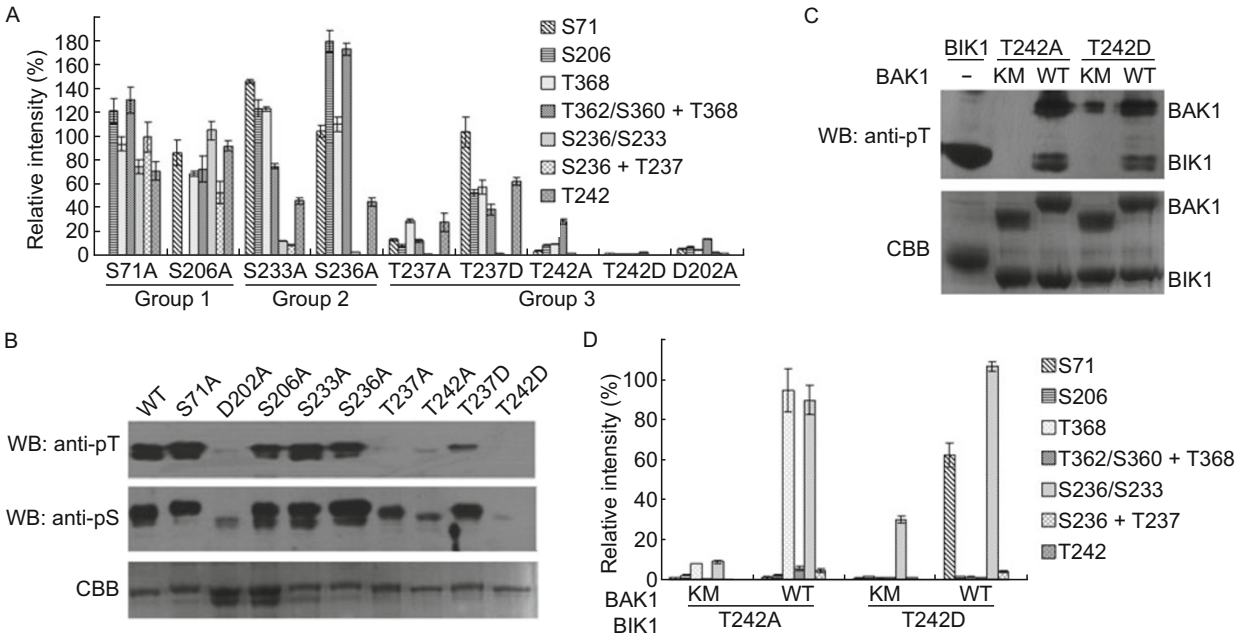

Figure 2. Phosphorylation residues of BIK1 have distinct impact on its autophosphorylation activity. (A) MS-based quantitation of specific phosphopeptides from BIK1 mutants relative to the wide-type (WT, defined as 100%). Peptides with distinct phosphorylation sites are indicated by columns of different patterns. The MS responses of phosphopeptides were normalized to the spiked-in casein peptide. Error bars are STD from three independent experiments. (B) Immunoblot probing overall Thr and Ser phosphorylation levels of BIK1 (upper); the protein loading control was shown by Coomassie Brilliant Blue (CBB) staining (lower). (C) BAK1-CD phosphorylates BIK1 T242A and T242D. The BIK1 mutant was incubated with BAK1-CD WT or BAK1-CD KM and its phosphorylation was probed by immunoblot (upper). (D) MS-based quantitation of site-specific phosphorylation in BIK1 T242A and T242D treated by BAK1-CD (WT or KM). The phosphorylation level of each peptide from BIK1 mutants was relative to that from BIK1 WT under the same treatment (defined as 100%).

wild-type). These results suggested that any change in these two critical residues is likely to disrupt the kinase activity. Group 2 consists of Ser-233 and Ser-236, for which S-to-A substitution mainly affected phosphorylation on Ser-233, Ser-236, Thr-237 and Thr-242. Their effect in mediating the kinase activity seems to be more restricted within the activation loop. Group 3 consists of residues Ser-71 and Ser-206, of which the mutation didn't significantly alter autophosphorylation at any site assessed.

Western blots probing the overall phosphorylation levels of BIK1 wild-type and various mutants were in general consistent with our MS-based quantitative comparison (Fig. 2B). The phosphomimic mutant T237D did rescue the autophosphorylation activity on both Thr and Ser residues to a certain extent, yet the other mutant T242D had no such effect, which agreed well with our findings by MS analysis. The phosphorylation levels of Group 2 mutants (S233A and S236A) didn't differ much from those of Group 1 mutants (S71A and S206A) in the Western blots. We infer that the abundance of phosphorylation on AL residues might not contribute significantly to the overall phosphorylation state of the kinase, thereby changes at these residues were difficult to assess by the anti-pSer/pThr antibodies. Alternatively, the antibodies could bias their specificity to the detection of certain residues, resulting in difficulties in detecting changes at those "overlooked" residues. Our MS-based approach, therefore, provides a means of measuring phosphorylation variation complementary to classical methods.

Given that mutants T242A and T242D displayed strong phosphorylation *in vivo* upon specific elicitation (Lu et al., 2010a; Laluk et al., 2011), yet they showed very weak autophosphorylation in our assays, we performed an *in vitro* kinase assay to see whether the two mutants could be phosphorylated by the active BAK1. Western blot demonstrated that the active BAK1-CD rather than BAK1-CD KM was able to phosphorylate the two BIK1 mutants (Fig. 2C). Further MS analysis revealed transphophorylation occurred on different sets of residues in the two mutants (Fig. 2D and Table S6).

Phosphorylation residues of BIK1 have differential impact on its kinase activity toward BAK1

The reciprocal phosphorylation between BAK1 and BIK1 allows BIK1 to be not only a substrate but also a kinase of BAK1 (Lu et al., 2010a) (Fig. 1A). We chose BAK1 as the substrate to further unveil the roles of BIK1 phosphorylation in mediating its kinase activity. Because of the strong autophosphorylation activity of BAK1-CD *in vitro*, the BAK1-CD KM was used as the substrate in the kinase assay.

BIK1 turned out to phosphorylate BAK1-CD KM on most of the residues that were autophosphorylated in BAK1-CD *in vitro*

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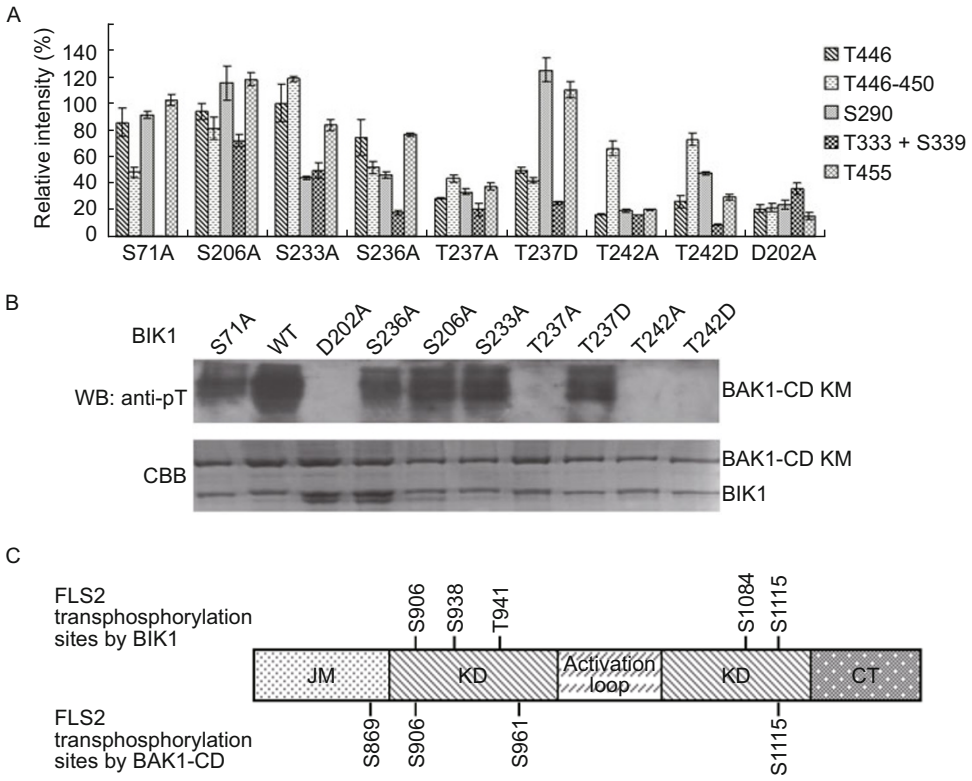

Figure 3. Phosphorylation residues of BIK1 have distinct impact on its phosphorylation activity toward BAK1. (A) Phosphorylation on specific sites of BAK1-CD KM treated by a BIK1 mutant was relative to that treated by BIK1 WT (defined as 100%). BAK1-CD peptides with distinct phosphorylation residues are indicated by columns of different patterns. The MS responses of phosphopeptides were normalized to the spiked-in casein peptide. Error bars are STD from three independent experiments. (B) Immunoblot of BAK1-CD KM treated by BIK1 WT and mutants (upper); the protein loading control was shown by CBB staining (lower). (C) Phosphorylation sites identified in FLS2-CD treated by BIK1 (upper) or BAK1-CD (lower). KD, kinase domain; CT, C-terminus; JM, juxtamembrane region.

(Yan et al., 2012), including the four Ser/Thr residues in its AL also phosphorylated *in vivo* upon brassinolide treatment (Wang et al., 2008). Notably, Thr-455 in BAK1 AL phosphorylated by BIK1 was shown to be critical for BAK1 kinase activity as well as its function in BR and flagellin signaling (Wang et al., 2008). It indicates a potential role of BIK1 in enhancing BAK1 activity through transphosphorylation. Site-directed mutagenesis on individual phosphorylation sites of BIK1 differentially affected its kinase activity toward BAK1 substrate, revealed by our quantitative MS analysis (Fig. 3A and Table S7) as well as immunoblot (Fig. 3B). The impact of the six selected residues on BIK1 activity of phosphorylating BAK1 was generally in accord with their impact on BIK1 autophosphorylation activity. Mutation on Group 1 residues (T237A and T242A) significantly reduced phosphorylation on almost all seven quantified sites in BAK1, and T237D was able to restore phosphorylation on Ser-290 and Thr-455 in BAK1 to the wild-type level (Fig. 3A). Mutants of Group 2 and Group 3 residues showed intermediate to minimal effect in suppressing phosphorylation of BAK1, with a few or no sites affected. Unexpectedly, the activity of mutant S71A was disrupted to a larger extent in regard to phosphorylation of the substrate than autophosphorylation. Tri-phosphorylation on Thr-446, Thr-449 and Thr-450 by this mutant was significantly reduced, and phosphorylation on Thr-333 and Ser-339 was completely abolished (Fig. 3A).

BIK1 and BAK1 phosphorylate a bacterial PAMP-receptor FLS2 at different sets of residues

BIK1 and BAK1 are regarded as two signaling partners of the flagellin receptor FLS2 which collectively initiate plant immune defenses against bacterial infection (Chinchilla et al., 2007; Lu et al., 2010a; Zhang et al., 2010). The cytoplasmic kinase domains of the two RLKs were reported to phosphorylate FLS2 *in vitro* (Lu et al., 2010a; Yan et al., 2012) (Fig. 1A). Whether they modify the same residues of FLS2 is unknown. Herein we identified the residues in FLS2-CD phosphorylated by BIK1 and compared with those by BAK1 reported by our previous study (Yan et al., 2012). It turned out that the two kinases showed differential selectivity in substrate sequences, as summarized in Fig. 3C (detailed MS data in Table S8 and Fig. S3). Two residues (Ser-906 and Ser-1115) in the kinase domain of FLS2 were phosphorylated by both kinases, and other residues were distinctly targeted by a specific kinase. All of these

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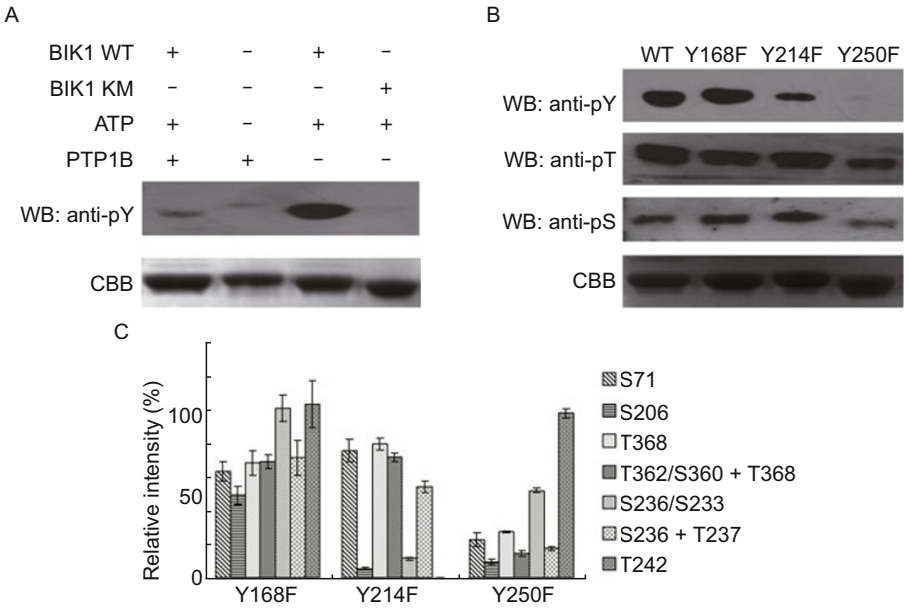

Figure 4. BIK1 is autophosphorylated on Tyr residues. (A) Detection of Tyr phosphorylation in BIK1 by immunoblotting with antiphosphotyrosine (anti-pY) antibodies. Pretreatment of BIK1 with a phosphotyrosine-specific phosphatase (PTP1B) eliminated the immunoblot signal, indicating specificity of the detection. BIK1 KM did not autophosphorylate on Tyr as indicated by the lack of anti-pY signals. Protein abundance was visualized by CBB staining. (B) Immunoblot probing overall Tyr, Thr and Ser phosphorylation levels of BIK1 (upper). (C) MS-based quantitation of specific phosphopeptides from BIK1 mutants relative to the wide-type MS-based quantitation of specific phosphopeptides from BIK1 mutants relative to the WT (defined as 100%). Peptides with distinct phosphorylation residues are indicated by columns of different patterns. The MS responses of phosphopeptides were normalized to the spiked-in casein peptide. Error bars are STD from three independent experiments.

phorphorylation sites are located in the intracellular kinase domain of FLS2 (out of the AL).

Identification and functional analysis of BIK1 tyrosine phosphorylation

In our LC-MS/MS analysis of phosphopeptides derived from the active BIK1, we were excited to identify three peptides distinctively phosphorylated on Y168, Y214 and Y250 in multiple replicates (Fig. 1B). The high-accuracy mass measurement (mass error <10 ppm) as well as high-quality MS/MS spectra strengthened our confidence in the assignment of modification sites (Table S9 and Fig. S4). Manual inspection of the MS spectra further confirmed the presence of phosphates on Y168 and Y214, yet phosphorylation on Y250 was not unambiguously assigned (Fig. S4C). Because the initial analysis was performed on purified peptides mostly carrying Ser/Thr-phosphorylation, we then biased the enrichment scheme using an anti-phosphotyrosine antibody (Boersema et al., 2010) and were able to repetitively identify peptides carrying phosphates on Y214 and Y250 (data not shown). Therefore, our confidence in identification of tyrosine phosphorylation was increased by the specificity in peptide enrichment.

To further validate these identified phosphoTyr sites and investigate their specific roles, we carried out biochemical assays and quantitative MS analysis on the wild-type and different Tyr mutants. BIK1 wild-type strongly cross-reacted with a widely-used anti-phosphoTyr antibody (Oh et al., 2009, 2010), and both the inactive mutant (D202A) and pre-treatment with a protein tyrosine phosphatase (PTP1B) eliminated the recognition by the anti-phosphoTyr antibody (Fig. 4A). These results confirmed tyrosine autophosphorylation occurring in BIK1 which depended on its kinase activity. Substitution of Y250 with Phe strongly inhibited the kinase activity, indicated by complete loss of cross-reactivity with anti-phosphoTyr antibodies and reduced recognition by anti-phosphoSer and anti-phosphoThr antibodies (Fig. 4B). Furthermore, relative quantitation by MS analysis revealed that autophosphorylation on the majority of Ser or Thr residues were all significantly reduced in this mutant except on Thr-242 (Fig. 4C and Table S10). Notably, the biochemical data alone cannot fully address whether this residue is an autophosphorylation site because of the suppressed kinase activity. Our high-quality MS data, however, provided complementary evidence to support the notion that phosphorylation on Y250 plays an important role in BIK1 activation. Substitution of Y214 with Phe reduced tyrosine autophosphorylation but retained a significant portion of kinase activity on serine and threonine residues, shown by immunoblot (Fig. 4B). MS quantitation suggested the kinase activity of this mutant was partially disrupted in that autophosphorylation on Ser-202, Ser-233/236 and Thr-242 was significantly reduced or elimi-

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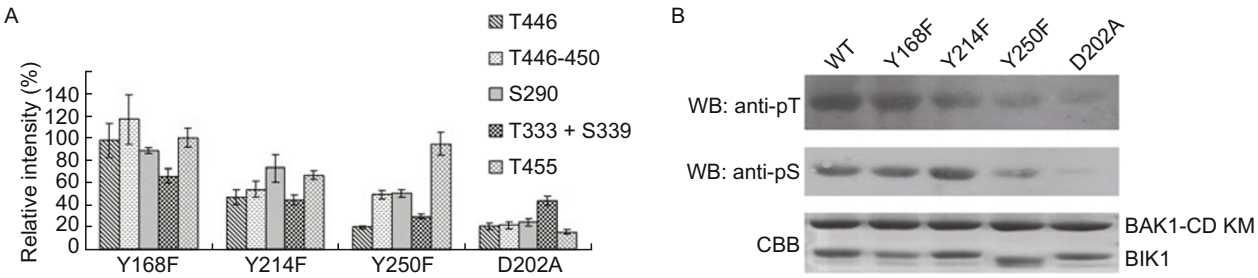

Figure 5. Mutating phosphotyrosine residues of BIK1 differentially impact its kinase activity toward BAK1-CD. (A) MS-based quantitation of phosphorylation on specific sites of BAK1-CD KM treated by a BIK1 mutant relative to that treated by BIK1 WT (defined as 100%). BAK1-CD peptides with distinct phosphorylation residues are indicated by columns of different patterns. The MS responses of phosphopeptides were normalized to the spiked-in casein peptide. Error bars are STD from three independent experiments. (B) Immunoblot of BAK1-CD KM treated by BIK1 WT and mutants (upper); the protein loading control was shown by CBB staining (lower).

nated. The remaining signal in the phosphoTyr immunoblot on Y214F indicated there is one (or multiple) additional phosphotyrosine residue(s) which could include Y250. Substitution of Y168 with Phe resulted in little change of either phosphoTyr signal or phophoSer/Thr signal by immunoblot (Fig. 4B), consistent with the quantitative MS analysis showing no significant suppression of phosphorylation on Ser or Thr residues (Fig. 4C). These results suggested this tyrosine residue is not essential for the autophosphorylation activity. Although the immunoblot data by itself indicated Y168 is not a major site of tyrosine phosphorylation, our confident identification suggested this modification may have evaded detection by the antibody due to low stoichiometry.

The impact of tyrosine phosphorylation on BIK1 activity toward BAK1-CD KM was less evident than that on BIK1 autophosphorylation. Both mutants Y214F and Y250F significantly reduced phosphorylation of BAK1-CD KM on specific residues such as Thr-446, Thr-333 and Ser-339 (Fig. 5A and Table S11), and the overall inhibitive effect of Y250F was more profound than of Y214F (Fig. 5B). The third mutant Y168F phosphorylated the substrate on each residue almost as sufficiently as the wild-type (Fig. 5A).

DISCUSSION

The phosphorylated Ser/Thr residues of BIK1 selected for mutagenesis are highly conserved among RLCK VII subfamily members whereas other Ser/Thr residues identified in our study are not (Fig. 6A). As we anticipated, the loss of autophosphorylation activity in different mutants generally correlated with the loss of their kinase activity toward a natural substrate BAK1 (Fig. 3A), suggesting the differential impact mostly stems from variation in the kinase activity. An exception, however, was the mutant S71A which retained autophosphorylation activity on all the residues assessed yet lost its capability to phosphorylate certain residues in the substrate (e.g. Thr-333, Ser-339 in BAK1-CD). This finding may support a previous notion that phosphorylation of KD residues could confer specificity in the substrate sequence by creating docking sites in addition to directly mediating the kinase activity, particularly for those residues in the less conserved N- and C-terminal regions (Laluk et al., 2011).

BIK1 is an RD-kinase and phosphorylation of the activation loop often is required for the activation of RD-kinases (Nolen et al., 2004). Our study identified autophosphorylation on the AL residues Ser-236, Thr-237 and Thr-242 which were found to be important for the *in vivo* kinase activity as well as for BIK1 functions in a variety of plant immune responses (Laluk et al., 2011). It merits a note that we found D substitution of Thr-242 didn't restore BIK1 auto- or trans-phosphorylation patterns *in vitro*. Furthermore, another mutant (Y214F) with completely removed phosphorylation on Thr-242 was still able to phosphorylate itself on several residues although the T242A mutant completely abolished autophosphorylation on all major sites. Based on these results, we speculate that the side chain of Thr-242, probably its hydroxyl group, is more likely to play a key role in maintaining the kinase activity than its phosphate moiety. Interestingly, the phosphomimic mutant T242D was shown to retain *in vivo* kinase activity and enhance plant resistance to microbial infection (Laluk et al., 2011). Furthermore, previous results for the induced *in vivo* kinase activity of T242A were controversial, depending on the type of elicitor (Lu et al., 2010a; Laluk et al., 2011). Because our data suggested both mutants T242A and T242D abolished kinase activity *in vitro*, we speculate their phosphorylation observed *in vivo* might be mediated by other RLKs (such as BAK1) involved in the signaling complex. Our study demonstrated that active BAK1-CD was able to transphophorylate BIK1 T242A and T242D (Fig. 2C and 2D) on different sets of residues.

Although the crystal structure of BIK1 remains undetermined, the sequence homology between BIK1 and BAK1 as well as their overlapping functions in signal integration from multiple receptors enables us to discuss the plausible function of these phosphorylation residues based on a modeled three-dimensional structure of BIK1 (Fig. 6B). Thr-242, which is the counterpart of Thr-455 in BAK1, plays an essential role in the kinase activity of BIK1. This position is highly conserved in plant Ser/Thr kinase family and its phosphorylation is known to

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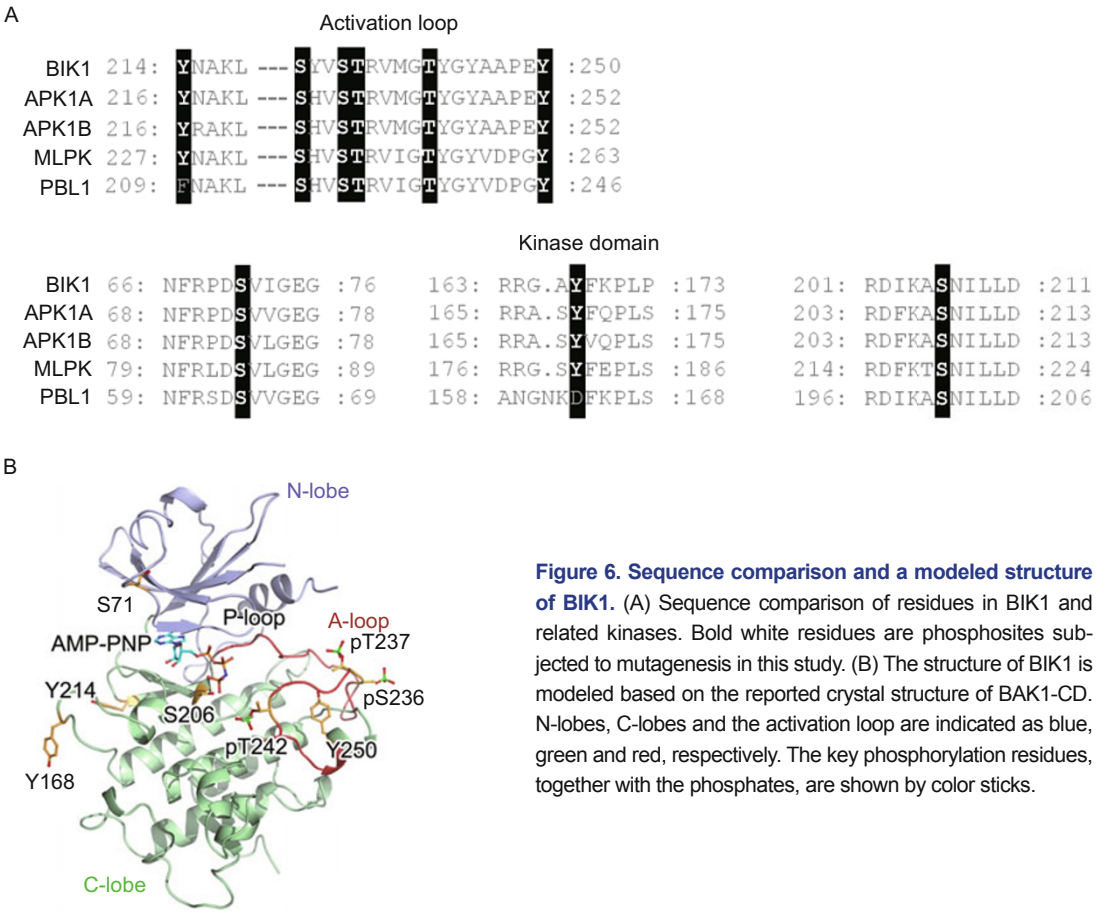

Figure 6. Sequence comparison and a modeled structure of BIK1. (A) Sequence comparison of residues in BIK1 and related kinases. Bold white residues are phosphosites subjected to mutagenesis in this study. (B) The structure of BIK1 is modeled based on the reported crystal structure of BAK1-CD. N-lobes, C-lobes and the activation loop are indicated as blue, green and red, respectively. The key phosphorylation residues, together with the phosphates, are shown by color sticks.

be critical for BAK1 kinase activity (Wang et al., 2008; Yan et al., 2012). Moreover, the phosphate moiety of Thr-455 makes crucial contribution to stabilizing the conformation of the activation loop as well as potentially mediates substrate binding of BAK1 (Yan et al., 2012). By contrast, this highly conserved threonine residue in another two Ser/Thr kinases IRAK-4 (Thr-351) and PHK (Thr-186) is not phosphorylated and still interacts with the relevant catalytic residues (Lowe et al., 1997; Wang et al., 2006). Taken together, it is a reasonable speculation that Thr-242 in BIK1 contributes to the kinase activation in the same way as Thr-455 in BAK1, and its phosphate may be responsible for fine-tuning the substrate specificity. Ser-236 is the counterpart of Thr-450 in BAK1, which also carries a phosphate in the BAK1 crystal structure. Our biochemical data suggested Ser-236 plays a less important role than Thr-237 for BIK1 activation *in vitro*, though their counterparts in BAK1 are equally essential for the kinase activity (Yan et al., 2012). An authentic 3D structure of BIK1 is expected to shed more light on the structural relations and functional roles of these two phosphorylated residues. In addition, our structural model suggests Ser-71 may be located in the N-lobe and closed to the P-loop of BIK1 (Fig. 6B), phosphorylation of this residue could possibly affect the conformation of the P-loop as well as the substrate specificity of BIK1.

Autophosphorylation on tyrosine residues of BIK1 *in vitro* was directly identified by our MS analysis and verified by biochemical analysis of site-directed mutants. These phosphoTyr residues (Tyr-168, Tyr-214 and Tyr-250) are highly conserved among RLCK VII subfamily members (Fig. 6A). Tyrosine phosphorylation in two other functionally-important plant LRR-RLKs, BAK1 and BRI1, have been shown to be critical for regulating their functions in multiple signaling pathways for plant growth and immune defenses (Oh et al., 2009, 2010). Sequence alignment showed Tyr-250 in BIK1 was also conserved in BAK1 (Tyr-463) and BRI1 (Tyr-1057). When this tyrosine was substituted with Phe, tyrosine phosphorylation was eliminated and the overall kinase activity was largely suppressed in BAK1 and BRI1 (Oh et al., 2009, 2010), agreeing well with our finding on BIK1 Y250F. It remains unclear, however, whether the phosphate group or the hydroxyl group of this particular tyrosine plays an important role in kinase activation. Substitution of another phosphorylated residue Tyr-214 with Phe significantly suppressed autophosphorylation on several residues including Thr-242, while retaining kinase activity toward most other residues. According to the structural model, the long distance from these tyrosine residues to the catalytic site and the substrate-binding site indicates that they are less likely to be directly involved in the catalytic reaction. Instead, they might play certain

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roles in BIK1 interaction with other components in the signaling complex.

Our study focusing on the *in vitro* phosphorylation profile of BIK1 has raised a few interesting questions for future study: 1) Does the *in vitro* result reflect the *in vivo* phosphorylation of BIK1? 2) What is the stoichiometry of phosphorylation of each site? 3) Although FLS2 doesn't show kinase activity by itself, would it be possible that the presence of BIK1 or BAK1 activate FLS2 in the complex? Profiling BIK1 *in vivo* phosphorylation patterns presumably induced by different endogenous or environmental cues and solving its actual 3D structure would significantly further our understanding of how phosphorylation of specific sites contributes to BIK1 activation and mediates downstream signaling specificity.

MATERIALS AND METHODS

Plasmid constructs and generation of recombinant proteins

Arabidopsis BIK1, *BAK1*, and *FLS2* genes were amplified by PCR from Col-0 cDNA library. The open reading frame of *BIK1* (encoding residues 39–372) was cloned into pET-28a vector with an N-terminal 6× His tag. The DNA sequences that encode the cytoplasmic domain of FLS2 (residues 841–1173, FLS2-CD) and BAK1 (residues 259–583, BAK1-CD) were cloned into a modified pET-28b, pGEX-6p-1, respectively. BIK1 and BAK1 point mutations were generated using a site-specific mutagenesis kit (TransGen Biotech, Beijing, China). The primer sequences for BIK1 and BAK1 point mutations are listed in Table S1. The plasmid was transformed into *E. coli* strain BL21 (DE3), and the transformed cells were cultured at 37°C in LB media containing 100 mg/L kanamycin or ampicillin. After OD_{600} reached 0.8, the culture was cooled to 16°C and supplemented with 0.2 mmol/L IPTG. After overnight induction, the cells were harvested through centrifugation at 6000 *g* and the pellets were resuspended in specific lysis buffer: 20 mmol/L HEPES (pH 7.0), 300 mmol/L NaCl, 4 mmol/L $MgCl_2$ and 5% Glycerin for preparing BIK1; 20 mmol/L Tris-HCl (pH 8.0), 1 mol/L NaCl, 4 mmol/L $MgCl_2$ and 5% Glycerin for preparing FLS2; 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 4 mmol/L $MgCl_2$ and 5% Glycerin for preparing BAK1. Cell lysates were homogenized at 4°C using an ultra-high-pressure cell disrupter (JNBIO, Guangzhou, China) mounted on an ultrasonic cell disruption system (SCIENTZ, Ningbo, China). The insoluble material was removed through centrifugation at 14,000 *g*. The fusion protein BIK1, its kinase-dead mutant (BIK1 KM, D202A) and FLS2-CD were first purified by Ni-NTA affinity chromatography and eluted with imidazole in the corresponding lysis buffer. Then the protein was further purified by passage through a Heparin column (GE Healthcare, USA) for BIK1 or a Superdex-200 column (GE Healthcare) for FLS2 preparation. BAK1-CD wild-type and kinase-dead mutant (BAK1-CD KM, N421A) were first purified with glutathione agarose beads, exchanged to the lysis buffer and then further purified using a Mono-Q ion-exchange column according to the manufacturer's instructions (GE Healthcare). All purified proteins were concentrated to 10 mg/mL by ultrafiltration using 10-KD Amicon Ultra (Millipore, USA) and stored at −80°C.

In vitro kinase reaction, phosphatase treatment and Western blot analysis

The recombinant BIK1 and its mutants (1 μg/μL) were incubated with ATP (0.1 mmol/L) at 30°C for 3 h to fulfill autophosphorylation. For the kinase activity assay, BAK1 KM or FLS2-CD (1 μg/μL) were incubated with BIK1 wild-type or mutants (0.1 μg/μL) in the kinase buffer (20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $MgCl_2$, 5 mmol/L EDTA, 100 mmol/L NaCl and 1 mmol/L DTT) supplemented with 0.1 mmol/L ATP at 30°C for 3 h with gentle shaking. After the kinase reaction, proteins were separated on 12% SDS-PAGE and the overall phosphorylation was analyzed using an anti-phosphoSer (Millipore, USA), anti-phosphoThr or anti-phosphoTyr antibody (Cell Signaling, USA). For BIK1 dephosphorylation reaction, it was incubated with a phosphotyrosine phosphatase PTP1B in 50 mmol/L HEPES, 10 mmol/L NaCl and 10% glycerol (pH 7.2) at 25°C for 2 h, then the mixture was separated on SDS-PAGE and immunoblotted using an anti-phosphoTyr antibody. PTP1B was prepared according to a published protocol (Hoppe et al., 1994).

Sample preparation and nanoUPLC-MS/MS analysis

After the kinase reaction, the protein was treated with DTT (10 mmol/L, at 30°C for 1 h) followed by iodoacetamide (40 mmol/L, for 30 min in the dark) to reduce and alkylate cysteine residues. Heat-denatured protein was in-solution digested with sequencing-grade trypsin (Promega, USA) at a ratio of 1:50 (E:S) overnight. Beta-casein digest was spiked into the BIK1 tryptic digest as the internal standard for MS quantitation. Phosphopeptides in the tryptic digests were enriched with TiO_2 microcolumns (GL Sciences, Japan) according to the manufacturer's instruction. Briefly, the protein digest was mixed with loading buffer (25% lactic acid, 80% acetonitrile, 0.3% trifluoric acid) and loaded into the conditioned TiO_2 microcolumn through repetitive pipetting and centrifugation (1000 *g*, 10 min). After washing the microcolumn with buffer B once and with buffer A twice, the bound phosphopeptides were first eluted with 50 μL elution buffer I (20 μL 28% ammonium hydroxide in 480 μL ddH_2O), followed by elution with 50 μL and 30 μL elution buffer II (500 mmol/L NH_4OH/60% acetonitrile). Each elution was conducted through centrifugation at 1000 *g* for 5 min. The eluates were dried in a speed vacuum and reconstituted in 0.1% formic acid/H_2O for MS analysis. Typically 5 μg of protein digest was prepared and enriched, with 1/3 of the total amount injected for nanoLC-MS^E analysis.

Peptide samples were first loaded onto a Waters Symmetry C18 trapping column (300 μm i.d. × 1 cm length) using Waters NanoAcquity UPLC system. After desalting and preconcentration, peptides were separated by in-line gradient elution onto a 100 μm i.d. × 10 cm column packed with 1.7 μm BEH C18 material (Waters, Milford, USA) at a flow rate of 400 nL/min using a linear gradient from 2% to 35% B over 30 min (A = 0.1% FA in H_2O, B = 0.1% FA in ACN). The Waters Synapt Q-IM-ToF G1 mass spectrometer was operated in high-definition MS^E mode (high- and low-collision energy switching every 1.0 s), and the data were processed with ProteinLynx Global Server (PLGS version 2.4, Waters) to reconstruct MS/MS spectra by combining all masses with a similar retention time. MS/MS spectra were searched against an in-house database consisting of the sequences of BIK1-CD, BAK1-CD and FLS2-CD and the entire proteome sequences of *E. coli* using PLGS with the following parameters: peak width, 0.3 min; MS and MS/MS tolerance, automatic (typical mass error <10 ppm for MS and <30 ppm for MS/MS); trypsin missed cleavages, 1; fixed modification, carbamidomethylation; variable modifications, Met oxidation and phosphorylation of Ser, Thr, or Tyr; at least 5 independent product ions assigned for a peptide identification. The false positive rate was <1%

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at both the protein and peptide levels.

Phosphosite identification had to meet the following requirements: (1) phosphopeptides were identified with a confidence >95% and a PLGS peptide score >6; (2) the mass error of the peptide precursor was below 10 ppm; (3) the MS/MS spectrum was manually inspected to confirm neutral loss and specific fragment ions critical for assigning the modification sites; and (4) phosphorylation sites were assigned consistently in at least three experimental replicates.

Label-free quantitation of relative changes of phosphorylation on specific sites

The quantitative responses of phosphopeptides were measured based on the peak areas of the extracted ion chromatograms (XICs) with defined peptide precursor *m/z* values. Peptide XIC extraction and peak area calculation were performed using MassLynx software (Waters), with peptide precursor mass tolerance of 0.02 *m/z* and retention time shift less than 0.15 min. We selected the most abundant peptide variant of a given charge state instead of combining variants of different charge states for quantitation as pointed out by Xian and coworkers (Gunawardena et al., 2011). To demonstrate the consistency of quantitation between one variant-based and multiple variants-based methods, we analyzed phosphorylation levels of a BIK1 mutant (S233A) relative to the wild-type using two methods and acquired almost identical quantitation data for all phosphopeptides (Table S2 and Fig. S1). The doubly-charged phosphopeptide FQpSEEQQQTEDELQDK from beta-casein (at 1031.415 *m/z*) was used as the internal standard to normalize the XIC peak response of the other phosphopeptides in the sample. For BIK1 autophosphorylation analysis, the normalized XIC responses of specific phosphopeptides from BIK1 mutants were compared with the counterparts from the wild-type in order to calculate percentage of changes. For the analysis of BAK1-CD KM phosphorylated by BIK1, the normalized XIC responses of specific phosphopeptides from BAK1-CD KM treated by individual BIK1 mutants were compared with the counterparts from BAK1-CD KM treated by the wild-type BIK1 to calculate percentage of changes. We defined more than 2-fold changes of phosphorylation to be significant (i.e. above 200% or below 50% of the wild-type). Three independent replicates were performed to calculate CV% of the relative quantitation.

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ABBREVIATIONS

AL, activation loop; BAK1, BRI1-associated receptor kinase 1; BIK1, botrytis-induced kinase1; BRI1, brassinosteroid-insensitive 1; PAMPs, pathogen-associated molecular patterns; PRRs, pattern-recognition receptors; RLKs, receptor-like kinases; RLCKs, receptor-like cytoplasmic kinases

COMPLIANCE WITH ETHICS GUIDELINES

Jinhua Xu , Xiaochao Wei, Limin Yan, Dan Liu, Yuanyuan Ma, Yu Guo, Chune Peng, Honggang Zhou, Cheng Yang, Zhiyong Lou and Wenqing Shui declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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