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Proteomics shed light on the brassinosteroid signaling mechanisms

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Abstract

Large numbers of receptor-like kinases (RLKs) play key roles in plant development and defense by perceiving extracellular signals. The mechanisms of ligand-induced kinase activation and downstream signal transduction have been studied for only a few RLK pathways, among which the brassinosteroid (BR) pathway is the best characterized. Recently, proteomics studies identified new components that bridge the last gap in the genetically defined BR-signaling pathway, establishing the first complete pathway from an RLK to transcription factors in plants. Furthermore, analyses of phosphorylation events, mostly by mass spectrometry, provided insights into the mechanistic details of receptor kinase activation and regulation of downstream components by phosphorylation. This review focuses on recent progress in understanding BR signal transduction made by proteomics studies.

Introduction

Signaling through cell-surface receptors is essential for survival of unicellular organisms and for development of multicellular organisms. In plants, the major class of receptors is the receptor-like kinases (RLKs), with over 600 members in *Arabidopsis* and about 1000 in rice [1]. Each RLK contains an extracellular domain that usually binds ligands, a single transmembrane domain, and a cytoplasmic kinase domain that initiates intracellular signal transduction. Many RLKs have been shown to play key roles in signaling pathways that regulate developmental or defense responses in plants [2]. One of the best-studied RLKs is BRASSINOSTEROID INSENSITIVE 1 (BRI1), the receptor of brassinosteroids (BRs), a steroid hormone that regulates a wide range of developmental and physiological processes in plants [3]. Mutations of BRI1 or other components of the BR biosynthetic or signaling pathway cause severe growth abnormalities such as dwarfism, photomorphogenesis in darkness, male sterility, and delayed flowering and senescence. Genetic studies have identified additional key components of the BR signaling pathway, including the coreceptor BRI1-Associated Receptor Kinase 1 (BAK1), the GSK3-like kinase BIN2 (Brassinosteroid-insensitive 2), the bri1-suppressor 1 (BSU1) phosphatase, and the Brassinazole-Resistant (BZR) family transcription factors [4]. Recent proteomics studies identified the BR-signaling kinases (BSKs) as substrates of BRI1 kinase [5^{**}], which bridges the gap between the receptor kinases on the plasma membrane and downstream cytoplasmic components [6^{**}]. Furthermore, mass spectrometry

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analyses identified phosphorylation sites involved in BR signal transduction [7*,8**,9]. The successful dissection of the BR signaling pathway will help in understanding of other RLK-mediated signaling pathways in plants. This review focuses on recent progress in proteomics and biochemical studies of the BR signal transduction mechanisms.

Activation of the BRI1 receptor kinase by brassinosteroid

BRI1 is a member of the leucine-rich repeat RLK (LRR-RLK) family [10,11], which include about 220 genes in *Arabidopsis* and 400 in rice [1]. The extracellular domain of BRI1 contains 24 LRRs and an island (ID) between LRR20 and LRR21, and its cytoplasmic region can be subdivided into a juxtamembrane (JM) domain, a Ser/Thr kinase domain and a C-terminal (CT) domain [4]. BRs directly bind to the ID–LRR21 domain to activate the BRI1 kinase [12], which initiates intracellular signal transduction by phosphorylating cytoplasmic target proteins. Several mechanisms have been shown to contribute to the ligand-induced activation of BRI1. In the absence of BR, the BRI1 kinase remains inactive, partly due to inhibition by its unphosphorylated CT domain [13] and by binding of the inhibitory protein BKI1 [14]. BR binding induces a number of molecular events that potentially contribute to kinase activation. These include homodimerization and autophosphorylation of BRI1 [11,13], disassociation of BRI1 Kinase Inhibitor 1 (BK1) [14], and association/transphosphorylation with the BAK1 coreceptor kinase [8**,9]. The exact sequence and causal relationship of these events were addressed in recent studies [8**].

BRI1 interaction with partners of the receptor complex

BAK1 was identified as a BRI1-interacting RLK that suppresses weak alleles but not strong alleles of *bri1* [13,15,16]. Several lines of evidence support that the BR-induced association between BRI1 and BAK1 is mediated by an initial activation of the BRI1 kinase activity. First, mutant BRI1 without kinase activity showed diminished binding to BAK1 *in vitro* and *in vivo*, whereas mutation of the BAK1 kinase only slightly reduced the interaction [15,16]. Second, BR can induce association between a kinase-dead mutant BAK1 and wild type BRI1, but not between a mutant BRI1 and wild type BAK1 [8**]. Third, BR cannot increase BAK1 phosphorylation in the *bri1-1* mutant, but can increase phosphorylation of BRI1 in the *bak1*, *bkk1* double mutant, though to a lesser extent than in wild type. Furthermore, *in vitro* kinase assays showed that BAK1 phosphorylation of BRI1 enhances its phosphorylation of a substrate peptide [8**]. It appears, therefore, that the BR-induced phosphorylation of BAK1 is strictly dependent on BRI1 kinase activity, whereas phosphorylation by BAK1 enhances BRI1 transphosphorylation of its substrate [8**]. Nevertheless, the strong dwarf phenotype caused by overexpression of a dominant-negative mutant *bak1* indicates that BAK1 is essential for BR signaling [15,16].

Regulation by sequential phosphorylation on specific residues

Many phosphorylation sites of BRI1 and BAK1 have been identified, mostly by mass spectrometry (Figure 1) [7*,8**,9]. Analysis of BRI1 immunoprecipitated from plant extract by liquid chromatography–tandem mass spectrometry (LC–MS/MS) identified six *in vivo* phosphorylation sites in the JM (S838, S858, T872 and T880), kinase (T982) and CT (S1168) domains, in addition to phosphorylation of at least three unspecified sites in the activation loop [8**,9]. Analysis of *in vitro* phosphorylated recombinant BRI1 kinase domain provided evidence for additional phosphorylation sites, including T842 and T846 in the JM, S887 in the kinase domain, and S1162, S1166 and T1180 in the CT domain [7,8,13,21]. BRI1 was considered a Ser/Thr protein kinase. Interestingly, two *in vivo* tyrosine phosphorylation sites (Y831 and Y956) and one additional *in vitro* phosphorylation site (Y1072) were identified in BRI1 using phospho-tyrosine specific antibodies [22*]. In addition to BRI1, BAK1 and six additional LRR-RLK were found to autophosphorylate on Tyr *in vitro*.

Mass spectrometry analysis also identified *in vitro* and *in vivo* phosphorylation sites of BAK1 and its homologs SERK1, SERK2, SERK4 and SERK5 [7*,8**,9]. Five phosphorylated residues were identified both *in vitro* and *in vivo* in the kinase domain of BAK1, including S290 in subdomain I, T312 in subdomain II, and three residues in the activation loop of subdomains VII/VIII (T446, T449 and T455). Four additional sites (S286, T450, S604 and S612) were identified *in vitro* but not *in vivo* [7*,8**].

The functions of the identified or predicted phosphorylation sites were analyzed using site-directed mutagenesis followed by *in vitro* assays of kinase activities and *in vivo* analysis of the ability to rescue *bril* or *bak1* mutants. Such analysis revealed the importance of phosphorylation in the activation loop, JM and CT domains for BRI1 function, and phosphorylation of the activation loop for BAK1 function. Mutation of some phosphorylation sites in the activation loops of BRI1 (S1044A or T1045A, T1049A and Y1052) and BAK1 (T455) reduced or abolished the kinase activities *in vitro* and signaling function *in vivo* [8**, 9,22*]. The JM and CT regions are important for BRI1 function. Deletion of the JM region abolished BRI1's signaling function and individual phospho-blocking mutations in the JM domain (S838A, T842A, T846A or S858A) reduced the substrate phosphorylation, whereas phospho-mimicking mutations of these residues (S838D, T842D, T846D and S858D together) enhanced BRI1 phosphorylation of an artificial substrate peptide [8**]. Deletion of the C-terminal 41 amino acids or simultaneous mutations of several serine/threonine residues to acidic amino acids in this region increased BRI1's kinase activity and signaling function, suggesting that phosphorylation alleviates the inhibitory effect of the CT domain [8**,13]. These results indicate that phosphorylation in the activation loop is essential for the kinase activity of both BRI1 and BAK1, and phosphorylation of BRI1 in the JM and CT domains quantitatively enhances BRI1 activity.

By incubating wild type BRI1 with the kinase-dead mutant BAK1 *in vitro*, LC-MS/MS analysis showed that BRI1 trans-phosphorylates kinase-dead BAK1 in the activation loop (T446, T449, T450 and T455) and subdomains I and II (S290 and T312). By contrast, BAK1 trans-phosphorylates BRI1 in the JM (S838, T846 and S858) and CT domains (S1166 and T1180) [8**]. These results support a sequential phosphorylation model in which BR binding induces BRI1 autophosphorylation in the activation loop, which activates the BRI1 kinase above a basal level. Active BRI1 kinase then interacts with BAK1 and phosphorylates BAK1 in the activation loop, leading to activation of BAK1. BAK1 in turn phosphorylates the JM and CT domains of BRI1 to enhance its phosphorylation of substrates.

While most phosphorylation sites have positive effects on the receptor kinase function, phosphorylation of some residues appears to be inhibitory. The T872A mutation on BRI1 resulted in a ~ 10-fold increase of substrate trans-phosphorylation activity [9], and the phospho-mimicking S286D substitution in BAK1 completely abolished BAK1's kinase activity *in vitro*, and enhanced dwarf phenotype of a weak *bril* allele, *bril-5 in planta* due to a presumed dominant-negative effect of the inactive BAK1 [8**]. Phosphorylation at these residues might negatively regulate BRI1 or BAK1 function. The mutant BAK1 S286A showed normal kinase activity and rescued the *bak1* mutant. It is possible that BAK1 is self-inactivated as a mechanism of desensitization or inactivated by an unknown kinase under certain conditions.

Signaling crosstalk and multitasking of RLKs

BAK1 is also named SERK3 and is one of the five members of the somatic embryogenesis receptor kinase (SERK) family, which is a subclass of LRR-RLKs containing five LRRs in the extracellular domain [15,16]. Recent genetic studies revealed multi-functions for combinations of the SERK family members in distinct pathways. These include the co-receptor function of BAK1/SERK3, SERK1 and SERK4 in the BR pathway [17*,18], co-receptor function of BAK1/SERK3 in flagellin/FLS2 signaling [19], an essential role of BAK1/SERK3 and

SERK4/BKK1 in suppressing cell death [18], and a role of SERK1 and SERK2 in male fertility [20]. BAK1, SERK1 and possibly SERK4 appear to play the same role as BRI1 co-receptor, as they all interact with BRI1 and their loss-of-function mutations contribute additively to a BR-insensitive phenotype [17,18].

Some phosphorylation sites appear to mediate a subset of specific downstream responses. BAK1 also functions as a coreceptor of the flagellin receptor FLS2 and regulates plant defense response [19,23]. Treatment with flagellin (flg22) inhibits seedling growth of wild type *Arabidopsis* but not the *bak1-4* mutant. The mutant BAK1 containing T450A substitution was able to rescue the BR-related phenotypes of *bri1-5* and the *bak1-4/bkk1-1* double mutant, but failed to rescue the flg22-insensitive phenotype, suggesting that phosphorylation of BAK1 T450 has a specific role in regulation of FLS2 signaling [8**]. In addition, expression of a mutant BRI1 containing Y831F substitution specifically affects leaf size and flowering time, suggesting that individual phosphorylation sites can have specific signaling functions [22*]. Quantitative proteomics studies of the dynamics of BRI1 and BAK1 phosphorylation after BR treatment will lead to a more comprehensive understanding of the mechanisms of receptor kinase activation and signal output.

Identification of BR responsive proteins by 2-DE based proteomics

Many BR-signaling components have been identified by molecular genetic studies in *Arabidopsis*. However, genetic approaches have limitations due to genetic redundancy and there are still gaps in knowledge of the BR-signaling pathway. A number of quantitative proteomics studies have been carried out to identify BR-responsive proteins that may include novel BR-signaling components as well as downstream BR targets that mediate cellular responses.

Two-dimensional gel electrophoresis (2-DE) separates thousands of proteins by charge in isoelectric focusing (IEF) and then by size in SDS-PAGE. In principle, change of protein phosphorylation can be detected on 2-DE gel as spot shift along the IEF dimension. In the improved two-dimensional difference gel electrophoresis (2-D DIGE), up to three samples are labeled with spectrally-distinct fluorescent dyes (Cy2, Cy3 or Cy5) and separated together in one 2-DE gel [24]. This greatly reduced gel-to-gel variation associated with traditional 2-DE, and allows detection and accurate quantification of changes in protein abundance as well as post-translational modifications [24].

A comprehensive analysis of total protein samples using 2-D DIGE identified a large number of proteins that responded to a relative long time of BR treatment (3–24 h) [25]. While these late BR-responsive proteins are likely to mediate downstream cellular responses, the study failed to detect any of the known BR-signaling components, which are phosphorylated or dephosphorylated within minutes of BR treatment. The primary BR-signaling proteins, such as BZR1, were not detected by 2-D DIGE in total protein samples probably because of their low abundance, and it was concluded that enrichment by fractionation is required to detect signaling proteins of low abundance [25]. Indeed, 2-D DIGE analysis of phosphoproteins enriched using Immobilized Metal Affinity Chromatography detected phosphorylation changes of the BZR1 as rows of > 24 spots [26**], consistent with 25 putative GSK3 phosphorylation sites in BZR1 [27]. In addition, BR-induced phosphorylation of BAK1 was detected in the plasma membrane fraction with a row of eight spots (Figure 2) [26**], comparable to the number of phosphorylation sites identified by mass spectrometry analyses [7,8**]. A number of other early BR-responsive phosphoproteins and plasma membrane proteins were also identified, including the plasma membrane protein DREPP shown to promote growth *in planta* [26**].

Similar to BAK1, two closely related (60% amino acid identity) protein kinases, BSK1 and BSK2 [5**], were identified as BR-responsive protein spots that shift to the acidic side upon BR treatment. There are 12 BSK homologous proteins in *Arabidopsis*, which belong to the Receptor Like Cytoplasmic Kinase XII (RLCK XII) family. Overexpression of BSK1, BSK3 or BSK5 in *bri1-5* mutants significantly suppressed the mutant phenotypes, whereas a T-DNA knockout mutant of BSK3 showed reduced response to exogenous applied brassinolide. Overexpression of BSK3 can also partially rescue the null mutant *bri1-116* but not *bin2-1* homozygous mutant, suggesting BSKs function downstream of BRI1 and upstream of BIN2 in the BR-signaling pathway. LC-MS/MS analysis of *in vitro* BRI1-phosphorylated BSK1 identified S230 as a major BRI1 phosphorylation site. Substituting S230 to Ala greatly reduced the phosphorylation of BSK1 by BRI1. Such a single phosphorylation site is consistent with the pattern of BR-induced spot shift of BSK1 in 2-D DIGE (Figure 2) [5**]. The data demonstrate that BSK is a new BR-signaling component that functions as a BRI1 substrate that transduces the BR signal to downstream components [5**].

Connecting the BR signaling pathway

Downstream BR-signaling components include a GSK3/SHAGGY-like protein kinase BIN2, a Kelch-repeats-containing phosphatase BSU1, and transcription factor BZR1 and BZR2 (also named BES1) (Figure 3). In the absence of BR, BIN2 negatively regulates BR-signaling pathway by phosphorylating BZR1 and BES1/BZR2 [28,29]. Phosphorylation by BIN2 inactivates BZR1 and BES1/BZR2 in several ways, including inhibition of the DNA binding and nuclear localization, and promoting degradation by the proteasome [28–32]. Particularly, phosphorylation of S173 of BZR1 causes binding to the 14-3-3 proteins, which promote transport to and retention of BZR1 and BZR2/BES1 in the cytoplasm [31,32]. In contrast to BIN2, the BSU1 phosphatase promotes dephosphorylation of BES1/BZR2 *in planta* [33]. How upstream BR-signaling regulates BSU1 and/or BIN2 has been an outstanding question until recently.

BSU1 was previously proposed to mediate dephosphorylation of BES1/BZR2 [33]. However, a recent study provides both biochemical and genetic evidence that BSU1 acts directly downstream of BSKs and upstream of BIN2 [6**]. Instead of dephosphorylating BES1/BZR2 or BZR1 directly, BSU1 inactivates BIN2 to prevent the phosphorylation, and it does so by dephosphorylating a phospho-tyrosine (pY200) residue of BIN2. This tyrosine residue is conserved in all GSK3 kinases and its phosphorylation has been shown to be essential for full kinase activity. The Y200F mutation of BIN2 greatly reduced its kinase activity *in vitro* and growth-inhibiting activity *in planta*. Dephosphorylation of pY200 of wild type BIN2 was detected *in vitro* after incubation with BSU1 (more effective if BSU1 is immunoprecipitated from BR-treated plants) and *in vivo* upon BR treatment or in BSU1-overexpression plants. However, such dephosphorylation was not observed for the *bin2-1* mutant protein, indicating that the *bin2-1* mutation causes the BR-insensitive phenotype by blocking BSU1 dephosphorylation of pY200. It has been shown that BR induces proteasomal degradation of BIN2 [34*]. Overexpression of BSU1 also reduced BIN2 accumulation, suggesting that degradation is a consequence of BSU1-mediated inactivation of BIN2 kinase [6**]. Consistent with the biochemical data, overexpression of BSU1 partially suppressed *bri1* but not homozygous *bin2-1*, confirming that BSU1 acts downstream of BRI1 but upstream of BIN2 [6**].

BSU1 not only directly interacts with BIN2 but also with BSK1 *in vitro* and *in vivo* [6**]. The binding of BSU1 to BSK1 is significantly enhanced when BSK1 is phosphorylated by BRI1 and nearly abolished by mutation of the BRI1 phosphorylation site (S230A) in BSK1. BSK1 is likely to activate BSU1 by phosphorylation, as BR treatment increases BSU1's activity of inhibiting BIN2 [6**]. These results demonstrate a complete BR-signaling pathway, from

sequential activation of BRI1, BSK1 and BSU1 to inhibition of BIN2 and accumulation of unphosphorylated BZR1 and BZR2/BES1 in the nucleus (Figure 3) [6**]. This represents the first completed pathway that physically links ligand perception by an RLK to downstream transcription factors in plants, with one possible missing piece: the protein phosphatase that dephosphorylates the BZR transcription factor. Genetic suppression of nearly all *bri1* phenotypes by *bzr1-1D* or *bes1-D* mutations suggests that this BRI1-to-BZR1/BES1 pathway is responsible for most BR responses. However, branch pathways may exist, as suggested by additional BRI1 substrates potentially involved in non-transcriptional regulation [35,36].

Conclusion

As a result of decade-long extensive studies by many laboratories, the BR signal transduction pathway provides a paradigm for understanding RLK-mediated signal transduction in plants. Proteomics enabled by mass spectrometry provides powerful tools that complement genetic analysis. In particular, sample prefractionation followed by 2D-DIGE is a powerful approach to identifying signal-induced changes in protein phosphorylation, as shown for BZR1, BAK1, BSK1 and BSK2 [5**,26**]. Mass spectrometry analysis of protein phosphorylation can provide mechanistic details of signal transduction processes. The case of the BR signal transduction pathway demonstrates not only how signals are transduced through RLK pathways, but also how signal transduction can be studied effectively using combinations of multiple approaches that include genetics, biochemistry and proteomics.

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Figure 1. *In vivo* and *in vitro* phosphorylated residues on BRI1 and BAK1 intracellular domains JM, juxtamembrane domain; CT, C-terminal domain. Activation loop within the kinase domain is highlighted. Red letters indicate the phosphorylated residues that are critical for BRI1 or BAK1 kinase activity. S/T to A or Y to F substitution of these residues reduces or abolishes BRI1 or BAK1 kinase activity. Residues at which phosphorylation seems to inhibit the kinase activity are in blue color. BRI1 and BAK1 transphosphorylated residues are underlined.

Figure 2.

BR-induced post-translational modifications of BAK1 (A) and BSKs (B) were detected as shifted spots in 2-D DIGE of plasma membrane fractions. Plasma membrane proteins of untreated control sample labeled with Cy3 (green) and BR-treated samples labeled with Cy5 (red) were separated in a 2-D DIGE gel. White arrows show spots that disappear (green spots) or and black arrows show spots that appear (red spots) after BR treatment. (A). An area of the 2-D DIGE gel showing BAK1 spots. (B) An gel area showing the two rows of spots that contain BSK1 and BSK2. From left to right are acidic to basic pH.

Figure 3.

A diagram of the BR signal transduction pathway in Arabidopsis. The left side of the dashed line (A) shows the inactive pathway when BR level is low, and the right side (B) shows the pathway when activated by BR. Although there is evidence for the formation of heterotetramer between BRI1 and BAK1, for simplicity a simple heterodimer model is shown. Components are in blue color when inactive and red color when active. (A) When BR level is low, BRI1 is associated with BKI1, which inhibits BRI1, as well as BSK1, which is to be phosphorylated upon activation of BRI1. BAK1 and BSU1 remain inactive, whereas BIN2 phosphorylates BZR1 and BZR2/BES1, which inhibits DNA binding, promotes cytoplasmic localization through the 14-3-3 proteins, and accelerates degradation by the proteasome. (B) BR binding

to the extracellular domain of BRI1 activates BRI1 through disassociation of BKI1 and oligomerization/transphosphorylation between BRI1 and BAK1. Activated BRI1 phosphorylates Ser230 of BSK1, which then interacts with and presumably activates BSU1. BSU1 dephosphorylates BIN2 at Tyr200 to inactivate BIN2 and stop phosphorylation of BZR1/2, leading to accumulation of unphosphorylated BZR1/2, likely with help of an unknown phosphatase (PPase) that dephosphorylates BZR1/2. Unphosphorylated BZR1/BZR2 accumulate in the nucleus, where they directly regulate BR-responsive gene expression and plant development.