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Multiple mechanisms modulate Brassinosteroid signaling

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Abstract

Brassinosteroids are essential hormones for plant growth and development. Genetic studies have identified key components of the BR signaling pathway, including the cell-surface receptor kinases that perceive BR, an intracellular kinase and a phosphatase, and nuclear transcription factors. Subsequent biochemical studies have revealed many details about signaling events from BR perception at the cell surface to gene expression in the nucleus. Recent studies have identified the 14-3-3 proteins as BR signaling components and elucidated multiple mechanisms by which phosphorylation modulates the BR transcription factors. In addition, BRI1 signaling from the endosomes and BR-independent functions of BAK1 have been observed. However, a major gap still exists in the current BR signaling pathway between the receptor complex and downstream signaling events.

Introduction

About a decade ago, the discovery of brassinosteroid-deficient Arabidopsis mutants established brassinosteroids (BRs) as an essential plant hormone and stimulated extensive studies of BR signal transduction. Since then, genetic studies of BR-insensitive mutants and their suppressors have identified several key components of the BR signal transduction pathway, including the leucine-rich-repeat (LRR) receptor-like kinases (RLK), BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1); the glycogen synthase kinase-3 (GSK3)-like kinase BRASSINOSTEROID-INSENSITIVE 2 (BIN2); the phosphatase *bri1* SUPPRESSOR 1 (BSU1); and two transcription factors BRASSINAZOLE-RESISTANT 1 (BZR1) and BRASSINZAZOLE RESISTANT 2 (BZR2)/*bri1*-EMS-SUPPRESSOR1 (BES1). Molecular biochemical studies of these components have established a model of the BR signaling pathway leading from BR perception at the cell surface to regulation of transcription in the nucleus. Namely, BRs directly bind the extracellular domain of BRI1 to activate its kinase activity and promote heterodimerization with, and phosphorylation of, BAK1. BIN2 negatively regulates BR signaling by phosphorylating and inhibiting BZR1 and BZR2/BES1, while BSU1 positively regulates BR response by dephosphorylating them. BR activation of the receptor kinases leads to dephosphorylation of BZR1 and BZR2/BES1, possibly by inhibiting BIN2 or activating BSU1 through unknown mechanisms. Unphosphorylated BZR1 and BZR2/BES1 directly bind BR-responsive promoters causing transcriptional changes that increase growth and reduce BR biosynthesis.

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This review focuses on recent advances in our understanding of cellular dynamics of intracellular signaling components, function of BAK1 receptor kinase in disease resistance, and mechanisms of transcriptional regulation by phosphorylation. In addition, outstanding questions in BR signaling will be discussed.

BR signaling by the receptor kinases

BRI1 is a plasma membrane-localized LRR-RLK that transduces the BR signal across the plasma membrane [1]. Direct binding of BR has been demonstrated by photoaffinity cross-linking of a biotin-labeled analogue of castasterone (a biologically active BL precursor) to BRI1 in microsomal membrane fractions. Further binding assays using fragments of BRI1 protein expressed in *E. coli* identified a 90-amino-acid region of the extracellular domain of BRI1 as the BR-binding domain [2]. BR binding induces BRI1 autophosphorylation, and a number of *in vivo* phosphorylation sites have been identified and functionally studied [3,4]. BRI1 kinase activation involves phosphorylation and/or conformational changes of its own C-terminal (CT) domain, as deletion of the C-terminal 41 amino acids of BRI1 and mutations that mimic phosphorylation (Ser/Thr to Asp) in this region increased the kinase activity [5]. BR activation of BRI1 also involves dissociation of an inhibitory protein, BKI1 (BRI1 Kinase Inhibitor 1), that binds to the BRI1 kinase domain to inhibit the association of BRI1 with BAK1 necessary for proper BR signaling [6].

The BR-induced dimerization and cross-phosphorylation between BRI1 and BAK1 is believed to be important for receptor activation and/or transducing the signal to downstream components [4]. BRI1 and BAK1 interact with each other through the kinase domains in a kinase activity-dependent manner [7,8], and the interaction is induced by BR *in vivo* [4]. It is unclear whether BAK1's main function is to activate BRI1, to bridge BRI1 with the next downstream component, or to promote receptor endocytosis (described below) [9]. In addition to BAK1 and BKI1, two other proteins, Transthyretin-like (TTL) protein and the Arabidopsis homolog of TGF-beta receptor interacting protein (TRIP-1), have been identified as BRI1 substrates that positively regulate plant growth [10,11]. However, their precise roles in BR signaling remain unclear. No direct interactions have been observed between BRI1, or its interacting proteins, and downstream components of the pathway, such as BIN2 and BSU1. Thus, how the BR signal is transduced from the receptor kinases to the downstream components remains an outstanding question. Recently, BRI1 has been shown to contain a domain that functions as a guanylyl cyclase *in vitro*, suggesting that cGMP may have a role as a second messenger in BR signaling [12].

Endosomal signaling of BRI1

BRI1 and BAK1 are believed to function at the plasma membrane. Interestingly, coexpression of BRI1 and BAK1 leads to endocytosis of the two receptors in protoplasts of cowpea and Arabidopsis [9]. Receptor endocytosis has been widely observed in animals and yeast as a mechanism either for receptor inactivation and turnover or for activation of signaling [13]. The effect of endocytosis on BRI1 signaling was recently studied in Arabidopsis. Using a BRI1-GFP transgenic line that expresses the fusion protein at endogenous levels, Geldner *et al.* observed BRI1 in early endosomes in the root cells of Arabidopsis [14]. However, the distribution of BRI1-GFP on plasma membrane and endosomes is not affected by BR deficiency or BR treatment, suggesting that BRI1 endocytosis is independent of its activation state.

To determine whether BRI1 in endosomes can activate downstream signaling, plants were treated with Brefeldin A (BFA) to inhibit trafficking from early to late endosomal compartments and vacuoles. BFA treatment caused BRI1-YFP accumulation in endosomal aggregates and blocked BRI1-YFP turnover. Interestingly, like BR treatment, BFA treatment

of a highly BR-sensitive cell suspension culture induced strong dephosphorylation of BES1 and suppression of DWF4 gene expression. BFA also enhanced BR responses in a root culture system, although the effect was much weaker in intact Arabidopsis seedlings. These results demonstrate that BRI1 can signal from early endosomes [14]. However, it remains unclear whether endosomal BRI1 is constitutively active or requires BR binding for kinase activation. The lack of BKI1, which dissociates from PM BRI1 upon BR activation, in endosomes suggests that endosomal BRI1 receptors are constitutively active. Yet, the reason for endosomal BRI1 signaling is unclear. One possible explanation is that endosomes provide additional membrane surface for receptor action since plasma membrane might be limiting for large numbers of receptors and channels [14]. It is also possible that active BRI1 proteins are endocytosed for degradation in late endosomal vesicles and vacuoles as a mechanism of desensitization. Testing the effect of blocking BRI1 endocytosis on BR responses could distinguish these possibilities.

In contrast to endosomal BRI1, a mutant BRI1 receptor (bri1-9) retained in the endoplasmic reticulum (ER) cannot activate BR signaling, but it is functional when localized on the PM due to mutation of a component of the ER quality control system identified as the *bri1-9* suppressor *ebs1* [15]. While demonstrating an important physiological function of the plant ER quality control system, the study also indicates that BRI1 retained in the ER cannot mediate BR signaling, perhaps due to lack of a modification, function partner, or BR in the ER.

The dual roles of receptor kinases in BR signaling and defense responses

BAK1, also named SERK3, belongs to a small family of LRR-RLKs named somatic embryogenesis-related kinases (SERK1 to SERK5). Recently, three studies have provided convincing evidence for a BR-independent role for BAK1 in basal defense and programmed cell death regulation. Using immunoprecipitation, He *et al.* showed that a close homolog of BAK1, called BKK1 or SERK4, is also part of the BRI1 receptor complex and when overexpressed it can partially suppress a *bri1* mutant, similar to overexpression of BAK1. Yet, a double mutant between *bak1* and *bkk1* showed a seedling lethal phenotype with symptoms of programmed cell death, as monitored by trypan blue staining [16]. Kemmerling et al. also reported a role for BAK1 in pathogen induced cell death responses [17]. Biotrophic and necrotrophic pathogens were observed to cause spreading necrosis in the *bak1* mutants. Mutants in BR signaling components and co-treatment with BR and pathogen did not result in a change in the pathogen susceptibility suggesting that BAK1 plays a BR-independent role in restricting cell death [17]. BAK1's role in disease resistance was confirmed when Heese et al. and Chinchilla et al. found that BAK1 interacts with the flagellin receptor, FLS2, in a ligand dependent manner [18,19]. The *bak1* mutant showed reduced growth inhibition by flagellin, and both *bak1* mutant and *NbSerk3*-silenced *Nicotiana benthamiana* inhibited the flg22-induced burst of active oxygen species (AOS) similar to the *fls2* mutant or NbFLS2-silenced line. Additionally, the *NbSerk3*-silenced N. benthamiana line was more susceptible to pathogenic (*Pto* DC3000) and non-pathogenic (*Pto* DC3000 *hrcC*) biotrophs, confirming BAK1's role in basal defense. Interestingly, these plants were also less responsive to other pathogen-associated molecular patterns (PAMPs), including EF-Tu, INF1, and CSP22, which are not recognized by FLS2, suggesting that BAK1 also participates in FLS2-independent PAMP responses possibly by interacting with other pattern-recognition receptors [19]. The dual roles of BAK1 in both BR and defense signaling are similar to the animal TOLL receptor, which controls both development and innate immunity. Furthermore, the severe seedling lethal phenotype of the *bak1/bkk1* double mutant suggests that SERK is involved in signaling by an endogenous ligand required for survival under normal conditions.

These studies provide compelling evidence for functions of BAK1 in multiple signaling pathways, which raises questions about how specificity of each pathway is achieved while sharing a common component and how different pathways cross talk and interfere with each

other. Although previous reports have shown that BR treatment can promote cell death and flg22 treatment causes growth inhibition, each signal mainly activates a specific set of responses. A simple explanation is that BAK1 functions as an enhancer for various LRR-RLKs, rather than a signal transducer that regulates downstream components. It is likely that the pathway specific LRR-RLKs, such as BRI1 and FLS2, recognize the ligand and mediate signaling to downstream components to achieve specificity and fidelity of the pathway. It is also possible that specificity is achieved through receptor heterodimers. Different pathways that share the SERKs may antagonize one another by competing for SERKs, or activation of SERKs by one signal may enhance responses in another pathway (Figure 1).

Dual functions have been observed for several plant receptor kinases. In tomato, the BRI1 ortholog has been shown to be required for responses to both BR and the peptide hormone systemin, which activates the systemic wound responses [20,21]. In Arabidopsis, ERECTA not only controls inflorescence and fruit development but also regulates resistance to the bacterial pathogen *Ralstonia solanacearum* and the necrotrophic fungus *Plectosphaerella cucumerina* [22]. Similar dual functions are known for LRR receptors in animals [23]. It is interesting to note the evolutionary conservation of the dual functions for cell surface receptors in both development and immune response. Such dual functionality together with large numbers of LRR-RLK genes in plant genomes (over 230 in Arabidopsis) presumably allows plants to respond to diverse internal and external signals.

Regulation of BZR1 and BZR2/BES1 by BR-regulated phosphorylation

A genetic study of a mutant that is resistant to the BR-biosynthesis inhibitor brassinazole (brassinazole resistant 1, *bzr1-1D*) lead to the identification of the key transcription factors that mediate BR-responsive gene expression [24]. Mutations of a conserved proline to leucine in BZR1 and its close homolog BZR2, also named BES1, stabilizes the proteins and causes activation of BR responses and suppression of the *bri1* mutant [24,25]. BZR1 and BZR2/BES1 are two transcription factors that directly bind the promoters of BR regulated genes [26,27]. BR induces rapid dephosphorylation of BZR1 and BZR2/BES1, indicating that phosphorylation inhibits and dephosphorylation activates the transcription factors. BZR1 and BZR2/BES1 are phosphorylated by the BIN2 kinase [25,28] and potentially dephosphorylated by the BSU1 phosphatase [29]. Recent studies have revealed multiple mechanisms by which phosphorylation inhibits the transcription factors.

Initial studies showed that BR-induced dephosphorylation of BZR1 and BZR2/BES1 is followed by an increase of the protein levels in the nuclei of hypocotyl cells [24,25]. In the *bin2-1* mutant, which encodes a hyperactive kinase, BZR1 and BZR2/BES1 are hyperphosphorylated and accumulate at a lower level [25,28], while inhibiting proteasome activity causes accumulation of the phosphorylated BZR1. Thus, it has been proposed that phosphorylation promotes degradation and inhibits nuclear localization of BZR1 and BZR2/BES1 [25,28]. In contrast, it was later reported that phosphorylation inhibits the DNA-binding activity and transcriptional activity [30] but has little effect on the stability or nuclear-cytoplasmic distribution of BZR2/BES1 [30,31]. However, recent studies of Arabidopsis BZR1 and its rice homolog OsBZR1 confirmed the importance of BR regulated nuclear localization and identified the 14-3-3 proteins as new components of the BR pathway that mediate cytoplasmic retention of phosphorylated BZR1 and OsBZR1 [32,33].

14-3-3s are highly conserved phosphopeptide-binding proteins. In yeast and humans, 14-3-3 proteins play important roles in many signaling pathways by binding to large numbers of target proteins in a sequence-and phosphorylation-dependent manner. Large numbers of 14-3-3-binding proteins have been identified in plants, but the functions of these interactions remain mostly unknown. Using yeast two-hybrid screens and a range of protein-protein interaction

assays, Gampala et al. and Bai et al. demonstrated that BZR1 and OsBZR1 interact with 14-3-3 proteins in Arabidopsis and rice, respectively [32,33]. A conserved 14-3-3 binding site was found in BZR1, BZR2, and OsBZR1, and this site of BZR1 was shown to be phosphorylated by BIN2. Mutations of the 14-3-3 binding site of BZR1 and OsBZR1 abolished 14-3-3 binding, and expression of the mutant proteins caused BR-activation phenotypes similar to those of *bzr1-1D*, indicating an essential role for 14-3-3s in inhibiting the phosphorylated BZR1. The mutant proteins showed increased nuclear localization but normal levels of phosphorylation, accumulation, and inhibition of DNA binding by phosphorylation [32], suggesting that 14-3-3s inhibit phosphorylated BZR1 and OsBZR1 by increasing their cytoplasmic retention. These studies demonstrate a conserved mechanism by which 14-3-3 proteins mediate BR signaling by specifically inhibiting the function of phosphorylated BZR1 through cytoplasmic retention. This together with promoting degradation and inhibiting DNA binding provide multiple mechanisms, presumably through the large numbers of putative BIN2-phosphorylation sites (23 conserved in BZR1 and OsBZR1), that ensure precise control of transcription by BR signaling (Figure 2). It is conceivable that the use of various mechanisms is influenced by developmental stage or environmental conditions to fine-tune BR sensitivities [32,33], which most likely explains the conflicting observations about nuclear localization in previous reports.

Summary and prospect

After a decade of productive research, a complete picture of the BR signaling pathway from cell surface receptors to nuclear gene expression is emerging. The only major gap in the current BR signaling pathway is between the receptor kinases and BIN2 or BSU1. This gap is likely to be filled by continued molecular genetic or novel proteomic studies. A more daunting task is to understand how BR signaling regulates the wide range of developmental and physiological processes. Previous studies have focused on BR's action in cell elongation and plant size; however, the pleiotropic phenotypes of BR mutants indicate roles for BR in a wide range of developmental processes. For example, a link has been found between BR regulation of FLC expression and flowering [34], a role for BR in organ boundary formation has been revealed by studies of *bzr1-1D* (unpublished data), and cell type-specific actions of BR, such as epidermal control of plant growth, have been recognized [35]. Identification of all BZR1 direct target genes by chromatin immunoprecipitation-microarray will establish large numbers of molecular links between BR signaling and target physiological or developmental pathways. Proteomic studies will likely identify posttranscriptional targets of BR signaling, as well as new components that mediate BR signal transduction. A combination of such genetic, genomic, and proteomic approaches will bring our understanding of the BR signaling network to the systems biology level. Clearly, more exciting discoveries will be made in the next decade.

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for 14-3-3 Proteins in Brassinosteroid Signal Transduction in Arabidopsis. Dev Cell 2007;13:177–189. [PubMed: 17681130]•• BR induces nuclear localization of BZR1 and BZR2/BES1. BIN2-catalized phosphorylation of BZR1 inhibits DNA binding and promotes interaction with the 14-3-3 proteins. Mutation of a BIN2 phosphorylation site of BZR1 (S173A) abolished 14-3-3 binding, and caused BR-activation phenotypes and constitutive nuclear localization of BZR1. The study demonstrates an essential role of 14-3-3 proteins in regulation of BZR1 nuclear localization by BR-induced dephosphorylation.

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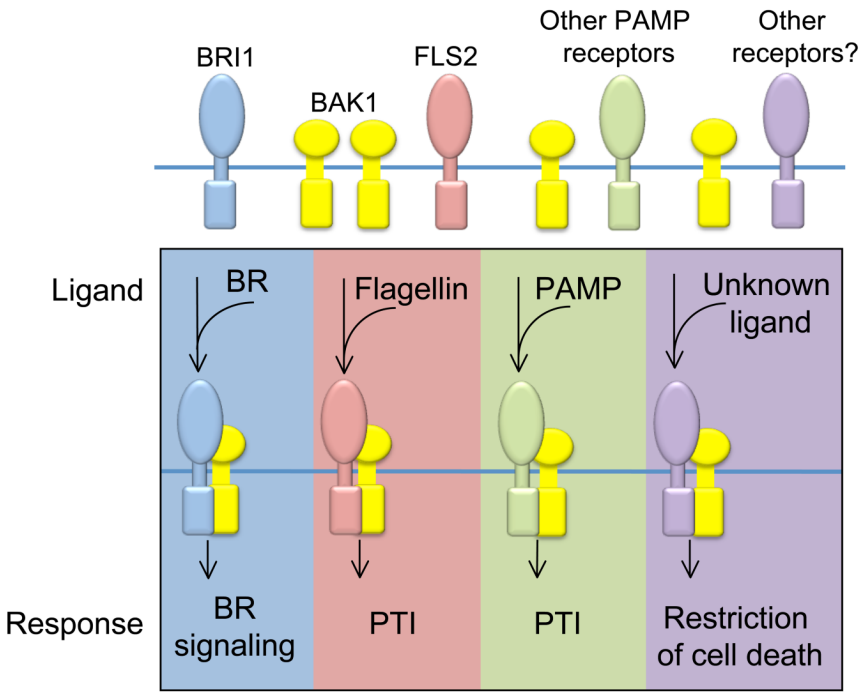

Figure 1.
Proposed functions of BAK1. Receptors exist as independent monomers in the absence of any ligand. BAK1 promotes BR and flagellin signaling through ligand-induced interaction with BRI1 and FLS2. BAK1 is likely also involved in signaling of other PAMPs to induce innate immunity responses and signaling of unknown endogenous signals to restrict cell death.

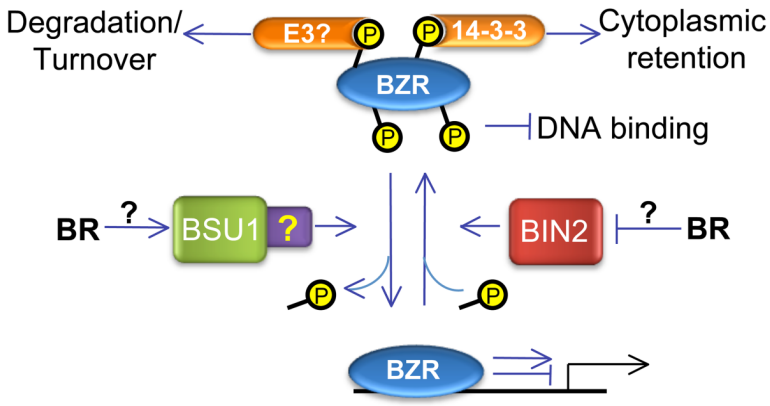

Figure 2.

Phosphorylation and dephosphorylation of BZR1 and BZR2/BES1 (BZR) are mediated by BIN2 and BSU1, either one of which might be regulated by BR. BSU1 likely needs an intermediate protein for function in planta (Purple). Phosphorylation (p) at multiple sites on BZR proteins regulates activity of the proteins through multiple mechanisms. Phospho-mediated 14-3-3 binding regulates cytoplasmic retention of the protein. Phosphorylation also inhibits the DNA binding activity and promotes proteasomal degradation, possibly through E3-mediated ubiquitination. When dephosphorylated upon BR signaling, BZR proteins are able to bind DNA and activate or repress BR-regulated genes. It is unclear how BR signaling from the receptor complex regulates BZR phosphorylation, though upstream signaling might regulate the activity of either BIN2 or BSU1, or modify BZR to change its affinity for BIN2 or BSU1.