Multiple mechanisms modulate brassinosteroid signaling
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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 regulation 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.

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 (PM)-localized LRR-RLK that transduces the BR signal across the plasma membrane [1]. Direct binding of BR has been demonstrated by photoaffinity crosslinking of a biotin-labeled analog 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 to inhibit the association of BRI1 with BAK1 [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 crossphosphorylation 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 BR1 substrates that regulate plant growth [10,11]. However, their precise roles in BR signaling remain unclear. No direct interactions have been observed between BR1, 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, BR1 has been shown to contain a domain that functions as a guanylyl cyclase \textit{in vitro}, suggesting that cGMP may have a role as a second messenger in BR signaling [12].

Endosomal signaling of BR1

BR1 and BAK1 are believed to function at the plasma membrane. Interestingly, coexpression of BR1 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 BR1 signaling was recently studied in Arabidopsis. Using a BR1-GFP transgenic line that expresses the fusion protein at endogenous levels, Geldner \textit{et al.} observed BR1 in early endosomes in the root cells of Arabidopsis [14*]. However, the distribution of BR1-GFP on plasma membrane and endosomes is not affected by BR deficiency or BR treatment, suggesting that BR1 endocytosis is independent of its activation state.

To determine whether BR1 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 BR1-YFP accumulation in endosomal aggregates and blocked BR1-YFP turnover. Interestingly, like BR treatment, BFA treatment of a highly BR-sensitive cell suspension culture induced strong dephosphorylation of BES1 and suppression of \textit{DWF4} gene expression. BFA also enhanced BR responses in a root culture system, though the effect was much weaker in intact Arabidopsis seedlings. These results demonstrate that BR1 can signal from early endosomes [14*]. However, it remains unclear whether endosomal BR1 is constitutively active or requires BR binding for kinase activation. The lack of BK1, which dissociates from PM BR1 upon BR activation, in endosomes suggests that endosomal BR1 receptors are constitutively active. Yet, the reason for endosomal BR1 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 BR1 proteins are endocytosed for degradation in late endosomal vesicles and vacuoles as a mechanism of desensitization. Testing the effect of blocking BR1 endocytosis on BR responses could distinguish these possibilities.

In contrast to endosomal BR1, a mutant BR1 receptor (br1–9) retained in the endoplasmic reticulum (ER) cannot activate BR signaling, but it is functional when localized on the PM. Mutation of a component of the ER quality control system, identified as the \textit{bri1–9} suppressor \textit{ebs1}, allows br1-9 to localize on the PM and suppresses the \textit{bri1} phenotypes [15*]. While demonstrating an important physiological function of the plant ER quality control system, the study also indicates that BR1 retained in the ER cannot mediate BR signaling, perhaps because of lack of a post-ER modification, functional 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 \textit{et al.} showed that a close homolog of BAK1, called BKK1 or SERK4, is also part of the BR1 receptor complex and when overexpressed it can partially suppress a \textit{bri1} mutant, similar to overexpression of BAK1. Yet, a double mutant between \textit{bak1} and \textit{bkk1} showed a seedling lethal phenotype with symptoms of programmed cell death, as monitored by trypan blue staining [16**]. Kemmerling \textit{et al.} also reported a role for BAK1 in pathogen-induced cell death responses [17**]. Biotic and necrotrophic pathogens were observed to cause spreading necrosis in the \textit{bak1} mutants. Mutants in other 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 \textit{et al.} and Chinchilla \textit{et al.} found that BAK1 interacts with the flagellin receptor, FLS2, in a ligand-dependent manner [18**,19**]. The \textit{bak1} mutant showed reduced growth inhibition by flagellin, and both \textit{bak1} mutant and \textit{NhSerk3}-silenced \textit{Nicotiana benthamiana} inhibited the \textit{flg22}-induced burst of active oxygen species (AOS) similar to the \textit{fls2} mutant or \textit{NbFLS2}-silenced line. Additionally, the \textit{NhSerk3}-silenced \textit{N. benthamiana} line was more susceptible to pathogenic (\textit{Pto DC3000}) and non-pathogenic (\textit{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-recog-
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 SERKs 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 crosstalk 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, *br1-1D*) led 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 *br1* 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...
Multiple mechanisms modulate brassinosteroid signaling Gendron and Wang 439

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.

important roles in many signaling pathways by binding to large numbers of target proteins in a sequence-dependent 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 thaliana and rice, respectively [32**,33**]. A conserved 14-3-3-binding site was found in BZR1, BZR2/BES1, 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 br1-1D, 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**], indicating 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 probably 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 brsr1-1D (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 networks of molecular links between BR signaling and target physiological or developmental pathways. Proteomic studies will probably identify post-transcriptional 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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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


In this study, a mutant BRI1 (bri1–9) was found to be retained in the ER. A mutant BRI1-GFP was observed in the endosomes in root cells of Arabidopsis grown in the dark, the double mutant shows a weak de-etiolation when overexpressed, suggesting a similar function as BAK1 in BR signaling. It was shown that phosphorylation of BZR2/BES1 inhibited DNA-binding activity of BZR1 and BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell 2002, 109:181-191.


In this study, BR1-GFP was observed in the endosomes in root cells of transgenic Arabidopsis, independent of BR levels. Blocking trafficking from early to late endosomal compartments causes accumulation of BR1-GFP in endosomal compartments and activation of BR signaling. It was concluded that BR1 can signal from endosomes.

In this study, the bak1 mutant develops spreading necrosis upon pathogen infection, accompanied by production of reactive oxygen intermediates. BL rescues the growth defects of bak1 but has no effect on its disease phenotypes, suggesting that BAK1 has a BR-dependent function in plant cell death control.


In this study, the bak1 mutant shows reduced sensitivity to pathogen-associated molecular patterns, including flagellin and EF-Tu, but normal flagellin-binding activity in the plasma membrane. BAK1 interacts with the flagellin receptor FLS2 upon flagellin treatment, indicating that BAK1 is involved in flagellin signaling as well as BR signaling.


In this study, similar to the above study, BAK1 interacts with FLS2 upon flagellin treatment. Silencing BAK1 expression reduces defense responses to pathogen infection.
BR induces nuclear localization of BZR1 and BZR2/BES1. BIN2-catalyzed 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.

In this study, the rice homolog of BZR1 (OsBZR1) was shown to be essential for BR responses in rice. Yeast two-hybrid screens identified all eight rice 14-3-3 proteins as OsBZR1-interacting proteins. Mutation of a 14-3-3-binding site in OsBZR1 abolished the interaction with 14-3-3 proteins, and expression of the mutant OsBZR1, which has increased nuclear localization, suppressed bri1–5 phenotypes. The study demonstrates a conserved mechanism for BR signaling in dicots and monocots, and reveals a novel role for 14-3-3 proteins in BR signaling.

In this study, new bri1 alleles were identified as enhancers of the late-flowering autonomous-pathway mutant luminidependens. The late flowering phenotypes of bri1 and cpd mutants were shown to be because of increased expression of the flowering repressor FLC.

In this study, expression of BRI1 in the epidermis rescues bri1’s dwarf phenotype more effectively than expression in vascular tissues. The authors show that BR-mediated expansion of cells in the epidermis drives expansion of the whole plant.