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Brassinosteroid Signal Transduction: A Mix of Conservation and Novelty

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Abstract

Brassinosteroids (BRs) are a unique class of plant steroids that are structurally similar to animal steroid hormones and play important roles in plant growth and development. Unlike the animal steroids, which bind to classical intracellular steroid receptors that directly modulate gene activities after translocation into the nucleus, the plant steroids rely on transmembrane receptor kinases to activate a phosphorylation cascade to regulate gene expression. Recent genetic and biochemical studies have identified several critical BR signaling components and revealed a striking mechanistic similarity between the plant steroid signaling pathway and several well-studied animal signaling cascades involving a receptor kinase and glycogen synthase kinase 3 (GSK3). A working model for BR signal transduction proposes that BR initiates its signaling pathway by promoting heterodimerization of two transmembrane receptor-like kinases at the cell surface, leading to inhibition of a GSK3 kinase and subsequent stabilization and nuclear accumulation of two GSK3 substrates that regulate BR-responsive genes. Such a simple model provides a framework for continued investigation of molecular mechanism(s) of plant steroid signaling.

Key words: *Arabidopsis*; Brassinosteroid; GSK3; Leucine-rich-repeat receptor-like kinase; Receptor heterodimerization; Steroid signaling

Introduction

Brassinosteroids (BRs) are a unique class of plant polyhydroxysteroids that are structurally similar to the well-studied animal and insect steroids. When applied exogenously to plants, BRs affect a variety of physiological processes, including cell division, cell elongation, vascular differentiation, root growth inhibition, biotic and abiotic stress tolerance, reproductive development, and modulation of gene expression (Clouse and Sasse 1998). The essential function of endogenous BRs was revealed by dramatic morphological changes of mutants that are defective in BR biosynthesis in several plant species including *Arabidopsis*, pea, rice, and tomato (Hong and others 2002; Mori and others 2002; Fujioka and Yokota 2003). In *Arabidopsis*, BR-deficient mutants display a characteristic mutant phenotype, including a dwarf stature with dark green and rounded rosette leaves, delayed flowering and senescence, reduced apical dominance and male fertility, altered vascular structure and cytoskeleton organization, and abnormal skotomorphogenesis in the dark (Li and others 1996; Szekeres and others 1996). All of

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these growth defects can be suppressed by supplying BRs to the growth medium.

In animals, steroid hormone signaling is initiated mainly by intracellular steroid receptors that belong to a large family of structurally related nuclear receptors with a characteristic three-domain structure consisting of an N-terminal transcriptional activation domain, a middle DNA-binding domain, and a C-terminal ligand-binding/protein dimerization domain (Aranda and Pascual 2001). In the absence of steroids, these intracellular steroid receptors are locked into nonproductive forms by association with heat shock proteins and other chaperones. Upon steroid binding, the receptors dissociate from the heat shock protein complexes, move into the nucleus, and bind to regulatory DNA sequences of their target genes to either activate or repress gene expression, leading to various physiological and developmental changes. Alternatively, animal steroid signaling can be rapidly activated at the cell surface by the membrane-localized steroid receptors to induce the so-called ''nongenomic'' steroid effects on cell physiology independent of gene regulation (Losel and Wehling 2003). Candidates for such membrane steroid receptors include the membrane-associated classical steroid receptors (Shaul 2002), G-protein coupled receptors (GPCRs, Grazzini and others 1998), and a novel family of membrane steroid-binding proteins that are structurally similar to GPCRs (Hammes 2003). It is known that calcium, cAMP, heterotrimeric G proteins, and protein phosphorylation are involved in the membrane-initiated steroid signaling pathways (Cato and others 2002).

By contrast, plants seem to lack the classical steroid signaling pathway, and the completely sequenced *Arabidopsis* genome does not encode any protein that shows significant sequence homology to the highly conserved nuclear steroid receptors (Arabidopsis Genome Initiative 2000). Instead, plant steroids are mainly, if not exclusively, recognized by cell surface receptors to initiate their signaling pathways to regulate a variety of physiological processes (Li 2003). Recent advances in the study of BR signaling have dramatically increased our understanding of how the plant steroid signal is perceived on the cell surface, transmitted into the cytosol, and finally transduced into the nucleus to regulate gene activities (Clouse 2002a, 2002b). This review highlights the characterization of several *Arabidopsis* proteins that are believed to be involved in plant steroid perception and signal transduction. Biochemical and genetic studies of these BR signaling components are beginning to reveal the mode of steroid action in plants.

BRI1: A Critical Component of a Membrane BR Receptor

BRI1 (Brassinosteroid-Insensitive 1), the first known component of the plant steroid signaling pathway, was initially discovered in a genetic screen for *Arabidopsis* mutants that showed normal root growth in the presence of high concentrations of BR (Clouse and others 1996). Repeated genetic screens for loss-of-function BR-insensitive mutants only resulted in the identification of additional *bri1* alleles, indicating that BRI1 is an essential nonredundant component of the plant steroid signaling pathway (Kauschmann and others 1996; Li and Chory 1997; Noguchi and others 1999; Friedrichsen and others 2000). *bri1* mutants are morphologically indistinguishable from BR-deficient dwarf mutants but cannot be rescued by exogenous BR application (Kauschmann and others 1996; Li and Chory 1997). Interestingly, these mutants also accumulate high levels of brassinolide (BL), the most active BR, and its biosynthetic precursors, with the more severe *bri1* mutants accumulating higher levels of endogenous BRs, suggesting that BRI1 plays an important role in regulating BR homeostasis (Noguchi and others 1999). It was reported that BR insensitivity in *bri1* mutants was also observed at the molecular level (Kauschmann and others 1996). The BR-induced expression of two xyloglucan endotransglycosylase genes, *TCH4* and *meri5*, was absent in *bri1-2* mutants, although the GA-induced *meri5* expression was still observed. Taken together, these genetic and physiological studies strongly suggest that *BRI1* encodes an essential component of the plant steroid signaling pathway.

The *BRI1* gene was cloned by a map-based strategy and was found to encode a leucine-rich-repeat receptor-like kinase (LRR–RLK) that consists of an LRR-containing extracellular domain, a single transmembrane segment, and a cytoplasmic Ser/Thr kinase domain (Figure 1) (Li and Chory 1997). The extracellular domain is composed of several discrete regions, including an N-terminal signal peptide, a leucine-zipper motif that might mediate protein–protein interaction, and two pairs of cysteine residues flanking 25 tandem LRRs that are disrupted by a 70 amino acid (70AA) island domain between the 21st and 22nd LRRs (Figure 1). Analyses of various *bri1* mutant alleles identified several regions essential for *in vivo* BRI1 function (Figure 1) (Li and Chory 1997; Noguchi and others 1999; Friedrichsen and others 2000). The *bri1-5* mutant contains a Cys69Tyr substitution in the first cysteine pair that might be important for mediating disulfide-linked dimerization of BRI1 with itself or its action part-

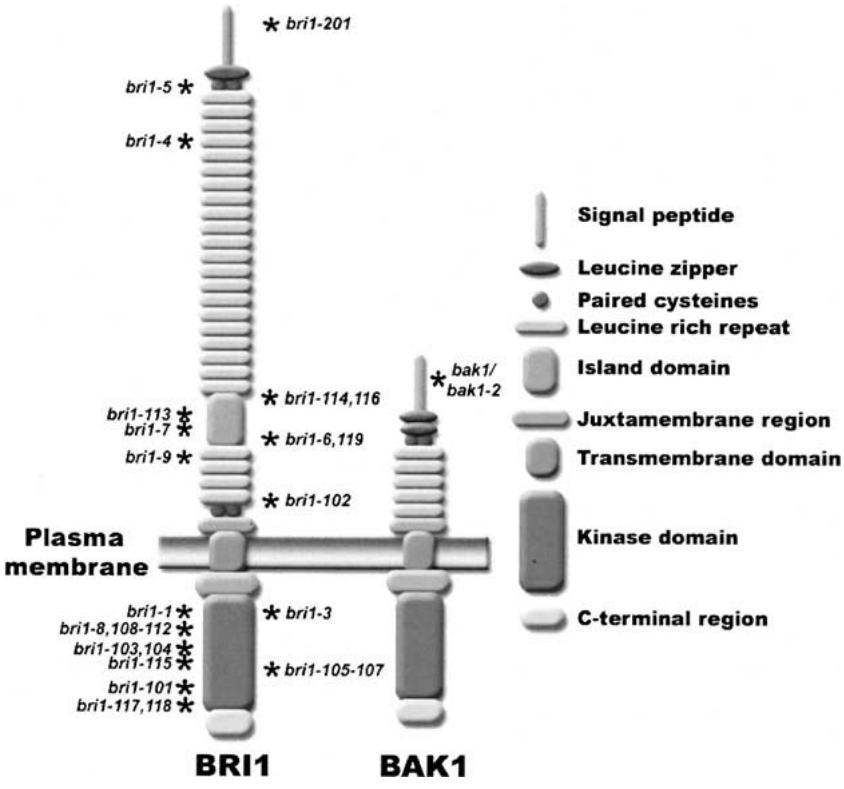

Figure 1. Schematic structure of BRI1 and BAK1 receptor kinases. Both receptor kinases are made up of similar structural domains and sequence motifs, but BAK1 is a much smaller protein, containing only 5 LRRs and missing the 70AA island domain and the second cysteine pair. Asterisks represent known mutations found in BRI1 and BAK1.

ners, while the *bri1-9* mutant harbors a Ser662Phe missense mutation in the 22nd LRR that is immediately downstream from the 70AA island. The *bri1-102* mutation changes Thr750 to Ile, which is located between the last LRR and the second cysteine pair. Five mutations were identified in the 70AA island domain, including two nonsense (*bri1-114* and *bri1-116*) and three missense mutations (*bri1-6/bri1-119, bri1-7,* and *bri1-113*), each involving a change of a glycine residue. The clustering of mutations in this domain led to a hypothesis that the 70AA island might be directly or indirectly involved in binding the plant steroids. As expected from its similarity with animal RLKs, allele sequencing also confirmed the essential role of the kinase domain for transmitting BR signals. Among 28 *bri1* alleles sequenced to date, 15 alleles contain mutations in the kinase domain (Li and Chory 1997; Noguchi and others 1999; Friedrichsen and others 2000). These kinase mutations might affect receptor dimerization, recruitment of downstream signaling proteins, or the catalytic activity of the kinase domain, thus blocking the propagation of BR signaling.

The deduced BRI1 protein sequence predicts that BRI1 spans across the cell membrane. Although there is no immunohistochemical data to prove this prediction and to determine the topology of the BRI1 protein, confocal microscopic analysis of a BRI1:GFP fusion protein revealed that BRI1 is localized at the plasma membrane (Friedrichsen and others 2000). The BRI1 cytoplasmic domain is expected to contain serine/threonine kinase activity that is essential for transmitting BR signal into the cytosol. Biochemical analyses of an *E. coli*-expressed calmodulin-binding peptide (CBP)–BRI1 kinase fusion protein and a C-terminal HA-tagged full-length BRI1 protein expressed in human cell cultures confirmed that BRI1 is indeed a functional kinase *in vitro* (Friedrichsen and others 2000; Oh and others 2000). Phosphoamino acid analyses revealed that the *in vitro* autophosphorylated BRI1 protein contains both phospho-Ser and phospho-Thr but no phospho-Tyr (Friedrichsen and others 2000; Oh and others 2000), thus proving that BRI1 belongs to the serine/threonine kinase family. Matrix-assisted laser desorption/ionization–mass spectrometry analyses with an *in vitro* phosphorylated CBP–BRI1 kinase fusion protein identified at least 12 autophosphorylation sites, including 5 in the juxtamembrane segment, 5 in the core kinase domain, and 2 in the carboxyl terminal region (Oh and others 2000). These phosphorylated residues may participate in regulating the kinase activity of the BRI1 protein or serve as docking sites to recruit different downstream BR signaling proteins into an activated

BR receptor complex. Thus, BRI1 is a membrane-associated receptor kinase that plays a key role in BR signaling through Ser/Thr phosphorylation.

The importance of BRI1 in BR signaling was further supported by the molecular genetic characterization of BR-insensitive dwarf mutants in other plant species. Two allelic rice dwarf mutants with erect leaves, *d61-1* and *d61-2*, were found to be less sensitive to BR treatment and accumulated higher-than-wild-type levels of BRs. Molecular cloning experiments revealed that a Thr989Ile mutation in the kinase domain and a Val491Met mutation in the 18th LRR of a rice BRI1 homologue were responsible for the BR-insensitive dwarf phenotype of the *d61-1* and *d61-2* mutants, respectively (Yamamuro and others 2000). In pea, three BR-related dwarf mutants were known (Nomura and others 1997, 1999; Schultz and others 2001), including two BR-deficient mutants, *lk* and *lkb*, and a BR-insensitive mutant, *lka*. It was reported that the *lka* mutant, which is morphologically similar to *lk* and *lkb* mutants but could not be rescued by exogenous BR treatment, contains a missense mutation in a pea BRI1 homologue (Normura and others 2001). Similarly, two BR-insensitive tomato mutants, *curl3* (Koka and others 2000) and *abs1* (Montoya and others 2002), were recently found to contain a nonsense mutation and a missense mutation in the extracellular domain and the kinase domain, respectively, of a tomato BRI1 homologue, tBRI1 (Montoya and others 2002). Surprisingly, tBRI1 was found to be identical to SR160, a putative membrane receptor for a peptide hormone systemin involved in wounding response (Scheer and Ryan 2002), suggesting that tBRI1 might be able to recognize two distinct ligands: a steroid and a peptide (Szekeres 2003).

Although there is no direct demonstration that purified BRI1 proteins can bind BL or its active analogues, several lines of experimental evidence support that BRI1 is crucial for a membrane BR receptor activity. He and others (2000) provided indirect evidence showing that BRI1's extracellular domain is involved in BR sensing. The extracellular domain, the transmembrane α-helix, plus a small intracellular juxtamembrane segment of the BRI1 protein were fused to the cytoplasmic kinase domain of Xa21, a rice LRR–RLK involved in disease resistance (Song and others 1995), to generate a BRI1:Xa21 chimeric receptor. Upon treatment with BL, this chimeric receptor can initiate a typical plant defense response in rice cell cultures, including rapid cell death, oxidative burst, and the expression of pathogenesis-related genes. Both the BRI1 portion and the Xa21 kinase domain are necessary for the activity of the BRI1:Xa21 chimeric receptor because a mutation in either domain completely abolished the BR-activated defense responses. These results clearly demonstrated the essential role of BRI1's extracellular domain, especially the 70AA island, for BR binding. Further support for BRI1 being a membrane BR receptor came from several BR-binding assays. Wang and others (2001) showed that transgenic *Arabidopsis* plants overexpressing *BRI1* exhibited a higher BR sensitivity and contained a higher BR binding activity in their membrane fractions. Consistent with previous genetic data, mutations in the extracellular domain eliminated such a BR binding activity, whereas mutations in the kinase domain had no effect on BR binding. Importantly, such a BR binding activity can be coimmunoprecipitated with BRI1. In line with the hypothesis that BRI1 is a crucial element of a BR receptor, BL treatment stimulated BRI1 autophosphorylation in wild-type *Arabidopsis* seedlings but not in *bri1* mutants containing a kinase-dead mutation. Based on these data, it was suggested that BR binding, either directly or indirectly, to the extracellular domain of BRI1, would activate the intrinsic kinase activity of BRI1 to propagate the plant steroid signaling.

BAK1: A BRI1-Interacting Receptor Kinase

It is well known that animal receptor kinases are activated by ligand-induced homo- or heterodimerization. Thus, it would be reasonable to assume that BRI1 is activated in a similar fashion by BR via the formation of homo- or heterodimers. The latter model is much more favored by the recent discovery of BRI1-associated receptor kinase 1 (BAK1) as a potential coreceptor for BRI1 (Li and others 2002; Nam and Li 2002).

BAK1 was identified independently by a yeast two-hybrid screen as a specific BRI1-interacting protein (Nam and Li 2002) and by a gain-of-function activation-tagging screen as an extragenic suppressor of *bri1-5*, a weak extracellular *bri1* mutation mentioned above (Noguchi and others 1999). Like BRI1, BAK1 is also an LRR-containing receptor kinase (Figure 1) and belongs to a small subfamily of the *Arabidopsis* LRR–RLK (LRRII, Shiu and Bleecker 2001) that contains 13 other members including the *Arabidopsis* *S*OMATIC *E*MBRYOGENESIS *R*ECEPTOR *k*inase *1* (AtSERK1), which was known to enhance somatic embryogenesis when overexpressed (Hecht and others 2001). However, BAK1 contains only 5 LRRs in its extracellular domain and

lacks the 70AA island domain, a characteristic feature of BRI1 and its homologues. Northern blot analysis revealed that BRI1 and BAK1 share similar gene expression domains, and confocal microscopic analysis of transgenic plants expressing a BAK1:GFP fusion protein showed that BAK1 is also a membrane-localized protein. Coimmunoprecipitation experiments were performed to demonstrate that BRI1 and BAK1 interact physically in yeast cells and in plants (Li and others 2002; Nam and Li 2002). These data strongly suggest that BAK1 and BRI1 have the potential to form a heterodimer at the cell surface.

Genetic studies with both gain-of-function and loss-of-function approaches showed that BAK1 is also involved in BR signaling (Li and others 2002; Nam and Li 2002). Overexpression of the *BAK1* gene not only suppressed the *bri1-5* mutation, but also rescued, in a dose-dependent manner, the mutant phenotype of *bri1-301* containing a weak kinase mutation, indicating that BAK1 becomes a rate-limiting factor when the activity of BRI1 is reduced. In addition, *BAK1* overexpression in a wild-type background gave rise to a phenotype that was previously observed in transgenic *Arabidopsis* plants overexpressing *BRI1* (Wang and others 2001) or *DWARF4* (*DWF4*) that encodes a P450 steroid hydroxylase catalyzing a rate-limiting step of BR biosynthesis (Choe and others 1998, 2001). In contrast, two null *bak1* mutations, caused by T-DNA insertions in either the first exon or the third intron of the *BAK1* gene, resulted in a weak *bri1*-like phenotype with a semidwarf stature, a compact rosette, shortened inflorescent stems, and reduced sensitivity to BR in inhibiting root elongation (Li and others 2002; Nam and Li 2002). The weak phenotype of the two null *bak1* mutants was not surprising at all given the fact that there are 13 BAK1-like receptor kinases in *Arabidopsis*. Some of the BAK1 homologues, especially those sharing similar expression patterns with BRI1, might also heterodimerize with BRI1 to mediate BR signaling and contribute to signal specificity.

The demonstration that BAK1 participates in BR signaling and that BAK1 physically interacts with BRI1 in plants suggests that BAK1 might function as a coreceptor for BRI1 in initiating BR signaling. Such a hypothesis was supported by several lines of genetic evidence. *BAK1* overexpression can only suppress weak *bri1* mutations but fails to rescue the mutant phenotype of a null *bri1* mutant or a *bri1-5 det2* double mutant with the second mutation affecting BR biosynthesis (Li and others 2002), indicating that the BR signaling activity of BAK1 depends on a (at least partially) functional BR11 and the presence of the plant steroids. In addition, a null *bak1* mutation could enhance a weak *bri1* mutation but apparently had no significant effect on a null *bri1* mutation (Nam and Li 2002). Furthermore, overexpression of a kinase-dead BAK1 in the weak *bri1*-5 mutant led to a more severe dwarf morphology (Li and others 2002), most likely due to dominant-negative effect of the mutated BAK1 on the formation of the presumed signaling-active BRI1/BAK1 dimers.

Although the genetic studies concluded that BRI1 and BAK1 could function together as a heterodimer in mediating plant steroid signaling, biochemical analyses of the interaction between the two receptor kinases led to two different heterodimerization models to explain how they are activated in response to BR binding. One model closely resembles the receptor tyrosine kinase model in which ligand binding would trigger receptor dimerization to activate its intrinsic kinase activity through transphosphorylation, thus initiating a phosphorylation cascade (Schlessinger 2000). The other is modeled after the TGFβ receptor, which is composed of a homodimeric type I TGFβ receptor and a homodimeric type II TGFβ receptor. TGFβ binding to the type II receptor promotes the formation of a heterotetramer to allow the constitutively active type II receptor to phosphorylate and activate the type I receptor, leading to propagation of the TGFβ signal (Attisano and Wrana 2002).

Nam and Li (2002) hypothesized that BRI1 and BAK1 exist mainly as inactive monomers that are in equilibrium with a small pool of active heterodimer. BR binding promotes the formation of the BRI1/BAK1 heterodimer, resulting in activation of both receptor kinases through transphosphorylation and initiation of a phosphorylation-mediated BR signaling cascade. Such a model, similar to the receptor tyrosine kinase model, is based on the biochemical studies of the two receptor kinases expressed in yeast cells as full-length proteins. Both BRI1 and BAK1 were inactive when they were expressed alone but became activated when they were coexpressed and interacted with each other. However, neither BRI1 nor BAK1 was phosphorylated when one of them was inactivated by site-directed mutagenesis, which had no effect on their physical interaction. Because yeast cells do not produce bioactive BRs, these phosphorylation assays indicated that the dimerization between BRI1 and BAK1 is sufficient to activate the two interacting receptor kinases but their activation requires transphosphorylation by their partners. Consistent with the hypothesis that a BR-binding protein is needed for BR to bind a BRI1-containing steroid

receptor (Li and Chory 1997), BR treatment of yeast cells coexpressing BRI1 and BAK1 did not increase the phosphorylation levels of the two receptor kinases or enhance their physical interaction (Nam and Li 2002). On the other hand, BR treatment was known to stimulate BRI1 autophosphorylation in plants. Additional genetic and biochemical studies will determine whether BR activates the BRI1 kinase activity by triggering the dimerization of BRI1/BAK1 in plants and whether the BRI1/BAK1 dimerization is sufficient to activate downstream BR signaling events.

Li and others (2002) proposed that BRI1/BAK1 interaction is more similar to the interaction between the type I and type II TGFβ receptors, based on the biochemical studies of the recombinant cytoplasmic domains of the two receptor kinases expressed in *E. coli*. Unlike the yeast-expressed full-length proteins, the *E. coli*-expressed BRI1 or BAK1 fusion protein was an active kinase when tested individually by *in vitro* autophosphorylation assays. Interestingly, the phosphorylation level of BAK1 was significantly increased when it was mixed with BRI1; however, no BRI1-stimulated BAK1 phosphorylation was observed if BAK1 was inactivated, suggesting that the increased BAK1 phosphorylation was likely a result of BAK1 autophosphorylation after it was stimulated by BRI1. On the contrary, the phosphorylation level of BRI1 remained the same regardless of whether it was incubated with an active or an inactive BAK1. These results suggested a unidirectional phosphorylation model in which an activated BRI1 phosphorylates and activates BAK1 to initiate a BR signaling cascade. The main difference between this model and the TGFβ receptor model is that BRI1 has to be activated first by the plant steroid, whereas the type II TGFβ receptor is a constitutively active kinase. Although BR treatment did increase the autophosphorylation level of BRI1 in plants, one has to propose a different dimerization model, such as BRI1 homodimerization, or a completely new mechanism to explain how BRI1 itself is activated in response to BRs.

Protein Coligands for BR to Bind Its Receptor?

Although the 70AA island in the extracellular domain of BRI1 is critical for BR binding, such a domain is surrounded by LRR motifs that are best known for mediating protein/protein interactions. This suggests that a protein coligand might be needed to help the plant steroid to interact with the 70AA island (Li and Chory 1997). The *Arabidopsis* genome encodes several proteins that are highly similar to animal sex steroid binding proteins, which are thought to mediate membrane-initiated steroid signaling (Rosner and others 1999). A recent structural study also implicated a steroid carrier function for Bet v 1 (Markovic–Housley and others 2003), a major birch pollen allergen that belongs to a large family of pathogenesis-related plant proteins (PR-10). Bet v 1 was found to contain a large Y-shaped hydrophobic cavity that can accommodate two molecules of deoxycholate and display a specific noncovalent interaction with BL. *Arabidopsis* contains at least 40 proteins sharing the conserved Bet v 1 structural domain, and some of them could function as protein coligands for BL to bind to its receptors. The involvement of putative BR-binding proteins in BR perception was further supported by the discovery of BRS1, a putative serine carboxypeptidase, in a genetic screen for extragenic suppressors of *bri1-5* (Li and others 2001a). *BRS1* overexpression suppressed only the extracellular *bri1* mutation but had no effect on a *bri1* kinase mutation. In addition, the phenotypic suppression of *bri1-5* by *BRS1* overexpression required the presence of active BR. Thus, BRS1 most likely functions before the activation of a BR receptor, possibly by converting an inactive precursor of a putative BR-binding protein to its active form, which can then facilitate the binding of BR to the BRI1-containing BR receptor at the cell surface.

BIN2: A Cytoplasmic Kinase Negatively Regulating BR Signaling

Extensive genetic screens for *Arabidopsis* mutants showing the BR-deficient phenotype but displaying no response to BR treatment resulted in the identification of the second *BRASSINOSTEROID-INSENSITIVE* locus, *BIN2/DWARF12* (Li and others 2001b; Choe and others 2002), which is allelic to the *UCU1* gene uncovered in genetic screens for leaf development mutants (Perez–Perez and others 2002). In contrast to known *bri1* alleles that are recessive loss-of-function mutations, all known *bin2/dwarf12/ucu1* mutants carry semidominant gain-of-function mutations, including the weakest *ucu1–3* mutant that was previously thought to contain a recessive loss-of-function mutation (Li and others 2001b; Choe and others 2002; Perez–Perez and others 2002; Peng and Li, unpublished data). Tetraploid analysis indicated that the *bin2* mutant phenotype is likely caused by hypermorphic mutations that increase the production or activity of the BIN2 protein. A

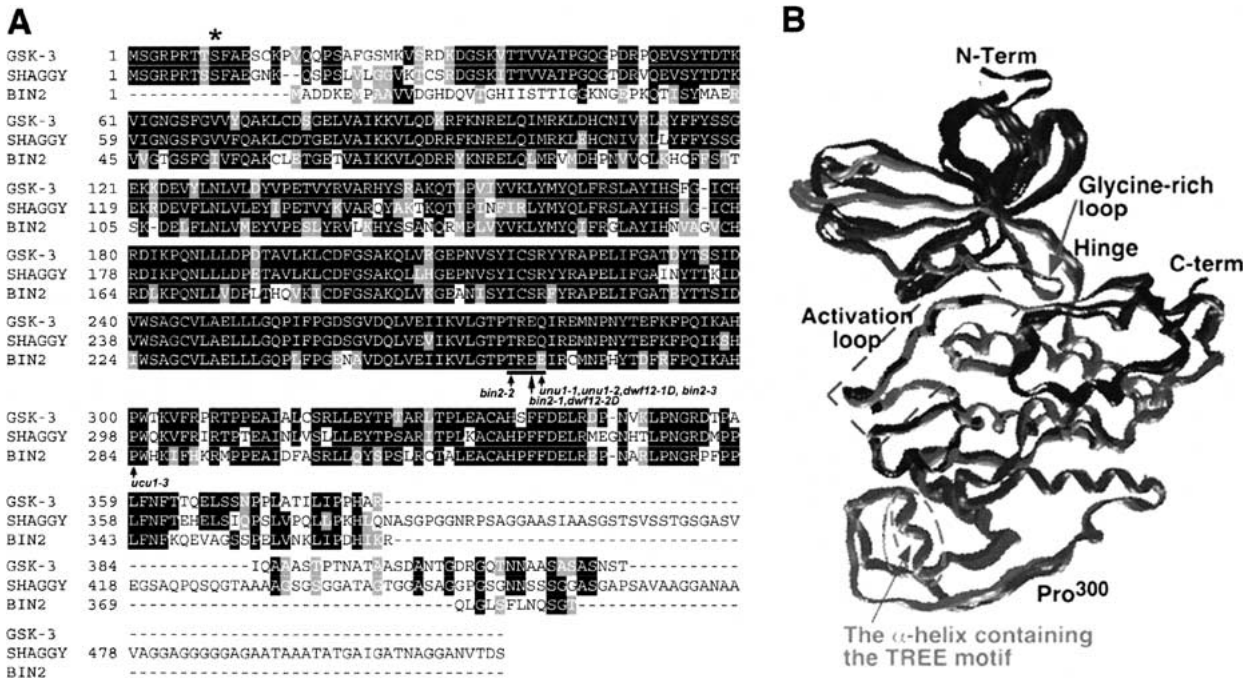

Figure 2. Sequence alignment and structural modeling of the *Arabidopsis* BIN2 protein. (**A**) Sequence comparison between BIN2 and the human GSK3β and *Drosophila* SHAGGY kinases. Black shading indicates identical amino acids, while gray shading denotes similar residues. The TREE motif in BIN2 is underlined by the black bar and the known *bin2/dwarf12/ucu1* mutations are indicated by arrows. The Ser9 residue in human GSK3β, critical for regulation, is highlighted by the asterisk. (**B**) The modeled structure of BIN2 is superimposed onto the three known GSK3 structures in the Protein Data Bank (PDB ID: 1I09, 1GNG, and 1H8F). The structure modeling was performed at the Swiss-Model automated knowledge-based protein-modeling server (http://www.expasy.org/swissmod/SWISS-MODEL.html). The locations of N-terminus, C-terminus, activation loop, glycine-rich loop, the hinge that links the N-terminal β-strand domain with the C-terminal α-helical domain, and the short α-helix containing the TREE motif are indicated.

root growth inhibition assay indicated that *bin2* mutants are insensitive only to BR but display a hypersensitivity to ABA and retain normal sensitivity to other plant hormones, including GA, auxin, cytokinin, and ethylene (Li and others 2001b). Like *bri1* mutants, *bin2* mutants were found to accumulate a higher level of BL and its biosynthetic precursors (Choe and others 2002), suggesting that BIN2 also participates in regulating BR homeostasis. Taken together, these data suggest that BIN2 is an important negative regulator in the plant steroid signaling pathway.

BIN2 encodes a cytoplasmic Ser/Thr kinase that displays 70% sequence identity within the catalytic domain to those of the mammalian GSK3 and the *Drosophila* SHAGGY kinases (Figure 2A) (Li and Nam 2002), which play key roles as negative regulators in a wide variety of signaling processes controlling cell proliferation, cell differentiation, cytoskeleton dynamics, and programmed cell death (Frame and Cohen 2001). In the absence of extracellular signals, GSK3 is a constitutively active kinase that phosphorylates a variety of protein substrates to block signal transduction through direct inhibition of their biochemical activities, interfering with their subcellular localization, or promoting their degradation (Frame and Cohen 2001). In response to extracellular signals, GSK3 becomes inactivated, thus relieving its inhibitory effect on its downstream targets.

Consistent with the genetic results suggesting that *bin2* mutants carry hypermorphic *bin2* alleles, the recombinant BIN2 kinase harboring the *bin2-1* mutation was more active towards a peptide substrate than the wild-type recombinant kinase (Li and Nam 2002). In addition, it was shown that overexpression of the wild-type *BIN2* gene gave rise to a *bri1*-like phenotype in transgenic plants, with a higher *BIN2* expression level leading to a more severe dwarf phenotype. Furthermore, decreasing the *BIN2* expression via a cosuppression strategy could suppress a weak *bri1* phenotype. These results further supported the hypothesis that BIN2 functions as a negative regulator in BR signaling and implied that the inhibition of BIN2 kinase activity would constitute a key regulatory

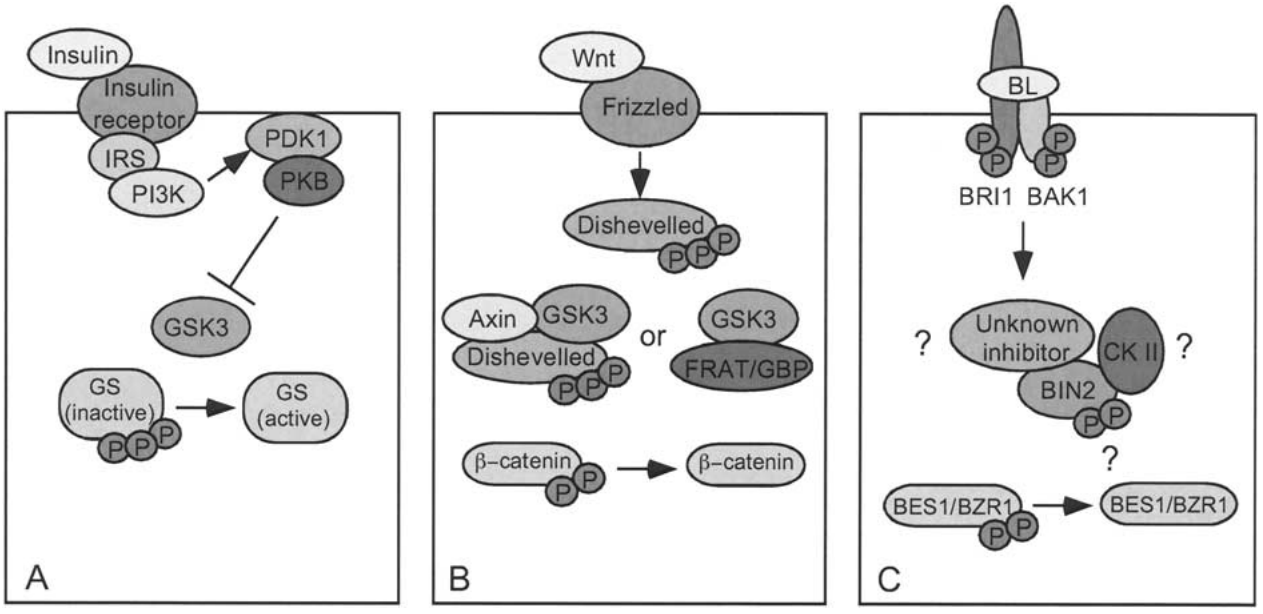

Figure 3. Regulation of GSK3 kinases in different signaling pathways. (**A**) The binding of insulin to its receptor initiates a phosphorylation cascade involving insulin receptor substrate (IRS), phosphoinositide-3-kinase (PI3K), and phosphoinositide-dependent kinase 1 (PDK1), which leads to the activation of protein kinase B (PKB) that can inhibit GSK3β by phosphorylating the Ser9 residue of the latter kinase. As a result, glycogen synthase (GS) becomes hypophosphorylated and active. (**B**) In the Wnt signaling, Wnt binds to its receptor, Frizzled, leading to the activation of a cytoplasmic protein, Disheveled. The activated Disheveled can directly interfere with the formation of the GSK3β/Axin/β-catenin destruction complex or recruit another cytosolic protein, FRAT/GBP, which can compete with β-catenin for binding GSK3β, thus preventing GSK3β from phosphorylating β-catenin. (**C**) A hypothetical model of BIN2 regulation in plants. In response to BR signals, both BRI1 and BAK1 are activated, leading to the activation of an unknown BIN2-binding protein or an alleged CKII that phosphorylates BIN2 at the suspected TREE motif, which can then inhibit the BIN2 kinase activity.

step after the plant steroids activate the BRI1-containing BR receptor.

In animals, the GSK3 kinase is generally regulated by two different mechanisms (Figure 3A, 3B). One involves protein phosphorylation and the other depends on protein/protein interaction. In the insulin signaling pathway, insulin binding to its receptor triggers a phosphorylation cascade that eventually phosphorylates GSK3 at a serine residue near its N-terminus (Figures 2A, 3A) (Cross and others 1995). The phosphorylated serine residue binds to a conserved substrate-binding site, thus preventing GSK3 from phosphorylating its downstream targets (Dajani and others 2001; ter Haar and others 2001). In the Wingless pathway, GSK3 phosphorylation of β-catenin, a critical regulator for expression of many Wnt-responsive genes, requires the formation of a multiprotein complex that includes GSK3, β-catenin, and an adapter protein named Axin (Ikeda and others 1998). Wnt proteins transduce their signals through Disheveled proteins to inhibit the GSK3 activity by the physical displacement of the kinase from the GSK3/Axin/β-catenin complex or the recruitment of FRAT/GBP, a GSK3-binding protein, to prevent the GSK3/β-catenin interaction (Dominguez and Green 2001), leading to increased accumulation of β-catenin and consequent upregulation of many proliferative genes (Figure 3B).

It remains to be determined how BIN2 is regulated by the steroid signals after the activation of both BRI1 and BAK1 receptor kinases. BIN2 lacks the N-terminal serine residue that is essential for the regulation of many animal GSK3 kinases (Figure 2A). Neither BRI1 nor BAK1 was able to phosphorylate BIN2 and no direct physical interaction was detected between BIN2 and these two transmembrane receptor kinases (Li and Nam 2002; Nam and Li, unpublished results), suggesting that additional BR signaling protein(s) are necessary for BIN2 inhibition in response to BR. On the other hand, the clustering of 6 out of all known 7 *bin2/dwarf12/ucu1* mutations in a highly conserved 4-amino-acid Thr-Arg-Glu-Glu (TREE) motif (Choe and others 2002; Li and Nam 2002; Perez–Perez and others 2002) suggests the involvement of protein–protein interaction and/or protein phosphorylation in BIN2 regulation (Figure 3C). Based on a recently resolved

crystal structure of the human GSK3β kinase, the TREE motif is part of a short α-helix that is exposed on the surface of the protein (Figure 2B) (Dajani and others 2001; ter Haar and others 2001). Thus, it is possible that this domain is critical for the interaction between BIN2 and a yet to be identified GSK3-binding protein essential for BIN2 regulation. However, the lack of mutations at other positions within the short α-helix favors a different inhibitory mechanism. The TREE motif is highly similar to the consensus phosphorylation motif S/TxxD/E (D, E, S, T, and x stand for aspartic acid, glutamic acid, serine, threonine, and any other amino acids, respectively) of casein kinase II (CKII), which also prefers acidic residues at −2 to +5 positions relative to the phosphorylation site, with the +1 and +2 positions being the most important ones (Meggio and Pinna 2003). The mutations of the threonine and the two glutamate residues in TREE motif would prevent the phosphorylation of BIN2 by such an acidic-directed protein kinase, uncoupling the *Arabidopsis* GSK3 kinase from a BR-activated regulatory mechanism. We are currently investigating these two possible mechanisms for BIN2 regulation and searching for missing components that link the activation of BRI1 and BAK1 to the inhibition of BIN2.

BES1 and BZR1: Two Nuclear Components of BR Signaling

The discovery of the cytoplasmic kinase BIN2 provided a great opportunity for identifying additional BR signaling components. In the absence of BR signal, BIN2 is an active kinase that would phosphorylate downstream targets to block the further transduction of BR signaling in the cytosol. Using a yeast two-hybrid method, we have identified two novel *Arabidopsis* proteins (originally named BIS1 and BIS2 for BIN2 SUBSTRATE 1 and 2, respectively), sharing 88% sequence identity with each other, as potential substrates for the BIN2 GSK3 kinase (Zhao and others 2002). Interestingly, BIS1 and BIS2 were also discovered as bri1-EMS-suppressor 1 (BES1) and brassinozole-resistant 1 (BZR1) in two different genetic screens for mutations that suppress a weak *bri1* mutant (Yin and others 2002a) or resistant to brassinozole, a specific BR biosynthesis inhibitor (Wang and others 2002), respectively. In yeast, BES1 and BZR1 displayed specific interactions with BIN2. In addition, both proteins contain multiple copies of a conserved GSK3 phosphorylation motif (S/TxxxS/T) and were phosphorylated by BIN2 *in vitro* (He and others 2002; Yin and others 2002a; Zhao and others 2002), apparently through a phosphorylation mechanism distinct from those of the known animal GSK3 kinases (Zhao and others 2002; Peng and Li, unpublished results). Furthermore, the BIN2-catalyzed phosphorylation of either BES1 or BZR1 could be specifically inhibited by lithium (Zhao and others 2002), a known inhibitor for GSK3 kinases (Ryves and Harwood 2002). These biochemical data strongly suggest that BES1 and BZR1 are substrates for the BIN2 GSK3 kinase and might play a role in the plant steroid signaling.

The involvement of BES1 and BZR1 in BR signaling was supported by several pieces of genetic evidence. First, a Pro233Leu mutation in BES1 was repeatedly identified as a suppressor for three different *bri1* mutations (Yin and others 2002a; Zhao and others 2002), while the corresponding mutation, Pro234Leu, in BZR1 was able to rescue a de-etiolation phenotype of dark-grown seedlings caused by brassinazole treatment, a BR-deficient mutation, or a *bri1* mutation (Wang and others 2002). Second, not only the mutated BES1 and BZR1 but also the overexpression of the wild-type BZR1 protein was capable of suppressing the *bin2* mutant phenotype (He and others 2002; Zhao and others 2002). Third, the *bes1* mutation not only suppressed the *bri1* mutant phenotype but also gave rise to a phenotype, characterized by long and bending petioles, curly leaves, and accelerated senescence, that was previously observed in transgenic plants overproducing BR or BRI1 (Choe and others 2001; Wang and others 2001), indicating that the *bes1* mutation led to a constitutive BR response, most likely through upregulation of many BR-responsive genes (Yin and others 2002a). In contrast, the Pro234Leu mutation in BZR1 caused a dramatic reduction in expression of the *CPD* gene (Wang and others 2002), which was known to be inhibited specifically by BR treatment (Mathur and others 1998), and led to a weak dwarf phenotype in the light, suggesting a role of BZR1 in regulating BR homeostasis in addition to its function in promoting cell elongation. Given the fact that both *bes1* and *bzr1* mutations increase protein stability and nuclear accumulation of the two proteins (Wang and others 2002; Yin and others 2002a), these genetic results demonstrate that BES1 and BZR1 act downstream of BRI1 and BIN2 as two positive regulators of the BR signal transduction pathway with overlapping yet distinct physiological functions.

Consistent with the *in vitro* BIN2–BES1/BZR1 phosphorylation results and the hypothesis of BR-mediated BIN2 regulation, BR treatment resulted in dephosphorylation of both BES1 and BZR1 proteins

in wild-type *Arabidopsis* seedlings, leading to enhanced protein stability and increased nuclear accumulation (He and others 2002; Yin and others 2002a). In contrast, exogenous BR application had little or no effect on the phosphorylation level or protein stability of either BES1 or BZR1 in *bin2* mutants, presumably containing mutated BIN2 proteins resistant to a BR-initiated inhibitory mechanism. A pharmacological study with a proteasome inhibitor indicated that the phosphorylated BZR1 proteins are degraded by a proteasome-mediated process (He and others 2002). These biochemical data strongly suggest that BZR1 and BES1 are physiological substrates for the BIN2 GSK3 kinase and that the BIN2-catalyzed phosphorylation targets both BZR1 and BES1 for proteasome-mediated protein degradation. Further genetic and biochemical studies are needed to confirm that BIN2 is the *bona fide* kinase that phosphorylates both BES1 and BZR1 in the absence of BR signal.

These genetic and biochemical studies revealed a striking similarity between the plant steroid signaling pathway and the Wnt signaling cascade. In the absence of BR, BIN2 is an active kinase that would phosphorylate BES1 and BZR1, leading to their degradation in the cytosol to block the transduction of BR signal into the nucleus. When the plant steroid hormone binds the BRI1-containing BR receptor, BIN2 becomes inactivated by an unknown regulatory mechanism, and the hypophosphorylated BES1 and BZR1 proteins move into the nucleus to regulate gene expression (Figure 4) (He and others 2002; Yin and others 2002a). Similarly, under resting conditions, GSK3 phosphorylates β-catenin, leading to its ubiquitin-mediated proteolysis (Aberle and others 1997; Hart and others 1999). In response to Wnt binding to a Frizzled receptor, GSK3 is inhibited and β-catenin accumulates in the cytosol and translocates into the nucleus to regulate gene expression by association with other transcriptional factors (Nusse 1997; Hecht and Kemler 2000). However, significant difference exists between the two pathways. First, there is no sequence similarity between BRI1/BAK1 and Frizzled, a seven-transmembrane G protein-coupled receptor (Bhanot and others 1996), or between BES1/BZR1 and β-catenin that contains 7 armadillo (ARM) repeats implicated in protein–protein interactions (Peifer and Wieschaus 1990). Second, the phosphorylation mechanism of BIN2 is different than that of the GSK3 kinase, in spite of their high sequence homology and similar effects on their substrates. In the Wnt signaling, the phosphorylation of β-catenin by GSK3 requires a scaffold protein to bring the kinase and the substrate together in a multiprotein complex (Ikeda and others 1998) and a priming phosphorylation (at the C-terminal S/T site of the S/TxxxS/T motif by a CKII kinase) to increase the affinity of β-catenin for the GSK3 kinase (Liu and others 2002). By contrast, the BIN2-catalyzed BZR1 phosphorylation is independent of either a scaffold protein or a priming phosphorylation but requires a direct kinase/substrate docking interaction through a dedicated BIN2-binding motif at the C-terminal end of the substrate (Zhao and others 2002; Peng and Li, unpublished data). Further studies should determine whether the two GSK3-mediated signaling pathways share additional mechanistic similarities or whether the plant steroid signaling pathway has additional unique features.

Concluding Remarks and Future Directions

The rapid progress over the past three years has greatly broadened and deepened our understanding of BR signaling mechanism. The emerging model of BR signal transduction in plants reveals a striking mechanistic similarity to several well-studied signaling pathways in animals. A simple working model for the plant steroid signaling involves the signal perception/initiation by the heterodimerization of BRI1 and BAK1 at the cell surface, signal relay through yet to be identified component(s) that lead to the inactivation of the BIN2 GSK3 kinase, and subsequent stabilization and nuclear accumulation of BES1 and BZR1 proteins that regulate an array of BR-responsive genes (Figure 4). However, this is a rather oversimplified linear signaling model and more questions remain to be answered.

With respect to signal perception, it is still not clear whether BR binds to the BRI1/BAK1 receptor complex directly or indirectly with the aid of a secreted BR binding/carrier protein that might require a BRS1-mediated proteolysis for maturation. The genetic and biochemical characterization of several putative *Arabidopsis* steroid-binding proteins mentioned before might provide some useful information to answer this question. It was hypothesized that BR would induce or stabilize BRI1/BAK1 heterodimerization, but this process has not yet been directly demonstrated *in planta*. With the help of several newly developed techniques, such as fluorescence resonance energy transfer (FRET) and protein fragment complementation assay (Rossi and others 2000; Subramaniam and others 2001; Shah and others 2002; Wehrman and others 2002), one could monitor the BRI1/BAK1 dimerization upon

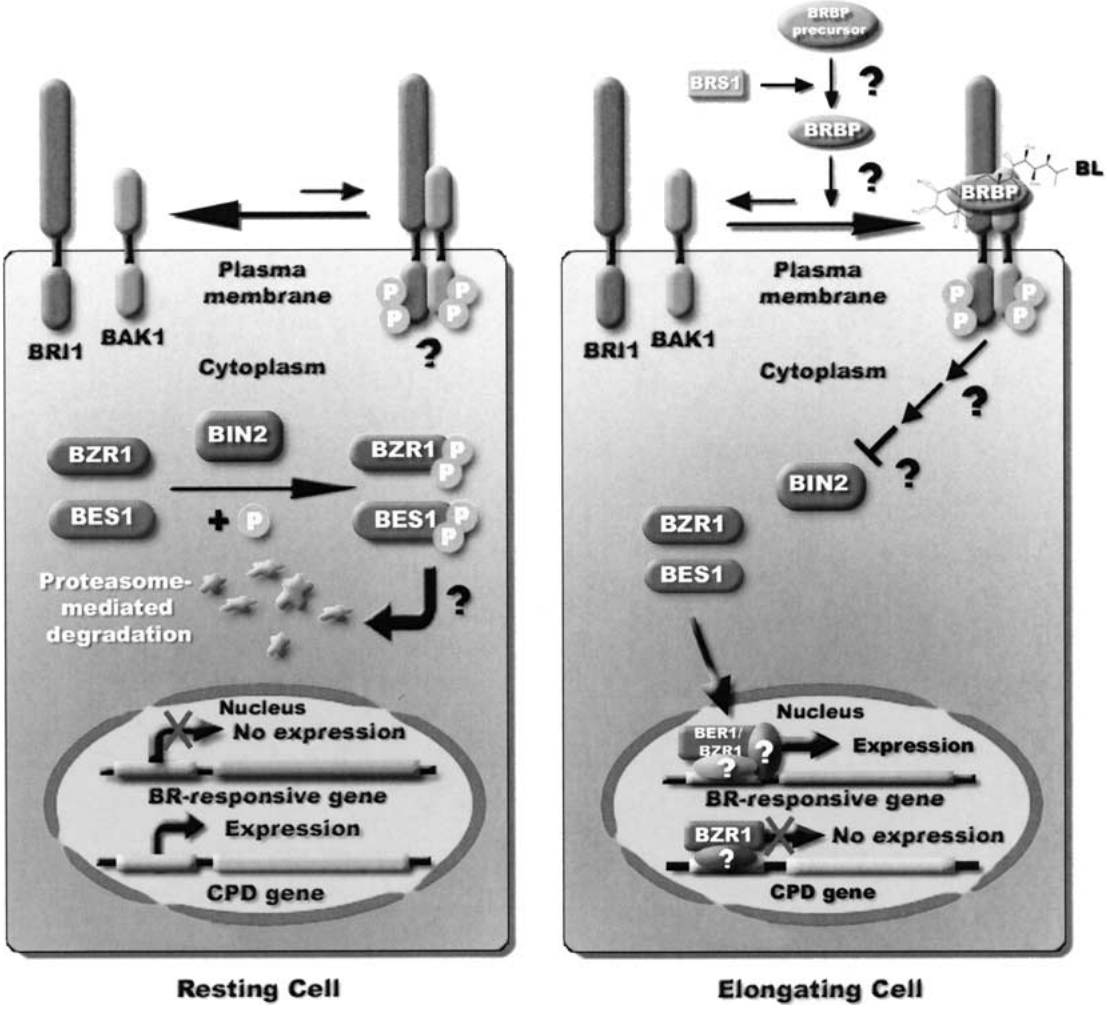

Figure 4. A hypothetical BR signaling pathway in *Arabidopsis*. In the resting cell (left), BRI1 and BAK1 mainly exist as inactive monomers that are in equilibrium with a tiny pool of BRI1/BAK1 heterodimers. The constitutively active BIN2 phosphorylates BES1 and BZR1, earmarking them for proteasome-mediated protein degradation. As a result, no BR signal reaches the nucleus and cell elongation is inhibited. BR binding (right), presumably facilitated by a BR-binding protein (BRBP) that might require BRS1 for proteolytic maturation, shifts the equilibrium to the formation of BRI1/BAK1 dimers, thus activating both receptor kinases and transmitting the BR signal into the plant cell. The BR signal is subsequently transduced by yet to be identified signaling components to inhibit the BIN2 kinase activity (see Figure 3C for two possible regulatory mechanisms), allowing the accumulation of both BES1 and BZR1 in the nucleus to regulate gene expression, possibly through association with other transcriptional factors that contain DNA-binding motifs. Question marks indicate predicted yet uncharacterized components or steps.

BR binding in living plant cells and study the detailed dynamics of the whole dimerization process. Additional studies are also needed to determine whether BAK1 is required for BR binding activity or just functions as a signaling component of a BR receptor.

The BRI1/BAK1 dimerization is alleged to trigger the autophosphorylation of this receptor kinase pair, which is critical for the activation of the receptor complex and the initiation of downstream signaling events. Thus, identification of the BR-dependent autophosphorylation sites on each receptor kinase would help us to understand the mechanism(s) of BR-induced receptor kinase activation. Another important aspect of the receptor kinase is the signaling output, which is essential for transmitting the extracellular BR signal into the cytosol or directly regulating membrane activity to control cell growth. Therefore, identification of putative substrates of BRI1 and BAK1 using the yeast two-hybrid assay and other biochemical approaches, such as purification of a BRI1-containing BR re-

ceptor complex, would greatly enrich our understanding of the signal relay.

Another intriguing question is how BR perception is regulated throughout plant development. Although *BRI1* seems to be expressed ubiquitously in adult plants, it is still possible that its expression or protein activity is regulated by various endogenous developmental signals. Three BRI1 paralogues were found and at least two of them were shown to be functional BR receptors (Yin and others 2002b), but they have more restricted expression profiles compared with BRI1 (Yu and Li, unpublished data). Therefore, the competence to respond to BR might be determined by the differential expression patterns of BRI1 and its homologues during plant development. The hypothesized BRI1/BAK1 heterodimerization in mediating BR perception and the fact that *Arabidopsis* contains 13 BAK1-like proteins add another layer of complexity to the regulation of BR sensitivity. Genetic studies with mutants containing a *bak1* mutation in combination with mutations in various BAK1 homologues or using RNA interference strategy should shed some light on this question.

The signaling mechanism after the activation of the BRI1/BAK1 receptor kinase pair shares similar logic with that of the Wnt signaling pathway in animals: The signal transmitted from the receptor inactivates a GSK3 kinase, which leads to the dephosphorylation and stabilization of nuclear signaling protein(s), allowing them to accumulate in the nucleus to control gene activities (Figure 4). Like its animal counterparts, BIN2 plays a key role in controlling the amount of the plant steroid signal that can reach the nucleus. A better knowledge of the biochemical mechanism of BIN2 inhibition in response to the plant steroid signal is crucial for a clear understanding of BR signaling. The first step towards this goal is to determine at what level BIN2 is regulated, such as protein stability, subcellular localization, or kinase activity. At the same time, effort should be made to investigate the biochemical consequence of the known *bin2* mutations. Although it was reported that the *bin2-1* mutation results in a 30% increase in its kinase activity toward a short peptide substrate, such a moderate increase in the BIN2 activity might not be sufficient to account for the severe dwarf phenotype, which could be phenocopied only by the accumulation of extremely high levels of the wild-type *BIN2* transcripts in transgenic plants (Li and Nam 2002). Thus, understanding how these known mutations affect the BIN2 activity could help to unlock the secret of BIN2 regulation in BR signaling. A clear understanding of BIN2 regulation would also require the identification of the missing components that link the activation of the BR receptor and the inhibition of the BIN2 kinase, which could be identified by genetic suppressor/enhancer screen, the yeast two-hybrid method, or purification of a BIN2-containing multiprotein complex.

BES1 and BZR1 represent another similar feature between BR and Wnt signaling pathways. Like β-catenin, both proteins are regulated by similar GSK3-initiated protein degradation processes and are important for controlling nuclear activities (Figure 4). Further investigation of both BES1 and BZR1 might show whether they share additional similar features with regard to the biochemical mechanisms of their degradation in the cytosol and their actions in the nucleus. In the absence of the Wnt signal, the phosphorylated β-catenin is recognized by β-TrCP, an F-box subunit of a ubiquitin ligase complex (SCF) that ubiquitinates and targets β-catenin for proteasome-mediated proteolysis (Hart and others 1999). It has been shown that the phosphorylated BZR1 is degraded by a proteasome-mediated process (He and others 2002), but it remains to be determined whether a similar F-box protein is required for the ubiquitination of both BES1 and BZR1 in BR signaling. In the presence of the Wnt signal, the hypophosphorylated β-catenin translocates into the nucleus where it interacts with a DNA-binding protein of the TCF family of transcriptional factors to control an array of Wnt target genes (van de Wetering and others 1997; Cavallo and others 1998). It is quite possible that BES1 and BZR1 might also require DNA-binding proteins to exert their effects on gene regulation because neither protein contains a recognizable DNA-binding motif.

In comparison to the rapid progress in understanding how the plant steroids are transduced to affect gene activity (the genomic effects of BR), the nongenomic aspects of BR have largely been ignored. Although it was known that gene expression is important for many BR-elicited cellular processes, previous studies also indicated that plant steroids could also exert their effects on cell physiology independent of gene regulation. Both the membrane ATPase and vacuolar ATPase were implicated in BR-mediated cell growth (Katsumi 1991; Schumacher and others 1999), most likely by facilitating water uptake to generate turgor pressure to drive cell expansion. BR was also known to affect the reorganization of cortical microtubules independent of tubulin gene expression (Mayumi and Shibaoka 1995; Catterou and others 2001), which is essential for determining the direction of cell growth and plant morphogenesis (Williamson 1991; Cyr and

Palevitz 1995). Further studies are needed to reveal how an activated BR receptor can control the activities of the two different types of ATPases and the nucleation/organization of the microtubules.

The discovery of additional BR signaling components and subsequent detailed genetic and biochemical studies will definitely add more complexity and additional signaling branches to the current simple and linear BR signaling pathway. As an essential plant hormone that plays important roles throughout the plant life cycle, it is also expected that some BR signaling components might also be subject to regulations by other plant hormones and certain endogenous developmental programs. A better understanding of the BR signal transduction pathway will not only contribute significantly to our general knowledge of steroid hormone action but will also provide important insights into the cellular processes governing plant growth and development.

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