CHAPTER 3

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RECENT PROGRESS IN BRASSINOSTEROID RESEARCH: HORMONE PERCEPTION AND SIGNAL TRANSDUCTION

Brassinosteroids (BRs) are cholestane derivatives that show structural similarity to insect, animal and human steroid hormones. Like steroids, brassinosteroids are signaling molecules in plants, which play important roles in normal growth, development and differentiation. Continued genetic screening and analysis of *Arabidopsis* mutants has provided new insight into brassinosteroid signaling, and today study of brassinosteroid action is the priority of many research laboratories around the world. Molecular genetic studies have led to the cloning and characterization of a BR receptor complex, BRI1/BAK1, a transmembrane receptor serine/threonine kinase pair. Brassinosteroid binding via a BR-binding protein stabilizes heterodimmer formation, activates intrinsic kinase activities and initiates a BR signaling cascade. This results in regulation of the activity of downstream elements of the signal transduction pathways. Although downstream components of BR action are largely unknown, research effort in last two years has led to the identification of a negative cytoplasmic regulator BIN2, a GSK3-like kinase. In the absence of BRs, BIN2 phosphorylates two positive regulators, BES1 and BZR1, resulting in their degradation and preventing their accumulation in the nucleus. In turn, initiation of a BR signaling cascade inhibits BIN2 kinase activity by an unknown mechanism(s). BIN2 is not able to phosphorylate BES1 and BZR1 proteins in the presence of BRs and unphosphorylated BES1 and BZR1 accumulate in the nucleus triggering expression of a variety of BR-induced genes involved in regulation of plant growth.

INTRODUCTION

Brassinosteroids (BRs) are plant hormones that show structural similarity to insect, animal and human steroid hormones (Evans, 1988). Unlike many steroids identified in plants, only BRs are widely distributed over the plant kingdom (Mandava, 1988). The first brassinosteroid, called brassinolide, was identified in 1979 as the effective compound in a hydrophobic extract called brassin from pollen of *Brassica napus* (Mitchell *et al.*, 1970; Grove *et al.*, 1979). Brassinolide (BL) is the most active among BRs, which elicit various physiological responses such as stimulation of cell elongation and are essential for male fertility and xylem differentiation (Müssig and Altmann, 1999; Altmann, 1999). Relative to study of BR biosynthesis, investigation of BR signaling is a pressing research priority in many laboratories in recent years. Even very recent and excellent plant physiology or biochemistry books and textbooks are still lacking chapters dealing intensively with brassinosteroid signaling.

Using genetic techniques many BR-related mutants were selected and

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characterized. BR-insensitive mutants often show phenotypic traits similar to mutants affected in BR biosynthesis, such as extreme dwarfism, altered leaf morphology, reduced fertility or male sterility, delayed senescence, and altered vascular development (Clouse and Feldmann, 1999). However, unlike BR-deficient mutants, the phenotype of BR-insensitive mutants cannot be rescued by exogenous BR application, suggesting that the mutated genes play a role in BR signaling. BR-insensitive mutants have been isolated in rice (*Os-bri1*), pea (*lka*) and tomato (*curl-3*) (Nomura *et al.*, 1999; Yamamuro *et al.*, 2000; Koka *et al.*, 2000). In *Arabidopsis*, numerous alleles of the BR-insensitive mutant *bri1* were identified using a screen based on the inhibition of root growth by BR or on phenotypic similarities to BR-deficient mutants (Clouse *et al.*, 1996; Kauschmann *et al.*, 1996; Li and Chory, 1997; Noguchi *et al.*, 1999). *BRI1* was cloned and has many of the properties expected of a BR receptor (Li and Chory, 1997). Although *BRI1* homologues with high sequence similarity exist in the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000), BRI1 appears to be the major *Arabidopsis* brassinosteroid receptor. Following some recent excellent reviews dealing with brassinosteroid perception and signal transduction (Friedrichsen and Chory, 2001; Müssig and Altmann, 2001; Bishop and Koncz, 2002), here I summarize advances that have been made in study of BR perception and in identifying novel downstream components in BR signal transduction.

CELL SURFACE RECEPTOR AND BRASSINOSTEROID PERCEPTION

Structural similarities of BRs to insect and animal steroids led to predictions of similar signal transduction pathways, nongenomic and genomic. In animal systems, the term nongenomic steroid action is used for rapid steroid effects, i.e. taking seconds or minutes. The effects are not blocked by inhibitors of transcription or translation (actinomycin D, cyclohexamine) and are mediated by steroids transported into the cell via carrier proteins or in addition using membrane-binding sites (Wehling, 1997; Watson and Gametchu, 1999; Fleet, 1999; Schmidt *et al.*, 2000). Steroid signals for genomic action are generally mediated by receptors inside the cell, and genomic steroid effects are characterized by delayed responses (i.e. taking minutes or hours) because of time required for transcription and protein synthesis. Therefore, they can be affected by inhibitors of transcription or translation (Beato and Klug, 2000; Mangelsdorf *et al.*, 1995). In plants, direct experimental approaches and analysis of the completed *Arabidopsis* genome sequence have failed to provide any evidence for the intracellular receptor family (The *Arabidopsis* Genome Initiative, 2000), and brassinosteroids are believed to be perceived by plasmamembrane receptors, triggering genomic and nongenomic actions (He *et al.*, 2000; Becraft, 2001).

Positional cloning revealed that *BRI1* encodes a Leu-rich repeat (LRR) transmembrane receptor-like kinase (RLK) (Li and Chory, 1997; Friedrichsen *et al.*, 2000). BRI11 is a typical plasmamembrane associated LRR-RLK, whose localization at the plasmamembrane was demonstrated by expression of the fusion protein BRI1-GFP (green fluorescent protein) (Friedrichsen *et al.*, 2000). BRI1 consists of several typical domains (Fig. 1). It carries a N-terminal signal peptide followed by a putative Leu-zipper motif and cysteine pair. Then, the extracellular LRR domain of 25 imperfect Leu-rich

repeats follows. Interestingly, in the LRR domain of BRI1 a 70-amino acid island was found between repeats 21 and 22. Several *bri1* mutations resulting in single amino

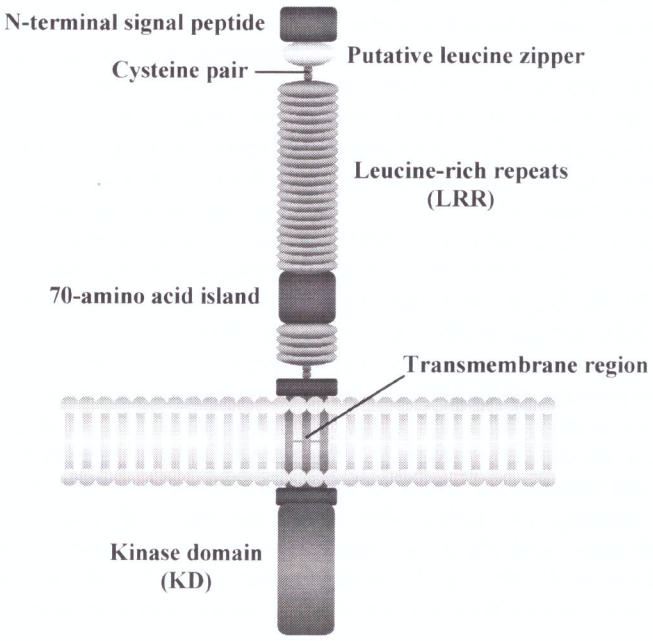

Figure 1. *Schematic structure of Arabidopsis BRI1. Source of primary sequence information: Li and Chory, 1997 (adapted from Bishop and Koncz, 2002)*

acid substitutions were found in the island and highlight its importance for the function of BRI1 (Noguchi *et al.*, 1999; Friedrichsen *et al.*, 2000; Friedrichsen and Chory, 2001). A predicted transmembrane region links extracellular domains with the intracellular Ser/Thr-kinase domain (KD) (Fig. 1). Several *bri1* missense mutations were also mapped to the KD (Friedrichsen and Chory, 2001; Bishop and Koncz, 2002).

A unique feature of BRI1's LRR-RLK is that it contains both the 70-amino acid island and a cytosolic KD. The 70-amino acid island is characteristic of a specific family of LRR receptor like proteins (LRR-R). It is observed in the Cf-9 (Jones *et al.*, 1994), CLAVATA2 (Jeong *et al.*, 1999), and TOLL LRR receptors (Hashimoto *et al.*, 1998). These LRR-Rs, however, lack a cytosolic KD (Bishop and Koncz, 2002). BRI1 also shows significant sequence identity with LRR-RLK ERECTA (Torii *et al.*, 1996) involved in regulation of organ size, to CLAVATA1 (Clark *et al.*, 1997) controlling meristem proliferation, or to Xa21 involved in pathogen resistance (Song *et al.*, 1995). These LRR-RLKs have cytosolic KDs, but in contrast to BRI1, they lack the 70-amino acid island

(Bishop and Koncz, 2002).

Several experimental results strongly support a critical role of *BRI1* in BR signaling. In rice, the product of the *Xa21* gene, conferring resistance to the bacterial plant pathogen *Xanthomonas oryzae* pv. *oryzae* race 6 *(Xoo)*, shows a structure similar to BRI1, contains 23 LRR, a transmembrane domain and Ser/Thr kinase domain (Song *et al.*, 1995). He *et al.* (2000) showed that after inoculation with an incompatible *Xanthomonas Xoo* strain, Xa21 could trigger a hypersensitive response in a rice cell-culture system. The authors then constructed a chimeric protein containing both the extracellular and transmembrane domain plus a short stretch of the intracellular domain of BRI1 fused to the Xa21 kinase domain. They showed that the chimeric protein was also able to elicit the hypersensitive response in rice cells after treatment with BL in the physiologically relevant concentrations of 10 nM to 2 mM. By contrast, the authors also showed that if the 70-amino acid island domain in BRI1 or Xa21 kinase domain was mutated, the hypersensitive response was blocked after treatment with BL (He *et al.*, 2000). By analysis of BL-binding to BRI1, further pieces of evidence were provided indicating that the extracellular domain of BRI1 perceives BR (Wang *et al.*, 2001). Binding of radio labeled BL to microsomal fractions was compared in wild type (WT) and transgenic plants overexpressing BRI1-GFP. The binding activity was dramatically increased in the membrane fractions of BRI1-GFP plants, and the number of BL-binding sites correlates with the amount of BRI1 protein. Unlike the nonspecific competitor ecdysone, the immediate and bioactive BL precursor, castasterone (Yokota, 1997; Bishop *et al.*, 1999), reduced BL binding. The authors also showed that BL-binding activity was abolished when the 70-amino acid island was mutated. In contrast, mutations on the KD of BRI1 have no effects on BL binding. These observations strongly indicate that the 70-amino acid island in the extracellular domain of BRI1 perceives brassinosteroids (Wang *et al.*, 2001).

Brassinolide is a steroid hormone, which classically bind to soluble ligand-activated transcription factors. As showed, BRI1 is an LRR-RLK. LRRs are known to recognize protein ligands. Extracellular LRR of G-protein-coupled receptors in animals are involved in the recognition of peptide hormones, such as gonodotropin, nerve growth factors, and thyroid-stimulating hormones (Kobe and Deisenhofter, 1994). Despite the large numbers of LRR-RLKs in plants, only a few of them have known biological function. For example, LRR-RLKs play a role in hormone perception, meristem signaling, or pathogen responses (Shiu and Bleecker, 2001). Even fewer LRR-RLKs have known ligands. Various peptide ligands were defined for receptors CLV1, FLS2, or PSK (Fletcher *et al.*, 1999; Gomez-Gomez *et al.*, 2001; Matsubayashi *et al.*, 2002). Very recently, a systemin receptor SR160 was identified, closely related to BRI1 (Sheer and Ryan, Jr., 2002). However, LRRs have not been shown to interact with organic compounds such as steroids, and BRI1 was the first LRR-RLK described to be involved in steroid signaling in plants. Therefore, BRI1 structure raised a new question about steroid signaling mechanisms and established a new paradigm for hormonal responses in plants. Although, Li and Chory (1997) admitted that BRs may fit in the cavity formed by the island of 70-amino acid, they proposed that BRI1 may only recognize protein ligands, such as putative steroid carriers, and thus additional factors may mediate BL binding.

In order to learn more about mechanism of BR perception and signaling, Li *et al.* (2001a) performed a gain-of-function suppressor screen with a weak *bri1* allele (*bri1-5*). The authors identified a single dominant *bri1 suppressor dominant* (*brs1-1D*) mutation that resulted in overexpression of a type II serine carboxypeptidase-like protein. However, the *brs1-1D* mutation suppresses only *bri1* alleles with a mutation in the extracellular LRR domain but cannot suppress the *bri1* phenotype due to mutation in cytoplasmic KD (Li *et al.*, 2001a). Also, the *brs1* did not suppress *clavata1* and *erecta* mutant phenotypes, which indicates specificity of BRS1 to BL signaling. Li *et al.* (2001a) suggested that BRS1 acts on a protein that is required for BL perception. The substrates of BRS1 might be steroid-binding proteins represented by genes, which have been identified in the *Arabidopsis* genome sequence (The *Arabidopsis* Genome Initiative, 2000). Suggested from the observation that the BRI1 product may potentially be processed (Wang *et al.*, 2001), BRS1 may alternatively use BRI1 to generate an activated BL receptor (Fig. 2). However, since BRS1 does not process functional BRI1-Xa21 chimera (He *et al.*, 2000) further investigation is necessary to define role of BRS1 in BR signaling (Li *et al.*, 2001a).

When a protein extract from transgenic BRI1-GFP seedlings treated by BL was immunoblotted with antibodies against the N-terminal region of BRI1 or GFP, the mobility of the BRI1 KD protein band in SDS-PAGE was reduced (Wang *et al.*, 2001). The authors suggest that this shift is likely a result of autophosphorylation of BRI1 in the presence of BL. This idea was supported by the observation that the intracellular cytoplasmic KD of BRI1 autophosphorylates on serine/threonine residues (Oh *et al.*, 2000). Li and Chory (1997) proposed that binding of BRs, either directly or via a steroid binding protein to the BRI1 receptor may dimerize BRI1 with other LRR receptors. Dimerization could mediate cell-to-cell interactions through LRRs, leading to activation kinase activity and triggering a phosphorylation cascade inside the plant cell. This was a logical proposal since receptor dimerization is a general mechanism for ligand-induced activation of receptor kinases in animals (Schlessinger, 2000). Although BRI1 is a component of a multiprotein BR receptor complex (Wang *et al.*, 2000), indirect evidence against BRI1 homodimerization as a mechanism for receptor activation has been provided (Clifford and Schupbach, 1994; Simin *et al.*, 1998; Friedrichsen and Chory, 2001; Clark, 2001; Nam and Li, 2002). Instead, it is hypothesized that BR binding results in formation of heterodimer (Fig. 2)(Schumacher and Chory, 2000; Friedrichsen and Chory, 2001).

Very recently, it has been confirmed that BRI1 receptor functions as a heterodimer. Results from two laboratories independently provided evidence that BRI1 forms heterodimer with distinct receptor kinase (Nam and Li, 2002; Li *et al.*, 2002). Using activation tagging, Li *et al.* (2002) identified a dominant genetic suppressor of *bri1*, *bak1-1D* (*bri1-associated receptor kinase1-1Dominant*), which encodes an LRR-RLK, distinct from BRI1. The BAK1 protein was independently identified in a yeast two-hybrid screen where the BRI1 kinase domain was used as bait (Nam and Li, 2002). Both groups showed that overexpression of BAK1 suppresses a weak *bri1* phenotype and results in elongated organ phenotypes, while a null *bak1* mutant is semidwarf and reduced in sensitivity to BRs. Li *et al.* (2002) showed that BAK1 and BRI1 have the same expression pattern, both are plasmamembrane localized, they can phosphorylate one another, and the autophosphorylation of BAK1 is stimulated by BRI1. Both groups further showed that

BAK1 and BRI1 proteins directly interact with one another *in vitro* as well as *in vivo*. On the basis of their genetic analyses, Li *et al.* (2002) and Nam and Li (2002) independently suggest that BAK1 is specifically involved in the BRI1-mediated BR signaling pathway. Li *et al.* (2002) hypothesize that when a ligand binds to BRI1, BRI1 activates BAK1, and the activated BAK1 then phosphorylates other downstream components. Nam and Li (2002) propose that BRI1 and BAK1 exist as inactive monomers (Fig. 2) that are in equilibrium with active dimers. BR binding via a BR-binding protein (BRBP) stabilizes or promotes active heterodimer formation, leading, via transphosphorylation, to activation of both receptor kinases and to initiation of a BR signaling cascade (Fig. 2).

Dephosphorylation by phosphatases plays an important role in the downregulation of receptor kinases in animals (Ostman and Bohmer, 2001). It has been observed that the 2C phosphatase, KAPP (kinase-associated protein phosphatase), can interact with a number of LRR-RLK, and may thus oppose the action of BRI1 KD (Fig. 2) (Schumacher and Chory, 2000). Overexpression of KAPP may thus lead to a *bri1*-like phenotype. So far, this type of experiment has been performed on a *clavata* mutant. The KAPP protein binds the phosphorylated form of LRR-RLK CLV1, and the KAPP overexpression results in a floral phenotype similar to that caused by weak *clv1* alleles (Williams *et al.*, 1997; Trotochaud *et al.*, 1999). Conversely, reduction of the KAPP transcript in the *clv1* mutant suppressed the mutant phenotype (Stone *et al.*, 1998). It suggests that KAPP could function as a negative regulator in the CLV1 signal-transduction pathway. KAPP was cloned and further interactions with RLKs were shown, indicating that KAPP may function in RLK-initiated signaling pathways (Stone *et al.*, 1994; Braun *et al.*, 1997), including BR signaling.

BR SIGNAL TRANSDUCTION

Until recently, nothing was known about steps which mediate transduction of the BR signal further into the cell. The inability to identify BR-signaling components encoded by genes different from *BRI1* was thought to be due to redundancy in downstream components or due to lethality of mutants with loss-of-function mutation(s) (Clouse, 2002). Screening of 150,000 EMS-mutagenized seeds led to isolation of only two alleles of a new mutant *bin2* (*brassinosteroid-insensitive 2*) (Li *et al.*, 2001b). The *bin2* is dwarf and semidominant. Homozygous *bin2* seedlings are extremely dwarf looking like *bri1* and lack transcriptional downregulation of the *CPD* gene (involved in BR biosynthesis) in the presence of BL. In the heterozygous configuration *bin2* is semi dwarf and shows approx. 50% reduction in CPD transcription level in comparison with the WT (Li *et al.*, 2001b). BIN2 has been cloned by a map-based approach, and was found to encode a cytoplasmic serine/threonine kinase (Li and Nam, 2002). Interestingly, BIN2 is an *Arabidopsis* ortholog of the *Drosophila* SHAGGY protein kinase and human glycogen synthase kinase-3 (GSK-3) (Li and Nam, 2002). SHAGGY/GSK-3-like kinases are widely distributed over eukaryotes and function often like negative regulators of signal transduction pathways (Kim and Kimmel, 2000). Similar to *bin2*, *Arabidopsis* mutant *ultracurvata1* (*Ucu1*), displaying aberrant leaf morphology, was mutated in the same kinase gene (Perez-Perez *et al.*, 2002). In both *bin2* and *Ucu1*, missense mutations

resulted in a semidominant phenotype, leading to the suggestion that, as for mammalian SHAGGY/GSK-3 kinases, BIN2 may function as a negative regulator of BR signaling (Li *et al.*, 2001b). Moreover, activity of the mutant *bin2* kinase is increased by approximately 33% in comparison to WT, and seedlings overexpressing BIN2 shows more pronounced dwarfism (Li and Nam, 2002). This suggests that the *bin2* mutation is hypermorphic. Also, overexpression of BIN2 in a weak *bri1* mutant background generated a more pronounced dwarf phenotype, whereas co-suppression of BIN2 transcription resulted in partial suppression of the weak *bri1* phenotype (Li and Nam, 2002). These data indicate that BIN2 is a negative regulator of BR signaling, and the authors speculate that BRI1 interacts with BIN2, phosphorylates and thus inactivates it (Li and Nam, 2002). Oh *et al.* (2000) showed that recombinant BRI1 KD could phosphorylate *in vitro* a conserved peptide motif and the authors deduced putative consensus sequence for peptide-substrate recognition by BRI1 KD. However, BIN2 lacks close homology to the consensus sequence, which would be likely phosporylated by BRI1. Therefore, BRI1 does not, most likely, interact directly with the BIN2 kinase. Instead, Li and Nam (2002) propose that, after BL binding to the BRI1, a signaling cascade is initiated and BRI1-containing complex interacts with and inactivates BIN2 (Fig. 2). Then, BIN2 is not able to phosphorylate and inactivate other pathway components such as positive regulators. In turn, in the absence of BRs, BIN2 is constitutively active and therefore phosphorylates and inactivates positive BR signaling proteins (Fig. 2).

DOWNSTREAM EVENTS OF BR SIGNALING: IDENTIFICATION OF THE POSITIVE REGULATORS

To elucidate new components of downstream BR signaling Yin *et al.* (2002a) screened EMS-mutagenized *bri1* seeds for mutant plants with suppressed *bri1* phenotypes. The authors isolated a semidominant mutant *bes1-D* (*bri-EMS-suppressor1-D*) which, along with the suppression of the *bri1* dwarf phenotype, also exhibited constitutive BR response phenotypes including long and bending petioles, curly leaves, and constitutive expression of BR-responsive genes encoding cell wall-modifying enzymes. The *BES1* gene [also called *BZR2* (Yin *et al.*, 2002a)] was cloned and found to encode phosphoproteins with nuclear localization signal and multiple consensus sites (S/TXXXS/T) for phosphorylation by GSK-3 type kinases (Yin *et al.*, 2002a). The *bes1-D* mutant phenotype and its gain-of-function nature suggests that BES1 acts downstream of BRI1 as a positive component in the BR signaling pathway. In WT, the BES1 protein was found in low levels in cytoplasm and nucleus, and its nuclear localization was rapidly and specifically enhanced by BR treatment. In contrast, the *bes1-D* mutant protein was found in the nucleus at high levels and BL had apparently no effect on nuclear bes1-D localization (Yin *et al.*, 2002a). The authors further showed that` BL treatment resulted in nuclear accumulation of BES1 in unphosphorylated form suggesting that the protein is regulated by a negative acting kinase. They provided evidence that BES1 interacts with BIN2, so that in the absence of BRs BIN2 phosporylates BES1 in vivo, resulting in low level of BES1 in the nucleus (Yin et al., 2002a).

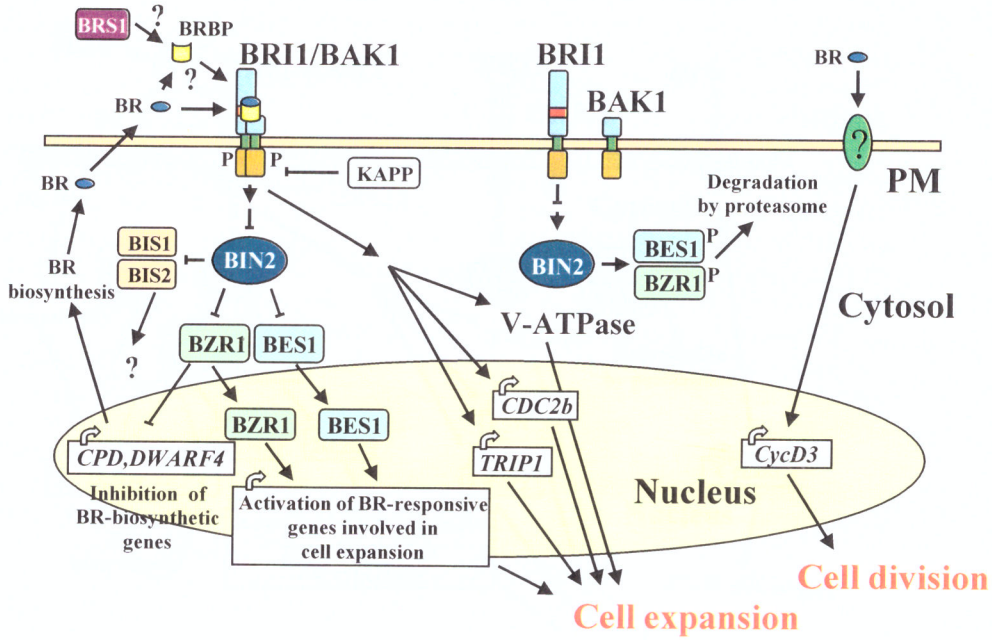

Figure 2 Model for BR signaling. BRI1 is the major BR receptor and is located on the plasma membrane. BRI1 and BAK1 (BRI1-associated receptor kinase) exist mainly as inactive monomers. BR binding through a putative BR-binding protein (BRBP) or directly to the amino acid island stabilizes or promotes active heterodimer formation, leading to activation of both receptor kinases via transphosphorylation. BRS1 may process BRBP and modulate the BRI1signaling pathway. KAPP (kinase-associated phosphatase) is a potential suppressor of receptor complex. Receptor activation leads to inhibition of the negative regulator BIN2 kinase. It allows stabilization and accumulation of unphosphorylated positive regulators BES1 and BZR1 in the nucleus (suppression of the BIN2 may also stabilize new positive regulators BIS1 and BIS2 recently identified with yet unknown functions). BES1 and BZR1 then activate BR-inducible genes encoding cell wall-modifying enzymes and other genes whose products are involved in regulation of cell expansion. In light, BZR1 also activates a negative feedback pathway that inhibits the BR-biosynthetic genes such as CPD and DWARF4, which leads to reduced cell elongation. BR-mediated activation of the BRI1/BAK1 complex leads to activation of other BR-induced genes, such as TRIP1 and CDC2b. These BR-induced genes work together with the V-ATPase pathway to promote cell growth. In the absence of BR, BRI1 is inactive. Negative regulator BIN2 is able to phosphorylate BES1 and BZR1 and target them for degradation by proteasome. Cyclin gene CycD3 is potentially activated through a separate BR pathway using unidentified BR receptor and affects the cell cycle to promote cell division. This model is adapted from Friedrichsen and Chory, 2001; Müssig and Altman, 2001; Bishop and Koncz, 2002; Clouse, 2002; He et al., 2002; Nam and Li, 2002, and Yin et al., 2002a.

Screening for mutants resistant to an inhibitor of BR biosynthesis, brassinazole, the research group of J. Chory isolated bzr1-D (brassinazole resistant1-D), a dominant mutation, and identified a second positive regulator of the BR signaling pathway (Wang et al., 2002). Like bes1-D, bzr1-D suppresses BR-deficient and BR-insensitive (bri1) phenotypes, and both *bzr1-D* and *bes1-D* dark-grown seedlings show resistance to brassinazole. The authors therefore suggested that *BZR1* might encode BES1-like protein. The BZR1 has been cloned and it shows 88% sequence identity to BES1, including the nucleus localization signal and multiple sites for phosphorylation by GSK3 kinases (Wang *et al.*, 2002; Yin *et al.*, 2002a). The authors found that in response to BRs, BZR1 accumulates in the nucleus of elongating cells in dark-grown hypocotyls and is stabilized by BR signaling and the *bzr1-D* mutation (Wang *et al.*, 2002; Yin *et al.*, 2002a). Consistent with this, in experiments using a proteasome inhibitor, MG132, He *et al.* (2002) revealed that in the presence of BRs BIN2 kinase activity is inhibited. As for BES1, BZR1 is not phosphorylated and accumulates in nucleus. In turn, in the absence of BRs, BZR1 is phosphorylated and destabilized (degraded) by the negative regulator BIN2 by the proteasome machinery.

Based on their results, Yin *et al.* (2002a) and He *et al.* (2002) proposed a model for BR signal transduction (Fig. 2). In the absence of BRs, BIN2 phosphorylates two positive regulators, BES1 and BZR1, resulting in their degradation and preventing their accumulation in the nucleus (Fig. 2). In the presence of BRs, the hormone binding to BRI1 via a BR-binding protein stabilizes or promotes active heterodimer formation, leading to transphosphorylation and activation of both receptor kinases (Nam and Li, 2002; Li *et al.*, 2002) and other signaling events (Fig. 2). Initiation of a BR signaling cascade inhibits BIN2 kinase activity by an unknown mechanism(s). BIN2 is not able to phosphorylate BES1 and BZR1 proteins and unphosphorylated BES1 and BZR1 accumulate in the nucleus. Nuclear localization of BES1 and BZR1 then activates expression of BR-induced genes such as those encoding cell-wall modifying enzymes required for cell elongation (Fig. 2).

In contrast to *bes1-D*, light-grown *bzr1-D* seedlings exhibit semi-dwarf phenotypes and reduced BR content (Yin *et al.*, 2002a). Therefore, even though BES1 and BZR1 have overlapping functions, they are not completely redundant since *bzr1-D* seems in addition to activate a BR feedback inhibition pathway (Wang *et al.*, 2002;Yin *et al.*, 2002a). The difference in phenotypes between *bes1-D* and *bzr1-D* mutants in light suggests that these two genes or gene products are differently regulated by light. BES1 and BZR1 therefore provide potential targets for crosstalk between the light and BR signaling pathways (Yin *et al.*, 2002a). There is evidence for regulation of BR levels by the phototransduction pathways via *BAS1* gene (Neff *et al.*, 1999), or for the crosstalk between light and BRs through a dark-induced small G-protein (Kang *et al.*, 2001). However, the function of *BES1* and *BZR1* genes in the control of photomorphogenesis and light-regulated gene expression is unknown.

Recently, two novel nuclear proteins BIS1 (BIN2 SUBSTRATE1) and its closest homologue BIS2 (BIN2 SUBSTRATE2), were identified and found to be phosphorylated by BIN2 *in vitro* by a novel phosphorylation mechanism (Fig. 2) (Peng *et al.*, 2002). The authors propose that BIS1/BIS2 are two nuclear components of BR signaling that are, in

addition to BES1 and BZR1, negatively regulated by BIN2. Functions of BIS1/BIS2 in BR-mediated responses have yet to be elucidated.

In addition to the BRI1 pathway, which results in targeting of genes involved in cell elongation and expansion, isolation and analysis of the *det3* mutant revealed other cellular components involved in BR-induced cell elongation. Interestingly, this component regulates cell elongation independently of gene expression, since mutations in *DET3* affect cell expansion in the absence of alterations of gene induction (Cabrera y Poch *et al.*, 1993). The *DET3* gene was found to encode a subunit of the vacuolar type H^+-ATPase (V-ATPase) (Schumacher *et al.*, 1999). It was reported earlier that BR-induced hypocotyl elongation in cucumber is dependent on membrane-bound ATPase activity, whereas hypocotyl elongation induced by gibberellin does not involve the H^+-ATPase (Mandava, 1988). Schumacher *et al.* (1999) propose that BR action triggers V-ATPase activity to initiate the uptake of water into the vacuole, and promote BR-induced cell elongation (Fig. 2). This is coordinated with changes in cell wall properties and changes in gene expression necessary to sustain growth responses.

BR-INDUCED GENES

Major brassinosteroid effects such as BR-induced growth are mediated through the genomic pathway. A limited number of BR-regulated genes have been identified hitherto. Most of them encode BR biosynthesis enzymes or affect cell expansion and proliferation.

A possible biological significance of the nuclear localization of BES1 is suggested by the fact that several genes encoding cell wall-modifying enzymes and involved in cell expansion are overexpressed in *bes1-D* mutant. Using a commercially available *Arabidopsis* Affymetrix Gene Chip, Yin *et al.* (2002a) revealed that 30 genes were induced by BL in WT and *bes1-D* seedlings, but not significantly changed in the *bri1* mutant. A time course of their BR-regulated expression correlated with BES1 nuclear accumulation. Among these 30 genes, 19 of them showed 2-fold basal expression in *bes1-D* in comparison to WT, but not greater induction by exogenous BL relative to WT. In a second group, 11 genes showed basal expression similar in *bes1-D* and WT, but they were hyper-responsive to BL treatment in *bes1-D* (Yin *et al.*, 2002a). Of the 30 BR-induced genes 7 encode putative cell wall-associated proteins, such as xyloglucan endotransglycosylases (XETs), endo-1,4-β-glucanases (EGases), polygalacturonase, pectin methylesterase, or expansin. All of these proteins are implicated in cell elongation or expansion (Nicol *et al.*, 1998; Darley *et al.*, 2001; Friedrichsen and Chory, 2001; Lamport, 2001). The increased expression of BL-induced genes in *bes1-D* or their hyperinduction by BL in the mutant provide molecular evidence for the constitutive BL-response phenotypes of the *bes1-D* mutant and suggests that the *bes1-D* phenotypes result from changes in gene expression.

The relatively small changes (2- to 4-fold) in expression of BR-induced genes possibly can be explained by tightly controlled BR biosynthesis by a feedback inhibition mechanism (Yin *et al.*, 2002a). It was found that expression of the important BL biosynthetic gene *CPD* (Szekeres *et al.*, 1996) is inhibited by exogenous BRs, and such feedback inhibition requires functional BRI1 (Fig. 2) (Mathur *et al.*, 1998). BL-mediated

downregulation of *CPD* was inhibited by the protein biosynthesis inhibitor cyclohexamine, indicating that *de novo* protein synthesis needs genomic effect of BR signaling (Mathur *et al.*, 1998). Similarly, another BL biosynthetic gene, *DWF4*, is negatively controlled by functional BR signaling pathway (Fig. 2) (Noguchi *et al.*, 2000). This possibility was also supported by an observation of Choe *et al.* (2001) who found that *DWF4* and *CPD* show derepressed expression in the *bri1-5* mutant. This is consistent with the accumulation of BR intermediates in *bri1* mutants (Noguchi *et al.*, 1999; Nomura *et al.*, 1999; Yamamuro *et al.*, 2000).

Interestingly, the onset of *CPD* gene expression in the cotyledons of etiolated *Arabidopsis* seedlings (Mathur *et al.*, 1998) correlated temporally with low expression of *BZR1* in cotyledons in seedlings of the same stage (Wang *et al.*, 2002) and, in contrast, with high accumulation of BZR1-GFP in the most actively expanding zone of the etiolated hypocotyls (Wang *et al.*, 2002). It seems that low BZR1 expression allows the high expression of CPD and BR biosynthesis in cotyledons. This mutually exclusive expression pattern of CPD and BZR1 is consistent with the role of BZR1 in BR-regulated cell elongation and feedback regulation of BR biosynthesis.

As mentioned above, BES1 and BZR1 appear to have overlapping functions, but they are not completely redundant since *bes1-D* and *bzr1-D* mutants differ in their-light grown phenotypes. Unlike *bes1-D* seedlings, which have long petioles, *bzr1-D* light-grown plants are semi-dwarf (Yin *et al.*, 2002a). Such differences in light-grown phenotypes are consistent with their different effects on feedback regulation of BR biosynthetic genes, since in contrast to *bes1-D*, the *bzr1-D* mutant has reduced BR levels and *CPD* gene expression (Yin *et al.*, 2002a). These results indicate that BES1 mediates downstream growth responses, but in contrast to BZR1, does not cause feedback regulation of BR biosynthesis (Fig. 2). The physiological significance of two such overlapping pathways seems to lie in the ability of the plant to fine-tune the levels of BR biosynthesis and sensitivity in different tissues and cells (Wang *et al.*, 2002).

Among other BR-induced genes, the expression of CDC2b cyclin-dependent kinase is upregulated by BR in dark (Fig. 2) (Yoshizumi *et al.*, 1999). CDC2b binds to its cyclin partner and promotes progression through the G2-M transition (D'Agostino and Kieber, 1999) suggesting its role in cell division. However, since CDC2b does not contain the conserved PSTAIRE motif, it is not believed to be involved in the cell cycle. Instead, CDC2b is rather important for cell elongation since *cdc2b* seedlings with antisense RNA are de-etiolated in the dark, reduced in cell elongation, but have WT cell number (Yoshizumi *et al.*, 1999).

None of the BR-response genes identified so far participate directly in signaling processes. Jiang and Clouse (2001) have identified a gene encoding the *Arabidopsis* homologue of TRIP1 (TGFβ receptor-interacting protein), which is rapidly induced by BL (Fig. 2). Interestingly, similarly to BRI1, TGFβ receptors are serine/threonine kinases, and in animals, TRIP1 is phosphorylated by TGFβ (Massagué, 1998). Antisense *trip1* *Arabidopsis* plants exhibit a broad range of developmental defects, including some that resemble the phenotype of BR-deficient and -insensitive mutants. TRIP in animals, yeast and plants is also a subunit of the eukaryotic translation factor eIF3 (Burks *et al.*, 2001;

Jiang and Clouse, 2001). This suggests that TRIP1 may mediate some of the molecular mechanisms underlying the regulation of plant growth and development by BRs (Fig. 2), and might establish a link between BR signaling and developmental pathways controlled by homologs of the eukaryotic translation initiation factor elF3 in plants (Jiang and Clouse, 2001; Bishop and Koncz, 2002).

Brassinosteroids have been found to regulate expression of other known or unknown genes in *Arabidopsis* or other plants, such as rice or tomato (Goetz *et al.*, 2000; Friedrichsen and Chory, 2001; Hu *et al.*, 2001; Bouquin *et al.*, 2001; Müssig *et al.*, 2002) but the upstream components which induce them and which are triggered by BRs have yet to be identified.

PERSPECTIVES

Significant progress has been made over the last few years in elucidating the BR signaling pathway. The discovery of BR-insensitive mutants led to isolation of *BRI1* gene. Molecular characterization of the BRI1 product revealed it to be a receptor-like transmembrane kinase (RLK) that transduces steroid signals across the plasma membrane. Discovery of *OsBRI1*, a *BRI1* homologue in rice (Yamamuro *et al.*, 2000), suggests that BR action through RLKs is a conserved mechanism in plant steroid signaling. In addition, a calcium-dependent protein kinase (CDPK) was identified in rice as another potential protein involved in BR action (Yang and Komatsu, 2000). Interestingly, using transgenic rice plants expressing the antisense strand of OsBRI1 transcript (Sharma *et al.*, 2001) revealed that the CPDK signaling cascade is parallel and independent to that via activation of BRI1 receptor. The authors speculate that there is involvement of additional receptor(s) other than BRI1 for BR responses in plants. The idea of an alternative BR receptor appeared also from work of Hu *et al.* (2000). The authors found that the cyclin gene *CycD3* can be induced by BRs, but they showed that the induction of CycD3 does not require functional BRI1. Hu *et al.* (2000) speculate that one of the several BRI1 homologues revealed in the *Arabidopsis* genome may serve as an additional BR receptor (Fig. 2) (The *Arabidopsis* Genome Initiative, 2000). In fact, in animals, hormone receptors are released from the cytoplasmic complexes after hormonal stimuli and are translocated to the nucleus (Mangelsdorf *et al.*, 1995). The CPDK activity observed in the study of Sharma *et al.* (2001) might involve a class of receptors similar to that reported in animals (Mangelsdorf *et al.*, 1995). So far, no evidence of a nuclear receptor has been found in plants, including the complete sequencing of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000). However, analysis of the rice genome, recently sequenced (Yu *et al.*, 2002; Goff *et al.*, 2002) could possibly reveal candidates of a putative nuclear receptor. It would be interesting to follow the idea of an alternative BR receptor(s), which could function in parallel with the BRI1 pathway. A newly identified receptor might mediate similar plant responses like BRI1, such as cell elongation, or it could trigger different responses, such as cell division (Fig. 2), or alternatively, both pathways may crosstalk, as in the system proposed for auxin in the control of cell elongation and cell division in tobacco leaves (Chen *et al.*, 2001; Chen, 2001). In addition, the proposed BR signaling shares many similarities to the Wnt signaling pathway (Cadigan and Nusse,

1997; Miller *et al.*, 1999; Polakis 2000; Clouse, 2002; Yin *et al.*, 2002a). It will, therefore, be interesting to determine whether other components in the BR pathway share homology to Wnt signaling elements, including a nuclear receptor, or if the two pathways split somewhere for some logical reason.

High homology among the BRI1, the systemin receptor SR160 (Scheer and Ryan, Jr., 2002), and PSK receptor (Matsubayashi *et al.*, 2002), and the fact that systemin and PSK are small peptides, whereas BRs are organic compounds provides unique opportunity to determine how these different ligands interact with very similar receptors. Also, identification of more downstream components for BR, and especially for systemin and PSK signaling will answer how specificity (BRs versus systemin) or similarity (BRs versus PSK?) of the signaling pathways are achieved.

Complete physiological and genetic characterization of already available BR-affected mutants (Yin *et al.*, 2002b), or further genetic screens for mutations that suppress or enhance the above described mutations, are expected to reveal new elements of BR signaling. Finally, because of existence of crosstalk among hormone signaling (Reid and Howell, 1995), we should pay attention to mutants recovered from genetic screens for other hormone-mutants. For example, some mutants screened originally for affected responses to cytokinin (Jang *et al.*, 2000) or auxin (Ephritikhine *et al.*, 1999), were later found to be altered in BR physiology. Physiological, genetic, and molecular studies of such mutants might result in identification of missing components, which link BR signaling with various hormone- or nonhormone-signaling pathways.

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