Journal of Plant Biology, March 2003, 46(1) : 1-9 **REVIEW**

Brassinosteroids: Modes of BR Action and Signal Transduction

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In humans and other animals, steroid hormones regulate transcription via membrane-bound receptors. These receptors have an extracellular ligand-binding domain and an intracellular domain that are responsible for transducing the signal to the next member along the signaling pathway. Brassinosteroids (BRs) are structurally similar to the animal steroid hormones found in vertebrates and insects. Plants also use steroids as signaling molecules. BRs regulate the expression of numerous genes associated with plant development, and require the activity of a serine/threonine (Ser/Thr) receptor kinase to realize these effects. Signaling in dicotyledonous (e.g., *Arabidopsis thaliana*) and monocotyledonous (e.g., *Oryza sativa*) models, is mediated by the receptor kinases BRI1 and OsBRI1, respectively. The extracellular domain of BRI1 perceives BRs, and the signal is then mediated via an intracellular kinase domain that autophosphorylates Ser and Thr residues and potentially, other substrates. BRI1 transduces steroid signals across the plasma membrane and mediates genomic effects.

Keywords: *Arabidopsis thaliana*, brassinosteroids, *Oryza sativa*, receptor kinase, signaling molecule

Brassinosteroids (BRs) comprise a class of over 40 polyhydroxylated sterol derivatives that appear to be distributed throughout the plant kingdom (Clouse and Sasse, 1998). These growth-promoting natural products are structurally similar to animal steroid hormones (Fig. 1), which have well-known functions in regulating embryonic and post-embryonic development and adult homeostasis.

Both plant and animal hormones rapidly alter gene expression before the onset of cellular and physiological changes. Like their animal counterparts, BRs impact the activity of complex metabolic pathways, contributing to the regulation of cell division and differentiation. They are also involved in modulating processes more specific to growth and development, e.g., photomorphogenesis, skotomorphogenesis, and cell expansion (Clouse and Feldmann, 1999). In many test systems (Clouse and Sasse, 1998), BRs have been shown to increase rates of cell division, particularly under conditions of limiting auxin and cytokinin. They can also accelerate senescence, cause hyperpolarization of membranes, stimulate ATPase activity, and alter the orientation of cortical microtubules. In addition to these direct effects on growth regulation, BRs also mediate abiotic and biotic stresses, including high salinity, drought, temperature extremes, and pathogen attack.

Molecular genetic analysis of BR action has begun. Genes regulated by BR have been cloned and mutants that are deficient in or insensitive to BR have been identified. This short review focuses on the most recent research results, and provides descriptions of the strong modes of BR action and signal transduction found in plants.

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MODES OF BRASSINOSTEROID ACTION

Cell elongation, critical for growth and differentiation in all plant organs, is controlled by coordinated alterations in wall mechanical properties, biochemical processes, and gene expression (Cosgrove, 1997). For turgor-driven expansion to proceed, the cell walls must transiently yield through breakage of the hemicellulose tethers, a process that is accompanied by the incorporation of new wall polymers to prevent thinning and weakening. Regulation of the synthesis and activity of wall-modifying enzymes, such as xyloglucan, sucrose synthase, and cellulose synthase, becomes an obvious target for hormones involved in elongation. In support of the proposed model, BR regulation of genes that encode XETs and expansins has been demonstrated in *Arabidopsis*, soybean, and tomato (Clouse, 1997). Likewise, BRs have been shown to promote wall loosening in soybean epicotyls (Zurek et al., 1994) and hypocotyls of *Brassica chinensis* and *Cucurbita maxima* (Wang et al., 1993; Tominaga et al., 1994).

Abbreviations: ABA, abscisic acid; *bri*1, *brassinosteroid insensitive* 1; BR, brassinosteroid; GA, gibberellin; TRIP-1, TGF-β receptor interacting protein.

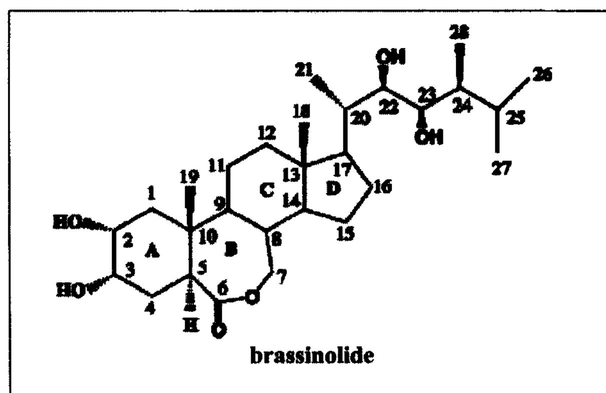

Figure 1. Structure of naturally occurring brassinosteroids. The configuration for brassinolide is presented with possible variations in the A and B rings.

When BRs are applied to elongating soybean epicotyls, plastic extensibility of the wall increases within 2 h, with a concomitant rise in the mRNA level of the *BRU1* gene (Zurek et al., 1994). The recombinant BRU1 protein exhibits xyloglucan-specific transglycosylation in vitro, and shares significant identity with numerous XETs from various plant species. In situ hybridization in apical epicotyl cross sections has revealed that *BRU1* is more highly expressed in inner vs outer stem tissues (particularly in phloem cells), in parenchyma cells surrounding the xylem elements, and in the starch sheath layer (Oh et al., 1998). In addition to the soybean, similar BR-regulated XETs have been characterized in *Arabidopsis* (Xu et al., 1996; Catala et al., 1997), rice (Uozu et al., 2000), and tomato (Koka et al., 2000). In all these species, induction of XETs is correlated with cell-wall loosening during BR-induced growth responses. Four XET-related (XTR) genes have been isolated and their expression patterns in rice plants determined. Of these, Os*XTR1* and Os*XTR3* have been found to be up-regulated by gibberellin and brassinosteroids (Uozu et al., 2000).

Arabidopsis TCH4 is an unusual gene in that its expression is up-regulated by a variety of seemingly unrelated stimuli, including mechanical perturbations, such as touch, temperature extremes, darkness, and the growth-promoting hormones BR and IAA. Induced *TCH4* expression is remarkably transient. Recently, Iliev et al. (2002) investigated which regions of the TCH4 locus contribute to its regulatory behaviors. Induction of expression by the diverse stimuli is conferred to reporter genes by the 102-bp 5-untranscribed TCH4 region. The *Arabidopsis* TCH4 gene encodes an XET with sequence similarity to *BRU1*, and environmental stimuli such as touch, darkness, and temperature affect its expression. *TCH4* is strongly expressed in expanding tissues, particularly in dark-grown hypocotyls, and vascular elements. Xu et al. (1995) have reported that *TCH4* is transcriptionally regulated by BR. Examination of cell files in wild-type *Arabidopsis* and *cbb*, *dwf4*, *cpd*, and dim mutant plants has provided direct physical evidence that longitudinal cell expansion is greatly reduced in BR mutants (Szekeres et al., 1996; Azpiro et al., 1998). As expected, the expression of genes associated with cell elongation, such as TCH4, is also reduced in BR mutants (Kauschmann et al., 1996). Besides such alterations in cell-wall properties, BRs may affect the transport of water via activity of a vacuolar H^+-ATPase, which is associated with cell elongation (Schumacher et al., 1999).

The results of second-internode bioassays in beans have suggested that BRs affect cell division as well as elongation (Steffens, 1991). Division has been stimulated by BRs (in the presence of auxin and cytokinin) in cultured parenchyma cells of *Helianthus tuberosus* (Clouse and Zurek, 1991), and in protoplasts of Chinese cabbage and petunia (Nakajima et al., 1996; Oh and Clouse, 1998). Preliminary results have also shown that brassinolide affects the kinetics of the cell cycle in synchronized cell cultures of tobacco, and also regulates the expression of genes associated with the S phase, including H_2B and High Mobility Group-1 protein. Moreover, the expression of *CDC2b* cyclin-dependent kinase is up-regulated by BRs in the dark, but remains unaffected in the presence of light (Yoshizumi et al., 1999). Treatment of *det2* cell suspension cultures with 24-epi-brassinolide increases transcript levels of the gene encoding cyclinD3 (*CycD3*), a protein involved in the regulation of G_1/S transition in the cell cycle (Jiang and Clouse, 2001). Similar treatments can also effectively substitute for the influence of zeatin on the growth of *Arabidopsis* callus and cell suspension cultures (Hu et al., 2000).

In addition to the well-known functions of auxins and cytokinins, much accumulated evidence supports the proposed role of BRs in the process of vascular differentiation. For example, in the *Zinnia* system, BRs regulate the expression of several genes associated with xylem formation (Fukuda, 1997). High levels of *BRU1* in paratracheary parenchyma cells that surround the vessel elements in soybean epicotyls also suggest the involvement of BRs, and XETs (Oh et al., 1998). In *Arabidopsis* mutants, BR plays a role in vascular differentiation. Specifically, the BR-deficient mutant *cpd* exhibits unequal division of the cambium, in which extranumerary phloem cell files are produced at the expense of xylem cells (Szekeres et al., 1996). Fur-

thermore, the spacing between vascular bundles can be joined without a separating layer of parenchyma cells (Choe et al., 1999).

Most BR mutants have extended life spans, and their senescence is delayed. One exception is the fertile *dwf*5-1 mutant (Choe et al., 2000). Whereas the administration of cytokinins in vitro has often been shown to retard the senescence of leaf and cotyledon tissue, 24-epi-brassinolide accelerates senescence in such systems (Ding and Zhao, 1995; He et al., 1996). The observed delay in senescence with *Arabidopsis* BR mutants also tends to indicate a role for BRs in accelerating senescence for normal plants. However, it is unclear whether they play a critical function in the intrinsic programming of senescence in vegetative tissue.

The quality, duration, and intensity of light profoundly influence development throughout a plant's life cycle. Upon exposure, stem elongation slows dramatically, the apical hook opens, and true leaves with mature chloroplasts develop (Chory et al., 1996). Numerous BR-deficient mutants in *Arabidopsis*, pea, and tomato show defective cell expansion in the dark. Depending on the severity of the mutant allele and the species, some of these mutants also exhibit other characteristics of light-grown plants in the dark, such as expanded cotyledons, the lack of an apical hook, and aberrant expression of light-regulated genes (Clouse and Feldmann, 1999). Neff et al. (1999) have shown that the *Arabidopsis bas*1 mutant, which inactivates brassinolide by overexpressing a C-26 brassinolide hydroxylase, is capable of suppressing the long hypocotyls phenotype in phytochrome B mutants. Such a result suggests that BR metabolism is possibly linked with phytochrome signaling (Neff et al., 1999). Kang et al. (2001) have reported that the dark-inducible, light-repressible small G protein, Pra2, interacts with and activates a cytochrome P450 C-2 hydroxylase that is involved in brassinolide biosynthesis. Because *Arabidopsis* contains a large multigene family of small G proteins, it would be interesting to study the possible interactions of these proteins with other cytochrome P450 involved in brassinolide biosynthesis. Very recently, several studies documented the extent of BR-regulated gene expression in *Arabidopsis*, as well as identified the first BR early response genes (Friedrichsen et al., 2002; Mussig et al., 2002; Yin et al., 2002). The Most direct evidence for the physiological significance of these small changes in gene expression comes from a recent study that identified three BR early response genes (Friedrichsen et al., 2002). These three genes encode closely related basic helix-loop-helix transcription factors, *BEE1*, *BEE2*, and *BEE3*, whose expression is induced within 30 min of BR treatment in the absence of new protein synthesis and requires a functional BR receptor. In contrast to the well-characterized numerous changes in gene expression following insect-steroid pulses, however, the magnitude of BR-mediated gene expression changes are small and appear to largely affect cell expansion processes. The identification of BR-responsive promoter elements would significantly enhance the molecular dissection of BR-regulated gene expression

SIGNAL TRANSDUCTION BY BRASSINOSTEROIDS

The majority of recent work on BR signal transduction has focused on *brassinosteroid insensitive* 1 (*bri*1), which affects the gene encoding a plasma membrane-bound leucine-rich repeat (LRR) receptor kinase (Clouse et al., 1996; Li and Chory, 1997). This mutant was first identified, in a screen of 70,000 EMS-mutagenized seedlings, by its ability to cause root elongation in the presence of 24-epi-brassinolide (Clouse et al., 1996). Its insensitivity to the inhibition of elongation is highly specific, and extends over a wide range of BR concentrations. The *bri*1 mutant does, however, retain sensitivity to auxins, cytokinins, GA, and ethylene, and shows hypersensitivity to ABA (Clouse et al., 1996). Numerous other BR-insensitive mutants have been identified in a variety of independent screens, but genetic analysis has shown all of them to be allelic to *bri*1 (Kausckmann et al., 1996; Li and Chory, 1997; Noguchi et al., 1999). The *bri*1 phenotype is among the most severe of the BR mutants, exhibited as extreme dwarfism, male sterility, delayed development, and reduced apical dominance (Fig. 2). BRI1 was originally identified by positional cloning, then verified by sequencing numerous mutant alleles (Li and Chory, 1997). The predicted protein is an 1196-bp amino acid containing the three major domains common to all animal and plant receptor kinases, the extracellular ligand-binding domain, the transmembrane domain, and the cytoplasmic kinase domain. The extracellular domain begins with an N-terminal single peptide, followed by a leucine zipper motif and 25 tandem copies of a 24-bp amino acid LRR with 13 potential N-glycosylation sites flanked by conservatively spaced cysteines. Both the leucine zipper and the LRRs are involved in protein-protein interactions, and many receptor kinases dimerize in response to ligand binding (Kobe and Deisenhofer, 1994; Heldin, 1995).

The molecular structure of the BRI1 extracellular

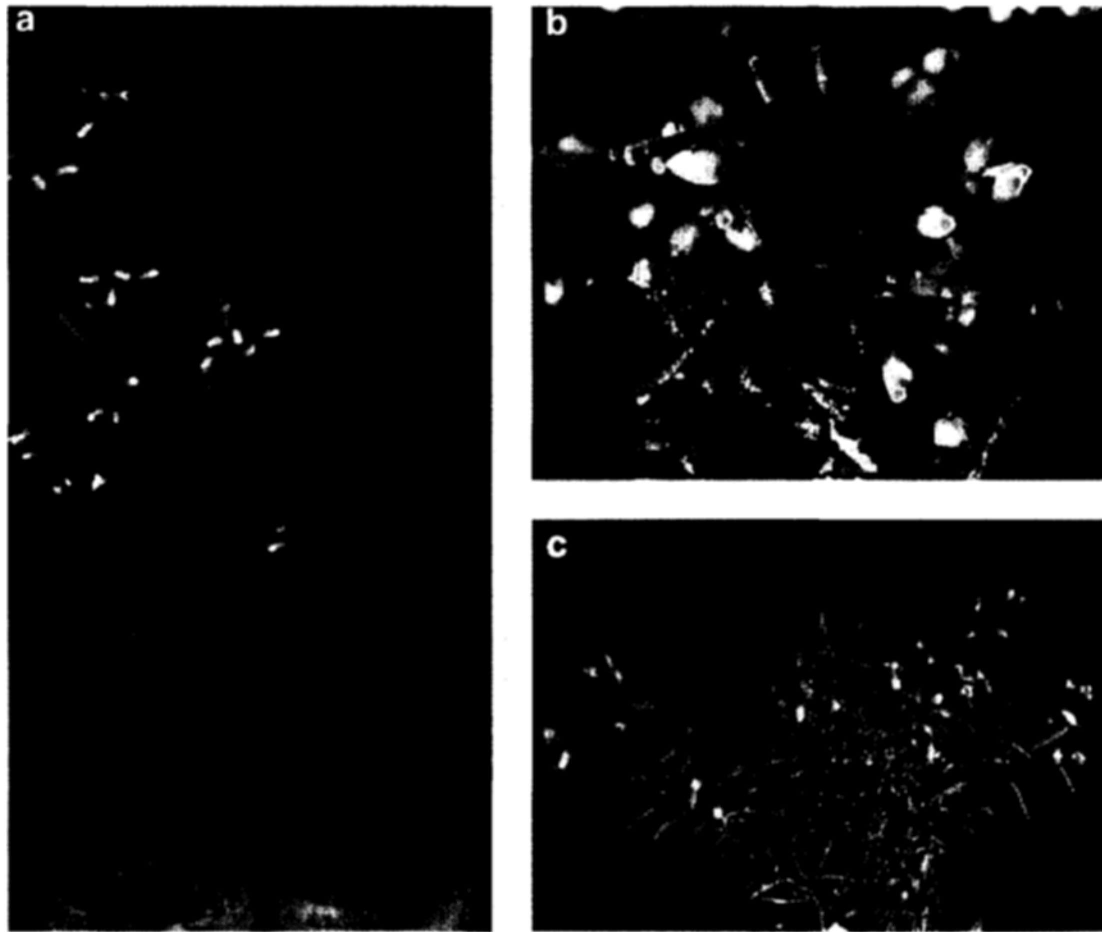

Figure 2. Phenotype of the bri1 mutant. Panel **A** shows 2-month-old mutant (left) and wild type (right) plants grown in 50 mL centrifuge tubes in a 23°C growth chamber (16 h light/8 h dark). Panels **B** and shows close-up view of a 2-month-old bri1 mutant plant. Panel **C** shows the plant after 4 months. Adapted from Clouse et al. (1996).

domain suggests its involvement in the formation of homodimers or heterodimers, although this has not been verified experimentally. A unique segment of this domain, which has been shown by mutant analysis to be critical for proper protein function, is a 70-bp amino acid island between LRR 21 and 22 (Li and Chory, 1997). Yamamuro et al. (2000) have cloned a rice gene, *OsBRI1* (*Oryza sativa BRI1*), with extensive sequence similarity to that of the *Arabidopsis BRI1* gene, which encodes a putative BR receptor kinase. The predicted OsBRI1 polypeptide contains several domains that are also present in *Arabidopsis* BRI1. Transgenic plants carrying the antisense strand of *OsBRI1* transcript show similar or even more severe phenotypes than those of the *d61* mutant, a rice dwarf mutant that is less sensitive than the wild type to BR. Yamamuro et al. (2000) have suggested that *OsBRI1* functions in various growth and developmental processes in rice, e.g., during internode elongation, bending of the lamina joint, and skotomorphogenesis.

Hundreds of putative receptor kinases that exist in *Arabidopsis* have been shown to function in diverse physiological processes, such as growth and development, embryogenesis, fertilization, abscission, disease resistance, and light-mediated responses (Lease et al., 1998). Mechanisms are required in order for signal transduction pathway-specific cytoplasmic components to bind to the intracellular domains of receptor kinases. BRI1 shares significant sequence identity with many plant receptor kinases, particularly those in the LRR family of extracellular domains. These include CLAVATA1 and ERECTA, both of which are involved in regulating different aspects of *Arabidopsis* development (Torii et al., 1996; Clark et al., 1997).

BRI1 is expressed in all organs of young, growing *Arabidopsis* plants, and is localized in the plasma membrane (Friedrichsen et al., 2000). He et al. (2000) have used a chimeric construct of the extracellular

domain of BRI1 and the kinase domain of the rice receptor kinase XA21 to demonstrate that the extracellular domain of BRI1 perceives BRs. Likewise, in direct confirmation that BRI1 is either the BR receptor or part of a BR receptor complex, Wang et al. (2001) have reported evidence that transgenic plants overexpressing the BRI1/green fluorescent protein have increased binding of tritiated BR in their plasma membranes, compared with wild-type plants. To gain further insights into BR signaling, Li et al. (2001a) have performed a gain-of-function suppressor screen with a weak *bri1* allele (*bri1-5*). Screening of 2,500 tagged lines has led to the identification of a single dominant (*brs1-1D*) mutation that results in overexpression of a serine carboxypeptidase-like protein. Li et al. (2001a) suggest that BRS1 might act on a protein that is required for BR perception. Other components of the BR signal transduction pathway have been identified by their gain-of-function phenotypes. Overexpression of *BAK1*, a gene encoding another leucine-rich repeat receptor kinases, partially suppresses the phenotype of a weak *bri1* allele (Li et al., 2002). BAK1 was also identified by its in vitro interaction with BRI1 and has been shown to modulate BR signaling (Li et al., 2002; Nam and Li, 2002). BAK1 has been proposed to act as a coreceptor for BRs, yet this remains to be shown. A second *brassinosteroid insensitive 2* (*bin2*) mutant, as a new component of BR signaling, has now been identified in *Arabidopsis* (Li et al., 2001b). In both *bin2* and *ultracurvata1* (*ucu1*), missense mutations have resulted in a semidominant phenotype. Therefore, Li et al. (2001b) have proposed that *bin2* represents either a gain-of-function neomorphic mutation or a hypermorphic mutation that defines a negative regulator function in BR signaling (Li and Nam, 2002). Two mutants, *bes1* and *bzr1*, were identified as suppressing *bri1* penotypes, as well as being resistant to brassinazole, a BR biosynthesis inhibitor (Wang et al., 2002; Yin et al., 2002). Identical dominant mutations identified in both genes stabilize the respective proteins and increase their accumulation in the nucleus in the absence BRs (Wang et al., 2002; Yin et al., 2002).

Studies by Friedrichsen et al. (2000) and Oh et al. (2000) on the kinase domain of BRI1 (BRI1-KD) have confirmed that it functions as an active kinase in vitro, and that specific serine (Ser) and threonine (Thr) residues within KD are autophosphorylated (Fig. 3). MALDI/MS analysis of BRI1-KD (Table 1) has identified five unique phosphorylation sites, as well as seven that have some remaining ambiguity because of multiple Thr or Ser residues within the peptide (Oh et al., 2000). Some of those residues of BRI1-KD, autophosphory-

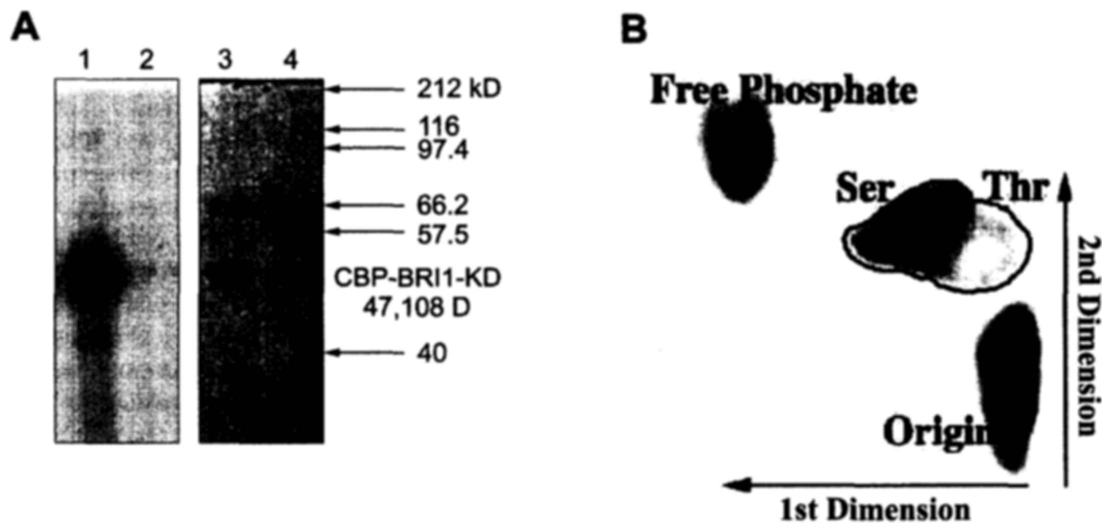

Figure 3. Autophosphorylation and phosphoamino acid analysis of recombinant BRI1-KD. **A)** Affinity purified calmodulin-binding peptide-BRI1 kinase domain recombinant protein (CBP-BRI1-KD, Lane 1) or the mutant CBP-BRI1-K911E (Lane 2) was incubated with 20 μCi [γ-^{32}P] ATP in kinase buffer for 1 h at ambient temperature, followed by PAGE. Lane 3 and Lane 4 represent the Coomassie Blue stained gel corresponding to the autoradiograph in Lane 1 and 2. Molecular mass of CBP-BRI1-KD was determined by MALDI/MS. **B)** CBP-BRI1-KD was autophosphorylated and transferred to PVDF membrane as described above. The membrane was digested with HCl and subjected to phosphoamino acid analysis by TLE with pSer, pThr, and pTyr standards. CBP-BRI1-KD autophosphorylated primarily on Ser residues with a minor Thr component and no detectable phospho-Tyr residues. Adapted from Oh et al. (2000).

Table 1. Phosphorylation sites of BRI1 kinase domain, as determined by MALDI-MS.

Amino acid sequence	Sites of phosphorylation
BRI1 kinase domain	
825-EAELEMYAEGHGNSGDR-841[a]	S-833
842-TANNTNWKLTGVK-854	S-842, S-846
855-EALSINLAAFEKPLR-869	S-858
870-KLTFA-874	T-872
886-DSLIGSGGFG-895	one site: S-887 or S-891
978-LNWSTR-983	one site: S-981 or S-982
1038-DTHLSVSTLAGTPGYVPPEYYQSFR-1,062	three sites: T-1039, S-1042, S-1044, T-1045, T-1049, S-1060
1,165-DSQSTIR-1,171	one site: S-1160, S-1168, T-1169
1,172-SIEDGGFSTIEMVDMSIK-1,189	one site: S-1172, S-1179, T-1180, S-1189

[a]Amino acid numbering corresponds to Li and Chory (1997) and GenBank accession no. AAC49810

lated in vitro, are conserved at corresponding positions in related plant Ser/Thr kinases. The region of the greatest conservation occurs in the peptide 1038-DTHLSVSTLAGTPGYVPPEYYQSFR-1062, which lies in the highly conserved activation loop of kinase subdomain VIII (Lease et al., 1998). Outside of that subdomain, the only other strongly conserved sites are in the positions equivalent to T-872 in the juxtamembrane region and T-982 in kinase subdomain VIa of the BRI1-KD (Oh et al., 2000). Autophosphorylation of subdomain VIII likely leads to kinase activation, which, if reflected in vivo, might indicate multiple interacting cytoplasmic partners for BRI1.

Identification of those binding partners and kinase domain substrates for BRI is critical if one is to comprehend downstream signaling events. A variety of molecular genetic and biochemical approaches can be employed to identify putative in vivo partners. These methods include yeast two-hybrid analysis (Gu et al., 1998), immunoprecipitation and purification of receptor-protein complexes (Trotochaud et al., 1999), and the use of synthetic peptides to understand the binding motifs and substrate recognition consensus sequences (Kuriyan and Cowburn, 1997).

Oh et al. (2000) have studied BRI1-KD phosphorylation recognition sequences in synthetic peptides. Their optimum test sequence was GRMKKIASVEMMKK. Using analogs of this peptide, they found that the positioning of residues for both the N- and C-terminals of phosphorylated Ser is critical for the optimal activity of BRI1-KD. A total of 109 hits was obtained when the preliminary consensus sequence [RK]- [RK]-X(2)-S-X(2)- [LMVIFY]-X-[RK]-[RK] was used to search the *Arabidopsis* non-redundant proteins database. Although some of these had obvious connections to signal transduction pathways, others were intriguing, given the connection between BRs and photomorphogenesis. Therefore, the BRI1-KD substrate recognition sequence may provide a valuable molecular tool for further analysis, although no direct evidence exists that these are true substrates in vivo.

TRIP-1 containing elements of the BRI1-KD recognition sequence have been identified via subtractive hybridization in a screen for BR-regulated genes (Jiang and Clouse, 2001). This WD-domain protein shares extensive sequence similarity with mammalian TRIP-1 (TGF-β receptor interacting protein), which is a kinase domain substrate of the TGF-β type II receptor (Chen et al., 1995). Surprisingly, TRIP-1 also has a dual function as a subunit of the eukaryotic translation initiation factor, eIF3. Enhanced expression of TRIP-1 by BR, as observed in bean, tobacco, and *Arabidopsis* (Jiang and Clouse, 2001), might lead to increased protein translation and serve as a general mechanism for BR-promoted growth. TRIP-1 in vertebrates is a known substrate of the TGF-β ser/thr receptor kinase. It is conceivable that BRI1, or one of the many other plant receptor kinases involved in developmental control, might phosphorylate TRIP-1, thereby enhancing its competence to initiate assembly of the eIF3 complex. Recombinant *Arabidopsis* TRIP-1 has been found to be strongly phosphorylated by BRI1-KD in vitro, predominately on Thr residues (Clouse et al., unpublished data). The correlative evidence for BR regulation of TRIP-1 transcript levels, and the similarity in characteristics between α-TRIP-1 plants (antisense transgenic lines) and BR mutants, suggests a direct link between TRIP-1 and BR signaling. Efforts are currently under way to monitor TRIP-1 protein levels and localization in wild type and mutant plants, and to identify the TRIP-1 binding partners in *Arabidopsis* (Jiang and Clouse, 2001). Such experiments may clarify the possible role of TRIP-1 in BR signaling and increase our understanding of how BR affects plant development.

CONCLUSION AND FURTHER PERSPECTIVES

This review focused on very recent examinations of the modes for BR action and signal transduction in plants. Preliminary results on the promotive effect of BRs in cell division is likely to lead to new areas of molecular research. The use of new technology, e.g., microarrays, to study various aspects of BR-regulated gene expression also can be expected to increase. The basic design of a BR signaling pathway, linking events at the plasma membrane to changes in gene expression in the nucleus, is beginning to be elucidated, yet several gaps in our knowledge remain. Several other questions remain, which could be answered through biochemical analysis using advanced techniques. Does BRI1 form homo- or heterodimers? What are the major signaling components that act between BRI1 and BIN2? What proteins do BES1/BZR1 interact with to regulate gene expression in the nucleus? Are accessory steroid-binding proteins required for BR binding? What are the in vivo autophosphorylation sites and specific phosphorylation recognition sequences in the kinase domain substrates? And finally, where does the specificity of brassinolide action come from? Further analysis of BRI1 and TRIP-1 will also be required for a more complete understanding of BR signal transduction. Studies of these biochemical and protein interactions will help researchers identify the signaling partners of BRI1 and BIN2 kinases.

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