Brassinosteroid Signal Transduction: Clarifying the Pathway from Ligand Perception to Gene Expression

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Recent genetic screens for novel components of brassinosteroid signaling have revealed proteins with cell surface, cytoplasmic, and nuclear localization that function as either positive activators or negative regulators of the brassinosteroid response. Initial microarray experiments have expanded the number of known brassinosteroid-regulated genes, providing a useful resource for better understanding terminal events in signal transduction.

Introduction
Animal steroid hormones have diverse, well-characterized roles in embryonic and postembryonic development and adult homeostasis. A number of plants have been shown to contain some of the same steroids as animal systems, including ecdysteroids, androgens, estrogens, and corticosteroids (Geuens, 1978). Among plant steroids, however, only brassinosteroids (BRs) are ubiquitously distributed throughout the plant kingdom and play essential roles in modulating the growth and differentiation of cells at nanomolar to micromolar concentrations (Clouse and Sasse, 1998). Like their animal counterparts, BRs regulate the expression of numerous genes, impact the activity of complex metabolic pathways, and help control overall developmental programs leading to morphogenesis. They are also involved in regulating plant-specific processes including photomorphogenesis and cell expansion in the presence of a potentially growth-limiting cell wall. BR mutants in Arabidopsis show a characteristic phenotype in the light (Figure 1) that includes dwarfism, dark green, rounded leaves, development delayed, reduced fertility, and altered vascular structure. Dark-grown mutants exhibit some features typical of wild-type light-grown plants, including shortened hypocotyls and open cotyledons. In BR-deficient mutants, all of these phenotypic defects are rescued to wild-type by exogenous application of BR (Altmann, 1999; Clouse and Feldmann, 1999), demonstrating an essential function for plant steroids in normal plant growth and development.

BRs and animal steroid hormones are products of the isoprenoid biosynthetic pathway and it is generally accepted that the steps leading from mevalonate to squalene-2,3-epoxide are conserved between animals and plants. However, the subsequent conversion of squalene-2,3-epoxide to steroid progenitors differs. In animals, squalene-2,3-epoxide is converted to lanosterol, the precursor of cholesterol and steroid hormones, while in plants it is converted to cycloartenol, the parent compound of all plant sterols, including campesterol, stigmasterol, and sitosterol (Benveniste, 1986). Cam-
esterol is the progenitor of brassinolide, the most active of the naturally occurring BRs (Figure 1). While structural variation in substrates is evident in the parallel pathways of animal and plant steroid biosynthesis, many of the enzymes are functionally equivalent. For example, mammalian and Arabidopsis steroid 5α-reductases share 80% of known conserved residues, including a specific glutamate that is required for enzyme activity (Li et al., 1996). When expressed in human embryonic kidney cells, recombinant plant 5α-reductase reduces testosterone to dihydrotestosterone. Conversely, overexpression of human 5α-reductases in BR-deficient transgenic plants lacking 5α-reductase activity results in a wild-type phenotype without exogenous BR application (Li et al., 1997).

The classical signaling pathway for animal steroid hormones is rather abbreviated and involves binding of the steroid to an intracellular receptor consisting of a variable N-terminal domain often associated with transcriptional activation, a highly conserved DNA-binding domain with two zinc fingers, and a multifunctional domain that mediates ligand-binding, dimerization, and ligand-dependent transcriptional activation (Evans, 1988). The receptor-ligand complex recognizes specific sequences in the promoters of steroid-responsive genes resulting in altered gene expression and steroid-mediated changes in cell physiology. While accessory components such as heat shock proteins, immunophilins, and interacting transcription factors complicate the picture somewhat, the overall signal transduction logic is fairly simple (Beato et al., 1995). In contrast, cell surface receptors for peptide ligands such as transforming growth factor-β (TGF-β), epidermal growth factor, and insulin have multiple steps between ligand perception and gene activation, in which phosphorylation status of intermediates plays a critical role (Heldin, 1995; Massague, 1998). Plants have apparently adapted aspects of both signaling pathways. BRs, which are structurally related to animal steroid hormones, are perceived by plasma membrane receptor kinases, with general structural similarities to both TGF-β receptors and receptor tyrosine kinases. Animals also perceive steroids at the cell surface, in addition to intracellular receptors, but the molecular nature of these plasma membrane receptors has not been fully characterized (Falkenstein et al., 2000). A survey of the completed genome sequence of Arabidopsis thaliana suggests that plants do not contain members of the superfamily of intracellular steroid receptors, and thus cell surface recognition is the predominant, if not only, form of plant steroid perception (Friedrichsen and Chory, 2001).

BR1, a Leucine-Rich Repeat Receptor Kinase Involved in BR Perception

Until very recently, almost all BR signal transduction research focused on Brassinosteroid Insensitive 1 (BR1), a single genetic locus in Arabidopsis encoding a leucine-rich repeat receptor kinase. The bri1 mutant was initially identified by a root growth inhibition screen for BR insen-

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Figure 1. Structure of Plant and Animal Steroids and the Phenotype of a Brassinosteroid Signaling Mutant

Brassinolide, the most active brassinosteroid, is derived from campesterol via cycloartenol. It contains a four-member steroid ring similar to animal steroid hormones such as testosterone, which is derived from cholesterol via lanosterol. Brassinolide is perceived at the cell surface by a complex including BrassinosteroidInsensitive 1 (BRI1), a Leucine-rich repeat receptor kinase. Mutations in the BRI1 gene (bri1 alleles) result in multiple developmental defects, including severe dwarfism and dark green curled leaves.

sitivity (Clouse et al., 1996) and several independent genetic screens revealed over 2 dozen alleles of bri1, most of which exhibited the extreme dwarfism and other phenotypic characteristics of severe BR-deficient mutants (Kausschmann et al., 1996; Li and Chory, 1997; Noguchi et al., 1999). In contrast to the biosynthetic mutants, bri1 cannot be rescued by BR treatment, consistent with its essential role in a signaling pathway. Plant genomes have been shown to encode hundreds of receptor-like kinases, many of which have a proven functional role in the regulation of plant growth, morphogenesis, disease resistance, and responses to the environment (Lease et al., 1998). Both animal and plant receptor kinases exhibit similar organization of functional domains and include an extracellular ligand-binding domain, a single-pass transmembrane sequence, and an intracellular kinase domain (Cock et al., 2002; Shiu and Bleecker, 2001).

The BRI1 protein (Figure 2) consists of a putative signal peptide for delivery to the plasma membrane, followed by a leucine zipper and 25 leucine-rich repeats that are flanked by short sequences containing paired cysteines. Between repeat 21 and 22 lies a 70 amino acid island that is critical for biological function, based on mutational analysis (Friedrichsen et al., 2000; Li and Chory, 1997). Downstream of the extracellular domain lies a short hydrophobic transmembrane domain, followed by the juxtamembrane region, and the cytoplasmic kinase domain containing all twelve conserved subdomains found in eukaryotic kinases. Point mutations in the kinase domain often result in severe bri1 phenotypes, including lesions in subdomain II at an invariant Ala residue and in subdomain VIII, containing the highly conserved activation loop required for kinase activity (Friedrichsen et al., 2000; Lease et al., 1998; Noguchi et al., 1999).

The role of BRI1 as at least one component of the BR receptor was demonstrated by binding studies with radiolabeled brassinolide and microsomal fractions of wild-type and mutant plants, as well as from transgenic plants expressing BRI1-green fluorescent protein (GFP) fusions (Wang et al., 2001). BRI1-GFP fusions were also used to demonstrate the plasma membrane localization of BRI1 (Friedrichsen et al., 2000). The BR-binding activity associated with BRI1 was of high affinity ($K_d = 7.4 \pm 0.9 \text{nM}$), consistent with physiological concentrations of BR, and was eliminated by mutations in the extracellular domain but not the kinase domain of BRI1. The binding studies confirmed previous experiments using chimeric receptor kinases that indicated the extracellular domain
Brassinosteroid Insensitive1 (BRI1) and BRI1 Associated Receptor Kinase (BAK1) may form a heterodimer on the cell surface and are involved in BR signaling. Asterisks represent known mutations that affect protein function.

The mechanism of action of many animal receptor kinases is well characterized and generally involves ligand-mediated homo- or heterodimerization of the receptor followed by autophosphorylation of the intracellular kinase domain. Kinase activation results in recognition and phosphorylation of downstream components of the signal transduction pathway, leading ultimately to alterations in gene expression (Heldin, 1995). Plant receptor kinases are not as thoroughly understood as their animal counterparts, but evidence is accumulating that plants also follow the same general paradigm of receptor kinase action (Shah et al., 2001a; Trotochaud et al., 1999; Wang et al., 2001). With respect to BRI1 and BR signaling, it is essential to understand the role of receptor dimerization and kinase domain autophosphorylation, including specific phosphorylation sites and their BR dependence. Identification of kinase domain substrates and their downstream interacting partners, along with specific transcription factors and their interaction with BR response elements in the promoters of BR-regulated genes, will also be required for a complete picture of BR signaling. Several groups are using genetic and biochemical approaches to address these issues and significant progress has been made recently in understanding several stages of BR signal transduction.

**BAK1, a Second Leucine-Rich Repeat Receptor Kinase, Interacts with BRI1**

A yeast two-hybrid screen for proteins putatively interacting with BRI1 identified a second leucine-rich repeat receptor kinase, termed BAK1 (BRI1 associated receptor kinase 1), that shares similar structural organization to BRI1 (Nam and Li, 2002). In contrast to BRI1, BAK1 has only five leucine-rich repeats in its extracellular domain and lacks the 70 amino acid island of BRI1. BAK1 shares 80% sequence identity to *Arabidopsis* somatic embryogenesis receptor kinase 1 (AtSERK1), which is...
transiently expressed during embryogenesis (Shah et al., 2001b). Unlike AtSERK1, BAK1 is expressed in all tissues of the plant, similar to the global expression pattern of BRI1. Furthermore, confocal laser microscopy of transgenic plants expressing a BAK1-GFP fusion shows plasma membrane localization in a pattern similar to BRI1-GFP. Direct physical interaction between BRI1 and BAK1 was confirmed both in yeast cells and Arabidopsis plants by coimmunoprecipitation experiments with tagged proteins (Li et al., 2002; Nam and Li, 2002).

A role for BAK1 in BR signaling was demonstrated by a number of genetic experiments. A dominant mutant of BAK1, bak1-1D, was uncovered in a gain-of-function activation tagging screen for extragenic suppressors of bri1-5, a weak allele of bri1 with a semi-dwarf phenotype (Li et al., 2002). Inflorescences in the double mutant are twice as long as in the bri1-5 single mutant, and the petiole length in the double mutant is nearly the same as wild-type. BAK1 mRNA levels are 30-fold higher in bak1-1D than in bri1-5 or wild-type, suggesting that overexpression of BAK1 leads to the suppressed phenotype of bri1-5. This was confirmed by phenotypic suppression of bri1-5 and bri1-301, another weak allele bri1, in transgenic mutant plants overexpressing BAK1 (Li et al., 2002; Nam and Li, 2002). Phenotypic suppression was not observed in double mutants of bak1-1D and null mutants of bri1, indicating that partial BRI1 activity is required for suppression. Moreover, BR itself is necessary, since bri1-5 bak1-1D plants were not suppressed when crossed into a BR-deficient mutant background. Knockout mutants of BAK1 were identified that showed a weak bri1-like phenotype and also decreased sensitivity to BR in a root inhibition assay (Li et al., 2002; Nam and Li, 2002). The weak phenotype of bak1 null mutants compared to severe bri1 mutants might be explained by functional redundancy, since BAK1 is a member of a multigene family consisting of at least 13 other members. Finally, overexpression of a kinase-deficient mutant form of BAK1 in bri1-5 led to a severe dwarf phenotype, suggesting a dominant-negative effect, most likely by the poisoning of a possible heteromeric complex between BRI1 and BAK1 (Li et al., 2002). Thus the genetic evidence strongly supports the biochemical experiments that show a direct in vivo interaction between BRI1 and BAK1 in plant membranes.

The demonstration that BRI1 may form a heterodimer with a second leucine-rich repeat receptor kinase represents a potential contribution toward understanding the molecular mechanism of BR perception and signal transduction. As mentioned above, BRI1 is at the very least an essential component of a complex that binds BR at the cell surface. It has not, however, been demonstrated that purified BRI1 binds BR alone. It should now be possible, using tagged proteins and radiolabeled BR, to determine whether or not BAK1, as has been previously demonstrated for BRI1, is an essential component of the BR receptor complex. Moreover, in a cytoplasmic environment, leucine-rich repeats are better known for creating a surface-mediating protein-protein interaction than they are for direct binding of small molecules such as steroids (Kobe and Deisenhofer, 1994). The unique 70 amino acid island between repeat 21 and 22 has been proposed as a BR binding site, and while numerous bri1 mutants cluster in this island, a direct binding of BR to this moiety has not been proven. It is also possible that leucine-rich repeats in the plant extracellular environment might bind BR directly. However, a secreted steroid-binding protein has also been postulated as an additional component of the complex, although no direct evidence for such a BR-binding protein is available (Li et al., 2001a).

In mammals, high-affinity binding proteins for sex steroids have been identified that are not in the classical superfamily of steroid receptors. These include putative membrane progesterone receptors (Falkenstein et al., 1996; Gerdes et al., 1998) and soluble sex hormone-binding globulins (Forest and Pugeat, 1986). The Arabidopsis genome encodes several predicted proteins with significant sequence identity to these mammalian sequences. Interestingly, these proteins contain potential processing sites for a putative secreted serine carboxypeptidase, encoded by BRS1 (BRI1 suppressor dominant). BRS1 was identified as a suppressor of bri1-5 in the same type of activation tagging screen that yielded BAK1. It was proposed that BRS1 cleaved a steroid-binding protein that then became competent to bind BR, and the BR-protein complex subsequently interacted with the extracellular domain of BRI1 (Li et al., 2001a). With BAK1 now in the picture, the question to be addressed is whether or not BR binding, either directly or via a protein-BR complex, affects dimerization and subsequent autophosphorylation of the BRI1 and BAK1 receptor kinases.

The expression of the cytoplasmic domain of a receptor kinase in the absence of the extracellular domain generally leads to constitutive kinase activity and this has been demonstrated previously for BRI1 (Friedrichsen et al., 2000; Oh et al., 2000). Specific sites of in vitro autophosphorylation were identified by mass spectrometry and included the expected Ser and Thr residues in the activation loop of the kinase and sites in the juxtamembrane region and carboxy terminus that might be involved in docking of downstream signaling components (Oh et al., 2000). Thus, BRI1 can autophosphorylate in the absence of BAK1, at least in vitro. However, coexpression of full-length BRI1 and BAK1 in yeast cells showed that transphosphorylation between BRI1 and BAK1 predominated and that expression of either receptor kinase alone did not result in appreciable autophosphorylation (Nam and Li, 2002). Moreover, mutant analysis showed that the kinase activities of both BRI1 and BAK1 were required for phosphorylation, even though physical association in a heterodimer still occurred in mutant kinases. Application of BR to yeast cells did not enhance phosphorylation, but it is possible that yeast cells lack an accessory protein, perhaps the steroid binding protein/carboxypeptidase combination described above. Recombinant cytoplasmic domains of BRI1 and BAK1 purified from bacterial cells also showed transphosphorylation of each other in vitro, with BAK1 showing a noticeable increase in phosphorylation when incubated with BRI1 (Li et al., 2002).

If dimerization of BRI1/BAK1 occurs in association with BR perception, it would suggest similarities to both animal receptor tyrosine kinase and TGF-β receptor activities. Receptor tyrosine kinases generally exist in monomeric form in the absence of ligand, with some notable exceptions including the insulin receptor family,
which exist as covalent heterotetramers (Heldin, 1995; Hubbard and Till, 2000). Ligand binding either promotes or stabilizes oligomerization, which can be either homomeric or heteromeric, depending on the receptor family. For many receptor tyrosine kinases, ligand-induced oligomerization results in transphosphorylation of a tyrosine in the activation loop of the kinase domain, followed by further phosphorylation of docking sites within the cytoplasmic domain, and subsequent phosphorylation of downstream signaling molecules. The TGF-β receptor complex consists of a heterotetramer of type I and type II receptor pairs. Type II receptors can homodimerize in the absence of ligand and show constitutive kinase activity. Ligand binding induces formation of the heterotetramer and phosphorylation of the type I receptor by the type II kinase on specific Thr and Ser residues in a region immediately preceding the kinase domain. The activated type I kinase propagates the signal by phosphorylating signaling proteins, termed Smads, which translocate to the nucleus where they form complexes with transcription factors (Roberts, 1999).

Nam and Li (2002) proposed a model for BR signaling in which BRI1 and BAK1 exist as inactive monomers in equilibrium with an active heterodimer. BR binding stabilizes the heterodimer and results in transphosphorylation of each cytoplasmic domain by its partner, leading to active kinases that recognize and phosphorylate currently undefined downstream components. This model more closely resembles the receptor tyrosine kinase paradigm than the TGF-β model. In this model an interesting question arises with respect to the relative activity and specificity of the BRI1 and BAK1 kinase domains. On the other hand, Li et al. (2002) hypothesize that BRI1/BAK1 interaction is more reminiscent of the TGF-β signaling pathway. They suggest BR binds to BRI1, which then activates BAK1 by transphosphorylation. The activated BAK1 would then phosphorylate downstream components. Both of these models are based in part on data from kinase activity of BRI1 and BAK1 expressed in yeast and bacterial cells. To clarify the true nature of BRI1/BAK1 interaction it will be necessary to examine specific phosphorylation sites and mechanisms in planta and their dependence on BR.

Substrates of the BRI1/BAK1 Receptor Kinases

Fully characterized interactions between cytoplasmic proteins and the intracellular domains of plant receptor kinases are currently in short supply. Kinase-associated protein phosphatase (KAPP) has been shown to interact with numerous recombinant kinase domains in vitro and was shown to have a biological role in the CLAVATA1 receptor complex involved in balancing cell proliferation and organ formation in plant meristems (Stone et al., 1998; Trotochaud et al., 1999). An arm repeat containing protein (ARC1) was shown to be a substrate of an S locus receptor kinase governing self-incompatibility in Brassica (Stone et al., 1999). Substrates of BRI1 (besides BAK1, which still has not been shown to be phosphorylated in planta) have not been identified in vivo. A study of BRI1 kinase activity on synthetic peptides identified a putative phosphorylation recognition sequence, which had a requirement for Arg or Lys residues at P-3 and/or P-4 relative to the phosphorylated Ser or Thr (Oh et al., 2000). Since this recognition motif has not been verified by identification of BRI1 phosphorylation sites in kinase substrates in vivo, it is not certain if the peptide data will predict recognition sequences in the tertiary and quaternary environment of in planta protein complexes.

One interesting protein that contains elements of the recognition motif is the plant homolog of TGF-β receptor interacting protein (TRIP-1), a WD domain protein (Jiang and Clouse, 2001). Mammalian TRIP-1 binds to the cytoplasmic domain of the TGF-β type II receptor in a kinase-dependent manner and is phosphorylated on Ser and Thr residues by the receptor kinase (Chen et al., 1995). TRIP-1 functions as a modulator of TGF-β receptor signaling in vivo and also has a dual role as an essential subunit of the eukaryotic translation initiation factor eIF3 in yeast, vertebrates, and plants (Burks et al., 2001; Choy and Derynck, 1998). Expression of plant TRIP-1 is regulated by BR in Arabidopsis, tobacco, and bean under a variety of conditions. Moreover, antisense-TRIP1 plants exhibit some of the phenotypic characteristics of BR-insensitive and deficient mutants, suggesting a possible connection between TRIP-1 activity and BR signaling (Jiang and Clouse, 2001). No direct evidence is currently available showing that TRIP-1 is an in vivo substrate of BRI1, and demonstration of such an in vivo interaction will be required before further parallels between BR and TGF-β signaling mechanisms can be assumed.

Downstream Events in BR Signaling

Until recently no information was available on events downstream of the initial perception of BRs at the cell surface. The lack of non-BRI1 mutants in genetic screens for brassinosteroid insensitivity was accounted for by redundancy in downstream components, as can now be verified in the case of BAK1, or lethality when loss-of-function mutations occurred in one or more of the downstream elements. A reexamination of Arabidopsis dwarfs obtained in a genetic screen for brassinosteroid insensitivity revealed a new mutant, named brassinosteroid-insensitive 2 (bin2), that in the homozygous state closely resembles the bri1 phenotype (Li et al., 2001b). In contrast to known bri1 alleles, bin2 is a semidominant gain-of-function mutant. BIN2 encodes a cytoplasmic Ser/Thr kinase whose catalytic domain shares 70% sequence similarity with the Drosophila shaggy kinase and mammalian glycogen synthase kinase-3 (GSK-3) (Li and Nam, 2002).

GSK-3/shaggy-like kinases are found widely among vertebrates and invertebrates, where they often function as negative regulators of signal transduction pathways controlling metabolism and developmental events such as cell fate determination and pattern formation (Kim and Kimmel, 2000). In mammals, GSK-3 is involved in a receptor tyrosine kinase pathway. In the absence of insulin, GSK-3 phosphorylates glycogen synthase, inhibiting its activity. Insulin binding to the cell surface receptor initiates a signaling cascade resulting in the activation of Akt, a kinase that phosphorylates GSK-3. Phosphorylated GSK-3 is inactive, which allows non-phosphorylated glycogen synthase to accumulate and convert glucose to glycogen. GSK-3/shaggy kinases...
also play a prominent negative regulatory role in the Wingless/wnt signaling pathway of invertebrates and vertebrates by phosphorylating β-catenin, which promotes its proteasome-dependent degradation. Ligand binding to a Frizzled family seven-pass transmembrane receptor leads to GSK-3/shaggy kinase inactivation, resulting in an accumulation of unphosphorylated β-catenin, which is not targeted for degradation by the proteasome. β-catenin continues to accumulate in the cytoplasm and moves to the nucleus where it interacts with transcription factors to regulate the expression of genes essential for developmental pattern formation (Kim and Kimmel, 2000).

BIN2 has been shown by biochemical and genetic analysis to be a negative regulator of BR signal transduction. First, the severity of the bri1-like phenotype in transgenic plants expressing BIN2 was correlated with the level of BIN2 transcripts. Moreover, recombinant bin2 mutant kinase had greater activity in a peptide kinase assay than wild-type BIN2 recombinant protein (Li and Nam, 2002). Both of these observations could be explained if bin2 is a hypermorphic mutant whose increased GSK-3/shaggy kinase activity negatively impacts BR signaling. If BIN2 plays a regulatory role similar to GSK-3/shaggy in the Wingless/wnt pathway, it would be predicted that BIN2 phosphorylates and inactivates an important positive regulator of BR signal transduction. Inactivation of BIN2, and release of its negative control of BR signaling, would likely result from phosphorylation initiated by BR binding to BRI1 or a putative BRI1/BAK1 complex. Genetic and biochemical experiments have shown that BRI1 is unlikely to phosphorylate BIN2 directly, but this needs to be reevaluated in light of the BAK1 discovery. It is also possible that intermediate steps, as occur in the Wingless/wnt pathway, are required between receptor activation and shaggy kinase inactivation (Li and Nam, 2002).

Potential substrates of the BIN2 kinase have recently been identified in genetic screens for suppressors of bri1 mutants and for plants resistant to the BR biosynthesis inhibitor, brassinazole (Wang et al., 2002; Yin et al., 2002). These screens uncovered semidominant or dominant mutants, bes1-D (bri1-EMS-suppressor 1-D) and bzn1-D (brassinazole resistant 1-D), that lead to constitutive brassinosteroid responses including long petioles, curled leaves, accelerated senescence, and constitutive expression of BR-regulated genes. The BES1 and BZR1 proteins are closely related, sharing 88% amino acid identity, and both exhibit bipartite nuclear localization signals and multiple consensus sites (S/TXXXS/T) for phosphorylation by GSK-3 kinases. Studies with recombinant proteins demonstrated that BIN2 kinase strongly phosphorylates BES1 and BZR1 in vitro, and further experiments with transgenic plants expressing tagged BES1 and BZR1 proteins showed that BIN2 activity negatively affected the level of these proteins in vivo (He et al., 2002; Yin et al., 2002). Moreover, the phosphorylation state and cellular localization of both BES1 and BZR1 are rapidly and significantly affected by BR treatment. Unphosphorylated BES1 and BZR1 accumulate upon BR treatment resulting in increased nuclear localization of both proteins. This nuclear localization is associated with the overexpression of several genes encoding wall-modifying enzymes involved in cell expansion and is correlated with the most actively expanding zones of dark-grown Arabidopsis hypocotyls (Wang et al., 2002; Yin et al., 2002). BES1 and BZR1 have overlapping but as yet uncharacterized functions that include positive regulation of BR signal transduction. However, they are not completely redundant, since they differ in aspects of their light-grown phenotypes and in the extent that feed-back inhibition of BR biosynthesis occurs in the two mutants.

The accumulation and nuclear localization of BES1 and BZR1 after BR treatment, and their negative regulation by BIN2, a GSK-3/shaggy kinase, is reminiscent of the interaction of GSK-3 and β-catenin in the Wingless/wnt signaling pathways. A further mechanistic parallel between the plant and animal pathways was recently demonstrated by the observation that a specific inhibitor of proteasome activity led to the accumulation of the phosphorylated form of BZR1, suggesting that phosphorylation targets BZR1 for proteasome-mediated degradation (He et al., 2002). Thus, inactivation of the BIN2 kinase by BR signal transduction would likely lead to increased levels of unphosphorylated BES1 and BZR1, which then accumulate and are localized in the nucleus to promote BR-regulated gene expression. While the mechanisms may be similar, neither BES1 nor BZR1 has any sequence homology with β-catenin.

**BR-Regulated Gene Expression**

A number of genes regulated by BR either by transcriptional or posttranscriptional mechanisms have been identified, although a BR response element and interacting protein factors have not as yet been reported (reviewed in Bishop and Koncz, 2002; Clouse and Feldmann, 1999; Friedrichsen and Chory, 2001). Plant cell expansion is critical for growth and differentiation in all organs and results from alterations in gene expression and biochemical processes that affect cell wall mechanical properties, cell hydraulics, and osmotic potential (Cosgrove, 1997). Modulating the expression of genes encoding wall-modifying proteins is one mechanism by which plant hormones such as BRs, auxins, and gibberellins promote cell elongation and many of the known BR-regulated genes encode such proteins. BRs also promote vascular differentiation and cell division, and BR-regulated genes encoding proteins associated with these cellular processes have also been identified. Downregulation of genes encoding BR biosynthetic enzymes has been demonstrated in several cases, as has BR-regulated gene expression associated with environmental adaptation, pathogen attack, assimilate partitioning, biosynthesis of other plant hormones, and translation initiation (Bishop and Koncz, 2002).

Most of the genes mentioned above were identified by classical methods of studying differential gene expression such as subtractive hybridization. More recently, DNA microarray analysis has been employed to identify several novel genes that appear to be regulated by BR signaling. Examination of BR-regulated gene expression in the bes1-1D and bri1 mutants using Affymetrix Arabidopsis GeneChip arrays verified the BR regulation of numerous genes encoding wall-modifying proteins and showed that several genes associated with auxin signal transduction are also BR regulated (Yin et
Interestingly, an independent genetic analysis of the *Arabidopsis ucu1* mutants (allelic to *bin2*) suggests that the BIN2/UCU1 kinase may also be involved in auxin signal transduction, indicating a possible cross-talk between BR and auxin, two hormones with pronounced effects on cell elongation (Perez-Perez et al., 2002). A recent genetic screen for new BR-insensitive mutants, combined with Affymetrix GeneChip analysis, has identified *bin3* and *bin5* as putative subunits of an *Arabidopsis* topoisomerase VI that regulates the expression of numerous genes, including many of those that are also regulated by BRs (Yin et al., 2002). Finally, a global expression analysis using Affymetrix GeneChips and RNA from weak alleles of BR-deficient mutants grown under two conditions has identified a core set of BR-regulated genes involved in BR biosynthesis, auxin response, nitrogen transport, and transcriptional activation (Mussig et al., 2002).

In spite of the number of genes identified as BR regulated, only one report on the analysis of a BR responsive promoter has appeared to date (Iliev et al., 2002). The *Arabidopsis TCH4* gene encodes a xyloglucan endotransglycosylase whose activity is associated with cell wall modification during elongation and morphogenesis. BR-deficient and insensitive mutants in *Arabidopsis*, which are defective in cell elongation, show much reduced *TCH4* expression (Kauschmann et al., 1996). *TCH4* gene expression is transcriptionally regulated by both BR and auxin and by environmental stimuli such as touch, darkness, and temperature (Xu et al., 1995). Induction of expression by these diverse stimuli is conferred to reporter genes by the same 102 bp *TCH4* promoter region. However, upstream regions influence the magnitude and kinetics of expression and likely harbor regulatory elements that are redundant with those located in the 102 bp region. Substitution of the proximal regulatory region sequences in the context of distal elements does not disrupt inducible expression, making the identification of the specific BR response element difficult (Iliev et al., 2002).

The use of bioinformatics to analyze upstream regulatory sequences in coregulated genes identified by microarray experiments has become quite common and effective in discovering novel promoter elements in *Arabidopsis*. For example, Harmer et al. (2000) identified 450 circadian regulated genes using the Affymetrix GeneChip. Further analysis of upstream promoter regions in these genes revealed an absolutely conserved motif, AAAAAATCTCT, occurring 46 times in the promoters of 31 cycling genes. Experiments with transgenic plants showed that this “evening element” was required for rhythmicity of luciferase reporter gene expression. Since a large number of BR-regulated genes are becoming available through microarray analysis, such an approach may be very productive in identifying a BR response element.

Conclusions and Future Prospects

The emerging picture of BR signal transduction reveals a pathway that combines some of the mechanisms and signaling logic of several individual pathways in vertebrates and invertebrates (Figure 3). The ligand itself is closely related structurally to animal steroid hormones but apparently is not perceived by intracellular nuclear receptors. Instead, BR perception is at the cell surface and involves at the very least a leucine-rich repeat receptor kinase, BRI1. Ligand-induced or stabilized oligomerization is the norm in animal receptor kinases and has been demonstrated in other plant receptor kinases as well. The discovery of BAK1 and the genetic and biochemical evidence showing a role for BAK1 in BR signaling and a physical interaction with BRI1 is intriguing. However, direct demonstration of a BRI1/BAK1 heterodimer as a coreceptor for BR is lacking and will be required before models of BR-dependent heterodimerization and kinase activation can be confirmed.

The apparent absence of nuclear steroid receptors in plants clearly shows divergence in the evolution of steroid signaling, but perception of steroids at the cell surface by animal cells has received much greater attention in the last decade. It will be interesting to determine if any of these animal membrane steroid-binding proteins are receptor kinases or if they form heterodimers with this class of signal transduction molecules. A great deal has been learned about BR perception during the past 3 years, but much remains to be done. Identification of specific autophosphorylation sites and their BR dependence is critical along with experiments using purified extracellular domains to determine if BR binds BRI1 or BRI1/BAK1 directly or requires additional accessory proteins. Moreover, it is possible that BRI1 forms as yet undiscovered heterodimers with other receptor kinases, or receptor-like kinases, in addition to BAK1, possibly in a tissue-specific manner. Continued screens such as those uncovered BAK1 may reveal these putative partners, if they indeed exist.

After plasma membrane perception of BRs, the signaling pathway begins to resemble elements of the Wingless/wnt pathway. An unknown number of steps follow BR binding, leading to inactivation of the negative regulator BIN2, a GSK-3/shaggy kinase homolog. Inactivation of BIN2 allows the unphosphorylated form of BES1 and BZR1 to accumulate and translocate to the nucleus. These proteins are obviously involved in the regulation of BR-responsive genes, but the mechanism remains completely unknown. By analogy with β-catenin in the Wingless/wnt pathway, BES1 and BZR1 may complex with transcription factors, and yeast two-hybrid analysis may be effective in identifying such putative binding partners. Once BR response elements have been located in the promoters of BR regulated genes, it will also be possible to conduct yeast one-hybrid analysis to isolate transcription factors associated with BR signaling.

The gap between BRI1 and BIN2 represents a major deficiency in our understanding of BR signaling. Substrates for the BRI1 (and BAK1) kinase domain need to be definitively identified and their function characterized. Molecular genetic and biochemical approaches used in other receptor kinase studies to identify substrates include yeast two-hybrid analysis (Bower et al., 1996; Gu et al., 1998), interaction cloning (Stone et al., 1994), immunoprecipitation of receptor-protein complexes (Trotchaud et al., 1999), and the use of synthetic peptides to identify binding motifs and recognition con-
Figure 3. Model for Brassinosteroid Signal Transduction in Arabidopsis

BR either binds directly to BRI1 or first complexes with an unidentified steroid binding protein (SBP), which may require processing by a serine carboxypeptidase. BRI1 and BAK1 have been shown to interact in vivo, but it has not yet been verified whether or not a BRI1/BAK1 heterodimer acts as a coreceptor for BR. Ligand binding initiates a signaling cascade that inactivates the BIN2 kinase and allows accumulation and nuclear localization of the unphosphorylated forms of the positive regulators BES1 and BZR1. The BIN2 kinase shares 70% amino acid identity in the catalytic domain with GSK-3/shaggy kinases of vertebrates and invertebrates. These kinases are cytoplasmic and act to negatively regulate signaling by phosphorylating positive regulators of the pathway, targeting them for proteasome-mediated degradation. Steps that have a positive effect on signaling are shown in green, while those with a negative effect are in red. Question marks represent proposed but uncharacterized steps.

sensus sequences (Kuriyan and Cowburn, 1997). Isolation of receptor kinase substrates will allow a second round of screening for downstream interacting components. Proceeding from the opposite direction, a yeast two-hybrid screen using BIN2 as the bait may reveal components directly upstream of BIN2.

While a number of BR-regulated genes have currently been identified in Arabidopsis, preliminary microarray experiments using the 8,200 element GeneChip suggest that many more such genes are required to drive BR-promoted developmental processes that lead to normal plant growth. The recent availability of full-genome Arabidopsis GeneChips from Affymetrix (recognizing more than 24,000 distinct genes) provides a unique opportunity to characterize the full spectrum of BR-regulated genes in Arabidopsis. Like other plant hormones, the effect of BRs on growth are pleiotropic and the cataloging and functional analysis of newly discovered gene
sets may increase our understanding of the range of physiological events influenced by BRs. Comparative analysis of upstream sequences from large sets of genes uncovered by microarray analysis may yield motifs that can be tested by a variety of methods for their potential function as BR response elements. Moreover, the con-
tinued detailed promoter analysis of TCH4, a known BR-regulated gene, will also likely point to sequences necessary and sufficient for BR inducibility.

Based on the rate of recent discoveries, it is highly likely that the major steps of BR signal transduction, from cell surface perception to the activation of specific nuclear genes, will be revealed during the next few years. The dramatic phenotype of BR-deficient and insensitive mutants is clear evidence of the critical importance of BR activity in the growth and development of plants. A greater understanding of the molecular mechan-
isms of BR signal transduction will deepen our knowl-
edge of plant growth and development and will establish further similarities and contrasts between plant and ani-
mal steroid signaling pathways.

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