Molecular Mechanisms of Steroid Hormone Signaling in Plants

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Key Words

Arabidopsis, brassinosteroids, receptor, signal transduction, gene expression

Abstract

Brassinosteroids (BRs), the polyhydroxylated steroid hormones of plants, regulate the growth and differentiation of plants throughout their life cycle. Over the past several years, genetic and biochemical approaches have yielded great progress in understanding BR signaling. Unlike their animal counterparts, BRs are perceived at the plasma membrane by direct binding to the extracellular domain of the BRI1 receptor S/T kinase. BR perception initiates a signaling cascade, acting through a GSK3 kinase, BIN2, and the BSU1 phosphatase, which in turn modulates the phosphorylation state and stability of the nuclear transcription factors BES1 and BZR1. Microarray technology has been used extensively to provide a global view of BR genomic effects, as well as a specific set of target genes for BES1 and BZR1. These gene products thus provide a framework for how BRs regulate the growth of plants.

Contents

BRs: brassinosteroids

BL: brassinolide

S/T: serine/threonine

LRR-RLK: leucine-rich repeat receptor-like kinase

INTRODUCTION

Polyhydroxylated steroid hormones are widely distributed in nature. They have been identified in fungi, plants, and animals. The likelihood of an ancient origin for these molecules is underscored by the remarkable conservation in activity between plant and human forms of at least one key biosynthetic enzyme (Li et al. 1997). In recent years, many of the proteins required for steroid response in plants have been identified. Strikingly, almost every protein in the pathway appears to belong to plant-specific families, suggesting that the role of steroids as signaling molecules may have arisen multiple times on the road to multicellularity.

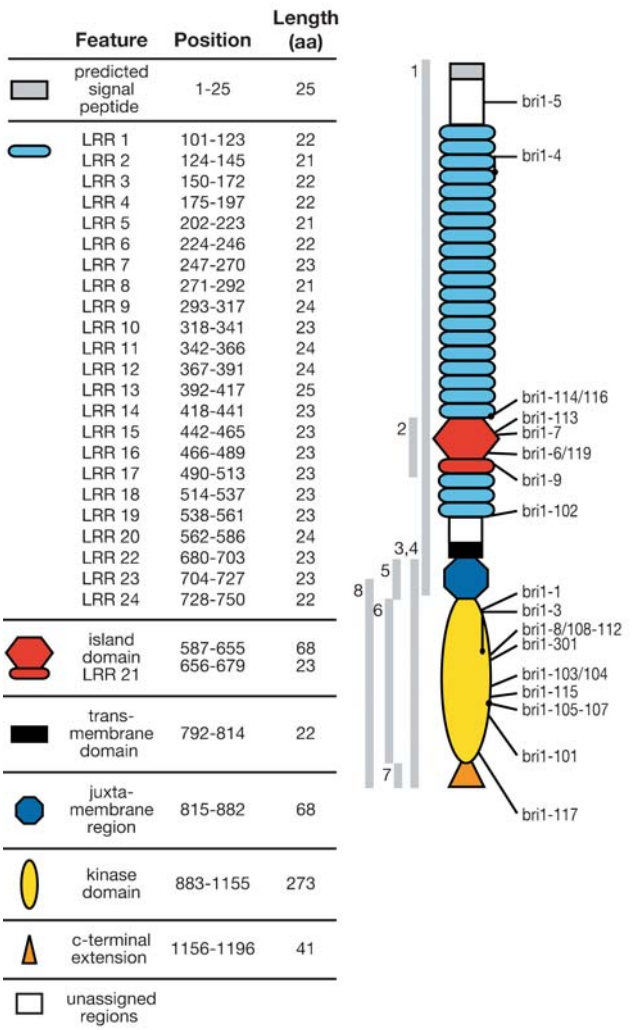
The BRs are important regulators of growth and differentiation in plants. BR biosynthesis is fairly well understood as a result of the identification of many BR-deficient dwarf mutants and numerous feeding experiments in cultured cells (Fujioka & Yokota 2003). In the past few years, tremendous progress has been made in *Arabidopsis* in understanding how BRs are perceived and how the information is transduced to promote genomic responses (Clouse 2002, Peng & Li 2003). In this review, we present a critical analysis of currently available data on BR signaling pathway components, highlighting the latest findings on the cell surface-localized BR receptor and on the specific control of gene expression by a novel family of transcription factors.

LIGAND PERCEPTION AND RECEPTOR ACTIVATION

Brassinosteroids are Perceived by a Receptor Serine/Threonine Kinase

In contrast to animal steroid signals, BRs are perceived by a plasma membrane-localized receptor kinase. This kinase is encoded by the *BRI1* gene, which was initially identified as a BL-insensitive mutant (Clouse et al. 1996) and is defined by a large number of recessive mutations (**Figure 1**). *bri1* mutants display a light-grown morphology in the dark, show extremely dwarfed growth in the light, and have numerous other phenotypes, all of which are also seen in strong BR biosynthetic mutants.

BRI1 is part of a large, plant-specific family of S/T LRR-RLKs, consisting of more than 200 members in *Arabidopsis* (Shiu & Bleecker 2001). The BRI1 extracellular region consists of more than 20 LRRs, interrupted by a stretch of amino acids termed the island domain. Initial annotations predicted a putative N-terminal leucine-zipper followed



Allele	Base pair change	Amino acid change	Allelic strength	Accession
bri1-1	GCT to ACT	Ala-909 to Thr	strong	Col-0
bri1-3	4-bp deletion	STOP 44 aa downstream	strong	Ws-2
bri1-4	10-bp deletion	STOP 13 aa downstream	strong	Ws-2
bri1-5	TGC to TAC	Cys-69 to Tyr	weak	Ws-2
bri1-6, 119	GGC to GAT	Gly-644 to Asp	weak	En-2
bri1-7	GGT to AGT	Gly-613 to Ser	weak	Ws-2
bri1-9	CTT to TTT	Ser-662 to Phe	weak	Ws-2
bri1-101	GAG to AAG	Glu-1078 to Lys	strong	Col-0
bri1-102	ACT to ATT	Thr-750 to Ileu	strong	Col-0
bri1-103, 104	GCG to ACG	Ala-1031 to Thr	strong	Col-0
bri1-105-107	CAA to TAA	Gln-1059 to STOP	strong	Col-0
bri1-8/108-112	CGG to CAG	Arg-983 to Gln	inter-mediate	Ws-2
bri1-113	GGA to GAA	Gly-611 to Glu	strong	Col-0
bri1-114,116	CAA to TAA	Gln-583 to STOP	strong	Col-0
bri1-115	GGT to GAT	Gly-1048 to Asp	strong	Col-0
bri1-117,118	GAT to AAT	Asp-1139 to Asn	strong	Col-0
bri1-301	not published	Gly-989 to Ile	weak	Col-0

Figure 1

(*Continued on next page*)

by 25 LRRs, with the island domain residing between repeats 21 and 22 (Li & Chory 1997). For this review, we have reannotated BRI1; the new annotation no longer predicts a leucine zipper. Furthermore, it now appears that BRI1 has 24 rather than 25 LRRs, with LRR21 (formerly LRR22) being an unusual methionine-rich repeat (**Figure 1**). The intracellular region can be subdivided into a JM, followed by a canonical S/T kinase and a short C-terminal extension (**Figure 1**). Thus by its overall structure, BRI1 is an archetypal receptor kinase (Li & Chory 1997), and several lines of evidence established BRI1 as a critical and limiting component for BR binding and perception. *BRI1* overexpression increases the number of BL binding sites, and this binding activity can be precipitated using specific antibodies (Wang et al. 2001). In competition experiments, binding affinities of these sites correlate with the bioactivity of the respective compounds. The

JM: juxtamembrane region

	Peptide aa positions	Number of sites	Possible positions
1	825-841	1	S-838
2	842-854	2	T-842, T-846
3	855-869	1	S-858
4	870-874	1	T-872
5	886-895	1	S-887, S-891
6	978-983	1	S-981, T-982
7	1038-1062	3	T-1039, S-1042, S-1044, T-1045, T-1049, S-1060
8	1165-1171	1	S-1166, S-1168, T-1169
9	1172-1189	1	S-1172, S-1179, T-1180
10	n.a.	1	S-1162
11	n.a.	1	T-1180

Kinase sub-domains and functional regions	aa position	Length
I	883-903	21
II	904-918	15
III	919-934	16
IV	935-949	15
V	950-980	30
VIa	981-1003	23
VIb	1004-1020	17
VII	1021-1037	17
VIII	1038-1058	21
IX	1059-1085	27
X	1086-1106	21
XI	1107-1149	43
ATP-binding signature	889-912	24
catalytic loop	1007-1014	8
activation loop	1027-1056	30

Number	Position	Length (aa)	Description	Reference
1	1-879	879	extracellular/ transmembrane/ juxtamembrane region (NGT-1)	He et al., 2000
2	580-673	94	BL-binding region (Island+LRR22)	Kinoshita et al. 2005
3	815-1196	382	region described as BRI1-KD or JKC	Oh et al., 2000 Wang et al. 2005
4	814-1196	383	region used for kinase and interaction assays	Li et al., 2002
5	815-882	68	juxtamembrane region	Wang et al. 2005
6	883-1155	273	kinase domain	Wang et al. 2005
7	1156-1196	41	c-terminal region	Wang et al. 2005
8	847-1196	350	region used for yeast-two-hybrid	Nam and Li, 2002

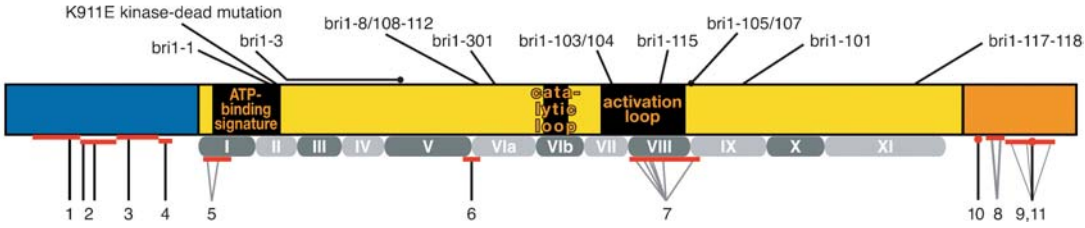


Figure 1

Compilation of BRI1 data. Features were predicted using the following web resources. Signal peptide and transmembrane domain: **www.cbs.dtu.dk/services/**; LRR repeats and kinase regions: **www.ebi.ac.uk/InterProScan/**. LRR repeats were refined manually. Kinase subdomains are based on Hanks & Hunter (1995). Alignments with cAPK (NP_00,2721), resources at **http://pkr.sdsc.edu/** and MSAs at **www.botany.wisc.edu/prkr/** were used to define kinase subdomain borders. Phosphorylation data are from Oh et al. (2000) and Wang et al. 2005. BRI1 mutation data are from Friedrichsen et al. (2000), Noguchi et al. (1999) and Nam & Li (2002). (Tables can be downloaded from Table S1. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**).

BRI1 N-terminal region, consisting of the extracellular domain, the transmembrane pass, and the JM (**Figure 1**), was shown to function as a BR signal-transducing module (He et al. 2000). By fusing this region to the kinase domain of a rice LRR-RLK involved in pathogen defense, BL-inducible defense responses could be transferred to transformed cells. However, because these experiments were done in rice, the presence of additional plant-specific factors involved in BR binding could not be excluded.

Recently, direct binding of BL to BRI1 was demonstrated with native and recombinant BRI1 proteins (Kinoshita et al. 2005). It was shown that a BR analog could be cross-linked to BRI1, both in microsomal preparations and in pull-down fractions highly enriched for BRI1-GFP, indicating that BRs and BRI1 directly interact. Moreover, recombinant proteins consisting of the island domain and the neighboring C-terminal LRR repeat (**Figure 1**) were sufficient to bind radioactive BL with an affinity comparable to that observed for full-length BRI1 from plants. In addition to BRI1, three highly similar homologs have been characterized (Cano-Delgado et al. 2004, Clay & Nelson 2002, Zhou et al. 2004). Two display high BL-binding affinity. Genetic analysis suggests that these receptors play a restricted and partially redundant role in BR signaling. Thus BRI1 apparently represents the single most important BR binding activity in *Arabidopsis*. Recently, the *BRI1* ortholog in tomato was shown to act as the receptor for systemin as well as for BRs (reviewed in Wang & He 2004). However, systemin, a small peptide signal involved in plant defense, is present only in a subgroup of higher plants, not including *Arabidopsis*. Why BRI1 was co-opted for this dual role is not known.

Possible Mechanisms of BRI1 Activation

How is ligand binding transduced across the membrane? Since the cloning of *BRI1*, numerous analogies to animal receptor pathways have been drawn (Peng & Li 2003, Wang & He 2004, Yin et al. 2002c). Such comparisons are inevitable and potentially useful. However, many receptor pathways have developed during the independent acquisition of multi-cellularity in plants and animals, and it is possible that mechanistic similarities between the BR and animal receptor pathways might merely represent random evolutionary convergences.

In animals, ligand-induced activation of single-pass transmembrane receptors is often associated with dimerization or multimerization of the receptor with itself and/or coreceptors. In mammalian cells, ligand-induced oligomerization was proposed to initiate downstream signaling by bringing intracellular kinase domains together and allowing their *trans*-phosphorylation (Schlessinger 2000). In many cases, however, this simple "induction by dimerization" model does not appear to apply. The insulin receptor, for example, exists as a constitutive, ligand-independent dimer (Jiang & Hunter 1999). Pre-formed dimers of epidermal growth factor (EGF) receptors were shown to exist in vivo (Gadella & Jovin 1995) and structures of the receptor's extracellular domain suggest a model whereby EGF binds with high affinity to dimeric receptor forms, leading to their stabilization rather than inducing their formation (Ferguson et al. 2003, Garrett et al. 2002, Ogiso et al. 2002). Current models suggest that ligand binding induces a reorientation of subunits with respect to each other. For the EGF receptor, some evidence supports a rotational rearrangement of subunits (Moriki et al. 2001). For Epo, another receptor tyrosine kinase, a scissor-like activation mechanism has been put forward (Jiang & Hunter 1999). In summary, it appears that dimerization is required but not sufficient for activation of single-pass transmembrane receptor kinases.

BAK1, an LRR-RLK with five extracellular LRRs, is a candidate for BRI1's coreceptor. BAK1 was independently found as a

FRET: fluorescence resonance energy transfer

gain-of-function suppressor of a weak allele of *bri1*, as well as a BRI1 yeast-two-hybrid interactor (Li et al. 2002, Nam & Li 2002). BRI1 and BAK1 expressed in yeast interact with each other and are able to mutually *trans*-phosphorylate. The phenotypes of BAK1 knockouts and kinase-dead, dominant-negative variants are consistent with its role as a positive component of BR-signaling. However, knockout phenotypes of *BAK1* are rather subtle compared with *BRI1* knockouts, indicating that BAK1 is either not strictly required or functions redundantly with the four other members of its subfamily (Hecht et al. 2001). It will be important to determine if multiple knockouts will eventually give rise to a *bri1*-like phenotype. Neither knockout nor overexpression of *BAK1* influences ligand binding to BRI1 (Kinoshita et al. 2005, Wang et al. 2005). Thus current data suggests that BAK1 is a coreceptor and/or downstream target of BRI1.

Recently, self-interaction of BRI1 was demonstrated by FRET using cell-culture transfection assays and by pull-down experiments in transgenic plants (Russinova et al. 2004, Wang et al. 2005). BR effects on interaction were not tested in the FRET experiments, but the pull-down experiments showed that BRI1 interaction increases upon BL treatment. In the future, it will be critical to address the oligomerization status of BRI1 and BAK1 together, in a functional but non-stimulated plant cell system. This can be done in the background of strong biosynthetic mutants or in the presence of high concentrations of BR biosynthetic inhibitors.

Thus initial BL binding to the island-LRR domain of BRI1 may occur on BRI1 monomers or with a preformed homo-oligomer. Current data cannot exclude the possibility that BL binds to a BRI1, which is part of a BRI1-BAK1 hetero-dimer or hetero-tetramer. The fact that BRI1 and BAK1 interact in yeast in the absence of ligand suggests that there may be pre-existing hetero-dimers or tetramers. For the animal BMP-receptors, all possible modes of receptor/coreceptor interaction states have been demonstrated in the absence of ligand (Gilboa et al. 2000). BL is a relatively small molecule compared with the ligands of most animal receptor kinases, and it is hard to imagine how BL could bridge two receptor molecules via bivalent interaction, as has been shown for ligands in animals (Schlessinger 2000, Wiesmann & de Vos 1999). To our knowledge, the family of TOLL-like receptors (TLRs) (but not TOLL itself) is the only example where smaller molecules (bacterial components) activate a single-pass transmembrane receptor in animals (Akira & Takeda 2004). Unfortunately, not much is known about the activation mechanism of TLRs. Rather than bridging subunits, BL could induce a conformational change that stabilizes a pre-existing dimer, as discussed for the EGF receptor. A consequent conformational change would then reorient the kinase subunits and allow for initial *trans*-phosphorylation, either between BRI1 subunits or between BRI1 and BAK1. *Trans*-phosphorylation is considered to be the critical initial event in receptor kinase activation, releasing the kinases from an auto-inhibited state of low activity (Hubbard 2004).

What is known about the regulation of BRI1 kinase activity? It has been demonstrated that BL treatment leads to BRI1 phosphorylation in planta (Wang et al. 2001, Wang et al. 2005). In yeast, BAK1/BRI1 *trans*-phosphorylation activity is apparently interdependent since neither of the two proteins can be phosphorylated if one is in its kinase-dead form (Nam & Li 2002). Slightly different results were obtained using recombinant BRI1 and BAK1 kinases (Li et al. 2002). The cytosolic parts were shown to interact and *trans*-phosphorylate each other, even if one partner was inactive, although not as efficiently. Thus isolated intracellular domains seem to be less dependent on each other than are full-length proteins. This might be explained by some topological restraints imposed on the full-length proteins. Therefore, back-and-forth signaling between BRI1 and

BAK1 is possibly needed for full activation of both.

In order to be activated, many kinases require phosphorylation in their activation loop, which increases kinase activity by a number of mechanisms (Johnson et al. 1996). The BRI1 kinase contains all the signatures of an activation-loop-dependent kinase, and it was shown that S/T residues in the activation loop are subject to auto-phosphorylation (Oh et al. 2000; **Figure 1**), suggesting that this might be an initial activation event for BRI1. Additional mechanisms of receptor auto-inhibition have been described, namely inhibition by C-terminal extensions or JM regions of the cytosolic domain (Hubbard 2004). Small insertion/deletions in the JM region of the RTK KIT, for example, lead to ligand-independent receptor activity (Hirota et al. 1998). Deletion of the BRI1-JM results in an inactive receptor, precluding conclusions about a possible role in auto-inhibition (Wang et al. 2005). Nonetheless, the JM domain is subject to BRI1 auto-phosphorylation in vitro (**Figure 1**), and it will be interesting to see if an in vivo function can be assigned to these phosphorylation sites.

The C-terminal extension of BRI1 appears to have an auto-inhibitory function (Wang et al. 2005). A BRI1 C-terminal deletion construct is functional and slightly hyperactive in vivo. Moreover, the deletion variant is less dependent on ligand, and a kinase domain lacking the C terminus displays increased kinase activity in vitro. The C terminus is phosphorylated at multiple sites (**Figure 1**), and "phosphorylation-mimic" mutations have similar effects as deleting the domain. Taken together, these results provide a first clue of how BRI1 kinase is auto-inhibited and activated by phosphorylation. However, because there is still a clear ligand dependency of C-terminally deleted BRI1, other mechanisms must provide additional layers of regulation. A fully phosphorylated receptor kinase will either directly phosphorylate downstream targets or simply interact with them, thereby recruiting them to their site of action.

Downstream Targets of BRI1

The direct targets of BRI1 in vivo are not known, but several candidates exist. As discussed, BAK1 and its homologs may be the main direct targets that initiate signaling events that ultimately inactivate the downstream kinase BIN2 (see below). Therefore, identifying BAK1 interaction partners promises to further our understanding of BR signaling. The second candidate for a direct BRI1 target, transthyretin-like protein (TTL), was identified in a yeast-two-hybrid with BRI1 (Nam & Li 2004). The interaction depends on BRI1 kinase activity, and TTL is phosphorylated by BRI1 in vitro. Genetic analysis, however, suggests that TTL is a negative modulator of BRI1 signaling. TTL is largely or completely associated with membranes. Therefore, TTL could be involved in recruitment of deactivating phosphatases or be necessary for receptor down-regulation.

BRI1 Deactivation

Understanding receptor deactivation is as important as understanding its activation because speed and mode of inactivation will determine the amplitude and duration of ligand-induced signaling. Virtually nothing is known about how the activated BRI1 receptor is turned off. Co-overexpression of BRI1, together with BAK1, in cowpea protoplasts leads to dramatic shifts of BRI1 localization toward endosomal compartments, and FRET between BRI1/BAK1 preferentially occurs in endosomes and at restricted plasma membrane sites. This suggests that BAK1 might somehow regulate BRI1 endocytosis (Russinova et al. 2004). It remains to be seen how this finding relates to the mechanism of BR signaling in planta and to BRI1 deactivation. Enzymes catalyzing inactivating hydroxylation reactions on BRs have been identified and shown to be important in BR homeostasis in vivo (Neff et al. 1999). If and how these enzymes act in deactivating receptor-bound BL is unknown. In animals, receptors can be

GSK3: glycogen synthase kinase-3

inactivated by pH-dependent ligand separation in the acidic endosomal compartments (Rudenko et al. 2002). This is unlikely to occur in plants, however, as the extracellular space already has a low pH. Therefore, a possible ligand/receptor separation in endosomes would have to occur by a different mechanism. Studies of BRI1 endocytosis and its turn-over rates upon ligand binding will help us to understand how BRI1 deactivation is achieved.

SIGNAL TRANSDUCTION

BIN2, a GSK3 Kinase Critical for BR Signaling

Downstream from BRI1/BAK1, a major signaling component in the BR pathway is defined by semidominant *bin2* gain-of-function mutations. These mutants are allelic to *dwarf12* (Choe et al. 2002) and *ucu1* (Perez-Perez et al. 2002), uncovered in genetic screens for BR-related dwarfism and altered leaf morphology, respectively. *bin2* mutants resemble *bri1* mutants, but are distinguished from *bri1* mutants by an extreme downward curling of the leaves. As in *bri1* mutants, the feedback down-regulation of the BR-biosynthetic gene *CPD* is lost in *bin2* (Choe et al. 2002, Li et al. 2001), accounting for the higher accumulation of BL and its precursors (Choe et al. 2002).

BIN2 encodes a protein kinase, 70% similar in its catalytic domain to the mammalian GSK3 (Choe et al. 2002, Li & Nam 2002, Perez-Perez et al. 2002). GSK3s are a group of highly conserved constitutively active S/T kinases implicated in numerous signaling pathways and controlling metabolism, cell fate determination, and tissue patterning in various organisms.

BIN2 is a negative regulator of the BR pathway. With a dominant mutant, unambiguous assignment of the affected gene to a given pathway is more difficult than with loss-of-function alleles. Indeed, as animal GSK3s are known to be fairly promiscuous in their substrates, a gain-of-function mutation in one family member could interfere with substrates of other GSK3s or unrelated kinases. Gene dosage analyses revealed that the *bin2-1* mutation was either hypermorphic or neomorphic (Li et al. 2001), whereas the *ucu1* mutation was likely to be antimorphic (Perez-Perez et al. 2002), although several studies argue in favor of the first hypothesis.

Three lines of evidence suggest that increased activity of BIN2 negatively regulates BR signal transduction. First, treatment of plants with Li^+, a known inhibitor of GSK3 (Klein & Melton 1996), provokes cell elongation and shows the typical BR-feedback down-regulation of *CPD* expression (J. Li, unpublished results), as well as dephosphorylation of a BIN2 substrate, BES1 (S. Mora-Garcia, unpublished results). This clearly indicates that one physiological function of GSK3s is to negatively regulate BR signaling. Second, BIN2 protein carrying the original *bin2-1* mutation displays a higher kinase activity in vitro toward both a GSK3-peptide substrate (Li & Nam 2002) and its substrate BES1 (Zhao et al. 2002), compared with activity of the wild-type BIN2 protein. Finally, overexpression of *BIN2* in the sensitized genetic background of a weak *bri1* mutant leads to either (*a*) severe dwarfing in plants with increased levels of *BIN2* or (*b*) wild-type-like plants resulting from co-suppression of endogenous *BIN2* (Li & Nam 2002). Though these observations point to a negative role of GSK3s in the BR signaling pathway, the function of BIN2 itself remains somewhat unresolved and will await the identification of a loss-of-function mutant for *BIN2*.

Are other GSK3s involved? Although well-characterized in animals, very little is known about plant GSK3s. In *Arabidopsis*, *BIN2* belongs to a 10-member family organized in four phylogenetic subclasses (Jonak & Hirt 2002). Plant GSK3s show a highly conserved S/T kinase domain, but divergent N- and C termini. The function of most

GSK3s remains largely unknown and may not be restricted to specific pathways. In mammals, GSK3β is indeed involved in diverse cellular processes such as phosphorylation of glycogen synthase and β-catenin in the insulin and Wnt signaling pathways, respectively, yet no cross-talk is observed between the two pathways.

Several lines of evidence suggest that plant GSK3s are involved in stress responses and developmental processes (Jonak & Hirt 2002). Interestingly, *BIN2* has been shown to be expressed and restricted to the suspensor cells and excluded from the hypophysis (Dornelas et al. 1999). Whether this specific expression pattern carries a BR-related function is unknown. Genetic evidence suggests a stress involvement for *ASK*τ (a close *BIN2* relative) that is ABA- and salt-induced and whose overexpression in plants enhances salt tolerance (Piao et al. 1999). It is not yet known if *ASK*τ acts in BR signaling, but it could represent a molecular link between BRs and their reported role in salt-stress tolerance (Anuradha & Rao 2001). Uncovering the degree of redundancy and specialization within the plant GSK3s awaits in-depth genetic and biochemical investigation.

Atypical regulation of BIN2 activity. In animals, GSK3s are usually constitutively active enzymes, tightly regulated by two major mechanisms: phosphorylation and protein-protein interactions.

Phosphorylation. Many GSK3 substrates need to be prime-phosphorylated by a different kinase at position $n + 4$ before being phosphorylated at position n by GSK3s. Also, GSK3s themselves are regulated by phosphorylation. For example, upon insulin binding to its receptor, protein kinase B (PKB)/AKT phosphorylates GSK3s at a highly conserved N-terminal serine residue (Cross et al. 1995). This mimicks a prime phosphorylation and therefore turns the GSK3 N terminus into a pseudosubstrate, blocking access to its catalytic site.

Multiprotein complex. The best characterized example is the canonical Wnt pathway, where GSK3β-binding proteins control access to its substrate β-catenin, generating a high degree of specificity in regulating GSK3β. In the absence of stimulus, the scaffold protein axin binds GSK3β and β-catenin, triggering the phosphorylation of β-catenin and thereby promoting its ubiquination and subsequent degradation by the proteasome (Aberle et al. 1997). Upon Wnt binding by the Frizzled family receptor, the GSK3-binding protein FRAT facilitates the disruption of the GSK3β-containing complex. This decreases the phosphorylation of β-catenin, which results in β-catenin accumulation and activation.

At present, the biochemical characterization of plant GSK3s is scarce. The absence of both plant PKB and of the highly conserved N-terminal serine residue in BIN2 suggests that BIN2 is regulated by a different mechanism than the one seen for insulin. Moreover, BIN2 activity has been shown to act following a new docking mechanism independently of prime phosphorylation and of a multiprotein complex formation (Zhao et al. 2002).

Neither BRI1 nor BAK1 physically interacts with or phosphorylates BIN2 (Li & Nam 2002, Peng & Li 2003), suggesting additional steps in the pathway. Out of seven alleles of *bin2/ucu1/dwf12* identified, six are gain-of-function mutations that cluster in the four-residue threonine-arginine-glutamic acid-glutamic acid (TREE) domain, highlighting its importance in BIN2 function (Choe et al. 2002, Li & Nam 2002, Perez-Perez et al. 2002). The TREE domain is part of a short α-helix at the surface of the protein (Peng & Li 2003) and could be part of a phosphorylation site for CK2. CK2 indeed phosphorylates a S/T residue in an environment of acidic residues (Meggio & Pinna 2003). In this sense, the different *bin2/ucu1/dwf12* mutations would affect either the target residue or its environment by substituting basic residues for acidic ones.

BRZ: brassinazole

Investigating a possible role for CK2 in the BR-signaling pathway may shed some light on how this key kinase is regulated by BRs.

BES1/BZR1, Two Nuclear Downstream Components of BR Signaling

Two independent genetic screens identified homologous proteins acting as positive regulators of the BR signaling pathway. The *bzr1* mutant was identified as resistant to the BR-biosynthesis inhibitor BRZ in the dark (Wang et al. 2002). A suppressor screen of a weak *bri1* allele identified the *bes1* mutant, which not only suppresses the dwarf phenotype of *bri1* but also leads to constitutive BR responses (Yin et al. 2002b). *BES1* and *BZR1* encode plant-specific proteins that are 88% identical at the amino acid level. BES1 and BZR1 belong to a family of six closely related members with unknown function in *Arabidopsis*. All contain a bipartite nuclear localization signal, a central region rich in S/T, including many consensus phosphorylation sites for GSK3s, and a proteolysis-related PEST domain that encompasses the same P to L substitution in both mutants.

BES1 and BZR1 are positive regulators. BES1 and BZR1 proteins exist as two different forms, visualized as a slow- and a fast-migrating band on a Western blot, corresponding to a difference in the phosphorylation status of the two proteins (He et al. 2002, Yin et al. 2002b). Following BL treatment, only the hypophosphorylated form of both proteins is detected, accumulating to higher level compared with that in non-treated plants. This post-transcriptional regulation by BR was recently shown for four other members of the BES1 family (Yin et al. 2005). Fusion of BES1 and BZR1 to fluorescent proteins indicates that the accumulation of the hypophosphorylated form of both proteins following BL treatment correlates with their accumulation in the nucleus (Wang et al. 2002, Yin et al. 2002b). The hyperphosphorylated form of BZR1 is stabilized in the presence of the proteasome inhibitor MG132, suggesting that the phosphorylation of BZR1 increases its degradation by the proteasome (He et al. 2002). In this sense, the respective mutations would uncouple the phosphorylation of both BES1 and BZR1 from their degradation. Phosphorylation appears necessary, but not sufficient, for the degradation of both proteins as both forms are detected in the cell under normal conditions. An additional modification of BES1 and BZR1, which could be from additional phosphorylations, may be required to efficiently target them for degradation. Consistent with this, the hyperphosphorylated form of the mutated BZR1 protein migrates as a faster band compared with that of the wild-type hyperphosphorylated BZR1 (He et al. 2002). These findings support a model where the BL-dependent accumulation of BES1 and BZR1 in their hypophosphorylated forms is regulated by a negatively acting kinase via proteasome degradation.

Although *bes1* and *bzr1* are gain-of-function mutations, several results argue for their specific involvement as positive regulators in the BR-pathway (He et al. 2002, Wang et al. 2002, Yin et al. 2002b, Zhao et al. 2002). Recently a loss-of-function dwarf phenotype was reported from RNAi knock-down plants for *BES1* and its relatives, further supporting the redundant role of these proteins in BR signaling (Yin et al. 2005).

One key question is why *bes1* and *bzr1* mutants, which share the same lesion in virtually identical proteins and result in similar BRZ-resistant phenotypes in the dark, exhibit opposite phenotypes in the light. For example, in the light, *bes1* displays constitutive BR responses, including long, bending petioles and pale green leaves reminiscent of *DWF4* or *BRI1* overexpressing plants (Choe et al. 2001, Wang et al. 2001). In contrast, *bzr1* displays a semidwarf phenotype and increased sensitivity to BRZ. *bzr1* shows reduced expression of the biosynthetic gene *CPD* (Wang et al. 2002),

a difference that may account for such phenotypic observations.

BES1 and BZR1: actual substrates of BIN2? BES1 and BZR1 proteins were shown to exist as two different forms and to specifically accumulate the hypophosphorylated form as early as 10 min after BL treatment (He et al. 2002). *bes1* and *bzr1* gain-of-function mutations, as well as *BES1* and *BZR1* overexpression, suppress the *bin2* dwarf phenotype, suggesting that BES1 and BZR1 act downstream from BIN2 (He et al. 2002, Yin et al. 2002b, Zhao et al. 2002). BIN2 was shown in vitro to interact with and to phosphorylate BES1 and BZR1 (He et al. 2002, Yin et al. 2002b, Zhao et al. 2002). Moreover, BES1 and BZR1 protein levels are low in the *bin2* gain-of-function background (Wang et al. 2002, Yin et al. 2002b). Finally, the drastic deletion of the central region of BES1, which contains the putative GSK3 phosphorylation sites, gives rise to constitutive BR responses (Yin et al. 2005). The next challenge will be the identification of the precise sites in BES1 and BZR1 that are phosphorylated by BIN2 in vivo and determining how this correlates with their biological activity.

BSU1, A Nuclear Phosphatase Promoting BES1 Dephosphorylation

A *bri1* suppressor screen by activation tagging led to the identification of the *bsu1-1D* mutant (Mora-Garcia et al. 2004). *BSU1* encodes a plant-specific protein with a long, Kelch-repeat-containing N-terminal region hooked up to a C-terminal S/T phosphatase domain. *bsu1* partially suppresses the dwarf phenotype of the *bin2* mutant. In addition, BES1 accumulates in its hypophosphorylated form in *bsu1* mutants, and in vitro BIN2-phosphorylated BES1 is dephosphorylated in the presence of BSU1 protein. Finally, RNAi knock-down plants show a compact phenotype resembling weak *bri1* alleles, providing additional support for a model where BSU1 directly counters the effects of BIN2 on BES1, and likely BZR1 (**Figure 2**).

Cracking the Code of the BES1/BZR1 Signaling Mechanism

Three distinct BR effects have been described for BES1/BZR1.

Dephosphorylation. The rapid conversion of the pool of BES1 to its hypophosphorylated form correlates with the first measurable changes in transcription of BR-responsive genes. The robustness of this response makes the disappearance of the hyperphosphorylated form of BES1 the best marker for BR signaling.

Accumulation. In some cases, a clear overall increase in BES1 protein levels can be observed, whereas in other experiments the total amount of protein appears unchanged, although shifted to the hypophosphorylated form. BES1 accumulation may reflect conversion of the BES1 pool to the more stable hypophosphorylated BES1 rather than to active stabilization, suggesting a minor role for protein accumulation in BR signaling.

Nuclear translocation. Nuclear accumulation of a BES1-GFP fusion protein was reported following BR treatment (Yin et al. 2002b). These data were interpreted as evidence of a nuclear translocation correlating with a shift from hypo- to hyperphosphorylated form, by analogy with what is known for β-catenin in the canonical Wnt signaling pathway (**Figure 3*a***). However, the data would also be consistent with stabilization of a constitutively nuclear protein. In agreement with this idea, mBES1 and mBZR1 mutant proteins, known to accumulate high levels of both hypo- and hyperphosphorylated forms, are detected exclusively in the nucleus (Wang et al. 2002, Yin et al. 2002b). One report described BES1 and BZR1 as constitutively nuclear proteins (Zhao et al. 2002), which also correlates with the nuclear localization

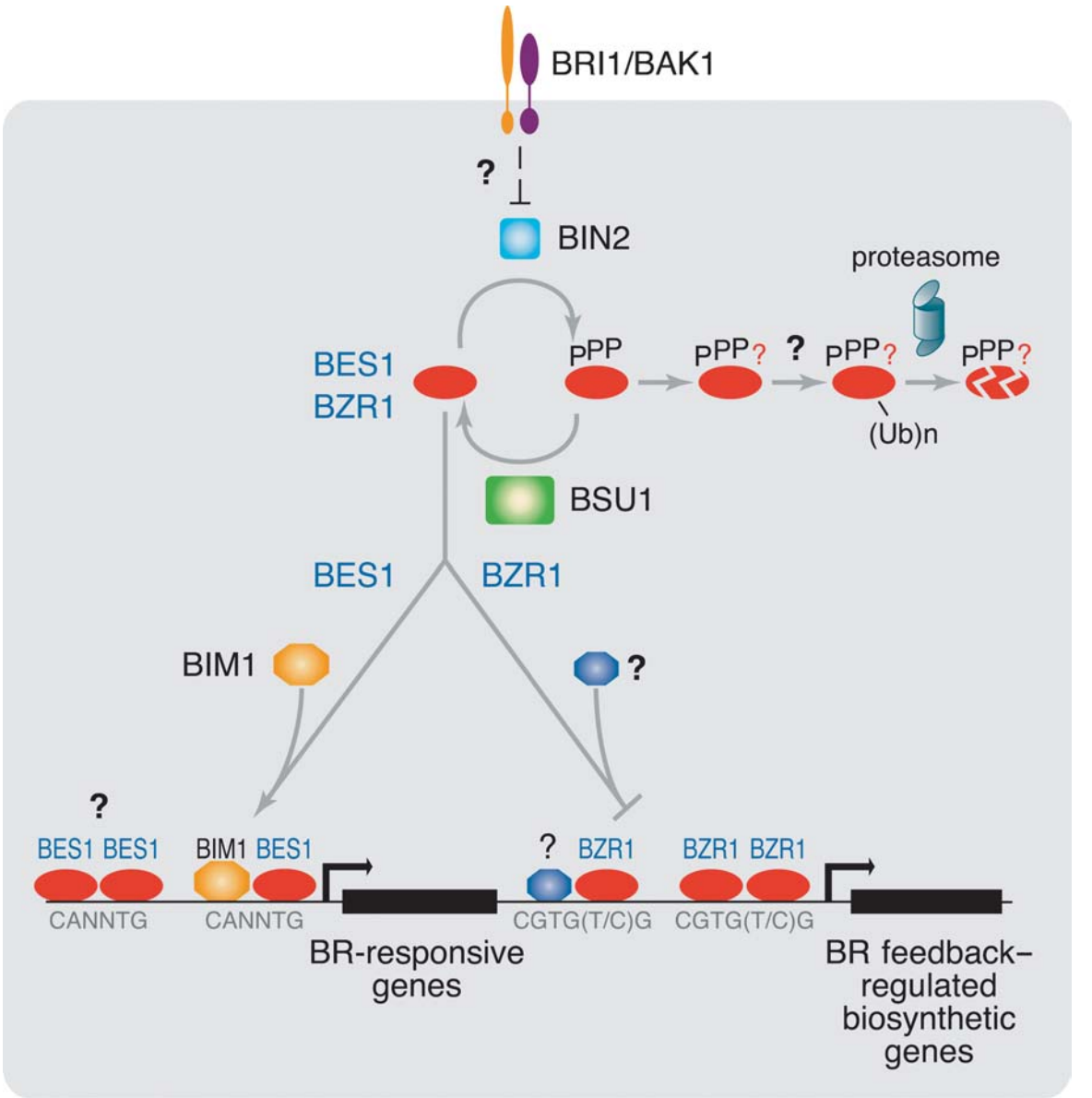


Figure 2

A model for downstream events in the BR signal transduction pathway. In resting cells, the BIN2 GSK3 kinase is active and phosphorylates the transcription factors BES1 and BZR1, targeting them for ubiquitination and subsequent proteasome-dependent degradation. In BR-stimulated cells, BRI1/BAK1 inhibits BIN2 and/or activates BSU1 activities by a yet unknown mechanism, leading to the conversion of the BES1/BZR1 pool to the hypophosphorylated form. BES1, in association with the bHLH transcription factor BIM1, promotes transcription of a subset of BR-regulated genes by binding to E-box motifs, CANNTG. BZR1 directly represses the transcription of BR feedback–regulated genes such as *CPD* to adjust BR homeostasis by binding to CGTG(T/C)G elements.

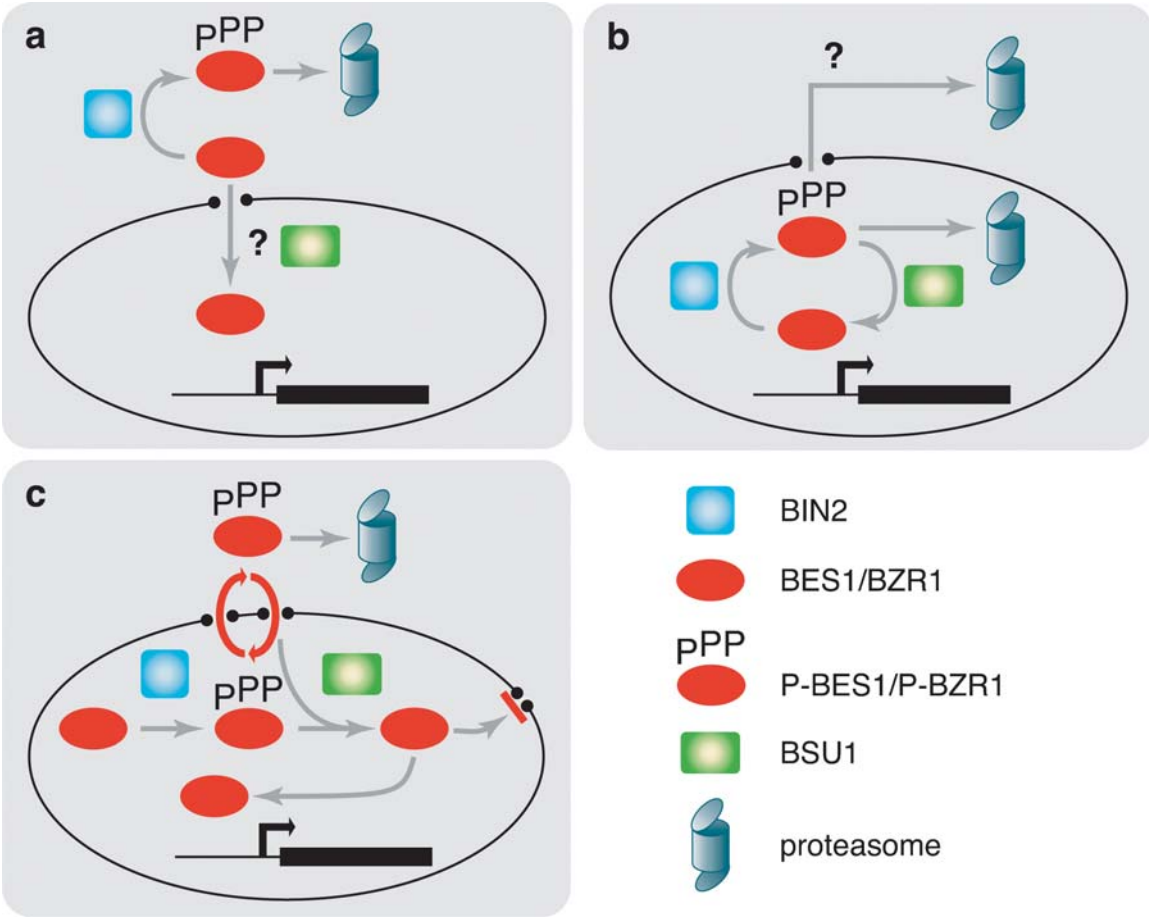


Figure 3

Models for downstream signaling. (*a*) Original nuclear translocation model. BIN2 phosphorylates BES1 and BZR1 in the cytosol. Upon BR stimulation, hypophosphorylated BES1 and BZR1 shuttle to the nucleus to promote BR responses (*b*) Nuclear model. BIN2, BES1, and BZR1 are constitutively in the nucleus; the activity of BES1 and BZR1 is primarily regulated by their phosphorylation status (*c*) Alternative nucleocytoplasmic model. Hyperphosphorylated BES1 and BZR1 are constantly cycling between the cytosol and the nucleus. Stimulation by BR triggers conversion of BES1 and BZR1 to their hypophosphorylated forms. This may lead to a greater affinity to DNA and/or prevent the two transcription factors from exiting the nucleus, thereby accounting for their accumulation in the nucleus.

of BSU1 (Mora-Garcia et al. 2004). Importantly, the subcellular localization of BIN2 is unknown to date and could help to solve this issue. Its placement in the cytosol was based on analogy with the Wnt pathway without supporting evidence, although there is evidence that plant GSK3s can be localized in the nucleus (Tavares et al. 2002).

These observations raise important questions about the overall design of the pathway and suggest that phosphorylation is the primary mode of regulation of BES1 protein activity. We therefore present a second model where BES1 phosphorylation and dephosphorylation events would occur exclusively in the nucleus, assuming that BIN2 could be

localized in this compartment (**Figure 3*b***). Whether the degradation of BES1 happens in the nucleus or in the cytosol is unknown, but nucleocytoplasmic transport and subsequent degradation in the cytosol could be involved, as described for p53, for instance (Liang & Clarke 2001). Alternatively, BES1 and BZR1 could undergo a rapid nucleocytoplasmic cycling between the two compartments even though the steady state of both proteins is in the nucleus (**Figure 3*c***). This phenomenon has been described for many transcription regulators such as ERF, SMADs, and STATs in the Ras/Erk, TGF-β, and JAK/STAT signaling pathways, respectively (Le Gallic et al. 2004, Marg et al. 2004, Nicolas et al. 2004, Pranada et al. 2004). Unraveling the relationship between BR-induced dephosphorylation of BES1 and BZR1 and their localization, uncovering the subcellular localization of the BIN2 protein, identifying the compartment where BIN2 interacts with BES1 and BZR1, as well as monitoring the possible dynamic distribution of all the players will be essential for determining the true architecture of the BR signaling pathway.

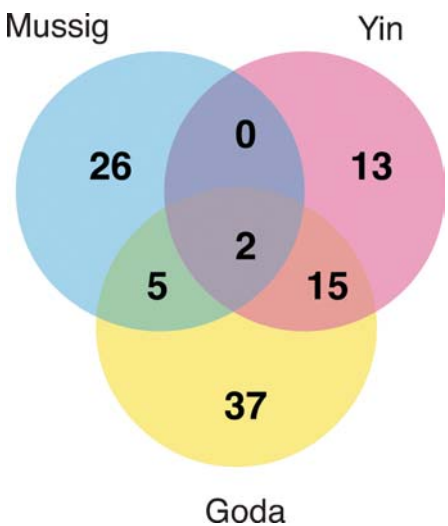


Figure 4

Early studies of BR-regulated gene expression have little overlap. Genes shown to be induced by BR treatment of seedlings are shown from three studies: Mussig et al. (2002), cyan; Yin et al. (2002b), magenta; Goda et al. (2002), yellow.

GENOMIC EFFECTS OF BRS

Studies have linked BRs to several nongenomic effects, including changes in wall extensibility (Zurek et al. 1994), osmotic permeability (Morillon et al. 2001), vacuolar function (Schumacher et al. 1999), and intracellular calcium fluxes (Allen et al. 2000). The best characterized direct effects, however, are the early transcriptional responses to BR treatment.

A High Confidence List of BR-Regulated Genes

Several recent reviews have described historical approaches to measuring BR responses (i.e., Mussig & Altmann 2003). The focus of this section is on the application of genome-scale tools to the question of the BR genomic response. In 2002, three groups published reports on short-term effects of BR treatment on gene expression, using Affymetrix chips representing approximately one third of the genome (Goda et al. 2002, Mussig et al. 2002, Yin et al. 2002b). Surprisingly, the findings from these groups showed little overlap in the genes identified (**Figure 4**), although similarities in the broad functional categories represented by each group's gene list could be observed. One important result common to all three reports was the modest nature of the BR response. Whereas studies on other plant hormones, such as auxin, have reported transcript-induction in excess of 10-fold (Zhao et al. 2003), few BR-regulated genes were shown to be induced by more than 2-fold. This is an interesting result from a biological perspective but also presents a challenge for current analysis methods. In an attempt to resolve the question of whether the results reported from each study reflected differences in experimental design or were largely attributable to varying analytical methods, we initiated a combined

analysis with new data from two of the original groups.

Both the Chory and Shimada groups had applied their original experimental conditions to the Affymetrix ATH1 microarray, representing approximately 22,000 genes. Importantly, to perform a joint analysis of the data from both laboratories, all differences, including experimenter, treatment, growth conditions, and age, were combined into a factor called lab effect (for details of analysis see Appendix A. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). Three replicates were available from the Chory laboratory, where 10-day-old seedlings grown on plates were submersed in 1 μM BL or mock treatments for 2.5 h (Nemhauser et al. 2004). Two replicates were available from the Shimada laboratory, where 7-day-old liquid-culture-grown seedlings were exposed to 10 nM BL or mock treatments for 3 h (**http://web.unifrankfurt.de/fb15/botanik/mcb/AFGN/atgenex.html**). To establish a high confidence list of BR-regulated genes, two diverse approaches were taken. In the first, linear models were used (Gentleman et al. 2004; limma library). Very few genes were found to be differentially expressed by linear models unless a term for lab effect was included. This lab effect was found to be significant for over half of the genes. Linear models identified 480 genes whose transcript levels increased following BR treatment and 386 genes whose transcript levels decreased at a false discovery rate (FDR) = 0.05.

A description of an alternative, nonparametric approach, called Rank Product, was recently published (Breitling et al. 2004; Gentleman et al. 2004; RankProd library of bioconductor). This approach was proposed to offer several advantages over linear modeling, including fewer assumptions under the model, no requirement to normalize all data together, and increased performance with noisy data and/or low numbers of replicates. At an FDR = 0.05, 681 transcripts increased following BR treatment, and 558 transcripts decreased. The overlap between the gene lists identified in these approaches is substantial (424 up-regulated genes and 332 down-regulated genes; Table S2, S3. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**).

With this high confidence list in hand, we returned to the original microarray data performed with the first-generation Affymetrix microarrays. All seedling data from these studies were used, including data from biosynthetic and signaling mutants. From the Altmann experiments, 20-day-old wild-type and weak BR-deficient *dwf1* seedlings were exposed to 300 nM epi-BL or mock treatments (Mussig et al. 2002). In the Shimada experiments, in addition to wild-type seedlings, weak mutants from either BR signaling (*bri1–5*) or biosynthesis (*det2*) pathways were exposed to 10 nM BL or mock treatments (Goda et al. 2002). Also, seedlings were exposed to BRZ. The Chory group published two papers on using BL treatment. One included arrays representing BL and mock treatments of BL-insensitive mutants *bin3* and *bin5*, subunits of topoisomerase VI (Yin et al. 2002a). In a second paper, strong BR-insensitive *bri1-116* mutants and *bes1*-hypersensitive mutants were exposed to 1 μM BL or mock treatments (Yin et al. 2002b). Both papers also had wild-type seedlings exposed to both treatments. All data were quantile-normalized within experiment (Gentleman et al. 2004; rma library of bioconductor), and then ratios were taken between important contrasts (i.e., WT + BL/WT + mock; mutant + BL/mutant + mock; mutant + BL/WT + BL; mutant + mock/WT + mock). The resulting 30 ratios were then clustered on the basis of the correlated expression of the 282 genes from the high confidence list, represented on the earlier version of the microarray (Table S4, S5. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). Very clear clusters

emerged, distinguishing up- and down-regulated genes, and clustering together experiments from different laboratories expected to have similar results (**Figure 5**). This analysis provides strong evidence that while BR genomic effects are undoubtedly affected by the various factors confounded in the lab effect, there are many genes with robustly detectable BR effects regardless of these factors.

One important result from this analysis is that more than 80% of consistently detected BR-regulated genes show estimated expression changes of less than twofold. All three original analyses used an arbitrary twofold cut-off in identifying differentially expressed genes. Determining whether such modest effects are biologically relevant will be a critical question for future studies of the BR response. Several alternative explanations have been proposed, including larger changes in a small subset of cells, highly responsive pathways, and the coupling of modest expression changes with large changes in protein stability or activity.

Biological Implications of BR-Regulated Gene Expression

Which pathways are clearly affected by BRs, as assayed by the genomic response? First, it should be stated that a large proportion of the genes identified by the analysis described above have no known function or only a vague hint without specific assignment to a biological process (e.g., DNA-binding domains). However, a few conclusions can be drawn with confidence. In support of decades of physiological data, BRs clearly initiate loosening of the cell wall and biogenesis of new cell wall material (Table S6. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). The strength of primary cell walls depends upon steel-like cables of cellulose microfibrils reinforced with cross-linking glycans (Reiter 2002). A gel-like pectin matrix surrounding this framework regulates porosity and other physiological properties. Structural proteins, such as the hydroxyproline-rich glycoprotein, extensin, and arabinogalactan proteins, contribute in largely undefined ways to cell wall architecture. One of the first genes identified as BR induced was *BRU1* in soybean, encoding a xyloglucan endotransglusylases/hydrolases (XTHs-formerly known as XETs) (Zurek & Clouse 1994). Consistent with their role in cell growth, many cell wall components and the enzymes that produce them are BR regulated, including extensins, arabinogalactans, and cellulose synthase subunits. Endo-glucanases and expansins are also up-regulated. Decreases in expression of several genes involved in cell division, including two cyclins, are also observed.

Interestingly, a number of genes involved in the production and secretion of very-long-chain fatty acids are also up-regulated following BR treatment (Table S6). This may reflect an increased requirement for waxy cuticle to cover rapidly elongating epidermal cells and could contribute to the biotic and abiotic stress protective effects of BR treatment (Krishna 2003). The cytoskeleton is also a target of BR regulation. In particular, two tubulin-encoding genes, *TUB1* and *TUB8*, are up-regulated by BRs (Table S6). Studies in the *bul1/dwf7-3* mutant suggest that one aspect of the dwarfing phenotype observed in BR mutants results from a defect in microtubule organization and concomitant loss of cellulose microfibrils (Catterou et al. 2001). BR treatment of the BR-deficient mutant induces correct orientation of cortical microtubules.

Connections with other hormones are plentiful, including components of both biosynthesis and signaling pathways (Table S7. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). A large number of genes previously identified as auxin-responsive has been noted by many groups, which reflects the close association of the BR and auxin genomic responses

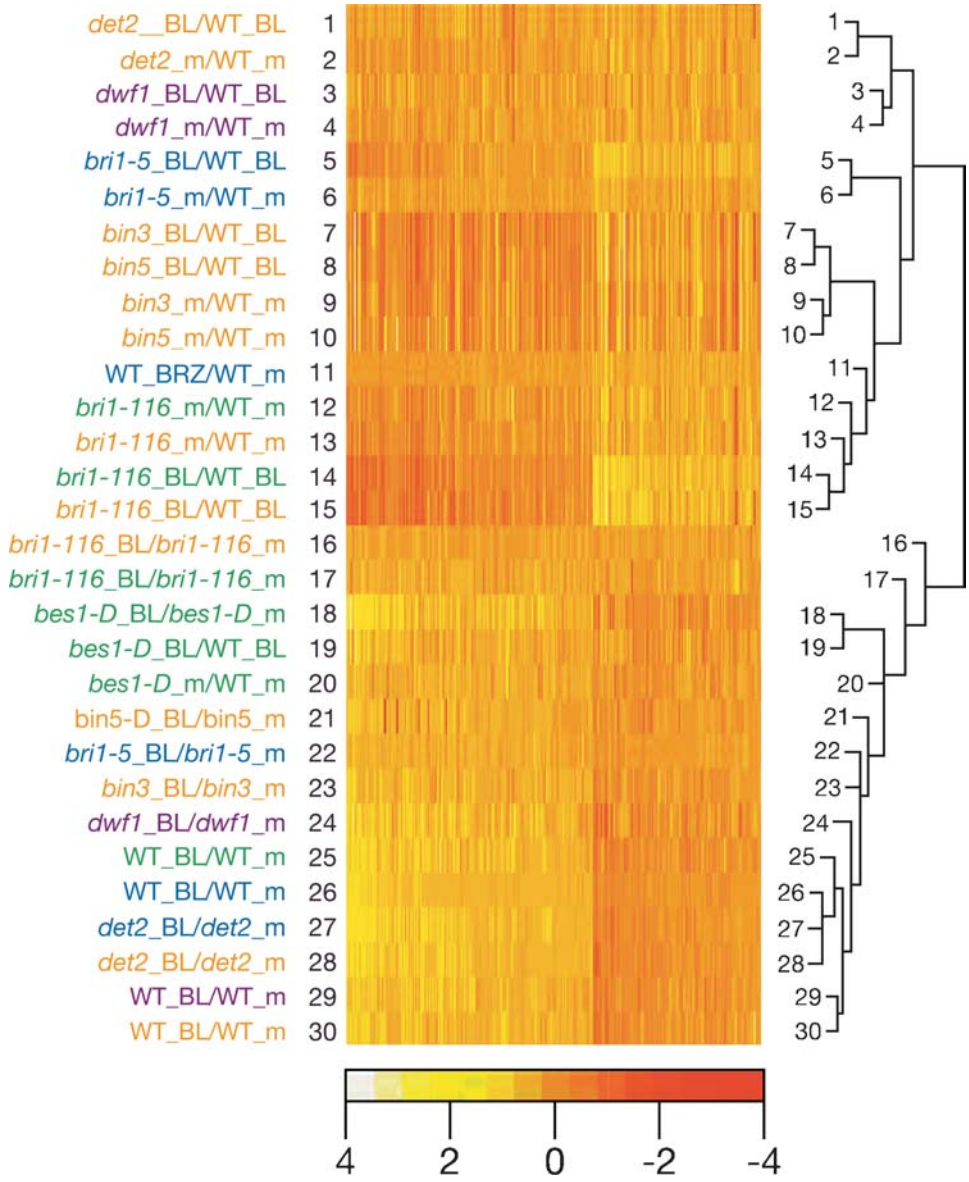


Figure 5

Re-analysis of microarray results. There are now many genes that behave consistently across BR microarray experiments. Log ratios were taken for relevant contrasts in four separate microarray experiments (e.g., WT treated with BL divided by WT mock-treated). These 30 ratios were clustered on the basis of correlated distance among the 282 genes identified as BR-responsive in our previous analysis (for details of analysis see Appendix A). A heat map is shown with each ratio represented as a row (described on the left) and each gene as a column. Columns represent up-regulated genes on the left and down-regulated genes on the right. The exact identity and order of these genes can be found in Tables S4 and S5. Experiments from Yin et al. (2002a) are shown in orange, from Mussig et al. (2002) in purple, from Goda et al. (2002) in blue, and from Yin et al. (2002b) in green. The results of the clustering analysis are shown at the right. Note that experiments from different groups are clustered together, suggesting that many genes behave reproducibly across laboratories.

(Goda et al. 2004; Nakamura et al. 2003a,b; Nemhauser et al. 2004). Several Aux/IAA transcriptional repressors are up-regulated by BRs and three ARF transcription factors are down-regulated. Multiple studies have demonstrated that both gene expression and growth effects of these two hormone pathways are interdependent (Bao et al. 2004; Nakamura et al. 2003a,b; Nemhauser et al. 2004). Genes involved in IAA homeostasis have also been found, and a number of genes involved in auxin transport are down-regulated, including members of the AUX1, PIN, and MDR families. Together, these effects might serve to reinforce local peaks in auxin concentration, perhaps as part of a canalization process. Effects of BRs on the ethylene biosynthesis enzymes ACC synthase (ACS) were observed many years ago in mung bean (*Vigna radiata*) (Yi et al. 1999). At least three ACS genes are up-regulated by BRs in our survey. Interestingly, one gene encoding an ACS was down-regulated by BRs. The *HOOKLESS1* (*HLS1*) gene, which encodes an N-acetyltransferase, is also down-regulated by BR treatment. HLS1 was recently shown to promote turnover of ARF2 protein in response to ethylene or light stimuli, perhaps providing another mechanism for regulation of the shared auxin:BR pathway (Li et al. 2004). Three type A response regulators, ARR3, ARR5, and ARR6, are down-regulated by BR treatment. These genes encode transcriptional repressors induced by cytokinin treatment and are thought to function as part of a negative feedback loop in that pathway (Suzuki et al. 2004).

A clear antagonistic relationship with the light response is also apparent in BR genomic responses (Table S8. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). Three photoreceptors, phototropin1 and phytochromes B and E, are down-regulated by BRs. Three other proteins connected with the light response, CIP7, DRT100, and an NPH3-like gene, are up-regulated by BRs, although their precise biological roles are not well established. Several papers have suggested that BR levels might be regulated by light, either through direct regulation of the DDWF1 BR biosynthetic enzyme or through BAS1-mediated hydroxylation/deactivation (Kang et al. 2001, Neff et al. 1999). Together, these findings suggest a complex web of interactions among both phytohormones and the light response modulating development and physiology.

A large number of transcription factors are regulated by BRs, including more than 10% of the BR down-regulated genes (Table S9. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). Among the 41 down-regulated genes predicted to encode transcription factors, 5 contain AP2 domains, 7 contain homeobox domains, and another 7 are predicted to contain Zn finger domains. Interestingly, several of the homeobox genes are expressed in vascular tissue, a developmental fate closely associated with BR response (Cano-Delgado et al. 2004). A major shift in transcriptional programs is likely to precede and support the significant changes in seedling morphology observed with changes in BR response.

From the Signal to Specific Target Gene Expression

Because BES1 and BZR1 share no significant homology to any known protein, the mechanism by which they control transcription was obscure until recently. DNA-binding activity and regulation of transcription were uncovered for both BZR1 and BES1.

A yeast two-hybrid approach using BES1 as a bait identified the bHLH transcription factor BIM1 (Yin et al. 2005). Gel shift experiments showed that both BIM1 and BES1 are able to bind CANNTG E-box motifs in the promoter of a *SAUR-AC1* BR-responsive gene and likely to form a heterodimer in a cooperative manner. Those E-box motifs are known binding sites for many bHLH

transcription factors (Toledo-Ortiz et al. 2003) and are also overepresented in the promoter of BR-induced genes (Nemhauser et al. 2004). BES1 binds DNA through its N terminus, which contains a highly basic domain as well as certain key residues of bHLHs predicted to form a helix-loop-helix type structure. BES1 appears to activate *SAUR-AC1* expression, although this remains to be directly shown using a heterologous system.

The actual involvement of BIM1 and its paralogs in the BR-signaling pathway comes from both gain- and loss-of-function mutants, although the mutant phenotypes obtained are weak. This suggests that other transcription factors likely act redundantly in the pathway or that the response mediated by BIMs affects only a subset of BR-responsive genes. The first candidates potentially acting in concert with BIMs are BEE1, BEE2, and BEE3, distantly related bHLHs previously shown to be positive regulators of the BR response (Friedrichsen et al. 2002). Importantly, direct binding of BES1 was shown only for the promoters of two SAUR-like genes and could not be detected on the promoter of a XET and BEE1, which are known to be BR responsive (Yin et al. 2005). This suggests that BES1 binding is not required on all BR-responsive promoters or that levels of BES1 protein on such promoters are quite low.

BZR1 was shown to act as a transcriptional repressor through direct binding to CGTG(T/C)G elements in the promoter of the BR biosynthetic gene *CPD*, a motif also conserved in the promoter of other biosynthetic genes such as *DWF4*, *ROT3*, and *BR6OX* (He et al. 2005). These observations strengthen the role of BZR1 in the control of BR homeostasis through the direct repression of BR feedback-regulated BR biosynthetic genes. In addition to its repressor role, a positive role of BZR1 on gene expression has been observed and is therefore likely dependent on its interaction with different partners that could switch BZR1 from a repressor to an activator depending on the context or, conversely, could simply be mediated by the repression of a BR-regulated transcriptional repressor.

These studies clearly argue for a direct role of BZR1 and BES1 in the repression of biosynthetic genes and promotion of BR responses, respectively, but do not completely explain the differences seen between *bes1* and *bzr1* gain-of-function phenotypes. Surprisingly, *DWF4* promoter activity, another target of the feedback regulation of BR biosynthesis by signaling, is down-regulated in both *bes1* and *bzr1* mutants (M. Lee, unpublished results). Consistent with this observation, the in-depth analysis done in the present study of previously published *bes1* microarray experiments (Yin et al. 2002b) indicates that several biosynthetic genes are down-regulated in *bes1*. This brings up the question about the opposite phenotype of *bes1* and *bzr1* in the light and gives rise to new questions at the molecular level. Does BES1 binding to the same promoter element that BZR1 is binding to directly repress *CPD* expression? Is BZR1 acting like BES1 to positively regulate BR response genes through E-box motifs? How do almost identical proteins act differently in the pathway? A comparative analysis of BES1 and BZR1 transcriptional activity should therefore be carried out using the same target promoters from both biosynthetic and other BR-regulated genes to determine their target specificity. Also, microarray analysis reveals that BZR1 transcripts are moderately induced by BR treatment and that two other family members, BEH1 and BEH2, show reduced transcript levels following BR treatment (Table S2, S3. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). This may reflect a more complex relationship among family members in promoting BR responses. A detailed analysis of spatial and temporal expression pattern of the entire family will also help clarify the apparent paradox of *bes1* and *bzr1* phenotypes.

CONCLUDING REMARKS

Despite significant progress in understanding the mechanisms of BR signaling, several fundamental questions remain unsolved. A major question is how the activity of BIN2 is regulated and whether this regulation involves BRI1/BAK1 directly. How BES1 and BZR1 and perhaps other family members coordinately regulate the large number of target genes is also unknown. In order to truly understand the role of BRs as developmental signals, we need to unravel the determinants of BR homeostasis: where and when BRs are synthesized and degraded, how they are transported out of the cell, and to what extent they are distributed in the plant. Finally, integration of BRs with other key signals, such as auxin and light, must be understood to gain further insight into the complexity of plant development.

SUMMARY POINTS

1. Brassinosteroids are perceived at the plasma membrane by direct binding to the extracellular domain of the BRI1 receptor. How ligand binding transduces the information across the membrane and activates BRI1, as well as the mechanism of receptor deactivation, is unknown.
2. BR-induced changes in gene expression are mainly achieved through the control of the phosphorylation state of the transcription factors, BES1 and BZR1.
3. The specific contribution of transcription factors in BR responses is emerging. Whereas the transcription factor BES1 is involved in the promotion of BR responses, BZR1 represses BR-biosynthetic genes.
4. The genomic response to BRs gives a good picture of their direct effects on growth and differentiation, which is correlated with physiological observations.

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