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The Mechanisms of Brassinosteroids' Action: From Signal Transduction to Plant Development

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ABSTRACT Brassinosteroids play diverse roles in plant growth and development. Plants deficient in brassinosteroid (BR) biosynthesis or defective in signal transduction show many abnormal developmental phenotypes, indicating the importance of both BR biosynthesis and the signaling pathway in regulating these biological processes. Recently, using genetics, proteomics, genomics, cell biology, and many other approaches, more components involved in the BR signaling pathway were identified. Furthermore, the physiological, cellular, and molecular mechanisms by which BRs regulate various aspects of plant development, are being discovered. These include root development, anther and pollen development and formation, stem elongation, vasculature differentiation, and cellulose biosynthesis, suggesting that the biological functions of BRs are far beyond promoting cell elongation. This review will focus on the up-to-date progresses about regulatory mechanisms of the BR signaling pathway and the physiological and molecular mechanisms whereby BRs regulate plant growth and development.

Key words: *Arabidopsis*; brassinosteroid; cross-talk; phosphorylation; plant development; signaling pathway; stresses.

INTRODUCTION

Brassinosteroids (BRs), a class of plant-specific steroid hormones characterized by their polyhydroxylated sterol structure, were first isolated and purified from *Brassica napus* pollen in 1979 (Grove et al., 1979). Although exogenous BRs are able to promote stem elongation and cell division (Mitchell et al., 1970), they were not widely recognized as a novel phytohormone until the 1990s, when a number of genes involved in BR biosynthesis and perception, including deetiolated2 (*DET2*), constitutive photomorphogenesis and dwarfism (*CPD*), and brassinosteroid insensitive 1 (*BRI1*), were identified. Loss-of-function mutation of these genes usually leads to severe growth defects, including short hypocotyl, dwarfism of seedlings and mature plants, short petioles, dark-green leaves, delayed flowering, and reduced male fertility (Li et al., 1996; Szekeres et al., 1996; Li and Chory, 1997). As a class of essential plant hormones, BRs play key roles in regulating broad aspects of plant growth and development, including vegetative and reproductive development, germination, senescence, and responses to various biotic and abiotic stresses.

A major BR biosynthetic pathway has been established in *Arabidopsis*. The precursor of BRs is campesterol, which is first converted into campestanol. Through early or late C-6 oxidation pathways, campestanol is transformed into castasterone (CS), which is finally converted into brassinolide (BL), the most biologically active among more than 50 natural BRs (see reviews of Altmann, 1998; Noguchi et al., 2000; Fujioka and Yokota, 2003).

Besides the levels of endogenous BRs in plants, the signaling pathway is also essential for BRs to regulate these biological processes (Figure 1). Since the finding of BRs, genetic screening has been widely used for identification and characterization of two major components involved in the BR signaling pathway: BRI1 and brassinosteroid insensitive 2 (BIN2). Recently, many laboratories have developed various approaches to identify a number of additional components in the BR signaling pathway. These approaches include well-designed mutant screening (such as suppressor screening), yeast two-hybrid screening, bioinformatics, proteomics, and mass spectrometry. These newly identified components include: BRI1 interacting proteins: BRI1-associated receptor kinase 1 (BAK1), BRI1 kinase inhibitor 1 (BKI1), transthyretin-like (TTL), and BR signaling kinases (BSKs); and downstream components: *bri1*-Suppressor 1 (BSU1) and 14–3–3 proteins; transcription factors: *bri1*-EMS

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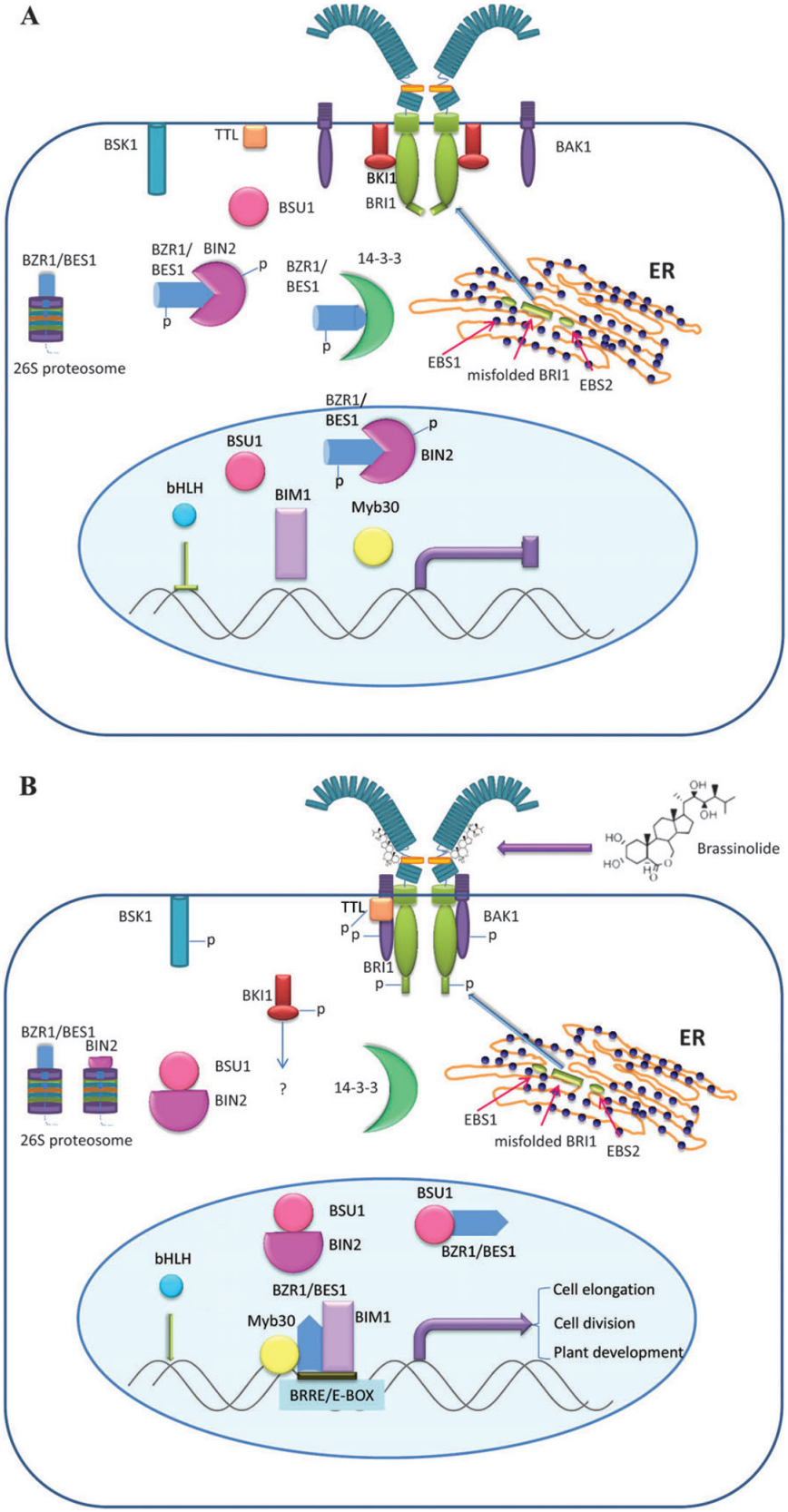


Figure 1. A Model of BR Signal Transduction Pathway.

(A) In the absence of BRs, the BR receptor BRI1 is inhibited by its carboxyl terminus and by a negative regulator BKI1 to prevent its interaction with other positive substrates, such as BAKI and BSKs. BSU1 is inactive and, as a consequence, BIN2 acts as an active kinase to phosphorylate BES1/BZR1, which is retained in the cytosol by 14–3–3 proteins or degraded by 26S proteosome. EBS1/EBS2 is required for correct folding of BRI1 and for BRI1's targeting to plasma membrane.

(B) In the presence of BRs, the extracellular domain of BRI1 perceives BRs, leading to the dissociation of BKI1 from plasma membrane, the association of BAK1 with BRI1, and their transphosphorylation to form a completely active BR receptor complex. After the phosphorylation of BSKs by BRI1 kinase, BSKs bind to BSU1, which may accelerate BSU1's activity, resulting in dephosphorylation and inhibition of BIN2. Subsequently, unphosphorylated BES1/BZR1 accumulates in the nucleus and recruits proteins such as BIM1 and Myb30 to form diverse transcriptional complexes, which will bind to E-Boxes of promoter regions of BR-response genes to regulate their expression. Meanwhile, bHLH regulates BR-response genes with an unknown mechanism.

suppressor 1 (BES1)/brassinazole resistant 1 (BZR1); and their interacting proteins: BES1-interacting Myc-like 1 (BIM1), Myb30, atypical basic helix-loop-helix (bHLH), and interact-with-spt6 1 (IWS1).These findings have provided essential materials and tools to discover the underlying mechanisms of BR regulating plant development, especially root development, anther and pollen development, and cellulose biosynthesis. In this review, we will discuss the current advances of BR signaling pathway from plasma membrane to nucleus and the new findings of the physiological, cellular, and molecular mechanisms by which BRs regulate various aspects of plant growth and development and responses to diverse environmental cues.

BR SIGNAL TRANSDUCTION PATHWAY

BR Receptor Complex and Its Activation

BRs are perceived by a plasma-membrane localized leucine-rich-repeat (LRR)– receptor-like-kinase (RLK) BRI1, standing for brassinosteroid insensitive 1. BRI1 was isolated and cloned following the identification of a large number of recessive mutant alleles on a single locus, which were from several intensive screenings for mutants insensitive to BRs in root inhibition (Clouse et al., 1996) or in hypocotyl elongation (Li and Chory, 1997). In general, BRI1 protein possesses three major domains with unique function in BR perception and receptor activation: a large extracellular domain, a small transmembrane domain, and an intracellular kinase domain. The extracellular domain of BRI1 contains an amino (N)-terminal signal peptide, a leucine-zipper motif, 24 LRRs, and an island domain located between the 20th and 21st LRRs (Li and Chory, 1997; Vert et al., 2005). The signal peptide and leucine-zipper motif are important for BRI1 targeted to plasma membrane and possible for dimerization and LRRs may function for protein–protein interaction (Li and Chory, 1997; He et al., 2000; Wang et al., 2001). Further dissection of the extracellular domain of BRI1 revealed a minimal BR-binding region consisting of a 70-amino-acid island domain and its carboxyl (C)-terminal flanking LRR21, which together define a novel steroid–protein binding element (Kinoshita et al., 2005). The intracellular domain can be further divided into a small intracellular juxtamembrane region (JM), a kinase catalytic domain, and a C-terminal tail. The JM domain is required for transducing signal from the outside to the inside of a cell, as indicated by the overexpression of a *BRI1* lacking this region failing to rescue the dwarf phenotype of *bri1-5*, a BR-perception mutant, although the subcellular localization and *in vitro* auto-phosphorylation activity of this truncated BRI1 were largely unaffected (Wang et al., 2005a). Many point mutations in the kinase catalytic domain of BRI1 result in a dead receptor, indicating the essential role of this domain for BRI1's basal activity (Friedrichsen et al., 2000). Recent studies have identified several Ser/Thr phosphorylation sites within the catalytic domain critical for BR signaling, which include T1049, S1044, and T1045 (Wang et al., 2005b). BRI1 kinase with mutation of S1049A or S1044A/T1045A completely lost its activity *in vitro*, and transgenic plants carrying these mutated *BRI1* also failed to rescue the dwarf phenotype of *bri1-5* (Wang et al., 2005b). The C-terminal tail of BRI1 apparently plays a negative role in keeping BRI1 at basal state before BR stimulus, because a BRI1 mutation without its C-terminal region has much stronger receptor kinase activity in plants and its kinase has much higher auto-phosphorylation activity *in vitro* (Wang et al., 2005a). Given that BRI1 can form homodimer in the absence and presence of BRs, it was proposed that an auto-regulatory mechanism is involved in the activation of BRI1: without BRs, BRI1 homodimer was kept at quiescent state by its C-terminal tail, BR binding induces the conformational change of its kinase domain, and subsequent auto-phosphorylation at a number of sites, including several S/T residues in the C-terminal tail to release its auto-inhibition (Wang et al., 2005a). In addition, a specific negative regulator, called BKI1, is also required to keep BRI1 at low and basal activity by preventing the interaction of BRI1 with other positive regulators (Wang and Chory, 2006; see more details in the section on BRI1's substrates).

In the BR receptor complex, besides BRI1, another receptor kinase BAK1 was also reported to be required in the activation of BRI1. BAK1 was identified by two independent groups through a yeast two-hybrid screen for BRI1 interacting proteins (Nam and Li, 2002) and through an activation-tagging screen looking for suppressors of *bri1-5* (Li et al., 2002), respectively. Both studies demonstrated that BRI1 and BAK1 can interact with each other *in vitro* and *in vivo* through their kinase domains. However, BRs cannot directly bind to BAK1, suggesting that BAK1 functions after BRI1's initial activation (Wang et al., 2005a). Recent studies further supported this hypothesis, because transphosphorylation between BRI1 and BAK1 greatly enhances the activity of BRI1 kinase (Li et al., 2002; Wang et al., 2008). After BR perception by the extracellular domain of BRI1, the kinase domain of BRI1 first phosphorylates and partially activates BAK1, then BAK1 in turn transphosphorylates BRI1 to further enhance the kinase activity of each other (Wang et al., 2008). Thus, a more comprehensive picture of the early signaling events proximal to the plasma membrane is emerging (Figure 1). Before BR binding, BRI1 is kept inactive by auto-inhibition of its C-terminal region and by a negative regulator BKI1. Upon BR perception, BRs induce a conformational change of the intracellular domain of BRI1 to auto-phosphorylate its C-terminal tail and phosphorylate BKI1 to release their inhibition on BRI1 activity (Wang and Chory, 2006). The pre-activated BRI1 will recruit BAK1 to its proximity to enhance each other's kinase activity via transphosphorylation and to form a fully activated receptor complex.

In a canonical view, plant receptor kinases are generally considered as Ser/Thr kinases; so is BRI1, according to previous studies. Using *in vitro* kinase assays and phenotypic analysis of transgenic *bri1-5* with different forms of mutated BRI1s, the functions of potential phosphorylation sites in the intracellular JM region, the activation loop, and the C-terminal tail are

revealed. Mutation of four out of seven autophosphorylation sites: S838, T842, T846, and S858 to Asp can enhance BRI1 kinase activity (Oh et al., 2000; Wang et al., 2005a, 2008). However, when S1044/T1045, T1049 was mutated, the mutated BRI1 kinase activity was abolished, and the mutated BRI1 cannot rescue the dwarf phenotype of *bri1-5* (Wang et al., 2005a, 2008). In the C-terminus of BRI1, substitutions of S1162, S1166, S1168, and T1180 to Asp can significantly enhance BRI1 kinase activity, so it was proposed that BR-induced phosphorylation of several residues in the C-terminus can release autoinhibition of BRI1 (Wang et al., 2005a, 2008). Interestingly, a recent study discovered that at least seven LRR-RLKs (among 24 tested), including BRI1 and BAK1, can auto-phosphorylate certain tyrosine residues (Oh et al., 2009). Site-specific mutation analysis of BRI1 *in vitro* and *in vivo* has pinpointed two tyrosine residues (Y1052 and Y831) important for BR signaling, suggesting that, like mammalian systems, tyrosine phosphorylation of receptor kinase may also be an important mechanism in regulating receptor kinase activity in plants (Oh et al., 2009).

A series of studies have revealed that endoplasmic reticulum-mediated quality control (ERQC) is involved in BRI1 plasma membrane localization. Studies on *bri1-5* and *bri1-9* mutants revealed that their dwarf phenotypes are both caused by retaining *bri1* proteins in endoplasmic reticulum (ER) (Jin et al., 2007; Hong et al., 2008). Mutation of EMS-mutagenized bri1 suppressor 1 (*EBS1*) gene, encoding a folding sensor of protein quality control in ER, or EMS-mutagenized bri1 suppressor 2 (*EBS2*) gene, encoding a multifunctional Ca^{2+}-binding protein participating in protein folding in the ER, can largely suppress the dwarf phenotype of *bri1-9* (Jin et al., 2007, 2009). However, Hong et al. (2008) reported that *bri1-5* can interact with calnexin and luminal binding protein (BiP) and two ER chaperones, resulting in degradation through a proteasome-independent endoplasmic reticulum-associated degradation pathway (ERAD). In addition, as a membrane-localized receptor, BRI1 can also undergo constitutive endocytosis. Treatment with Brefeldin A, a chemical agent that could prevent BRI1's movement from early to late endosomes, leads to an accumulation of BRI1–GFP in certain early endosomes and enhancing BR signaling outputs (Geldner et al., 2007).

Substrates of BRI1 Kinase

One of BRI1's substrates, BKI1 was identified via yeast two-hybrid screen using BRI1's kinase domain as bait (Wang and Chory, 2006). BKI1 acts as a negative regulator of BR-signaling as indicated by overexpression of *BKI1* causing a *bri1*-like dwarf phenotype and inhibiting BR signaling outputs (Wang and Chory, 2006). *In vitro* pull-down assays revealed that the interaction between BRI1 and BAK1 was severely reduced by additional BKI1 protein, suggesting that BKI1 inhibit BR signaling by preventing positive regulators, such as BAK1, from accessing BRI1. Interestingly, BR treatment can rapidly induce the dissociation of BKI1 from plasma membrane, and this process is dependent on a kinase-active BRI1 (Wang and Chory, 2006). A myristoylated BKI1 can constitutively associate with plasma membrane and lead to a further enhanced dwarf plant. *In vitro* kinase assay confirmed that BKI1 can be phosphorylated by BRI1 kinase, which may lead to the dissociation of BKI1 from BRI1 and plasma membrane through unknown mechanisms (Wang and Chory, 2006). However, the function of the phosphorylated and cytosolic BKI1 is still unknown.

Another BRI1 substrate, TTL, was also identified by a yeast two-hybrid screen using the kinase domain of BRI1 as bait (Nam and Li, 2004). *In vitro* kinase assay confirmed that BRI1 kinase can phosphorylate TTL. Similarly to *BKI1*, overexpression of *TTL* led to a dwarf plant, and knock-out of *TTL* and its closely related homolog led to larger plants. However, unlike BKI1, TTL can strongly interact with a kinase-active BRI1, rather than a kinase-inactive BRI1, suggesting that TTL may inhibit BRI1 signaling after its activation.

The recently identified BRI1-interacting proteins BSKs through proteomics can be phosphorylated by BRI1 kinase *in vitro* and interact with BRI1 *in vivo* (Tang et al., 2008). Interestingly, BR treatment weakens their interaction, suggesting that BSK1 may be released from BRI1 after phosphorylation (Tang et al., 2008). Ser^{230} is a major phosphorylation site of BSK1 for BRI1 kinase for the phosphorylation is reduced by 82% in a Ser^{230} to Ala^{230} mutant BSK1 (Tang et al., 2008). BSKs function as positive regulators of BR signaling, as indicated by a knockdown mutant of *bsk3-1*, a homolog of *BSK1* showing a reduced BR signaling output and overexpression of *BSK3* partially rescuing the dwarf phenotype of *bri1-5* (Tang et al., 2008). Their possible downstream components will be discussed in the later section.

Downstream Events of BR Signaling

The second BR-insensitive locus, *BIN2*, also called *UCU1* or *DWF12*, was identified by different groups through genetic screen for BR-insensitive mutants or for other morphological mutants in *Arabidopsis* (Li et al., 2001; Choe et al., 2002; Pérez-Pérez et al., 2002). *BIN2* encodes a GSK3/SHAGGY-like kinase, and *bin2-1* mutant shows a dramatic dwarf phenotype. The triple mutant *bin2-3/bil1/bil2* with knock-outs of *BIN2* and its two close homologs *AtSK2-2* and *AtSK2-3* shows a constitutively BR-enhanced phenotype (Vert and Chory, 2006; Yan et al., 2009). The protein encoded by *bin2-1* possesses significantly higher kinase activity than that of wild-type BIN2, suggesting that *bin2-1* is a gain-of-function mutation, and BIN2 acts downstream of BRI1 to negatively control BR signaling (Li et al., 2001; Li and Nam, 2002). BIN2–GFP is scattered over the entire cell, whereas the bin2-1–GFP is accumulated more in the nucleus. Furthermore, a *BIN2* fused with a nuclear localization signal can significantly enhance its inhibition on BR signaling, suggesting that BIN2 mainly phosphorylates its substrates BES1/BZR1 in the nucleus (Vert and Chory, 2006). The detailed phosphorylation mechanism will be discussed in the later section. The level of BIN2 protein can be regulated by BR signal likely through a proteasome-mediated protein degradation system, because the exogenously applied BRs can lead to a reduction of BIN2 proteins, and treatment with

a proteasome inhibitor, MG132, can promote the accumulation of BIN2 (Peng et al., 2008).

A protein phosphatase, BSU1, was originally identified from a mutant screen for suppressors of *bri1-5* by activation tagging (Mora-García et al., 2004). BSU1 is a constitutively nuclear-localized Ser/Thr phosphatase (Mora-García et al., 2004). Gain-of-function mutation of *bsu1-D* or overexpression of *BSU1* can greatly rescue the dwarf phenotype of *bri1-5* and promote the accumulation of dephosphorylated BES1, a downstream transcription factor (please see later sections for details about BES1) (Mora-García et al., 2004). In yeast, BSU1 can directly interact with BES1, suggesting that *BSU1* acts downstream of *BRI1* by directly dephosphorylating BES1. Other evidence to support that BSU1 may directly act on BES1 is that overexpression of *BSU1* can partially suppress the dwarf phenotype of heterozygous *bin2-1* (Mora-García et al., 2004). However, a recent study proposed that the phosphorylated BSKs can interact with BSU1, and BSU1 may directly dephosphorylate a conserved phosphorylation site pY200 of BIN2 to inhibit its kinase activity (Kim et al., 2009). In addition, overexpression of *BSU1* can partially rescue the dwarf phenotype of *bri1-5* but not *bin2-1* (Kim et al., 2009), which is different from the result of a previous study (Mora-García et al., 2004). Therefore, the authors proposed that BR perception can activate BRI1, BSKs, and BSU1 to inactive BIN2, resulting in the activation of downstream transcription factors (Kim et al., 2009).

A Class of BR-Activated Transcription Factors, BES1/ BZR1, and their Regulation

The expression of many BR-responsive genes is directly regulated by a class of plant-specific transcription factors that includes BES1, BZR1, and BES1/BZR1 homologs 1–4 (BEH1–4). *bes1-D* was originally identified from mutagenized *bri1-119* by ethyl methanesulfonate (EMS) with suppressed *bri1* dwarf phenotype (Yin et al., 2002). While *bzr1-1D* was isolated from EMS mutants that are insensitive to brassinozole (BRZ), a brassinolide biosynthesis inhibitor (Wang et al., 2002). Because both *bes1-D* and *bzr1-1D* are semi-dominant mutants and can rescue the dwarf phenotype of *bri1* and *bin2-1D*, it was proposed that BES1/BZR1 act downstream of BIN2 as positive regulators of BR signaling (Yin et al., 2002; Wang et al., 2002). Further experiments demonstrated that BES1/BZR1 can be directly phosphorylated by BIN2 kinase, and BR treatment can rapidly induce dephosphorylation of BES1/BZR1 *in planta*, which has been widely used as a biochemical maker to evaluate the strength of BR signaling outputs (Yin et al., 2002; Zhao et al., 2002; Wang et al., 2005a; Zhang et al., 2009b). Following several hydrophobic residues in the N-terminus of BES1/BZR1, there is a putative bipartite nuclear localization signal sequence (NLS), and a stretch of about 20 repeats of a consensus site (S/TXXXS/T, where S/T stands for serine or threonine and X stands for any amino acid) in the central region for phosphorylation by GSK-3 kinase (Wang et al., 2002; Yin et al., 2002). Furthermore, a PEST motif from amino acids 231–250 of BES1, where *bes1-D* point mutation is located, apparently plays an important role in regulating the stability of BES1 and BZR1 (Yin et al., 2002, 2005). In addition, BES1/BZR1 proteins contain a C-terminal domain, which may account for an interaction with BIN2 (He et al., 2002; Wang et al., 2002; Yin et al., 2002).

The activated BES1 by BR stimulus can bind to CANNTG sequences called E-Boxes, which are present in promoter regions of numerous BR-responsive genes, by interacting with BIM1 transcription factors (Yin et al., 2005). In addition, AtMYB30, another transcription factor, is also positively involved in BR signaling by promoting a subset of BR-responsive gene expression (Li et al., 2009). BES1 can interact with AtMYB30 both *in vitro* and *in vivo* to promote the expression of downstream target genes (Li et al., 2009). Recently, it was discovered that BES1 can also physically interact with IWS1, which participates in RNA polymerase II (RNAPII) post-recruitment and transcriptional elongation processes (Li et al., 2010b). An *iws1* mutant was identified from suppressor screening of a gain-of-function *bes1-D* mutant and loss-of-function mutation of *iws1* is less sensitive to BR in hypocotyl elongation, suggesting that factors, such as IWS1, involved in chromatin remodeling may also be required for BES1-regulated transcription process (Li et al., 2010b).

BZR1 is another nuclear-localized transcription factor, a homolog to BES1 (Wang et al., 2002). BZR1 can bind to a CGTG(T/C)G element, called BR-Response Element (BRRE) with its N-terminal domain to negatively feedback regulating the expression of genes involved in BR biosynthesis, such as *CPD*, *DWF4*, *ROT3*, and *BR6ox* (He et al., 2002, 2005). Apparently, BES1 may have a similar function in the feedback regulation of genes encoding BR-biosynthetic enzymes (Vert and Chory, 2006; Yin et al., 2005). Using transcript profiling and chromatin-immunoprecipitation microarray experiments, Sun et al. (2010) reported 953 BR-regulated BZR1 target genes, which function in BR promotion of cell elongation, cross-talk between BR and other hormonal and light-signaling pathways at multiple levels.

Nuclear accumulation of dephosphorylated BES1/BZR1 plays important roles in directly regulating the expression of BR-responsive genes. Early studies on the sub-cellular localization of BES1–GFP or BZR1–GFP in *Arabidopsis* showed that, without BRs, BES1/BZR1 are distributed in both nucleus and cytoplasm, and BR treatment can rapidly promote the accumulation of BES1/ BZR1 in nuclear in *Arabidopsis* hypocotyl cells (Wang et al., 2002; Yin et al., 2002). Later, another study showed that, under BL or BRZ treatment, the endogenous level of BES1 or the nuclear-localization pattern of BES1–GFP and BZR1–GFP was unchanged (Vert and Chory, 2006; Zhao et al., 2002), and proposed that dephosphorylated BES1 and BZR have higher binding ability to the promoters of *CDP* and small auxin up RNA 1 (*SAUR-AC1*) than their phosphorylated forms (Vert and Chory, 2006). However, more recently, by calculating the ratio of nucleoplasmic and cytoplasmic BES1–YFP and BZR–YFP using a protoplast system and by Western blot analysis of nuclear and cytoplasmic proteins, it was demonstrated

that BES1–GFP and BZR1–GFP can be localized in both cytoplasm and nucleus, and BR treatment can significantly induce a nucleus accumulation of dephosphorylated BES1–GFP and BZR1–GFP (Gampala et al., 2007; Ryu et al., 2007). Furthermore, a subset of 14–3–3 proteins, a class of phosphopeptide-binding proteins widely distributed and highly conserved in all eukaryotes, is found to play a key role in keeping phosphorylated BES1 and BZR1 in cytoplasm (Gampala et al., 2007; Ryu et al., 2007). BES1/BZR1 proteins mutated on a 14–3–3 binding site were accumulated more in the nucleus (Gampala et al., 2007; Ryu et al., 2007). However, it is still unknown whether 14–3–3 proteins promote nuclear export of BES1/BZR1 or prevent nuclear import of BES1/BZR1.

Besides the BES1/BZR1 family of transcription factors in BR signaling pathway, a class of a typical bHLH transcription factors was recently identified to participate in the BR-regulated gene expression. A gain-of-function mutant *atbs1-D*, caused by overexpression of a 93-amino-acid atypical bHLH *ATBS1*, suppressed the phenotype of a weak *bri1* mutant (Wang et al., 2009). Overexpression of allograft inflammatory factor1 (*AIF1*), which encodes another bHLH protein and can interact with ATBS1 both *in vitro* and *in vivo*, masked the suppression caused by *ATBS1* (Wang et al., 2009), suggesting that AIF1 may interact with a negative regulator in the BR signaling pathway. Similar bHLH proteins, named increased leaf inclination 1 (ILI1) and ILI1 binding bHLH 1 (IBH1), were also reported in rice (Zhang et al., 2009a). However, how these atypical bHLH proteins are involved in the BR signaling pathway is not clear.

During plant growth and development, the primary signaling pathway of BRs undergoes intensive interaction with many signaling pathways. BR and ABA can antagonistically co-regulate numerous developmental processes (Steber and McCourt, 2001; Chen et al., 2004; Finkelstein et al., 2008; Gao et al., 2008) and the expression of hundreds of genes (Nemhauser et al., 2006). Recently, we discovered that ABA may inhibit plant growth by suppressing BR signaling downstream of BR receptor and on/upstream of BIN2 (Zhang et al., 2009b). In addition, BR and auxin act synergistically in regulating multiple developmental and physiological processes. It was reported that overexpression of *YUCCA* genes involved in auxin biosynthesis can suppress the dwarf phenotype of a weak BR-perceptional mutant *bri1-301* (Kang et al., 2010). Another study suggests that the cross-talk between BR and auxin signaling pathways may through BIN2, which can phosphorylate and inactivate ARF2, a member of the auxin response factor family of transcriptional regulators (Vert et al., 2008).

THE CELLULAR AND MOLECULAR MECHANISMS OF BR REGULATING PLANT DEVELOPMENT

As crucial plant hormones in promoting plant growth, BRs participate in a large array of plant developmental progresses. At cellular levels, BRs can regulate cell elongation, cell division, and cell differentiation. At whole-plant levels, BRs can regulate hypocotyl elongation, shoot development, leaf development, root development, male fertility, senescence, and responses to biotic and abiotic stresses (Figure 2). In this section, we will discuss the current advances of how BRs participate in regulation of these developmental processes.

Cell Elongation

From more than 40 years ago, before Grove et al. (1979) isolated brassinolide, the most active brassinosteroids, from *Brassica napus* pollens, people have known that organic extracts from *Brassica napus* pollens have a key function in stimulating stem elongation in plants (Mitchell et al., 1970). In the 1990s, genetics studies confirmed that cell elongation was severely retarded in most BR-deficient and BR-insensitive mutants as indicated by dramatically reduced hypocotyl length in the dark and severe dwarfism in the light (Salchert et al., 1998). Feeding BR-deficient mutants, *cpd* and *dwf4*, with BRs but not other phytohormones could restore their dwarf phenotype to wild-type (Szekeres et al., 1996; Azpiroz et al., 1998). In another BR-deficient mutant, *bul1-1*, which encodes Δ7-sterol-C-desaturase functioning in BR synthesis, cell elongation was also inhibited and microscope observation of its cytoskeleton indicated that, compared to the wild-type, a parallel microtubule organization in the mutant was reduced (Catterou et al., 2001). A rice BR-deficient dwarf1 (*brd1*) mutant, which lacks activity of BR C-6 oxidase, exhibits abnormal organization and polar elongation of leaf and stem cells, leading to a drastic defect in development of many organs (Hong et al., 2002).

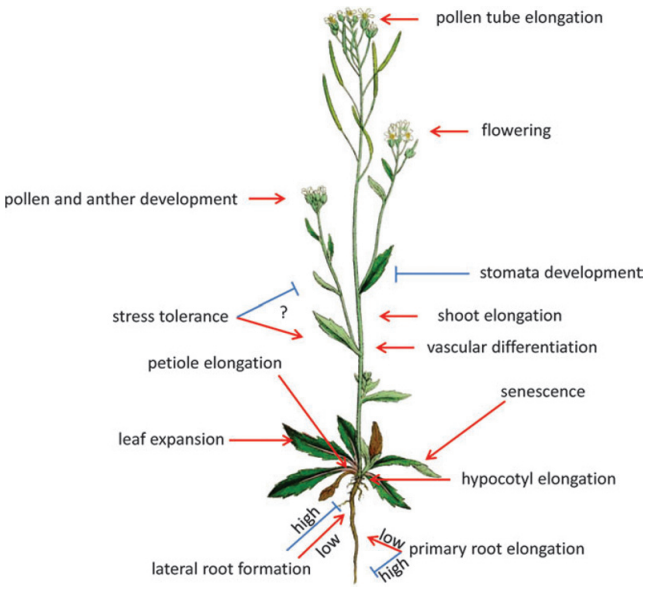


Figure 2. A Graph to Illustrate the Roles of BR in Regulating Plant Development.

Arrows in red indicate that BRs promote developmental processes and inhibition arrows in light blue indicate that BRs inhibit developmental processes. 'Low' stands for low-concentration BR and 'High' stands for high-concentration BR.

Genetic and molecular studies revealed that the BR-promoted cell elongation largely depends on the expression of xyloglucan endotransglycosylases (XETs), which function to incorporate new xyloglucan into the growing cell wall. *BRU1* in soybean and *TCH4* in *Arabidopsis*, both encoding XET proteins, are highly and rapidly induced by BR treatment during early stages of elongation (Zurek and Clouse, 1994; Xu et al., 1995). Recently, we found that BR-activated transcription factor BES1 can directly bind to the promoter regions of nearly all of 10 cellulose synthase genes in *Arabidopsis* to enhance their expression and biomass accumulation, which makes a significant contribution to cell elongation (unpublished data). In cotton, BRs can enhance fiber development by promoting fiber cell elongation (Sun et al., 2005). Compared to auxin, another growth-promoting hormone, the response in cell elongation to BRs is much slower. Auxin begins to promote cell elongation within 10–15 min and reaches its maximum rate within 30–45 min in several plant species (Taiz, 1984). In contrast, BRs show a lag time of cell elongation onset of at least 45 min and can continually promote elongation for several hours in soybean (Clouse et al., 1992). In contrast, a higher concentration of BRs can inhibit cell elongation, especially in primary root extension and lateral root formation (Sasse and Sasse, 1994).

Hypocotyl Elongation Is Caused by Cell Elongation

Arabidopsis hypocotyl is positioned below the cotyledon and connects the radical and BRs can regulate hypocotyl elongation together with many other phytohormones, such as auxins, gibberellins, and ethylene. During hypocotyl elongation, BRs regulate the expression of a number of genes functioning in auxin responses, especially auxin transport system in both dark-grown and light-grown seedlings (Nemhauser et al., 2004). Moreover, Katsumi (1985) reported that BRs cooperate with auxin but not gibberellins to promote hypocotyl elongation in cucumber. BRs were also found to accelerate hypocotyl elongation by enhancing the action of a growth factor that contributes to gravitropic growth in bean (Meudt, 1987). In addition, BRs may act downstream of ethylene, because exogenously applied BRs can suppress the insensitivity of *hookless* mutant to ethylene, which encodes an N-acetyltransferases and controls differential cell growth (De Grauwe et al., 2005). Interestingly, Deslauriers and Larsen (2010) reported that FERONIA (a receptor-like kinase required for normal pollen tube reception and cell elongation) may be involved in BR signaling and acts contrarily to ethylene in hypototyl elongation of etiolated seedlings. It was also reported that BRs can stimulate hypocotyl elongation in pakchoi through increasing cell wall relaxation (Wang et al., 1993).

Cell Division

The effect of BRs on cell division is somewhat controversial. On one hand, the dwarf phenotype of BR-deficient and BR-insensitive mutants is mainly caused by reduction in cell size rather than in cell number through microscopic examination, suggesting that BR may not promote cell division (Kauschmann et al., 1996). On the other hand, some studies suggested that BRs can promote cell division in cultured parenchyma cells and Chinese cabbage protoplasts (Clouse and Zurek, 1991; Nakajima et al., 1996), but a similar effect was not observed in cell cultures of carrots and tobacco (Sala and Sala, 1985; Roth et al., 1989). To explore the possible mechanism of BRs in regulating cell division, Hu et al. (2000) found that epi-BL treatment can up-regulate the expression of CycD3, a D-type plant cyclin gene, which may have a role in promoting cell division in *Arabidopsis* seedlings. However, how BRs regulate cell division is still poorly understood.

Shoot Development

BR-promoted shoot development has been reported in a large number of plant species, including *Arabidopsis*, soybean, mung bean, azuki bean, pea, rice, and tomato (Mandava, 1988). The mechanisms whereby BRs regulate stem development are mainly involved in promoting expression of genes responsible for cell elongation and wall extensibility (Zurek et al., 1994; Horvath et al., 2003). Overexpression of BR receptor *BRI1* or a BR synthetic enzyme *CPD* in the epidermis, but not in the vasculature, can rescue the dwarf phenotype of a null allele of either *bri1* or *cpd*, indicating that BRs may be transported from epidermal cells to inner layers, where they passively or actively corporate the expansion of epidermal cells, depending on mechanical stimuli (Savaldi-Goldstein et al., 2007). However, the mechanism of how BRs transport is unknown. Furthermore, it was reported that exogenously applied BL can induce the differentiation of vascular tissue in two major *in vitro* systems used for studying xylem differentiation: one is *H. tuberosus* explants and the other is isolated mesophyll cells of *Zinnia elegans* (Clouse et al., 1991; Fukuda, 1997). Because the number of vascular bundles in stems is fewer in BR-deficient and BR-insensitive mutants and is more in the enhanced BR signaling mutants than in the wild-type, BRs may play important roles for vasculature bundle formation in the stems (Ibanes et al., 2009). The homologs of *BRI1*, *BRL1*, and *BRL3* may also play specific roles in regulating vasculature differentiation, because overexpression of *BRL1* and *BRL3* driven by *BRI1* promoter can largely rescue *bri1* mutants and are specifically expressed in vascular tissue (Wang et al., 2001; Cano-Delgado et al., 2004). The authors also found an increase in the ratio of phloem to xylem in the *brl1* loss-of-function mutant, further suggesting the importance of *BRL1* and *BRL3* in regulating vascular tissue development (Cano-Delgado et al., 2004).

Leaf Development

The BR-related mutants in *Arabidopsis* also exhibit abnormal leaf phenotype, such as short petioles, dark-green, and cabbage-like leaves (Li et al., 1996; Szekeres et al., 1996; Wang et al., 2001; Li et al., 2002). Opposite phenotypes were observed in the *BRI1–GFP* overexpression line (Nam and Li,

2002) and *bes1-D* mutant (Yin et al., 2002). Nakaya et al. (2002) found that cell number per leaf blade in *det2* and *dwarf1* is lower than that in wild-type, suggesting that BRs may affect cell division in leaf to regulate its morphology. In addition, it was observed that stomata number was increased in the BR-deficient and BR-perceptional mutants, such as *bul-1*, *cpd*, and *bin2-1* with unknown mechanism (Catterou et al., 2001; Schlüter et al., 2002; Pérez-Pérez et al., 2004).

In monocots, such as rice, BRs have a specific role in regulating lamina joint inclination as applied BRs greatly enhancing the bending of lamina joint (Fujioka et al., 1998), and lamina joint inclination is significantly reduced in rice BR-deficient mutants, such as a dwarf mutant *d61*, encoding a rice homolog of *AtBRI1* (Yamamuro et al., 2000). At a cellular level, one possible mechanism of BR regulating lamina inclination is that BRs enhance cell enlargement at the adaxial side of the lamina joint; another possibility is that BRs disturb normal development of lamina joint with unknown mechanisms (Hong et al., 2004). Recently, it was reported that cell length at the center of adaxial side of lamina joint was reduced by 10–30% in the BR-related rice mutants (Nakamura et al., 2009). Leaf angle is an important trait to determine the architecture of several cereal crops. Rice mutants with erect leaves can be planted in high density to gain higher biomass and grain yield (Sakamoto et al., 2005).

Root Development

The first *bri1* mutant was isolated by looking for individuals with elongated roots under a high concentration of BRs (Clouse et al., 1996). However, it was also observed that a low concentration of BRs can promote root elongation in wild-type and BR-deficient mutants of *Arabidopsis*, and also in wild-type maize (Kim et al., 2000). The enhancement of root elongation by a low concentration of BRs was largely independent of auxin, because this effect was not altered by the applied auxin transport inhibitor, 2, 3, 5-triidobenzoic acid (Mussig et al., 2003). Recent studies on a brevis radix (*brx*) mutant, which has a reduced root growth, revealed a transcriptional feedback loop used by BRs to maintain root development (Mouchel et al., 2006). *BRX* encodes a plant-specific protein predicted to regulate transcription. BRs inhibit the expression of *BRX*, which leads to a decreased expression of *CPD* to reduce the levels of BRs, while auxin promotes the expression of *BRX* to maintain appropriate BRX activity for optimal root growth (Mouchel et al., 2006). Furthermore, BRs may act synergistically with auxin to accelerate lateral root formation, which may be partially mediated by the patatin-related phospholipases A (Rietz et al., 2010). It was found that lateral roots in BR-deficient mutants are fewer than in the wild-type and *DR5::GUS* expression in root tips of BR-deficient mutants was reduced compared to wild-type (Bao et al., 2004; Fukaki and Tasaka, 2009). Moreover, BR-enhanced lateral root formation and *DR5::GUS* expression were suppressed by treatment of auxin transport inhibitor N-(1-naphthyl) phthalamic acid (Bao et al., 2004; Fukaki and Tasaka, 2009). Similarly, primary root length and lateral root number are reduced in the BR-related mutants of pea (Ferguson et al., 2005). In addition, BRs may also regulate root hair development, as Kuppusamy et al. (2009) reported that the expression level and pattern of two master epidermal patterning regulators, *WEREWOLF* and *GLABRA2*, were regulated by BRs, and root hair development is abnormal in the BR-related mutants.

Reproductive Development

Several lines of evidence have indicated that BRs play a critical role in plant reproductive growth, specifically in regulating male fertility. First, pollen is a rich source of endogenous BRs and the first BR, brassinolide, was purified from oil rape pollens (Grove et al., 1979). Second, exogenous BL can promote pollen tube elongation *in vitro* (Hewitt et al., 1985). Third, BR-deficient and BR-perceptional mutants, such as *cpd*, *dwf4*, and *bri1*, are male sterile or with significantly reduced male fertility (Szekeres et al., 1996; Kim et al., 2005). In a traditional view, the reduced length of filament is the major reason to cause male sterility. Another study suggested that the reduced growth of pollen tube is a reason to reduce fertility as indicated by the exogenously applied BL being able to promote pollen tube elongation in an *in vitro* experiment (Hewitt et al., 1985) and pollen tube growth being slower in the *cpd* mutant (Szekeres et al., 1996).

Recently, the underlying molecular mechanism of BR regulating male fertility was systematically studied (Ye et al., 2010). This study discovered that slower growth of pollen tubes is not the major reason to cause reduced male fertility in BR-related mutants. In contrast, the development of tapetum and microspore in the BR-deficient and BR-insensitive mutants was abnormal, as indicated by a significantly reduced number of microspore mother cells, microspores, and pollens, and by an increased difficulty in releasing pollens from anther locule (Ye et al., 2010). Microarray and real time RT–PCR analysis indicated that most essential genes involved in anther and pollen development are down-regulated in the BR-related mutants. Further chromatin immunoprecipitation experiments demonstrated that the BR-activated BES1 can bind to the promoters of these genes, including *SPL/NZZ*, *TDF1*, *AMS*, *MS1*, and *MS2*, to directly regulate their expression (Ye et al., 2010). In addition, studies also suggested that fruit size was increased with the exogenously applied BR or by overexpressing BR synthetic genes in cucumber and tomato, indicating that fruit development is also positively regulated by BRs (Montoya et al., 2005; Fu et al., 2008).

Flowering Time and Senescence

Nearly all BR-deficient and BR-insensitive mutants exhibit late-flowering phenotype (Li et al., 2010a). Hanano et al. (2006) reported that the expression period of circadian rhythms of *CCR2*, *CAB2*, and *CCA1* that control flowering time was also altered by the applied BRs. In addition, *CPD* gene also undergoes a diurnal regulation, which may contribute to a diurnal accumulation of BRs (Bancos et al., 2006). These suggest that

BRs may use circadian rhythms to regulate flowering time. Recently, it was reported that the expression of *FLC*, a floral repressor, was enhanced in *bri1* mutant, which may provide a reasonable explanation for why the BR-related mutants are late-flowering (Domagalska et al., 2007). However, more experiments are needed to explore the underlying molecular mechanisms.

Delayed senescence in the BR-deficient and BR-insensitive mutants in *Arabidopsis* (Clouse et al., 1996; Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996) and early senescence in the *bes1-D* mutant had been observed (Yin et al., 2002). Applied BL can accelerate senescence of mung bean leaves (He et al., 1996). In contrast, ABA treatment can counteract the effect of BRs to delay senescence (Zhao et al., 1990). To explore the mechanism of BR regulating senescence, one study proposed that BRs regulate senescence via 'activated oxygen', because the activities of peroxidase, superoxide dismutase, and catalase were altered, and malondialdehyde was significantly increased by BR treatment in an *in vitro* assay (Ding and Zhao, 1995).

Stress Response

Many studies have suggested that BRs play essential roles in responding to various stresses, such as abnormal temperatures, drought, high osmotic pressure, and pathogen attack (Krishna, 2003). The cross-talks of BRs with stress-responsive hormones, such as abscisic acid, jasmonic acid, and ethylene, also indicate the importance of BRs in plant stress responses (Zhang et al., 2009c). Kulaeva et al. (1991) reported that BRs can increase plant resistance to low temperature by promoting plant growth and maintaining chlorophyll contents and promote resistance to high temperature by maintaining protein synthesis. The inhibitory effect of salt on rice seed germination was remarkably reduced under BR treatment, which maybe resulted from the enhanced level of nucleic acids and soluble proteins (Anuradha and Rao, 2001). Morillon et al. (2001) proposed that exogenously applied BRs can enhance plant resistance to drought by increasing osmotic permeability. Some studies also suggested that BR treatment can promote plants' resistance against many pathogens, such as fungal pathogen, bacteria, and virus pathogen (see review of Krishna, 2003). However, BR acts antagonistically with stress-responsive hormone ABA in promoting germination (Steber and McCourt, 2001; Zhang et al., 2009b), suggested that BRs may not simply increases plant resistance to various stresses. More detailed studies are needed to fully understand the cellular and molecular mechanisms of BR participating in responding to various stresses.

PERSPECTIVES

Through highly fruitful and intensive studies, a relatively holistic picture of the BR signaling pathway is emerging, including BR perception on plasma membrane, transduction in cytoplasm, and gene expression regulation in nucleus (Figure 1). All of the achievements potentially enlighten research in signal transduction for other phytohormones and provide us with a serviceable tool to study cross-talk amongst phytohormone signaling pathways and detail mechanisms of BR regulating plant development. As impressive as it sounds, there are still at least four major gaps in the current BR signaling model: the first is the desensitized mechanisms of many kinases involved in the pathway, such as BRI1, BAK1, BSK1, BIN2; the second is that the exact role of BKI1, particularly after its dissociation from the plasma membrane, remains elusive; the third is the unfilled gap between upstream components (e.g. BRI1, BAK1, BKI1, BSK1) and downstream components (e.g. BIN2, BSU1, BES1, BZR1); the fourth is how BES1/BZR1 regulates the expression of BR-responsive genes that participate in plant developmental and physiological processes. Although BRs play key roles in a wide range of developmental and physiological processes, only very few studies illustrate the molecular mechanisms whereby BRs regulate these biological processes (Figure 2). However, most of the molecular mechanisms of these processes have remained unknown. In addition, how BRs act together with other phytohormones to regulate a specific plant developmental process is another tier of major questions to answer. Identification of more components involved in the BR signaling pathway offers us powerful materials to investigate these mechanisms. We anticipate that more effective 'tools', such as bioinformatics, genomics, proteomics, modern cell imaging, system biology, and protein three-dimensional structure study, will be widely used to reveal the complete network of BR signal transduction pathway. In addition, considering many phosphorylation and dephosphorylation processes in the BR signal transduction pathway, large-scale phosphoproteome profiling, which has been widely used in chloroplast kinase network studies (Baginsky and Gruissem, 2009), may be used in future studies. To unravel the mechanisms of BR regulating development of certain tissues or organs, tissue-specific study on BR signaling should be broadly used. Microdissection combined with deep sequencing of chromatin immunoprecipitation products will become efficient tools to find direct targets regulated by BR signaling at tissue and organ levels.

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