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Regulation of the Nuclear Activities of Brassinosteroid Signaling

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Abstract

Brassinosteroids (BRs) are important plant growth hormones that largely rely on transcription factors (TFs) to regulate a variety of plant physiological/developmental processes. Past genetic and biochemical studies have identified two key TFs and interacting partners that play major roles in regulating many BR-responsive genes, while genome-wide microarray experiments have discovered at least 50 BR-regulated TFs. However, little is known how these TFs function or whether additional TFs are involved in BR signaling. In the past few years, genetic studies and yeast one/two-hybrid screens coupled with microarray and chromatin immunoprecipitation experiments not only revealed new roles of the key regulatory TFs but also implicated additional TFs and other nuclear proteins in regulating the nuclear activities of BR signaling in Arabidopsis and rice.

Introduction

Brassinosteroids (BRs) are important plant growth hormones that regulate a variety of physiological and developmental processes [1]. In the last decade, genetic and biochemical studies in Arabidopsis have elucidated a phosphorylation-mediated signaling pathway that regulates the stability, subcellular localization, and/or DNA binding activity of two highly similar transcription factors (TFs), bri1-EMS-Suppressor 1 (BES1) and Brassinazol Resistant 1 (BZR1) [2,3]. By contrast, little is known how BRs regulate gene activities in rice despite a recent discovery demonstrating a role of a rice BZR1 homolog, OsBZR1, in feedback regulation of BR biosynthesis [4]. Genome-wide microarray analyses in Arabidopsis have shown that BRs affect the expression of hundreds, if not thousands, of genes involved in a wide range of cellular processes, and identified at least 50 BR-regulated TFs that include members of all major families of plant TFs, such as basic helix-loop-helix (bHLH) proteins, MYB, GRAS, AP2, NAC, WRKY, homeodomain-leucine-zipper (HD-ZIP), and basic leucine zipper (bZIP) TFs [5]. However, nothing is known whether these TFs function dependently or independently of BES1/BZR1 to regulate expression of certain BR-responsive genes or whether BR signaling involves additional nuclear proteins. This article reviews studies of the last several years that uncovered additional roles of BES1 and its interacting factor in specific developmental processes and implicated other TFs and nuclear proteins in regulating BR signaling in both Arabidopsis and rice.

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Competing interest

I declare that I have no competing financial interest

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New roles of BES1 and BIM1 in pollen/anther development and embryogenesis

BR-deficient/signaling mutants are often male sterile, yet little is known about how BR regulates the pollen development/function, even though pollen is the tissue from which the first BR was discovered [6]. A systematic analysis of Arabidopsis BR-related mutants and transgenic lines revealed that BR signaling is important not only for tapetum and microspore development but also for pollen wall formation, which explains decreased number and reduced release efficiency of pollen in BR mutants [7]. Gene expression analyses and chromatin immunoprecipitation (ChIP) assays demonstrated that BES1 binds to the promoters of key regulatory TFs of anther/pollen development to control their expression (Figure 1A), thus revealing a molecular mechanism by which BR regulates anther/pollen development. BES1 is also involved in the BR-induction of several related receptor kinases, including FERONIA [8,9] that was previously known to mediate male-female interaction for releasing sperms from elongating pollen tubes [10], thus providing another possible explanation for reduced fertility of BR mutants (Figure 1A).

Despite its synergistic interaction with auxin, BR has so far not been implicated in embryogenesis that requires auxin signaling for pattern formation [11]. A recent study, however, uncovered a potential link between BR and embryogenesis (Figure 1B). BES1-interacting Myc-like 1 (BIM1) [12] physically binds and genetically interacts with two AP2-domain proteins, DORNROSCHE (DRN) and its paralog DORNROSCHE-LIKE (DRNL) [13] that are crucial for embryonic patterning by affecting cotyledon development in the apical embryo domain and cell division in the basal embryo domain [14].

Potential functions of ARF2, BRX, and DRN in the auxin-BR crosstalk

The auxin-BR interaction was thought to be mediated by auxin response factors (ARFs) that bind to the promoters of common target genes carrying the ARF core-binding element (TGTCT) [15]. Interestingly, BRASSINOSTEROID INSENSITIVE 2 (BIN2), an Arabidopsis GSK3-like kinase that inhibits BR signaling [16], was recently shown to interact with ARF2 [17], a known repressor of auxin signaling [18]. Genetic and microarray analyses suggested a role of ARF2 in BR signaling while biochemical studies demonstrated that BIN2 phosphorylates ARF2 *in vitro* to reduce its binding affinity to the *SAUR-AC1* promoter, a shared target of auxin and BR signaling [15], thus inhibiting its repressor activity. However, it remains unclear how the BIN2-catalyzed ARF2 phosphorylation contributes to the auxin-BR synergistic interaction. BR is known to negatively regulate the kinase activity and protein abundance of BIN2 [19,20], implying ARF2 would be an active suppressor when the BR signaling is active. Perhaps the BIN2-catalyzed ARF2 phosphorylation inhibits ARF2 interaction with other ARFs that bind to the common targets of the two signaling pathways.

In the Arabidopsis roots, the auxin-BR interaction could be mediated by BREVIS RADIX (BRX) (Figure 1C), which was originally identified as a regulator of root cell division/elongation [21] and is predicted to regulate transcription [22]. Auxin induces the *BRX* expression and promotes BRX movement from the plasma membrane into the nucleus [23] where it activates the root-specific expression of the *Constitutive Photomorphogenesis and Dwarf* (*CPD*) gene [24] encoding a rate-limiting BR biosynthetic C23-hydroxylase [25]. Another potential mechanism of the auxin-BR crosstalk might be mediated by the BIM1-interacting DRN [13] that acts upstream of auxin signaling/transport [14] but is also a direct target of MONOPTEROS (MP) [26], a well-studied ARF (ARF5) regulating embryonic patterning [27] (Figure 1B).

Regulatory roles of atypical and non-canonical bHLH proteins

Consistent with enrichment of E-box element on many BR-responsive promoters [15], several bHLH proteins were implicated in BR signaling, including BR Enhanced Expression 1 (BEE1) to BEE3 [28] and 3 BIMs [12]. It is known that transcriptional activities of bHLH proteins are often negatively regulated through heterodimerization with a non-DNA-binding HLH protein (often called atypical bHLH proteins in the plant literature) to inhibit DNA binding [29]. The Arabidopsis and rice genomes encode at least 27 and 26 atypical bHLH proteins, respectively [30]. Three recent studies discovered regulatory functions of such atypical bHLH proteins in BR signaling [31–33].

Activation-Tagged *bri1* Suppressor 1 (ATBS1), discovered as a suppressor of a weak BR receptor mutant *bri1-301* [33,34], is a 93-amino-acid protein homologous to 5 other Arabidopsis proteins, including KIDARI and Paclobutrazol Resistance 1 (PRE1) previously implicated in light and gibberellin signaling, respectively [35,36]. Overexpression of *ATBS1* or any of its 5 homologs suppressed *bri1-301*. Despite having a N-terminal basic region, all 6 proteins lack amino acids critical for DNA-binding [29] and are thus atypical bHLH proteins. Consistently, a truncated ATBS1 lacking the basic region still possess the *bri1-301*-rescuing activity, most likely via heterodimerization with one or more closely-related ATBS1-Interacting Factors (AIFs) (Figure 2A), which were discovered by a yeast two-hybrid screen and are also atypical bHLH proteins. Remarkably, overexpression of an *AIF* gene resulted in a severe dwarf phenotype indistinguishable from that of known BR-deficient mutants and suppressed the activation-tagged *atbs1* mutant, whereas RNA interference (RNAi)-mediated silencing of *AIF1* rescued *bri1-301*, suggesting their function redundancy in blocking BR signaling. Interestingly, both *ATBS1* and *AIF1* were previously discovered as BR-regulated genes with *ATBS1* being down-regulated and *AIF1* up-regulated by BR [15]. It remains to be determined how AIF1 and ATBS1 are regulated by BR and how AIF1 inhibits BR signaling. It is interesting to note that ATBS1 was recently discovered as a direct target of MP/ARF5 [26] (Figure 1B), revealing another potential mechanism mediating the auxin-BR crosstalk important for root initiation during embryogenesis.

Support for a role of ATBS1 in BR signaling came from independent studies on two rice genes encoding ATBS1/PRE1-like proteins [31,32] (Figure 2B). *BRASSINOSTEROID UPREGULATED 1* (*BU1*) was discovered as a BR-induced bHLH gene, while *INCREASED LAMINA INCLINATION* (*IL11*) was identified by its activation-tagged mutant phenotype. Overexpression of *BU1* or *IL11* resulted in enhanced lamina inclination [31], a characteristic phenotype of enhanced BR signaling in rice, whereas RNAi-triggered silencing of *BU1* and its homologs or antisense-mediated inhibition of *IL11* led to reduced lamina inclination and decreased BR sensitivity. How BU1 stimulates BR signaling remains unknown but genetic and biochemical studies of *IL11* revealed a potential mechanism of IL11 function that seems different from that of ATBS1 [32]. Despite the presence of several AIF homologs in rice, a yeast two-hybrid screen identified a typical bHLH protein as an IL11-binding bHLH protein (hence the name IBH1) that has a single homolog in Arabidopsis, AtIBH1. Biochemical assays showed that IL11 and its Arabidopsis homolog PRE1 interact with IBH1 and AtIBH1, while transgenic studies supported a role of the IL11/PRE1-IBH1/AtIBH1 interaction in modulating BR responses in both rice and Arabidopsis. ChIP and gene expression analyses indicated that BZR1/OsBZR1 binds to the promoters of *PRE1/IL11* and *AtIBH1/IBH1*, stimulating expression of *PRE1/IL11* but inhibiting transcription of *AtIBH1/IBH1*.

Given the interactions of ATBS1-AIFs, PRE1-AtIBH1, and a previously reported interaction of KIDARI with HFR1, a well-studied atypical bHLH protein involved in light signaling [37], it will be interesting to investigate if each member of the ATBS1/PRE1 family has specific interaction partners or whether heterodimerization between an ATBS1/PRE1 family member

with bHLH proteins is mainly dictated by their tissue/organ-specific expression patterns. Further studies are also needed to determine the relative importance of each member of the ATBS1/PRE1 or BU1/ILI1 family in BR signaling or in crosstalk with other signaling pathways.

Besides these canonical bHLH proteins, a recent study implicated a regulatory role for a non-canonical bHLH protein TCP1 in BR biosynthesis [38], which was previously known to regulate floral symmetry [39]. TCP1 is a member of the plant-specific TFs sharing a highly conserved ~60-amino-acid TCP domain with a bHLH-like structure involved in DNA-binding and protein dimerization [39,40]. Activation-tagging of *TCP1* suppresses the weak *bri1-5* mutation whereas overexpression of a dominant-negative form of TCP1 resulted in a *bri1-5*-like dwarf phenotype that could be rescued by BR application. Metabolic profiling and gene expression analysis suggested that TCP1 activates the *DWARF4* (*DWF4*) gene encoding a rate-limiting BR biosynthetic C22-hydroxylase [41], while a ChIP assay demonstrated a direct TCP1 binding to the *DWF4* promoter (Figure 2A). Interestingly, TCP1 was induced by BR but inhibited by brassinazol (Brz), a specific inhibitor of BR biosynthesis [42], suggesting that TCP1 is not involved in feedback regulation of BR biosynthesis but might play a role to regulate BR biosynthesis and plant growth in response to certain environmental and developmental signals.

Involvement of members of the GRAS, MADS, and MYB families in BR signaling

In addition to bHLH proteins, TFs of other families were recently implicated in BR signaling, which include a GRAS and three MADS proteins in rice and a MYB protein in Arabidopsis [43-45] (Figure 2B). The rice DWARF and LOW TILLERING (DLT) protein and its Arabidopsis homolog AtGRAS8 constitute a unique subgroup of the GRAS family of the plant-specific TFs [46]. Loss-of-function *dlt* mutation not only results in a phenotype resembling weak BR-deficient rice mutants, but also reduces BR sensitivity and impairs the BR feedback regulation likely via inhibiting OsBZR1 expression. Intriguingly, the *DLT* itself is negatively regulated by BRs and its promoter binds OsBZR1 *in vitro*, thus revealing a potential regulatory loop likely for maintaining appropriate levels of OsBZR1 and DLT. However, it remains unknown how DLT affects *OsBZR1* expression and whether the Arabidopsis homolog of DLT, AtGRAS8, plays a similar role in BR signaling.

Besides OsBZR1 and DLT, the BR signaling in rice might also involve three related MADS proteins [44,45] (Figure 2B) that are members of the SHORT VEGETATIVE PHASE (SVP)-subfamily of the MADS TFs, named after the Arabidopsis SVP known to regulate flowering time and floral meristem identity [47]. *Oryza sativa* MADS-domain-containing protein 1 (OsMDP1) was identified as a BR-repressed gene and its silencing resulted in enhanced BR signaling [45]. Mutations in one of its homolog, *OsMADS22*, had no detectable growth defect, but silencing of the other homolog, *OsMADS55*, led to an increased lamina inclination with normal sensitivity to exogenously BR [44]. Interestingly, simultaneously silencing of all three MADS genes gave rise to a larger angle of the lamina inclination with enhanced BR sensitivity, arguing for their redundant roles in regulating BR responses. Further studies are needed to investigate how they regulate BR signaling and to determine whether the involvement of the SVP-type MADS proteins in BR signaling is evolutionarily conserved in Arabidopsis.

As mentioned above, at least 50 TFs are regulated by BR in Arabidopsis [5]. Using a ChIP assay, Li *et al* showed that one of these TFs, AtMYB30, is a direct BES1 target [48]. Genetic, physiology, and microarray analyses suggested that AtMYB30 plays a role in regulating a subset of BR-responsive genes. Remarkably, AtMYB30 also interacts with BES1 and both proteins can bind to promoters of two well-studied BR-responsive genes, suggesting an

interesting model by which BES1 acts cooperatively with its target to amplify BR signaling (Figure 2B). Because AtMYB30 is involved in the hypersensitive response to pathogen attack [49], the BES1-mediated up-regulation of AtMYB30 provides a plausible mechanism by which BR enhances pathogen resistance. In addition to AtMYB30, BR also induces the expression of another well-studied MYB TF, WEREWOLF (WER) [50], to influence root hair formation in Arabidopsis [51], but it remains to be determined whether WER is another target of BES1.

BES1-mediated recruitment of other nuclear proteins

It is well known that eukaryotic gene regulation involves proteins other than TFs, such as transcription elongation factors and chromatin-modifying proteins. The nuclear regulation of BR signaling is no exception (Figure 3). It was shown recently that the full transcriptional activity of BES1 requires AtIWS1 [52], an Arabidopsis homolog of the yeast/human Interacting-With-Spt6 1 (IWS1) known to regulate mRNA export and histone modification [53]. A loss-of-AtIWS1 mutation alters expression of many BR-responsive genes, giving rise to a semi-dwarf phenotype with reduced BR sensitivity. AtIWS1 interacts not only with BES1 but also with the Arabidopsis homolog of the yeast Spt6, a well-studied transcription elongation factor [53], suggesting that AtIWS1 is likely recruited by BES1 to promote transcription elongation of BR-responsive genes. Indeed, AtIWS1 binds preferentially to the transcribed region of a BES1 target gene and that a loss-of-AtIWS1 mutant is hypersensitive to a transcription elongation inhibitor. Interestingly, the human IWS1 recruits a histone methyltransferase to the elongating RNA polymerase II complex, thus stimulating trimethylation of lysine 36 on histone 3 (H3K36me3) crucial for nucleosome reassembly during transcription elongation [54]. It will be interestingly to know if recruitment of a H3K36 methyltransferase by IWS1 is evolutionarily conserved in plants.

A role of histone modification in BR-regulated gene expression was revealed by a recent exciting discovery of enhancement of the BES1 transcriptional activity by ELF6 and REF6 [55], two similar proteins known to antagonistically regulate Arabidopsis flowering [56]. Both proteins contain the highly conserved Jumonji N/C domain present in a group of histone H3 demethylases that demethylate di or trimethylated lysine 9 or lysine 36 on histone H3 (H3K9me2/3 preventing transcription or H3K36me2/3 activating transcription) [57]. BES1 interacts with ELF6 and REF6 to regulate the expression of ~13% of the previously identified BR-induced genes, while a ChIP experiment detected elevated H3K9m3 levels in the promoter region of a BES1 target gene in both *elf6* and *ref6* mutants, supporting a role of ELF6 and REF6 in posttranslational H3 modification. The discovery of ELF6 and REF6 as BES1-interacting factors also suggested a potential molecular link between BR signaling and plant flowering [58].

Conclusions and perspectives

These studies revealed that BR-regulated gene expression is much more complicated than what is expected from a simple linear signaling pathway involving only two highly similar TFs. Some of the discussed TFs were potential targets of BES1 or BZR1/OsBZR1 based on ChIP experiments, but it is important to experimentally verify that the detected TF-promoter binding is necessary for the BR-regulated expression of a target gene of interest. It is expected that other TFs are likely regulated independently of BES1/BZR1 and further studies are needed to elucidate their regulatory mechanisms. For example, a preliminary assay showed that AIF1 was phosphorylated by BIN2 [33], but it remains unknown if AIF1 is phosphorylated by BIN2 *in vivo* and if so, how the BIN2-catalyzed AIF1 phosphorylation affects its inhibitory activity. Regardless of their regulatory mechanisms, a full understanding of BR signaling demands identification of the target genes of not only BES1 and BZR1 but also the BR-regulated TFs. The ChIP method coupled with the whole genome-tiling arrays (ChIP-CHIP) or next-

generation sequencing technologies (ChIP-SEQ) [59] will generate genome-wide binding profiles for each of these BR-regulated TFs and reveal interesting transcriptional networks of BR-regulated processes. It is anticipated that classical and novel genetic approaches, yeast one/two-hybrid screens, and proteomic experiments will uncover additional proteins that regulate the nuclear activities of the BR signaling.

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Figure 1.

The potential roles of BES1 and BR-related TFs in plant development. (A). In *Arabidopsis* flowers, BES1 likely binds to the promoters of known anther/pollen regulatory TFs and a female gametophytic receptor-like kinase *FERONIA* to affect anther/pollen development and pollen discharge, respectively. (B). In *Arabidopsis* embryos, BIM1 physically and genetically interact with DRN and PVH to regulate cotyledon development while an auxin-dependent MP directly activates the expression of *DRN* and *ATBS1* (encoding an atypical bHLH protein recently implicated in BR signaling [33]) to control embryonic cotyledon and root development, respectively. (C). In *Arabidopsis* root, auxin induces the *BRX* expression likely through an ARF and promotes the translocation of BRX from the cell surface to the nucleus where it activates the *CPD* expression crucial for BR biosynthesis.

Figure 2.

The involvement of bHLH proteins and TFs of other families in BR signaling. (A). In Arabidopsis, BR induces *AIF1* and *PRE1* but inhibits *ATBS1* and *AtIBH1* with *BZR1* binding to both *PRE1* and *AtIBH1* promoters. *ATBS1* binds *AIF1*, thus inhibiting *AIF1* dimerization with yet to be discovered bHLH proteins (X) that activate a subset of BR-responsive genes, while *PRE1* heterodimerizes with *AtIBH1* that functions as a negative regulator of BR-mediated cell elongation. BR also induces the expression of *TCP1*, which encodes a non-canonical bHLH protein that binds and activates the *DWF4* promoter to stimulate BR biosynthesis, and stimulate the *AtMYB30* gene, a directly target of *BES1*. *BES1* was shown to interact with *AtMYB30* on the promoters of some BR-responses genes to amplify BR signaling. (B). In rice, BR induces *BU1* and *IL11* but suppresses *IBH1* and *DLT* with *OsBZR1* binding to the promoters of *IL11*, *IBH1*, and *DLT*. *IL11* heterodimerizes with and thus inhibits *IBH1* that blocks BR signaling; while *DLT* promotes BR signaling and plays a role in feedback regulation, likely by activating BR-upregulated genes and *OsBZR1*. The BR signaling in rice might also be negatively regulated by three SVP-type MADS proteins that likely localize in the cytosol [60].

Figure 3. BES1-mediated recruitment of elongation factors and histone-modifying proteins. BES1 not only binds histone-modifying proteins ELF6 and REF6 in the promoters of BR-responsive genes to initiate their transcription, most likely through demethylating histone H3 and altering chromatin structure, but also recruits elongation factors to the phosphorylated C-terminal domain (CTD-P) of the RNA polymerase II (RNAPII) within the transcribed regions of BR-regulated genes to promote the production and export of their mRNAs.