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Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression

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Summary

A paradox of plant hormone biology is how a single small molecule can affect a diverse array of growth and developmental processes. For instance, brassinosteroids (BRs) regulate cell elongation, vascular differentiation, senescence and stress responses. BRs signal through the BES1/BZR1 (bri1-Ethylmethane Sulphonate suppressor 1/brassinazole-resistant 1) family of transcription factors, which regulate hundreds of target genes involved in this pathway, yet little is known of this transcriptional network. Through microarray and chromatin immunoprecipitation (ChIP) experiments, we identified a direct target gene of BES1, *AtMYB30*, which encodes an MYB family transcription factor. *AtMYB30* null mutants display decreased BR responses and enhance the dwarf phenotype of a weak allele of the BR receptor mutant *bri1*. Many BR-regulated genes have reduced expression and/or hormone-induction in *AtMYB30* mutants, indicating that AtMYB30 functions to promote expression of a subset of BR target genes. AtMYB30 and BES1 bind to a conserved MYB-binding site and E-box sequences, respectively, in the promoters of genes that are regulated by both BRs and AtMYB30. Finally, AtMYB30 and BES1 interact with each other both *in vitro* and *in vivo*. These results demonstrate that BES1 and AtMYB30 function cooperatively to promote BR target gene expression. Our results therefore establish a new mechanism by which AtMYB30, a direct target of BES1, functions to amplify BR signaling by helping BES1 activate downstream target genes.

Keywords: BES1, brassinosteroids, MYB30, target genes, transcription.

Introduction

Brassinosteroids (BRs) play important roles in many plant growth and developmental processes, including germination, cell expansion and division, photomorphogenesis, vascular differentiation, senescence and stress/disease resistance (Clouse, 1996; Li and Chory, 1999; Krishna, 2003). Mutants defective in BR biosynthesis or perception display dwarf phenotypes under both light and dark conditions (Clouse *et al.*, 1996; Li *et al.*, 1996; Szekeres *et al.*, 1996; Li and Chory, 1997).

Genetic and molecular studies in Arabidopsis have greatly advanced our understanding of the BR signaling pathway (Clouse, 2002; Thummel and Chory, 2002; Belkhadir and Chory, 2006; Li and Jin, 2007). The BR receptor was identified by many mutant alleles in a single gene, *BRASSINOSTEROID INSENSITIVE1* (*BRI1*), which encodes a leucine-rich repeat (LRR) receptor kinase (Clouse *et al.*, 1996; Li and Chory, 1997). BRI1 binds BRs through a novel 100 amino acid sub-domain embedded within the LRRs, and transduces the hormone signal to downstream targets through its intracellular kinase domain (Friedrichsen *et al.*, 2000; He *et al.*, 2000; Oh *et al.*, 2000; Wang *et al.*, 2001; Kinoshita *et al.*, 2005; Wang *et al.*, 2005a,b). In the absence of BRs, a novel protein, BKI1, binds to BRI1 and inhibits its function (Wang and Chory, 2006). BR binding to BRI1 leads to dissociation of BKI1 (Wang and Chory, 2006) and increased association of BRI1 with BAK1, another LRR receptor kinase (Li *et al.*, 2002;

Nam and Li, 2002). BRI1 may signal through BSKs (BR signaling kinases) to downstream targets (Tang *et al.*, 2008).

The output of the signaling pathway is the dephosphorylation of closely related transcription factors including BES1, BZR1 and four other family members in Arabidopsis (Wang *et al.*, 2002; Yin *et al.*, 2002; Zhao *et al.*, 2002). BES1 was identified by a gain-of-function mutation in the *BES1* gene, which suppresses *bri1* dwarfism. The gain-of-function mutant *bes1-D* has a constitutive BR response phenotype, including excessive stem elongation, early senescence and resistance to the BR biosynthesis inhibitor brassinazole (BRZ) in both dark- and light-grown seedlings, as well as up-regulation of BR-induced gene expression (Yin *et al.*, 2002), most likely due to increased BES1 protein levels. BZR1 was identified by a gain-of-function dominant mutation that leads to its over-accumulation; *bzr1-D* seedlings are resistant to BRZ in the dark but hypersensitive to BRZ in the light, due to increased feedback inhibition of BR biosynthesis (Wang *et al.*, 2002). Consistent with the difference between the mutant phenotypes in the light, BES1 was shown to be a transcriptional activator while BZR1 is a transcription repressor (He *et al.*, 2005; Yin *et al.*, 2005).

BES1 and BZR1 activities are regulated by a GSK3-like kinase BIN2 (Choe *et al.*, 2002; Li and Nam, 2002; Pérez-Pérez *et al.*, 2002). BIN2 phosphorylates BES1 and BZR1 and negatively regulates their function (He *et al.*, 2002; Yin *et al.*, 2002; Vert and Chory, 2006; Gampala *et al.*, 2007; Gendron and Wang, 2007; Ryu *et al.*, 2007). BR signaling through BRI1 inhibits BIN2 function by an unknown mechanism, leading to accumulation of non-phosphorylated BES1/BZR1 in the nucleus. The dephosphorylation of BES1 is facilitated by BSU1 phosphatase, which is required for accumulation of non-phosphorylated BES1 (Mora-Garcia *et al.*, 2004). The non-phosphorylated forms are capable of binding promoter elements in BR-regulated genes.

Several genome-wide microarray analyses performed in Arabidopsis have indicated that BRs regulate several classes of target genes, including many cell-wall organization enzymes required for cell expansion and division, genes involved in ethylene biosynthesis and many other metabolic pathways, transcription factors, signaling molecules and genes with unknown functions (Goda *et al.*, 2002; Mussig *et al.*, 2002; Yin *et al.*, 2002; Goda *et al.*, 2004; Nemhauser *et al.*, 2004). In light-grown seedlings, BR treatment for 2.5 h induces the expression of approximately 342 genes and represses the expression of 296 genes (Nemhauser *et al.*, 2004). Longer BR treatment (12–24 h) affected many more genes (Goda *et al.*, 2004). Reduction of BR levels in root tissues in the *brevis radix* mutant (*brx*, which has reduced root growth*)* affected approximately 4000 (approximately 15%) of the Arabidopsis genes, indicating a more profound effect of BRs on long-term gene expression (Mouchel *et al.*, 2006). How BRs regulate various subsets of target genes in various tissues, organs, developmental stages and environmental conditions is largely unknown.

BR-induced genes include more than 29 transcription factors (Nemhauser *et al.*, 2004), many of which are also up-regulated in the *bes1-D* mutant (our unpublished results). This observation raises the possibility that BES1 directly regulates some of these transcriptional factors, which either mediate or modulate BR target gene expression. Here, we report the characterization of one BES1-induced transcription factor, AtMYB30, which was previously found to be involved in cell death in the hypersensitive response during pathogen attack in adult Arabidopsis plants (Daniel *et al.*, 1999; Raffaele *et al.*, 2006, 2008). At the young seedling stage, knockout of the *AtMYB30* gene caused altered BR responses and target gene expression. We also found that AtMYB30 protein binds to a conserved MYB-binding site in BR target gene promoters and interacts with BES1. Thus, AtMYB30 is a node in a transcriptional circuit by which BRs regulate target gene expression.

Results

AtMYB30 *is a BES1 direct target*

Recently published microarray studies identified 342 BR-induced genes, of which 29 encode putative transcription factors (Nemhauser *et al.*, 2004). We have been studying the functions of several BR-regulated transcription factors. Here we present the characterization of *AtMYB30* (At3g28910), whose expression level increased approximately 1.5-fold after BR treatment in Arabidopsis seedlings (Nemhauser *et al.*, 2004). To confirm the microarray result, we examined *AtMYB30* expression levels using quantitative real-time PCR (Figure 1a). As expected, *AtMYB30* was induced approximately 1.5-fold in wild-type seedlings after treatment with BL (brassinolide), the most active BR. Moreover, in *bes1-D* mutants (in which BES1 protein levels are increased), *AtMYB30* expression levels were significantly higher than in wild-type plants treated with or without BL. In the dark, the expression level of AtMYB30 was approximately 2-folds as that of seedlings grown under light, and is reduced by BRZ, which specifically blocks BR biosynthesis at the C22 hydroxylation step, thereby decreasing the endogenous BR level (Asami *et al.*, 2000). The induction of *AtMYB30* by BRs has also been reported in the Arabidopsis gene expression eFP browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). All of these results indicate that AtMYB30 is regulated by BRs/BES1, and may function in the BR pathway.

Chromatin immunoprecipitation (ChIP) experiments were used to determine whether BES1 directly regulates *AtMYB30* expression levels. ChIP was performed using anti-BES1 antibody and a control antibody. TA3, a retrotransposable element in Arabidopsis, which does not respond to BRs or contain BES1-binding sites, was used as the internal control

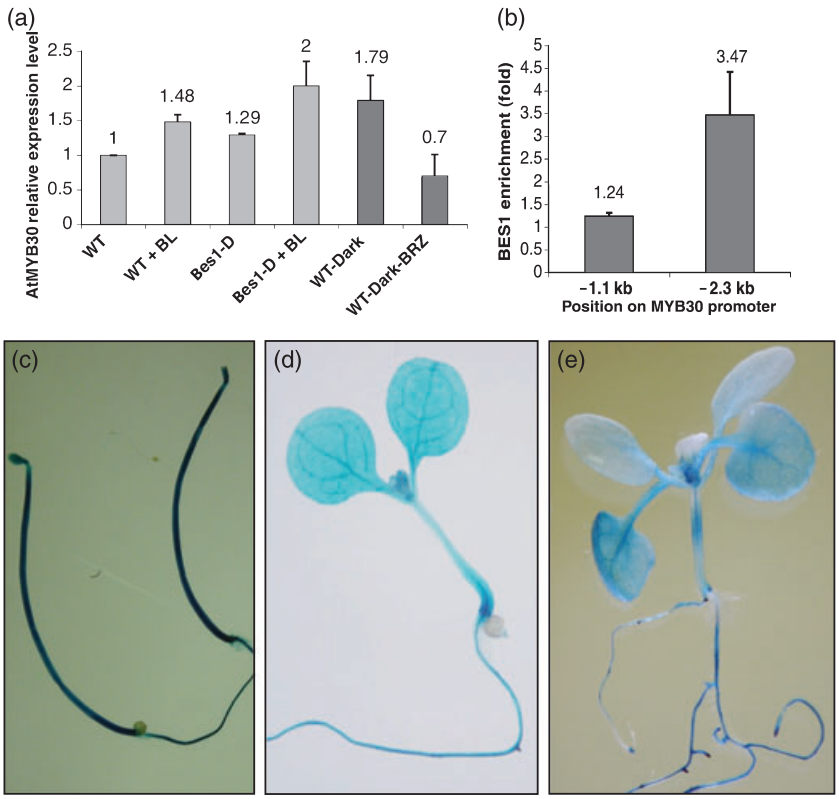

Figure 1. AtMYB30 is a BES1 direct target.
(a) The expression levels of *AtMYB30* were determined using quantitative real-time PCR with RNA prepared from 10-day-old light-grown Col-0 (WT) or *bes1-D* seedlings treated with or without 1 μM BL for 3 h, and 5-day-old dark-grown Col-0 (WT) in the absence or presence of 1 μM BRZ. The number on each column represents the relative expression levels compared to *UBQ5* (At5g15400) as an internal control.
(b) ChIP assay indicating that BES1 is associated with the promoter of AtMYB30 *in vivo*. BES1 antibody and GFP antibody (as control) were used to immunoprecipitate chromatin prepared from 10-day-old light-grown *bes1-D* seedlings. Quantitative real-time PCR was performed using primers from indicated positions in the *AtMYB30* promoter. The fold changes were calculated based on the change for anti-BES1 relative to anti-GFP, after normalization to a TA3 internal control.
(c–e) AtMYB30 expression patterns in 5-day-old dark-grown (c) or 5- (d) or 10-day-old (e) light-grown seedlings, detected by the GUS reporter gene.

(Yu *et al.*, 2008). It has been demonstrated that BES1 can bind to E-boxes (CANNTG) to regulate target gene expression (Yin *et al.*, 2005). Quantitative real-time PCR was performed using ChIP products and PCR primers flanking E-boxes at −2.3 and −1.1 kb with respect to the AtMYB30 promoter (Figure 1b). BES1 was enriched significantly at position −2.3 but not at −1.1 kb, suggesting that BES1 binds to a −2.3 kb promoter region *in vivo*. We therefore conclude that BES1 activates *AtMYB30* expression by directly binding to the *AtMYB30* promoter, probably through E-box sequences.

AtMYB30 *knockout mutants show altered BR response phenotypes*

To study the function of AtMYB30 in the BR pathway, we first determined its expression pattern using the GUS reporter gene fused to the AtMYB30 promoter fragment. Consistent with our previous quantitative PCR analysis (Figure 1a), AtMYB30 is strongly expressed in dark-grown seedlings (Figure 1c). In light-grown seedlings, *AtMYB30* was expressed strongly in roots, cotyledons and hypocotyls, and relatively weakly in leaves (Figure 1d,e). The strong expression of *AtMYB30* in young seedlings and lack of detectable expression in adult plants (Daniel *et al.*, 1999) suggest that the gene plays an important role during early stages of plant development. It is worth noting that BES1 is ubiquitously expressed throughout entire seedlings, overlapping with the AtMYB30 expression regions (Yin *et al.*, 2002).

To investigate the role of AtMYB30 in the BR response, we obtained two T-DNA knockout lines, *atmyb30-1*, with a T-DNA insertion located in the third exon, and *atmyb30-2*, with a T-DNA insertion in the 5′ UTR region (Figure 2a). No transcripts of the *AtMYB30* gene were detected by RT-PCR in either line (Figure 4, and data not shown), suggesting that they are null alleles. The knockout lines showed altered BR response phenotypes as measured by hypocotyl elongation assays in the absence or presence of BRZ (Figure 2). Because the main function of BRs is to regulate cell elongation, hypocotyl elongation assays are routinely used to determine BR activity (e.g. Li and Chory, 1997; Nemhauser *et al.*, 2004; Yin *et al.*, 2005).

In the dark, the knockout mutants had slightly shorter hypocotyls compared to the wild-type control (Figure 2b,c). More significantly, in the presence of 1 μM BRZ, the knockout lines were approximately 30% shorter than wild-type (Figure 2b,c). These experiments have been repeated more than five times with similar results. The differences are significant as determined by Student's *t* test ($P < 0.01$). The results indicate that AtMYB30 knockout lines are more sensitive to BRZ compared to wild-type seedlings, suggesting that AtMYB30 is involved in BR responses.

The light-grown seedlings of *AtMYB30* mutants were not obviously different from the wild-type plants; however, the mutants were more sensitive to BRZ, with shorter hypocotyls and darker green leaves (Figure 2d). Taken together,

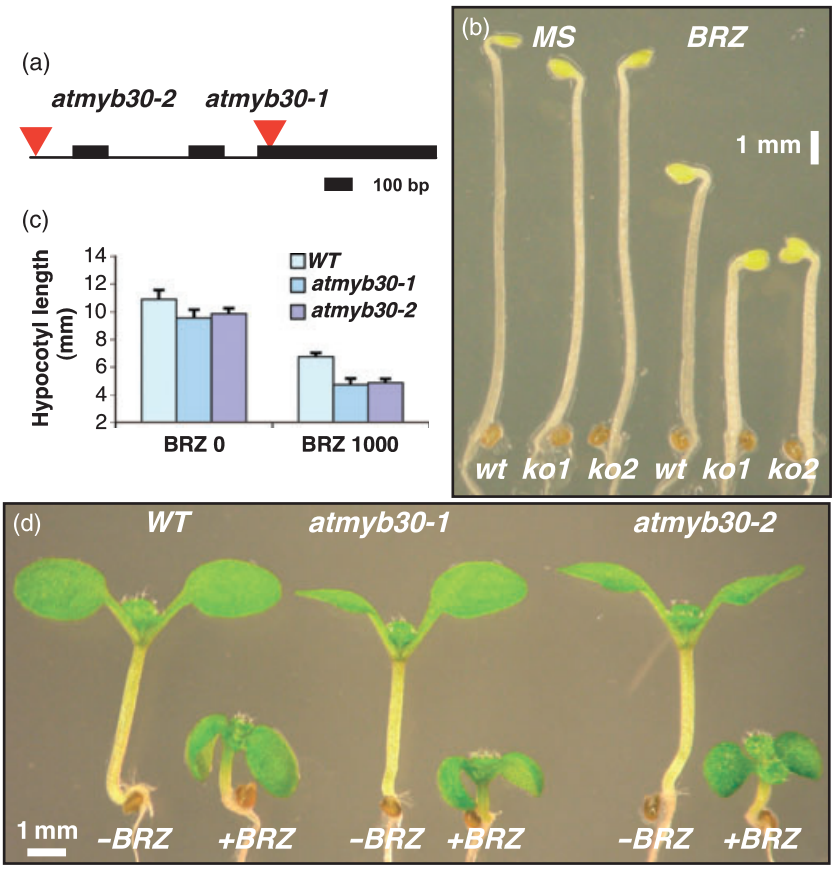

Figure 2. AtMYB30 knockout and over-expression lines show an altered BR response phenotype.
(a) Schematic representation of the two T-DNA knockout alleles of *AtMYB30* gene.
(b) Five-day-old dark-grown seedlings of Col-0 (WT), *atmyb30-1* and *atmyb30-2* grown on half-strength MS medium with or without 1 μM BRZ.
(c) The hypocotyl lengths of 5-day-old dark-grown seedlings in the absence or presence of 1 μM BRZ. Means and standard deviations were calculated from 15–30 seedlings.
(d) Six-day-old light-grown seedlings grown on half-strength MS medium with or without 1 μM BRZ.

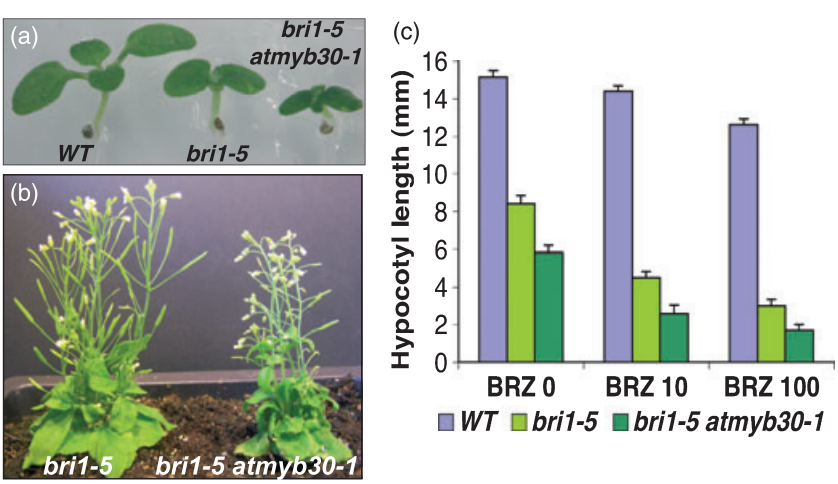

Figure 3. *AtMYB30* knockout mutants enhance the *bri1-5* mutant phenotype.
(a) Six-day-old light-grown WT, *bri1-5* and *bri1-5 atmyb30-1* seedlings grown on half-strength MS plates.
(b) Adult phenotypes of *bri1-5* and *bri1-5 atmyb30-1* in soil.
(c) Hypocotyl length measurements for 5-day-old dark-grown seedlings in the presence of various concentrations of BRZ (nM).

these results demonstrate that AtMYB30 plays a positive role in the BR signaling pathway.

To test whether there is any genetic interaction between BRI1 and AtMYB30, we crossed *atmyb30-1* with *bri1-5*, a weak allele of *bri1*, and generated a *bri1-5 atmyb30-1* double mutant (Figure 3). *bri1-5* has a mutation in the extracellular domain of the BR receptor BRI1, and displays a semi-dwarf phenotype (Noguchi *et al.*, 1999). The double mutant showed a significantly enhanced *bri1-5* phenotype, including reduced hypocotyl and leaf petiole length, and rounder, curlier and darker-green leaves under light conditions (Figure 3a,b and S1). The inflorescence stems of double mutants were shorter than those of *bri1-5* (Figure 3b), although the difference became much more subtle as plants matured (data not shown). We also tested the BRZ response with dark-grown seedlings of wild-type, *bri1-5* and *bri1-5*

atmyb30-1 double mutants. As shown in Figure 3c, the *bri1-5 atmyb30-1* double mutant was more sensitive to BRZ than *bri1-5* was. The results suggest that AtMYB30 is involved in BR pathway.

AtMYB30 regulates a subset of BR-induced genes

The fact that AtMYB30 is a direct target of BES1 and that loss-of-function mutants display altered BR response phenotypes suggests that AtMYB30 may function to modulate BR-regulated gene expression. We used Affymetrix Arabidopsis genome arrays to examine BR-regulated gene expression in *atmyb30-1* in the absence or presence of BRZ in the dark, conditions under which the mutants showed the clearest and most consistent phenotypes (Figure 2b). A Western blotting experiment indicated that non-phosphorylated BES1 (the active form) was reduced significantly in the presence of BRZ in the dark (Figure S2), suggesting that BRZ can reduce BES1 activity. In addition, previous microarray experiments indicated that there is a strong inverse correlation between BR- and BRZ-regulated genes (Goda *et al.*, 2002). We therefore considered the absence of BRZ as 'plus BR' and the presence of BRZ as 'minus BR' conditions, respectively. As BRs usually induce gene expression by a small multiple (1.4–5-fold) (Nemhauser *et al.*, 2004), we set the cut-off ratio to a twofold change and only chose genes with an expression level above 100 in wild-type without BRZ treatment for further analysis. Approximately 400 genes were down-regulated (greater than twofold reduction) by BRZ treatment in wild-type and therefore are probably BR-induced genes in the dark. More importantly, approximately 25% of those 400 genes were reduced at least twofold in *atmyb30-1* mutants compared with wild-type seedlings grown on either control or BRZ medium (data not shown). Nine genes that showed most significant changes were chosen for semi-quantitative RT-PCR with independent biological samples. All nine genes were clearly reduced in the *atmyb30* mutant, in good agreement with the microarray results (Figure 4). BRZ treatment also appeared to reduce target gene expression in light-grown seedlings. The expression level of *TCH4* (At5g57560), which is known to be involved in BR-regulated cell elongation (Xu *et al.*, 1995), was reduced in 6-day-old light-grown *atmyb30-1* seedlings with or without BRZ treatment (Figure S3). These results indicate that BES1 and AtMYB30 act cooperatively to activate expression of a subset of BR target genes in young seedlings.

We also examined BR responses using several BR marker genes in light-grown seedlings by quantitative real-time PCR (Figure 5). In general, the expression levels of BR-induced genes were reduced either in the absence or presence of BL in the *atmyb30* mutant. BL induction was significantly decreased in at least two genes tested (At5g23860 and At2g21140). Taken together, the gene expression studies

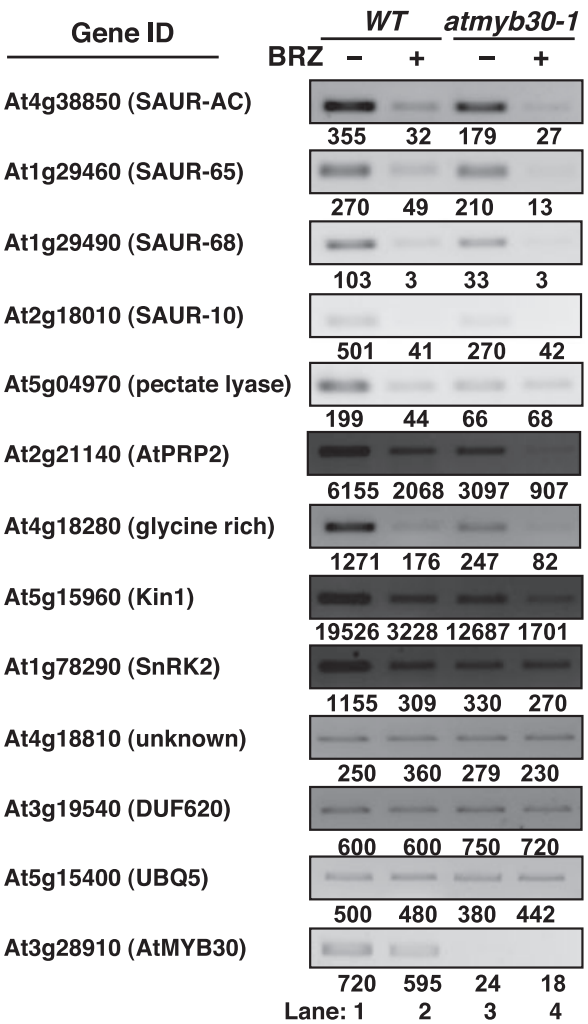

Figure 4. Expression of a subset of BR-regulated genes is reduced in *atmyb30*.
(a) RT-PCR was performed using a biological replicate of material used in microarray experiments with 5-day-old dark grown seedlings in the absence or presence of 1 μM BRZ. The results for nine BRZ-regulated genes, three control genes (At4g18810, At3g19540 and At5g15400/*UBQ5*) and *AtMYB30* are shown. The numbers below the gel panels (lanes 1–4) are the expression levels from the microarray experiments.

suggest that AtMYB30 is required for the optimal expression and/or BL induction of a subset of BR target genes in both dark- and light-grown seedlings.

AtMYB30 binds to a conserved MYB site in target gene promoters

To study how AtMYB30 regulates BR-induced (or BRZ-repressed) gene expression, we examined the promoter of *SAUR-AC1*, a well-established BES1 target gene (Yin *et al.*, 2005) that is also regulated by AtMYB30 (Figure 4). In the approximately 600 bp *SAUR-AC1* promoter region, there are five E-box sequences, including four CACATG E-boxes

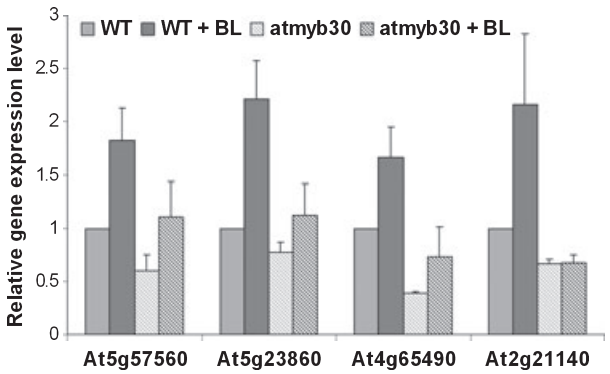

Figure 5. BR-induced gene expression was reduced in the *atmyb30* mutant. Quantitative real-time PCR was performed using 6-day-old light-grown *atmyb30-1* or WT seedlings treated with or without 1 μM BL for 3 h for At5g57560 (*TCH4*), At5g23860, At4g65490 and At4g21140. *UBQ5* was used as the reference gene. The gene expression levels were significantly reduced both in the absence and presence of BL in the *atmyb30-1* mutant, as tested by a two-way ANOVA *F* test ($P < 0.05$).

(designated E1) that have been previously shown to bind BES1 (Yin *et al.*, 2005) and one CACTTG E-box (designated E2) (Figure 6a). Adjacent to the E2 site, there is a putative MYB-binding site (AACAAAC, designated MYB1) predicted by AGRIS (http://arabidopsis.med.ohio-state.edu/AtcisDB/) (Davuluri *et al.*, 2003; Palaniswamy *et al.*, 2006) (Figure 6a). This observation raises the possibility that AtMYB30 directly binds to BR target gene promoters with BES1 to activate gene expression. We used gel mobility shift assays (GMSAs) to test this hypothesis. DNA probes containing both E2 and MYB1 sites or a mutated MYB1 site with six of the seven base pairs changed were first used in a binding assay with recombinant BES1 and AtMYB30 proteins, both fused to MBP (maltose binding protein) at their N-termini (Figure 6a). BES1 bound to the probe specifically, and its binding can be competed by unlabeled probe (Figure 6b, lanes 1–3) and super-shifted by anti-MBP antibody (lane 4). AtMYB30 also bound to the probe (lane 6), and the binding can be competed by unlabeled wild-type probe (lanes 7 and 8) but not by a mutant probe in which the MYB1 site is totally disrupted (lanes 9 and 10). AtMYB30 binding was super-shifted by anti-MBP antibody (lane 11). Moreover, when both BES1 and AtMYB30 were present, they appeared to bind to the probe independently (lane 12).

To further confirm the AtMYB30 binding site, we performed a competition assay with a set of mutants in which every two residues within the MYB1-binding site were mutated to AA (Figure 6c). Mutations of the residues within the predicted MYB1 site (mutant 1–4) significantly reduced their ability to bind AtMYB30, while a mutation (mutant 5) outside the MYB1 site (Figure 6c) did not affect its binding. These results indicate that AtMYB30 specifically binds to the AACAAAC site found in the *SAUR-AC1* promoter.

AtMYB30 and BES1 bind to and regulate target gene promoters in vivo

We then used a ChIP assay to test whether BES1 and AtMYB30 can bind to *SAUR-AC1* and other target gene promoters *in vivo* using *AtMYB30–Myc* transgenic plants (Figure 6d). We generated transgenic tagged lines containing AtMYB30–MYC driven by the strong and constitutive BRI1 promoter (Li and Chory, 1997). Anti-BES1 and anti-Myc antibodies were used to immunoprecipitate BES1- and AtMYB30-associated chromatin, respectively, with IgG as the antibody control. TA3 was used as the internal control. When dark-grown seedlings were used to prepare chromatin, the *SAUR-AC1* promoter was enriched in both anti-Myc and anti-BES1 samples compared to the IgG control (Figure 6d, left bars). On the other hand, as shown in Figure 6d (right bars), the *TCH4* gene promoter was clearly enriched in light-grown seedlings (in which *TCH4* is reduced in *atmyb30-1*, Figure 5) by both BES1 and MYB30. These results suggest that AtMYB30 and BES1 can bind to target gene promoters *in vivo*.

AtMYB30 interacts with BES1 in vitro *and* in vivo

The functional interaction of BES1 and AtMYB30 prompted us to examine whether they physically interact with each other. We first examined the interaction using a bi-molecular fluorescence complementation (BiFC) assay. In the assay, Enhanced Yellow Fluorescent Protein (EYFP) is split into N-terminal (YFP-N) and C-terminal (YFP-C) parts (Citovsky *et al.*, 2006). We fused YFP-C downstream of BES1 and YFP-N upstream of AtMYB30. When both constructs were transformed into Arabidopsis protoplasts, strong fluorescence was observed in the nucleus (Figure 7a,b), indicating reconstruction of the EYFP protein and a physical interaction between BES1 and AtMYB30 *in vivo*. Approximately 2–5% of the protoplasts we observed showed positive signals, depending on the quality of the protoplasts. As negative controls, BES1–YFP-C plus YFP-N or YFP-N–AtMYB30 plus YFP-C were also co-transformed into protoplasts and no positive signal was observed (Figure 7c).

To further test whether there is direct interaction between AtMYB30 and BES1, we performed a GST pull-down assay with purified proteins expressed from *Escherichia coli*. As shown in Figure 7d, GST–AtMYB30 can pull-down significantly more MBP–BES1 than the GST control (Figure 7d, top gel), suggesting that BES1 interacts with AtMYB30 directly *in vitro*. To identify the domain of BES1 required for the interaction, we examined the binding between AtMYB30–MBP and a series of truncated GST–BES1 proteins (Figure 7d, middle gel). While deletion to BES1 amino acid positions 89 and 140 had no effect on AtMYB30 binding, deletion to amino acid 198 of BES1 reduced the interaction. Further deletion of BES1 to amino acid 272 abolished the

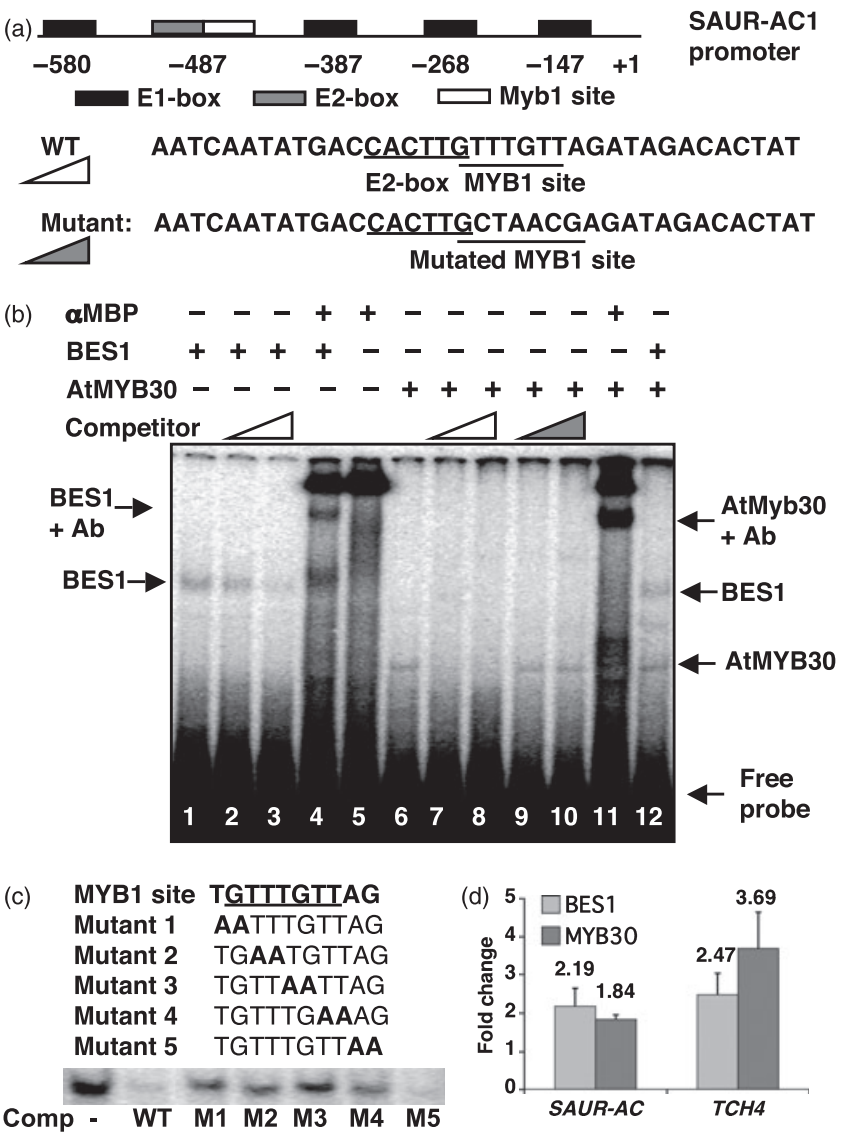

Figure 6. AtMYB30 and BES1 bind to promoters of target genes *in vitro* and *in vivo*.
(a) Schematic representations of *SAUR-AC1* gene promoters showing the E1 (CACATG) and E2 (CACTTG) boxes and the MYB1 (GTTTGTT/AACAAAC) binding site. Sequences of the wild-type probe with both E2 and MYB1 sites and the mutant probe with a mutated MYB1 site were shown.
(b) Gel mobility shift assays (GMSAs) with BES1 and AtMYB30 proteins using probe derived from the *SAUR-AC1* promoter. Arrows on the left and right indicate the BES1 and AtMYB30 bands. In lanes 4, 5 and 11, 5 μl anti-MBP antibody was added, and BES1 or AtMYB30 complexes with antibody (Ab) are also indicated. Anti-MBP alone produced a background band (lane 5) that was also present in lanes 4 and 11. The triangles represent competition with increasing concentrations (50× and 250×) of unlabeled wild-type (open) or mutant (filled) probes.
(c) Competition of AtMYB30 binding with various mutations in the MYB1 site. AtMYB30 protein bound to the wild-type probe (lane 1). The binding can be competed by 100× cold self-competitor (lane 2) and by mutant 5 with a mutation outside the MYB1 site, but not by mutants 1–4 with two consecutive residues mutated within the MYB1 site.
(d) Quantitative real-time ChIP PCR assay showing that AtMYB30 and BES1 interact with *SAUR-AC1* and *TCH4* gene promoters *in vivo*. Anti-Myc and anti-BES1 antibodies were used to precipitate chromatin prepared from 5-day-old dark-grown or 6-day-old light-grown seedlings. IgG was used as an antibody control. Primers from *SAUR-AC1*, *TCH4* or *TA3* (internal control) were used to detect the corresponding promoters in ChIP products. The fold changes were calculated based on the relative change in either anti-Myc or anti-BES1 compared with anti-IgG, after normalization to the TA3 internal control.

interaction with AtMYB30. These results suggest that the central part of BES1 (amino acids 140–272) is important for interaction with AtMYB30.

Discussion

BES1 is a key transcription factor in the BR signaling pathway, regulating the expression of many genes. However, relatively little is known about how BES1 regulates target gene expression and BR responses. In this study, we demonstrate that BES1 interacts with one of its target transcription factors to regulate the expression of a subset of BR-induced genes. We previously found that BES1 binds E-boxes with its partner BIM1, a bHLH protein (Yin *et al.*, 2002, 2005). A large number of E-box elements have been predicted in the promoter regions of Arabidopsis genes. However, only a small proportion of these genes are regulated by BES1, implying that BES1 requires other functional partners to regulate target genes at certain developmental or environmental stages. The requirement for AtMYB30 for optimal BES1-induced gene expression at the seedling stage provides an important mechanism to modulate BES1 function, i.e. a promoter needs to have both BES1 and AtMYB30 binding sites to achieve a high level of gene expression. The direct interaction of BES1 and AtMYB30 may explain, at least in part, the synergistic activation of the subset of BR target genes. As BES1 and AtMYB30 do not bind DNA cooperatively (Figure 6), their interactions may lead to synergistic transcriptional activation. Considering the rather weak direct interaction between BES1 and AtMYB30 *in vitro* (i.e. only approximately 2–3% of the input, Figure 6d), it is possible that additional transcriptional factors or co-factors are involved in the formation of a transcriptional activation complex for BR target gene expression, similar to the

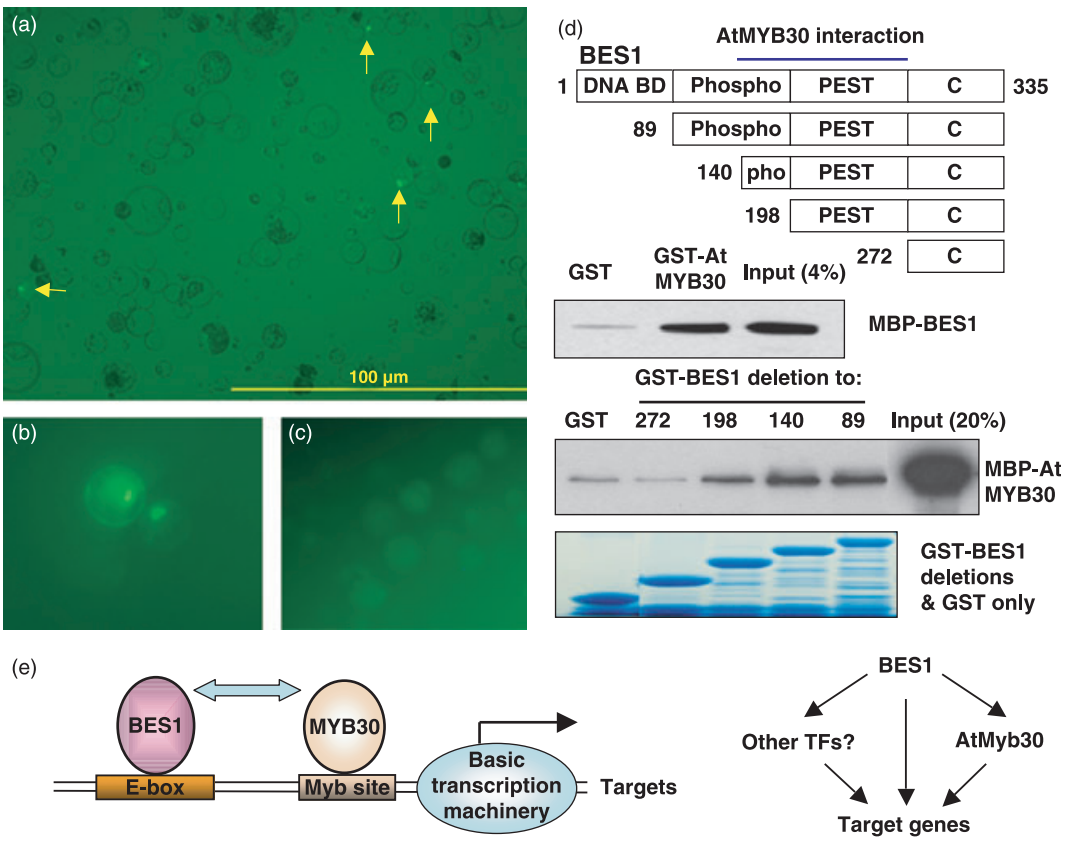

Figure 7. AtMYB30 interacts with BES1 *in vitro* and *in vivo*.
(a,b) Co-expression of BES1–EYFP-C and EYFP-N–AtMYB30 led to the reconstitution of EYFP activity as observed under an Olympus IX71 fluorescence microscope with a YFP filter. Protoplasts with positive signals in the nuclei are indicated by arrows.
(c) Co-expression of BES1–YFP-C and YFP-N did not produce any positive signal.
(d) BES1 interacts with AtMYB30 by GST pull-down assay. The full-length BES1 structure (amino acids 1–335), including the DNA binding domain (DNA BD), BIN2 phosphorylation domain (Phospho), PEST motif and the C-terminus (C), as well as various deletions, is shown. Approximately equal amounts of GST, GST–AtMYB30 and MBP–BES1 (top gel) or GST, GST–BES1 deletions and MBP–AtMYB30 (middle gel) proteins were used in the assays, as shown by a Coomassie-stained gel (bottom gel). The proteins were detected by Western blotting with anti-MBP antibody.
(e) Model of AtMYB30 function in BES1-regulated gene expression. BES1 activates the expression of AtMYB30, which binds to the target gene promoters together with BES1 to synergistically activate a subset of BR target genes. Other BES1-induced transcription factors (TFs) may function similarly to AtMYB30 to regulate other BR target genes.

enhanceosome that is involved in activation of IFNβ gene expression (Thanos and Maniatis, 1995). Several transcriptional factors (IRF-1, ATF-2/C-Jun, HMG and NF-κB) bind to various DNA elements in the IFNβ promoter to form a transcriptional activation complex (enhanceosome) for synergistic induction of the target gene.

MYB transcription factors are one of the largest transcription factor families in Arabidopsis, and are involved in a broad spectrum of physiological processes (Jin and Martin, 1999). MYB transcription factors are known to cooperate with other transcription factors to control developmental processes and hormone-regulated gene expression. In maize, MYB transcriptional factor C1 and bHLH factor R interact to regulate genes in the anthocyanin pathway (Grotewold *et al.*, 1998). In addition, AtMYB2 and AtMYC2 (bHLH) function together to activate genes in the abscisic acid (ABA) signaling pathway (Abe *et al.*, 2003). More recently, it has been reported that AtMYB77 interacts with auxin response factor ARF7 to synergistically activate target genes in the auxin response pathway (Shin *et al.*, 2007). Our study provides a new example of a situation in which an MYB factor (AtMYB30) functionally and physically interacts with an atypical bHLH transcription factor (BES1) to regulate BR target gene expression (Figure 7e, left).

Based on our observations that BES1 directly regulates AtMYB30 gene expression and that BES1 and AtMYB30 act cooperatively to regulate BR target gene expression, we propose that AtMYB30 functions as a BES1 target to amplify the BR signal (Figure 7e, right). This model correlates well with the kinetics of BR-regulated gene expression. Both auxin and BRs function to promote cell elongation; however, it has long been recognized that the kinetics of BR-promoted elongation is much slower than that of auxin (Clouse *et al.*, 1992; Zurek *et al.*, 1994). The model indicating that BES1

activates and cooperates with secondary transcription factors to activate target genes may partially explain the longer time required for maximum BR-regulated gene expression.

BRs appear to regulate various sets of genes under various conditions (Nemhauser *et al.*, 2004; Goda *et al.*, 2004). This differential gene expression can be achieved by interactions of BES1 or its family members with various partners. AtMYB30, the partner of BES1 identified in this study, appears to function during very early stages of plant development, which is in good agreement with a previous report that the mRNA level of AtMYB30 was relatively high in seedlings, but hardly detectable at adult stages (Daniel *et al.*, 1999). Consistent with this developmental expression pattern, the BR response phenotype in *AtMYB30* mutants is strongest in young seedlings (Figure 2), and there is no obvious phenotype difference between AtMYB30 knockout and wild-type plants at the adult stage (our unpublished observations). As BRs regulate cell elongation at both seedling and adult stages, other factors may function during the later stages to amplify the BR signal. In light of this, at least 28 other transcription factors are induced by BRs (Nemhauser *et al.*, 2004), and some of them may function in various tissues and/or developmental stages in a similar manner to AtMYB30 (Figure 7e).

AtMYB30 itself appears to act in various pathways at various developmental stages. AtMYB30 is involved in BR-regulated gene expression at early stages, but is implicated in the pathogen response in adult plants. Although AtMYB30 is not expressed at adult stages under normal conditions, its expression is rapidly induced during the hypersensitive response to bacterial pathogens (Daniel *et al.*, 1999). While over-expression of *AtMYB30* increases the hypersensitive response and pathogen resistance, suppression of the gene causes the opposite phenotype, indicating that AtMYB30 is a positive regulator of the hypersensitive response (Vailleau *et al.*, 2002). Recently, AtMYB30 has been shown to regulate the biosynthesis of very-long-chain fatty acids, which function as the cell-death messengers in plants (Raffaele *et al.*, 2008).

In summary, our study establishes a new mechanism by which BRs function through BES1 and one of its targets, AtMYB30, to cooperatively activate target gene expression and amplify the hormone signal. The results not only provide important insights into the network of BR-regulated gene expression, but also help explain the general mechanisms through which a specific signal can be amplified during growth, development and responses to a changing environment.

Experimental procedures

Plant materials, growth conditions and hypocotyl elongation assay

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control. T-DNA insertion mutants *atmyb30-1* and *atmyb30-2* were obtained from the Arabidopsis Biological Resource Center, and correspond to lines SALK_122884 and SALK_027644, respectively. Plants were grown in plates or soil under long-day conditions (15 h light/9 h dark) at 22°C with 70% humidity. BRZ was added to the half-strength MS agar medium during the assays. For the hypocotyl elongation assay, seeds collected from plants grown synchronously were planted in half-strength MS agar plates and kept at 4°C in the dark for 5 days. For hypocotyl measurement under dark treatment, seeds were exposed to light for 12 h before kept in the dark at room temperature for 5 more days. Hypocotyls were measured from the root of the seedlings to the base of cotyledons. Fifteen to thirty seedlings were measured in each assay, which were repeated more than three times. For BL treatment, approximately 100 mg of 6-day-old light-grown seedlings were treated with 1 μM BL or DMSO (as a control) in liquid half-strength MS medium for 3 h.

Plasmid construction

All the primers used in this study are listed in Table S1. For recombinant protein and GST pull-down assays, AtMYB30 and BES1 coding regions were amplified from Col-0 cDNA and incorporated into the pETMALc-H vector (Pryor and Leiting, 1997). AtMYB30 and BES1 deletion constructs were cloned into pET42a(+) (Novagen, http://www.emdbioscience.com/html/NVG/home.html). For transgenic over-expression plants, the AtMYB30 ORF was fused with a 2 × MYC tag flanked by the BRI1 promoter (Li and Chory, 1997) and the RBCS terminator in pZP211 (Hajdukiewicz *et al.*, 1994).

For the BiFC assay, constructs of the N- or C-termini of EYFP were used; these have been described previously (Yu *et al.*, 2008). The coding region of AtMYB30 and BES1 were inserted downstream of YFP-N or upstream of YFP-C, respectively.

Plant transformation and analyses of transgenic plants

Agrobacterium tumefaciens (stain GV3101) containing plasmid constructs was used to transform plants by the floral-dip method (Clough and Bent, 1998).

Transgenic lines were identified by selection in half-strength MS medium plus 50 mg/l kanamycin. Transgenic plants were further identified by semi-quantitative RT-PCR and Western blotting.

Gene expression analysis

Total RNA was extracted and purified from seedlings of various genotypes and for various treatments using an RNeasy mini kit (Qiagen, http://www.qiagen.com/) with on-column DNase digestion, according to the manufacturers' instructions. RNA samples were processed by the GeneChip facility at Iowa State University (http://www.biotech.iastate.edu/facilities/genechip/Genechip.htm/).

For RT-PCR, 2 μg of total RNA was reverse-transcribed by SuperScript II reverse transcriptase (Invitrogen, http://www.invitrogen.com/) as described by the manufacturer. Equal amounts of reverse transcription were used for PCR reactions with 25–31 cycles within the linear amplification range. PCR products were resolved by electrophoresis on a 2% agarose gel, and images were captured using the Alphalmager 3400 system (Alpha Innotech, http://www.alphainnotech.com). For quantitative real-time PCR, SYBR GREEN PCR Master Mix (Applied Biosystems, http://www.appliedbiosystems.com/) and an M × 4000 multiplex quantitative PCR system (Stratagene, http://www.stratagene.com/) were used as described by the manufacturer. Two biological replicates and two or three technical replicates (for each biological replicate) were used for

each treatment. The means and standard deviations were calculated from biological replicates.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described (Johnson *et al.*, 2002) with 5-day-old Col-0 or AtMYB30 over-expression lines. Antibodies against BES1, the Myc tag (for AtMYB30) and the IgG (Sigma) control were used for immunoprecipitation. Equal amounts of starting plant material and ChIP products were used for the quantitative real-time PCR reaction. The ChIP experiments were performed 2–4 times with biological replicates. The means and standard deviations were calculated from biological repeats.

Gel mobility shift assays (GMSAs)

Gel shift mobility assay were performed as described previously (Yin *et al.*, 2005). Briefly, oligonucleotide probes were synthesized, annealed, and labeled with ^{32}P-γ-ATP using T4 nucleotide kinase (NEB, http://www.neb.com). The binding reactions were carried out in 20 μl binding buffer (25 mM HEPES-KOH pH 8.0, 50 mM KCl, 1 mM DTT and 10% glycerol) with approximately 1 ng probe (10 000 cpm) and approximately 200 ng recombinant proteins purified from *E. coli*. After 30 min incubation on ice, the reactions were resolved by 5% native polyacrylamide gels with 1× TGE buffer (6.6 g/l Tris, 28.6 g/l glycine, 0.78 g/l EDTA, pH 8.7) and exposed to a phosphorimaging screen.

GST pull-down assay

GST pull-down assays were performed as described previously (Yin *et al.*, 2002). AtMYB30 and BES1 fragments fused to glutathione-*S*-transferase (GST) were purified using glutathione beads (Sigma). AtMYB30 or BES1 fused to maltose binding protein (MBP) were purified using amylose resin (NEB). Approximately 5 μg of proteins were used in the assay each time. The pull-down assays were repeated twice with similar results.

Bi-molecular fluorescence complementation (BiFC)

Arabidopsis mesophyll protoplasts were prepared and transformed by PEG-mediated transfection (Yoo *et al.*, 2007). Protoplasts were observed under an Olympus IX71 fluorescence microscope (http://www.Olympusamerica.com) with a YFP filter, 16–24 h after transformation. The assay was repeated more than three times, and various positive rates were obtained, depending on the quality of the protoplasts.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *atmyb30-1* enhances the *bri1-5* mutant phenotype: seedlings of Col-0 (WT), *bri1-5* and *bri1-5 atmyb30-1* double mutant grown on a ½ MS plate under light.

Figure S2. BRZ reduces active (non-phosphorylated) BES1 in both light- and dark-grown seedlings.

Figure S3. BRZ reduces the expression of *TCH4* in light-grown seedlings.

Table S1. Primers used in this study.

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