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A New Class of Transcription Factors Mediates Brassinosteroid-Regulated Gene Expression in *Arabidopsis*

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Summary

Brassinosteroids (BRs) signal through a plasma membrane-localized receptor kinase to regulate plant growth and development. We showed previously that a novel protein, BES1, accumulates in the nucleus in response to BRs, where it plays a role in BR-regulated gene expression; however, the mechanism by which BES1 regulates gene expression is unknown. In this study, we dissect BES1 subdomains and establish that BES1 is a transcription factor that binds to and activates BR target gene promoters both in vitro and in vivo. BES1 interacts with a basic helix-loop-helix protein, BIM1, to synergistically bind to E box (CANNTG) sequences present in many BR-induced promoters. Loss-of-function and gain-of-function mutants of *BIM1* and its close family members display BR response phenotypes. Thus, BES1 defines a new class of plant-specific transcription factors that cooperate with transcription factors such as BIM1 to regulate BR-induced genes.

Introduction

Plant steroid hormones called brassinosteroids (BRs) regulate many growth and developmental processes, such as cell elongation, vascular development, senescence, stress responses, and photomorphogenesis (Clouse, 1996; Li and Chory, 1999; Mandava, 1988). Loss-of-function mutants in the BR biosynthetic or signaling pathways display pleiotropic dwarf phenotypes (Clouse and Sasse, 1998; Li and Chory, 1999). In the dark, BR mutants are deetiolated with short hypocotyls, open cotyledons, and inappropriate expression of photomorphogenetic genes. Light-grown BR mutant plants have compact rosettes composed of dark green and epinastic leaves, with reduced elongation of hypocotyls, leaf petioles, and inflorescence stems and decreased apical dominance, senescence, and male fertility.

Unlike animal steroids that primarily bind to nuclear receptors to directly activate target genes (Mangelsdorf et al., 1995), BRs are perceived at the plasma membrane by a receptor complex that contains BRI1, a leucine-rich repeat (LRR) receptor kinase (Friedrichsen et al., 2000; Li and Chory, 1997). BRI1's extracellular domain is involved in the perception of BRs, and its intracellular serine/threonine kinase domain has been implicated in transducing the hormone signal to downstream targets (He et al., 2000; Wang et al., 2001). While BRI1 is ubiquitously expressed in most tissues and cells involved in cell elongation, two other BR receptors are expressed predominantly in vascular tissues and function together with BRI1 during vascular development (Caño-Delgado et al., 2004). BAK1, another LRR receptor kinase, interacts with BRI1 and has been proposed to act as BRI1's coreceptor (Li et al., 2002; Nam and Li, 2002).

Genetic screens have identified several signaling components that function downstream of BRI1. *bes1-D*, identified as a dominant suppressor of a weak *bri1* mutant, displays constitutive BR responses, including a complete suppression of the *bri1* dwarf phenotype, resistance to a BR biosynthesis inhibitor brassinazole (BRZ), excessive stem elongation, early senescence, and upregulation of BR-induced gene expression (Yin et al., 2002). *bzr1-D*, identified as a BRZ-resistant mutant, has a similar phenotype to *bes1-D* in the dark but a semidwarf phenotype in the light (Wang et al., 2002). *BES1*, *BZR1*, and four other *Arabidopsis* genes form a small plant-specific gene family whose members likely have partially redundant functions in BR signaling (Wang et al., 2002; Yin et al., 2002). The amino-terminal domain of these proteins includes a bipartite nuclear localization signal (NLS) and a highly conserved region without known function. The central region of these proteins contains 22–24 putative phosphorylation sites for the GSK3 kinase family, and several contain a putative PEST motif involved in protein degradation. In plants, BES1 and BZR1 are present at low levels in both multiply phosphorylated and dephosphorylated forms. In the presence of brassinolide (BL), the most active BR, BES1 and BZR1 accumulate in the nucleus as dephosphorylated forms in a BRI1-dependent manner. In addition, in *bes1-D* and *bzr1-D* mutants, BES1 and BZR1 proteins accumulate to high levels in the absence of hormone, presumably due to a mutation in the PEST domain that disrupts their degradation. Together, these results indicate that regulation of the protein levels of BES1 and its close relatives plays a critical role in BR signaling and that phosphorylation and dephosphorylation of these proteins are pivotal for this regulation.

Both the kinase and the phosphatase that regulate BES1 have been identified. *bin2* (*brassinosteroid insensitive 2*) was identified as a hypermorphic mutant that displays a typical BR dwarf phenotype (Li et al., 2001), suggesting that BIN2 is a negative regulator in the BR pathway. *BIN2* encodes a GSK3-like kinase, and the kinase activity is increased in the *bin2* mutant (Choe et al., 2002; Li and Nam, 2002; Pérez-Pérez et al., 2002). We found that the *bes1-D* mutant completely suppresses *bin2*'s dwarf phenotype and that dephosphorylated

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BES1 protein fails to accumulate in the *bin2* mutant (Yin et al., 2002), indicating that BIN2 functions upstream of BES1 to regulate BES1 protein levels. Consistent with these observations, BIN2 interacts with and specifically phosphorylates BES1 in vitro, suggesting that BES1 is a substrate of BIN2 kinase (Yin et al., 2002). BZR1 is also similarly regulated by BIN2 kinase (He et al., 2002). Based on these results, we proposed a model for BR signaling (Yin et al., 2002): in the absence of BRs, BES1 is phosphorylated by BIN2 kinase, and phosphorylated BES1 is targeted for protein degradation; BR signaling through BRI1 results in the inhibition of BIN2 kinase activity, leading to the accumulation of dephosphorylated BES1 in the nucleus, a process that may be facilitated by a nuclear-localized serine/threonine phosphatase called BSU1 (Mora-Garcia et al., 2004).

BRs are known to induce the expression of many genes, including many cell wall loosening enzymes required for cell elongation, transcription factors, and genes with unknown functions previously known to be induced by auxin (Coll-Garcia et al., 2004; Friedrichsen et al., 2002; Goda et al., 2002, 2004; Mussig et al., 2002; Nemhauser et al., 2004; Yin et al., 2002). Little is known about how BRs regulate target gene expression. Analysis of the promoter of the BR-induced *TCH4* gene, which encodes a xyloglucan endotransglycosylase (XET), revealed that a 102 bp promoter fragment confers responses to BRs and other stimuli (Iliev et al., 2002). However, the redundant elements in other regions of the *TCH4* promoter and relative small induction by BRs made the identification of specific BR response elements and interacting transcription factors difficult (Clouse, 2002).

Many BR-induced genes are upregulated in the *bes1-D* mutant (Yin et al., 2002), suggesting that BES1 protein is involved, directly or indirectly, in the activation of BR target genes. However, since BES1 and BZR1 do not have homology to other proteins with known functions, how BES1 activates target gene expression has remained unknown. In addition, both *bes1-D* and *bzr-1D* are gain-of-function mutants, and loss-of-function phenotypes of these genes have not been characterized. Here we show that reduction of BES1 and BZR1 levels leads to a typical BR dwarf phenotype. In addition, we show that BES1 is a transcription factor that binds to BR target gene promoters through a novel DNA binding domain to activate BR target gene expression. Moreover, BES1 interacts with a bHLH transcription factor BIM1 to synergistically bind to E box sequences that are present in many BR-induced gene promoters (Nemhauser et al., 2004). The results provide a framework to further understand how BRs differentially regulate gene expression in various organs and physiological conditions to control plant growth and development.

Results

BES1 and Homologs Function Redundantly in BR Signaling

In the *Arabidopsis* genome, four proteins, designated BEH1–4 (*BE*S1/BZR1 *h*omolog 1–4, At3g50750, At4g36780, At4g18890, and At1g78700, respectively), show high sequence identity with BES1 and BZR1. BES1 shares 88%, 59%, 56%, 45%, and 48% identity to BZR1 and BEH1–4, respectively. To investigate if these proteins are involved in BR signaling, the BEH1, 2, 3, and 4 proteins were tagged with green fluorescence protein (GFP) at their carboxyl termini and expressed in plants. As shown in Figure 1A, BES1 and BEH1–4 exist in plants in at least two forms, likely phosphorylated and dephophorylated forms, and accumulate as dephosphorylated forms when treated with BL, as shown previously for both BES1 and BZR1 (Wang et al., 2002; Yin et al., 2002). Mutation of a proline to leucine in the PEST motif of BES1 or BZR1 leads to the accumulation of these proteins and constitutive BR response phenotypes (Wang et al., 2002; Yin et al., 2002). BEH2 contains this conserved proline, and a mutant form with an analogous mutation accumulated to a high level even without BL treatment (Figure 1A). Plants that express BEH2m displayed a *bes1-D* phenotype (data not shown). These results suggest that BES1 functions with BZR1 and BEH1–4 in BR signaling. Consistently, a *BES1* T-DNA knockout line did not show

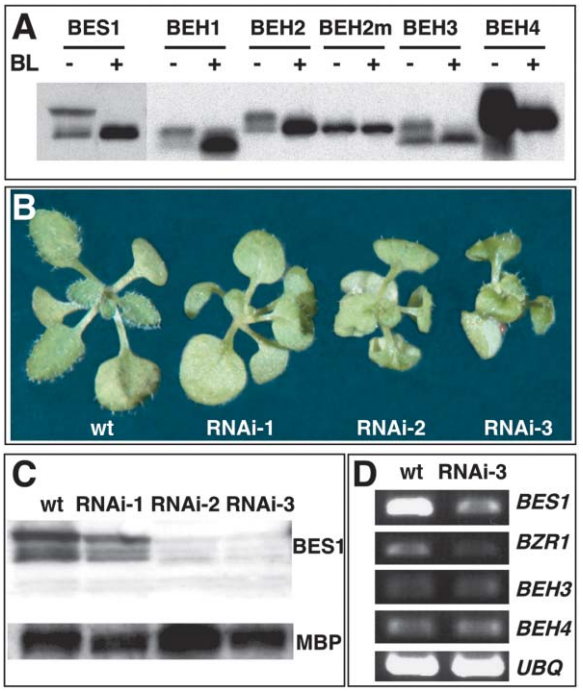

Figure 1. BES1 Family Members Function Redundantly in BR Signaling

(A) A Western blot with proteins from transgenic plants with *BES1-GFP* (Yin et al., 2002) and *BEH1–4-GFP* fusion genes and anti-GFP antibodies. BEH2m harbors a mutation in the PEST motif (from proline to leucine, same mutation as in *bes1-D* and *bzr1-D*). T1 seedlings were pooled and treated without (−) or with (+) 1 μM BL. The upper band likely represents the phosphorylated form, while the lower band represents the dephosphorylated form of the various proteins, as previously determined for BES1 (Yin et al., 2002).

(B) Representative T2 plant lines expressing *BES1* RNAi (RNAi-1-2 and -3) together with wild-type control (*wt*).

(C) A Western blot with proteins from *BES1* RNAi lines shown in (B) with anti-BES1 antibodies (Mora-Garcia et al., 2004). BES1 and a loading control band are indicated.

(D) RT-PCR analysis of *BES1*, *BZR1*, *BEH1* (*H1*), *BEH4* (*H4*), and *ubiquitin* (*UBQ*) genes in *BES1* RNAi-3 and wild-type plants. PCRs were performed with specific primers from each gene for 25 cycles.

any obvious phenotype (our unpublished data). To investigate the loss-of-function phenotype of the gene family, we transformed plants with a *BES1* RNAi (RNA interference) construct that includes the whole BES1 coding region. Approximately 25% of the transgenic lines displayed a semidwarf phenotype similar to weak BR loss-of-function mutants (Figure 1B). In the PCR condition we used, both *BES1* and *BZR1* transcripts were reduced in RNAi plants, while the transcripts of *BEH1* and *BEH4* did not appear to be affected (Figure 1D), consistent with the fact that *BES1* and *BZR1* are more closely related to each other than to other family members. *BEH2* and *BEH3* transcripts were not detected in the seedling tissues used for this experiment (data not shown). BES1 protein levels were also reduced in RNAi plants, and the extent of BES1 reduction correlated well with the phenotypes (Figures 1B and 1C). These results indicate that reduction of *BES1* and *BZR1* leads to a semidwarf phenotype. Together with the observation that dominant mutations in *BES1* and *BZR1* result in distinct phenotypes in light-grown plants, we conclude that BES1 and BZR1, and perhaps other family members, have partially redundant functions in BR signaling.

BES1 Functional Domains

BES1 protein contains a putative NLS, followed by a highly conserved amino-terminal domain (N) shared among BES1 and family members, a BIN2 phosphorylation domain (P), a PEST motif, and a carboxyl-terminal domain (C) (Wang et al., 2002; Yin et al., 2002) (Figure 2A). A mutation in the PEST motif stabilizes the protein in *bes1-D* mutants, suggesting that the PEST motif is involved in BES1 degradation (Yin et al., 2002). To determine the functions of the other domains of BES1, we generated deletion mutants for each of the N, C, and P domains in either wild-type *BES1* or *bes1-D* (PEST mutation) gene backgrounds (Figure 2A) and transformed the constructs into wild-type plants. Transgenic lines with roughly similar expression levels of the transgenes were chosen for further analysis (Figure 2B, lower panel). The accumulation of truncated proteins was analyzed by Western blotting. As reported previously, both wild-type BES1 and BES1-D are present in plants as phosphorylated and dephosphorylated forms and accumulate as the dephosphorylated form in the presence of BL, although mutant BES1-D protein accumulates to much higher levels than wild-type BES1 (Yin et al., 2002) (Figure 2B, lanes 1–4; upper panel shows long exposure, and middle panel shows shorter exposure). Deletion of the N, C, or P domains leads to BL-independent accumulation of truncated proteins in transgenic plants (Figure 2B, upper and middle panels, lanes 5–16), suggesting that these domains are all required for efficient BES1 turnover.

To determine the function of these domains in BR signaling, the phenotypes of the transgenic plants in the light (Figure 2C) or dark (Figure 2D) were analyzed in the absence and presence of BRZ, a BR biosynthesis inhibitor that reduces endogenous BR levels (Asami et al., 2000). While transgenic plants that express wild-type BES1 displayed a wild-type phenotype and were sensitive to BRZ, plants that express BES1-D displayed a long hypocotyl and curly leaf phenotype and were resistant to BRZ when grown in either dark or light conditions (Figures 2C and 2D), as previously reported (Yin et al., 2002). Expression of deletions of the N domain (BES1ΔN and BES1-DΔN) resulted in wild-type-like plants with somewhat longer hypocotyls and leaf petioles that were sensitive to BRZ (Figures 2C and 2D), suggesting that the BES1-N domain is required for full BES1 activity. *BES1ΔC* and *BES1-DΔC* plants were smaller than wild-type and were more sensitive to BRZ in the light (Figure 2C). The results suggest that the C domain is required for BES1 function and that truncated BES1 proteins lacking the C terminus function as dominant-negative forms. In contrast, expression of proteins that deleted BIN2 phosphorylation sites (P) (BES1ΔP and BES1-DΔP) produced plants with typical *bes1-D* mutant phenotypes that were resistant to BRZ in both light and dark growth conditions (Figures 2C and 2D), indicating that the P domain is involved in BES1 turnover. We conclude that, while the N, C, and BIN2 phosphorylation (P) domains are all required for BES1 turnover, the N- and C-terminal domains are both necessary and largely sufficient for BES1 function in promoting cell elongation.

BES1 Interacts with a Basic Helix-Loop-Helix Transcription Factor BIM1

To understand how each of the N, P and C domains are involved in BR signaling, we performed yeast two-hybrid screens with each domain and identified several BES1-interacting proteins. The characterization of BIM1 (*B*ES1-*i*nteracting *M*yc-like 1), a basic helix-loop-helix (bHLH) transcription factor that was identified using BES1-C domain as bait, is reported here. From approximately 10 million yeast clones screened, 11 clones contained cDNA fragments from the *Arabidopsis* gene At5g08130, here designated *BIM1*. There are two homologs of BIM1 predicted in the *Arabidopsis* genome, named *BIM2* (At1g69010) and *BIM3* (At5g38860). BIM1, BIM2, and BIM3 are also named *Arabidopsis* bHLH proteins EN 126, 125, and 127, respectively (Toledo-Ortiz et al., 2003). The protein sequence alignment of BIM1, BIM2, and BIM3 is shown in Figure 3A, and the protein structures are shown in Figure 3B. These proteins are predicted to have highly conserved DNA binding domains (Figure 3A, underlined) that are 76%, 69%, and 77% similar between BIM1/BIM2, BIM2/BIM3, and BIM1/BIM3, respectively. The predicted BIM1 bHLH domain shares 25% similarity with the bHLH domain of the Myc transcription factor (Nair and Burley, 2003). The C-terminal portions of these proteins have a stretch of 40 conserved amino acid residues (Figure 3A, denoted between two asterisks) that are 39%–51% similar between BIM1, BIM2, and BIM3. This domain does not have high similarity to any known proteins. BIM1 also has a predicted amino-terminal domain (about 200 amino acid residues) that is absent in BIM2 and BIM3. All the clones obtained from the yeast two-hybrid screen using BES1-C domain as bait contained the carboxyl portion of BIM1, indicating that these two proteins can interact with each other in yeast through their C-terminal domains.

To confirm that BIM1 and its homologs interact with

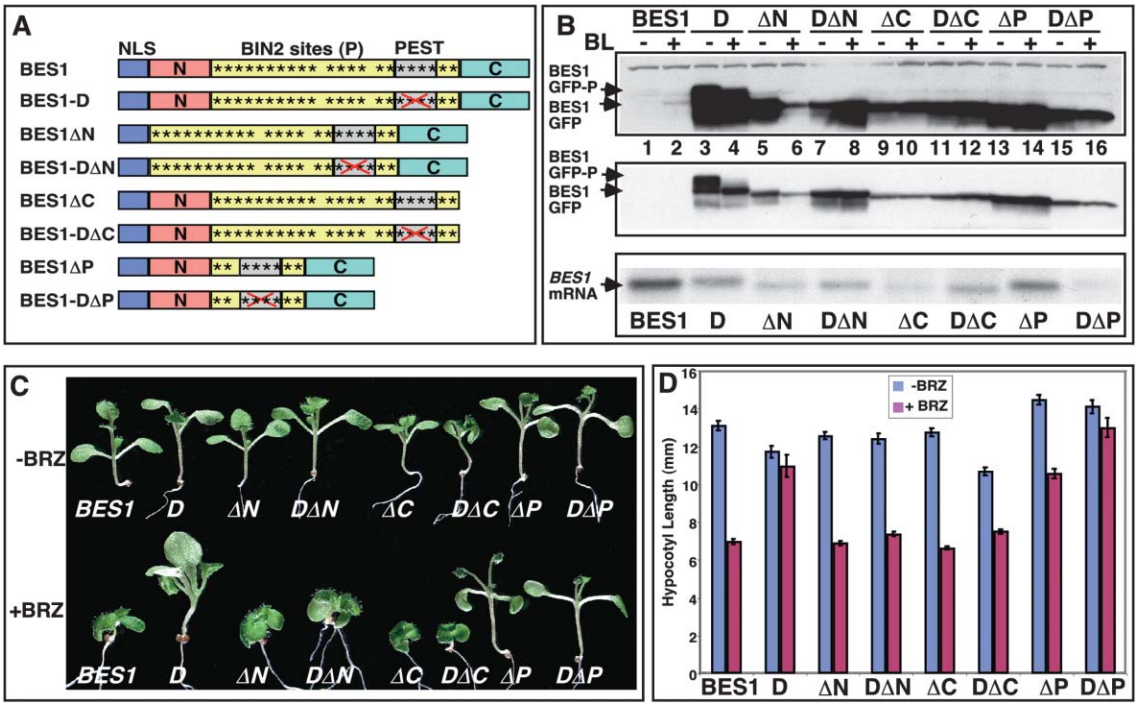

Figure 2. Functional Domains of BES1

(A) Schematic structures of BES1, BES1-D (with mutation in the PEST domain), and mutants with deletions of the amino-terminal domain (N), BIN2 phosphorylation sites (P), and the carboxyl domain (C). The amino acid residues for each domain are the following: N (42–98), P (99–267, 99–196 were deleted in ΔP constructs), PEST (231–250), and C (268–335). The putative BES1 nuclear localization signal (NLS, 21–41) was retained in all the deletion mutants. In (B)–(D), the BES1 label was used only in wild-type and was omitted in BES1-D (D) and all the deletion mutants, owing to space constraints.

(B) Northern and Western analysis of BES1 deletion mutant transgenic lines. (Lower panel) A Northern blot with representative transgenic lines from each construct using a GFP cDNA probe. Transgenic lines with approximately equal expression levels and representative phenotypes are shown. (Upper two panels) Western blot with *BES1*/*BES1-D* deletion transgenic lines and anti-GFP antibodies. Upper panel indicates a longer exposure, and middle panel indicates a shorter exposure of the same blot. A background band above the BES1 bands shown on the top panel indicates equal loading of the samples.

(C) The phenotypes of *BES1*/*BES1-D* deletion mutants in the absence or presence of 1 μM BRZ in the light.

(D) Hypocotyl length of *BES1*/*BES1-D* deletion mutants in the absence or presence of 1 μM BRZ in the dark.

BES1, we performed a GST pull-down experiment with purified proteins expressed in *E. coli*. As shown in Figure 3C, both BIM1 and BIM3 interacted with full-length BES1 or BES1-D. In addition, BIM1, BIM2, and BIM3 all interacted with truncated forms of BES1 that correspond to the N-terminal part of the protein (Figure 3C, indicated by lower arrow). Indeed, the N terminus of BES1 (including NLS and the N domain) interacted with BIM1, BIM2, and BIM3 (data not shown). We further found that the bHLH domain of BIM1 interacted with the N-terminal domain of BES1 (Figure 3D). The fact that BIM1 interacted much more strongly with full-length BES1 than with the N-terminal part of BES1 in vitro indicated that the C-terminal part of BES1 is also involved in interaction with BIM1 in vitro (Figure 3C), which is consistent with the fact these two proteins interact in yeast through their C-terminal domains. In conclusion, we found that BES1 interacts with BIM1 through two different domains: the N-terminal BES1 and bHLH domain of BIM1 as well as the C-terminal domains of BES1 and BIM1.

BIM1 Family Members Are Involved in BR Responses

To determine the functions of *BIM1* and closely related genes in BR signaling, we generated both loss-of-function and gain-of-function mutants. T-DNA insertion mutants for *BIM1*, *BIM2*, and *BIM3* were identified (Figure 3B). *bim1*, *bim2*, and *bim3* appeared to be null mutants since corresponding mRNA could not be detected in the mutants (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/120/2/249/DC1/). While none of the single mutants displayed any visible phenotype, the *bim1 bim2 bim3* triple mutant seedlings had shorter hypocotyls compared to wild-type controls in both light- and dark-grown seedlings (Figures 4A and 4B). In addition, the *bim1 bim2 bim3* mutant was about two times more sensitive to BRZ than wild-type seedlings in a hypocotyl elongation assay in the dark (Figure 4C), suggesting that BIM1, BIM2, and BIM3 are involved in BR signaling. Although transgenic plants that overexpressed the *BIM1* gene did not display obvious phenotypes (Figures 4A and 4B), these plants showed reduced sensitivity to BRZ, especially with lower concentrations (Figure 4C). Accordingly, overexpression of *BIM1* in a weak *bri1* mutant background partially suppressed the *bri1* dwarf phenotype, with the double mutant having more expanded leaves and longer leaf petioles compared to *bri1* (Figure 4D), further suggesting a positive role for *BIM1* in BR signaling.

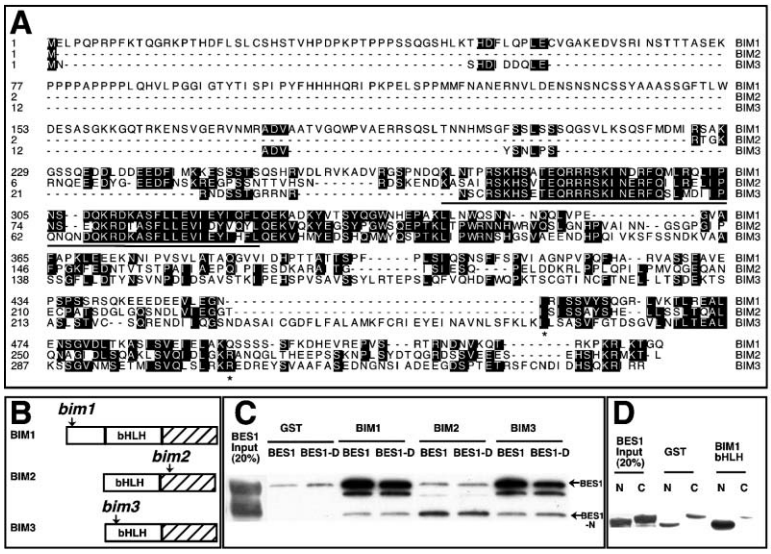

Figure 3. BES1 Interacts with BIM1, BIM2, and BIM3

(A) Alignment of predicted BIM1, BIM2, and BIM3 proteins using Mega Align program (Kumar et al., 2001). The highly conserved bHLH domains are underlined. A stretch of conserved amino acid residues in the carboxyl domains are indicated by two asterisks.

(B) Schematic protein structures of BIM1, BIM2, and BIM3. The locations of T-DNA insertion mutants are indicated by arrows.

(C) A GST pull-down experiment with GST, GST-BIM1, GST-BIM2, GST-BIM3, and BES1/BES1-D protein purified from *E. coli*. BES1 protein was detected by a Western blot with anti-BES1 antibodies. BES1 and a truncated form corresponding to the amino-terminal part of BES1 (BES1 NLS + N) are indicated by arrows.

(D) A GST pull-down experiment using GST and GST-BIM1 bHLH domain with either BES1 NLS + N or BES1-C domains. BES1 was detected with antibodies against MBP that was tagged to the BES1 NLS + N and BES1-C.

BES1 and BIM1 Synergistically Bind to and Activate a BR-Induced Gene Promoter

Based on the facts that BES1 and BIM1 interact with each other and that both are involved in BR responses, we hypothesized that BIM1 and BES1 may directly bind to the promoters of BR-induced genes. The promoter of the *SAUR-AC1* gene that was previously shown to be induced by BL and upregulated in the *bes1-D* mutant was chosen to test the hypothesis (Yin et al., 2002). The promoter contains three predicted E box sequences (CANNTG) that are known to be the binding sites for many bHLH transcription factors (Toledo-Ortiz et al., 2003). Three DNA fragments (approximately 30 base pairs each) containing each of the E boxes were synthesized, labeled, and used in DNA binding experiments with purified BES1 and BIM1 proteins expressed in *E. coli*. Similar results were obtained with all three probes, and the results for one of them (Ea) are shown (Figure 5). Both BES1 and BIM1 can bind to the promoter individually in a protein concentration-dependent manner, perhaps as homodimers (Figure 5A, lanes 1–6; BES1 and BIM1 are indicated by arrows on the left, see below for details). Interestingly, BES1 and BIM1 together bind to the promoter synergistically (Figure 5A, lanes 7–9; BES1 + BIM1 bands are indicated by arrows). Since heterodimers usually migrate somewhere between homodimers (for example, see Spinner et al. [2002]), the position of the BES1::BIM1::DNA complex is consistent with the notion that BES1 and BIM1 form a heterodimer. The increased complex formation with increased BIM1 protein concentrations (while BES1 was kept constant) supports the idea that the complex contains BIM1 protein (Figure 5A, lanes 7–9). The BES1::BIM1::DNA complex was supershifted to a higher position (BES1 +

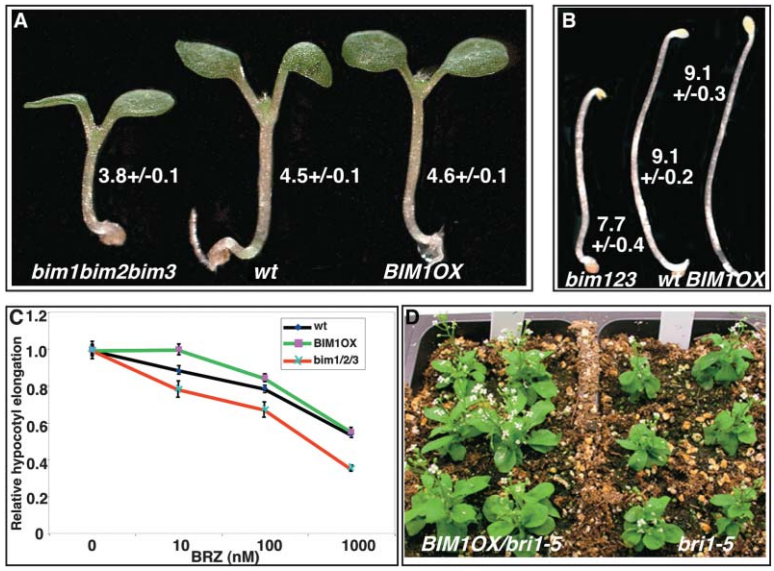

Figure 4. BIM1, BIM2, and BIM3 Are Involved in BR Responses

(A) One-week-old seedlings grown in short day conditions. The numbers indicate the average hypocotyl length (mm) and standard error with at least 15 seedlings measured for each genotype. The results were repeated with three batches of seeds, and similar results were obtained.

(B) Four-day-old seedlings grown in the dark.

(C) The effects of BRZ on hypocotyl elongation in wild-type (wt), *bim1 bim2 bim3* (*bim1/2/3*), and *BIM1* overexpresser (*BIMOX*). The concentrations of BRZ for half-maximal responses are 150, 70, and 200 nM for wt, *bim1 bim2 bim3*, and *BIMOX*, respectively.

(D) Eight-week-old plants of *bri1-5* and *BIM1* overexpresser in *bri1-5* background (*BIM1OX/bri1-5*).

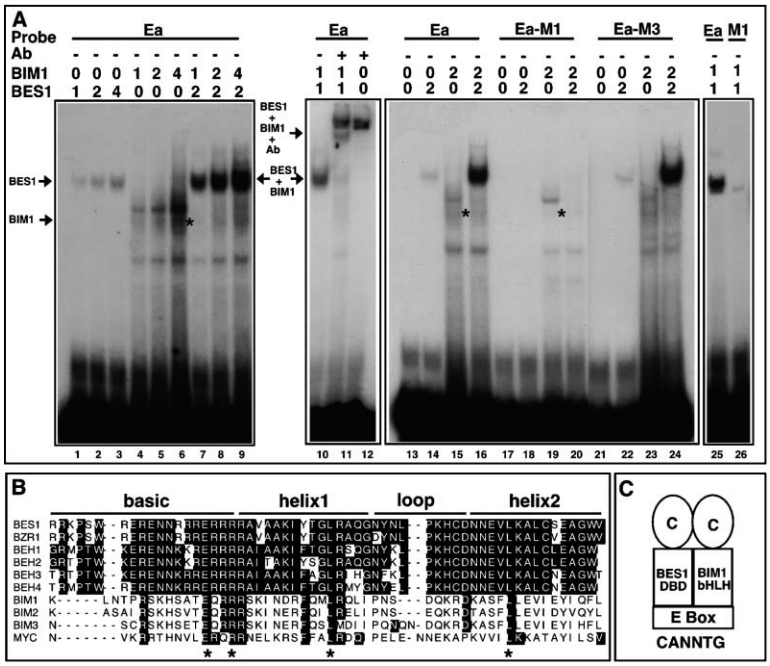

Figure 5. BES1 and BIM1 Bind to a BR Target Gene Promoter

(A) Electrophoretic mobility shift assays (EMSAs) with BES1 and BIM1 proteins using Ea, Ea-M1, or Ea-M3 as probes. MBP-BES1 and 6HIS-BIM1 were purified from *E. coli*, adjusted to 50 ng/μl, and used in DNA binding experiments (numbers indicate microliters used in binding assay). The BES1, BIM1, and BES1 + BIM1 complexes are indicated by arrows. The specific BIM1 complex bound to the E box is also indicated by asterisk (see text for details). In lanes 11 and 12, anti-MBP antibodies were added to the reaction. While the upper bands in lanes 11 and 12 indicate background binding from anti-MBP preparation, the lower band in lane 11 indicates BIM1/BES1/Ab (anti-MBP):DNA complex (indicated by an arrow). In lanes 25 and 26, MBP-BES1 NLS + N and 6HIS-BIM1-bHLH domains were used in the DNA binding assays.

(B) Alignment of putative BES1 DNA binding domain with corresponding domains in BES1 homologs, bHLH domains of BIM1 family, and MYC. Glu-13Arg-16, which determine the E box (CANNTG) binding specificity in the basic region of bHLH transcription factors and the conserved leucine residues in helix1 and helix2, are indicated by asterisk.

(C) A model indicating that BES1 and BIM1 form a heterodimer and bind to an E box (CANNTG).

BIM1 + Ab, indicated by an arrow) by antibodies, against MBP which was used to tag BES1 (Figure 5A, lanes 10–12), indicating that the complex contains BES1 as well.

To determine if BIM1 or BES1 binds to the E box in the probe, two probes that harbor mutations either in the E box (Ea-M1: CANNTG to TCNNAA) or outside of the E box (Ea-M3) were used in the binding experiments (Figure 5A, lanes 13–24). BES1 binding was completely abolished in Ea-M1 (Figure 5A, compare lanes 18 and 14) but not in Ea-M3 (Figure 5A, compare lanes 22 and 14), suggesting that BES1 binds to the probe through the E box sequence. There are several BIM1 complexes that bound to the Ea probe (Figure 5A, lanes 4, 5, 6, and 15), and at least one of them (indicated by asterisk) bound to the probe through the E box, since it was reduced in the E box mutant Ea-M1 (Figure 5A, compare lanes 19 and 15). The upper complex in lanes 4–6, 15, and 19 might represent less-specific binding of BIM1 to other non-E box sequences present in the probe. More importantly, the synergistic binding of BES1 and BIM1 to the probe was completely abolished in the E box mutant Ea-M1 (Figure 5A, compare lanes 20 and 16) but not in the mutant with a mutation outside of the E box (Figure 5C, compare lanes 24 and 16). These results suggest that BES1 and BIM1 synergistically bind to the E box sequence, likely as a heterodimer.

The BES1 N-terminal domain contains a bipartite NLS followed by a highly conserved motif (N), which together may serve as a DNA binding domain. This BES1 putative DNA binding domain can be aligned with sequences of BES1 homologs, as well as bHLH domains of the BIM1 subfamily and MYC (Figure 5B). BES1 is predicted to contain a highly basic region that is very similar to the basic regions of other bHLH proteins. The Glu-13Arg-16 pair (Figure 5B, indicated by asterisks) that determines E box binding specificity of bHLH proteins (Toledo-Ortiz et al., 2003) is conserved in BES1 and BES1 family members. Although the sequence following the BES1 basic region is less conserved, it can form a helix-loop-helix structure as predicted by the Chou-Fasman method (Chou and Fasman, 1978) and helical wheel analysis (http://cti.itc.virginia.edu/cmg/Demo/wheel/wheelApp.html) (Figure 5B and data not shown). In addition, two leucine residues (indicated by asterisks) that are highly conserved in all plant bHLH proteins (Toledo-Ortiz et al., 2003) are also present in BES1 family members (Figure 5B). Indeed, the BES1 DNA binding domain (NLS plus N) and BIM1's bHLH can bind to the Ea probe synergistically, and such binding was abolished with Ea-M1 as probe (Figure 5A, lanes 25 and 26). Our results therefore suggest that BES1 is a transcription factor with a unique DNA binding domain that can form a heterodimer with the bHLH domain of BIM1 to bind to the three E box sequences present in the SAUR-AC1 promoter (Figure 5C).

BES1 and BIM1 Bind to and Activate BR Target Gene Promoters In Vivo

Chromatin immunoprecipitation (ChIP) experiments were performed to test if BES1 binds BR target gene promoters in vivo using *BES1::GFP* and *BES1-D::GFP* transgenic plants and anti-GFP antibodies (Figure 6A). While no significant amount of *SAUR-AC* promoter DNA was immunoprecipitated from the wild-type control, a detectable amount of the promoter was pulled down from the *BES1::GFP* transgenic plants in the presence of BL. Consistent with the fact that BES1-D protein accu-

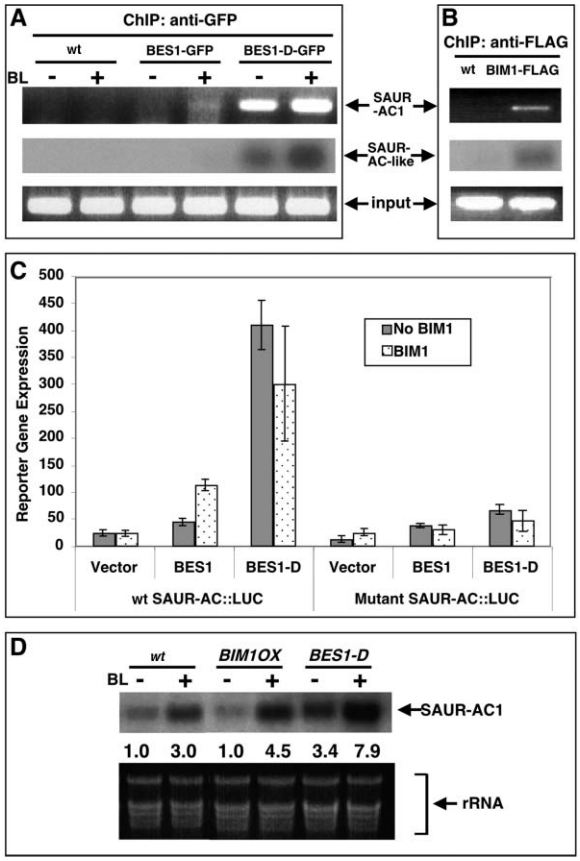

Figure 6. BES1 and BIM1 Bind to and Activate BR Target Gene Promoters In Vivo

(A) Chromatin imunoprecipitation (ChIP) with wild-type (wt), *BES1-GFP*, or *BES1-D-GFP* transgenic plants using anti-GFP antibodies. The *SAUR-AC1* (At4g38850) and *SAUR-AC1*-like (At1g29490) promoters were detected by PCR amplification and observed by ethidium bromide staining or Southern blotting, respectively. The lower panel indicates PCR reactions with aliquots of samples before immunoprecipitation (inputs) with *SAUR-AC1* primers.

(B) ChIP with wild-type or *BIM1-FLAG* transgenic plants using anti-FLAG antibodies.

(C) Coexpression of BES1 and BIM1 activate wild-type but not a mutant form of the *SAUR-AC1* promoter:luciferase gene. The data were normalized as described in Experimental Procedures. The mutant promoter has all three E boxes mutated.

(D) A Northern blot showing the expression of *SAUR-AC1* gene in wild-type, *BIM1* overexpresser (*BIM1OX*), and *bes1-D* mutant plants without or with BL treatment (1 μM, 2.5 hr). Lower panel indicates approximately equal amount of RNA (10 μg) loaded.

mulates to high levels in plants, more *SAUR-AC1* promoter was found to be associated with BES1-D proteins; this was further stimulated by BL treatment (Figure 6A, upper panel). Similar results were obtained with a *SAUR-AC1*-like gene promoter, which is also regulated by BR application (Nemhauser et al., 2004) (Figure 6A, middle panel). At the same time, several other BR-induced promoters tested, including At4g25810 (encoding a XET) and At1g18400 (*BEE1*) (Friedrichsen et al., 2002), appeared not to associate with BES1 or BES1-D in vivo (see Supplemental Figure S2 on the *Cell* web site). These results support the interpretation that BES1 is a DNA binding transcription factor that is associated with a subset of BR-induced gene promoters in vivo. *BIM1::FLAG* transgenic plants were used to test if BIM1 was also associated with the BR target gene promoters in vivo. As shown in Figure 6B, anti-FLAG antibodies pulled down a significant amount of *SAUR-AC1* promoter fragment (upper panel) and *SAUR-AC1*-like promoter (middle panel) from BIM1::FLAG transgenic lines but not from the control plants, indicating that BIM1 is also associated with BR target gene promoters in vivo.

Since BES1 is implicated in the activation of BR-induced genes (Yin et al., 2002) and both BES1 and BIM1 bind to the *SAUR-AC1* promoter, we tested whether BES1 and BIM1 activate transcription in a transient expression assay with *SAUR-AC1* and mutant *SAUR-AC1* promoter::luciferase reporter constructs. As shown in Figure 6C, with a wild-type *SAUR-AC1* promoter-luciferase reporter, expression of BES1 and especially BES1-D led to increased reporter gene expression (gray bars in groups 1–3), indicating that BES1 protein activates the *SAUR-AC1* promoter in vivo. Although BIM1 did not significantly activate the *SAUR-AC1* promoter by itself, coexpression of BIM1 and BES1 enhanced BES1-mediated activation of reporter gene expression (Figure 6C, groups 1 and 2), consistent with the fact that these two proteins bind to the E boxes in the *SAUR-AC1* promoter synergistically (Figure 5A). When all three E boxes were mutated in the *SAUR-AC1* promoter, BES1- and BIM1-mediated reporter gene activation was largely abolished, and this was especially notable in experiments that involved BES1-D (Figure 6C, groups 4–6). These results demonstrate that BES1 and BIM1 activate BR target gene expression through the E box sequences present in a BR-induced gene promoter.

To determine how BES1 and BIM1 proteins regulate *SAUR-AC1* expression in plants, *SAUR-AC1* transcript levels were detected in wild-type, a *BIM1* overexpression line, and *bes1-D* mutant plants (Figure 6D). In wild-type plants, *SAUR-AC1* was induced 3-fold by BL treatment, consistent with our earlier microarray results (Figure 6D) (Yin et al., 2002). Overexpression of BIM1 increased BL induction of *SAUR-AC1* gene expression (from 3-fold to 4.5-fold), although the basal *SAUR-AC1* expression was not significantly increased (Figure 6D). As reported previously (Yin et al., 2002), accumulation of BES1 (in *bes1-D*) led to the upregulation of basal *SAUR-AC1* gene expression, but the degree of BL induction did not change (Figure 6D). The extent of *SAUR-AC1* upregulation correlated with the facts that BIM1 was only slightly overexpressed in the transgenic line used (data not shown) and that BES1 accumulated to very high levels in the *bes1-D* mutant (Yin et al., 2002). In the *bim1 bim2 bim3* triple mutant, the accumulation of *SAUR-AC1* mRNA after BL treatment was slightly reduced compared to wild-type (about 25% reduction) and was similar to a weak allele of *bri1* (Supplemental Figure S3 and data not shown). Together with the transient expression studies, these results indicate that BES1 and BIM1 directly bind to and regulate *SAUR-AC1* gene expression in vivo.

Discussion

The mechanisms by which nuclear receptors for animal steroids regulate gene expression are well-established

(Beato and Klug, 2000; Mangelsdorf and Evans, 1995). Upon ligand binding, nuclear receptor superfamily transcription factors bind directly to target gene promoters as homo- or heterodimers and recruit transcription cofactors to regulate gene expression. However, how plant steroids regulate gene expression through their membrane-localized receptors has remained a mystery. We previously identified BES1 as an important signaling component that transduces the BR signal to the nucleus (Yin et al., 2002). Since BR-induced genes are upregulated in a gain-of-function *bes1-D* mutant, we proposed that BES1 was involved, either directly or indirectly, in the activation of BR target genes (Yin et al., 2002). Here we establish BES1 as a novel transcriptional activator that directly binds to BR target gene promoters both in vitro and in vivo. Furthermore, BES1 interacts with the bHLH transcription factor BIM1 to synergistically bind to E box sequences to activate several BR-induced gene promoters. The reduced BR response phenotype of the *bim1 bim2 bim3* triple mutant and suppression of *bri1* phenotype by *BIM1*overexpression suggest that a BES1::BIM1 interaction is required for normal BR responses.

BES1, BZR1, and other predicted *Arabidopsis* proteins (BEH1–4) share close similarity and likely perform partially redundant functions in BR signaling. In this study, we provide two pieces of evidence to support this hypothesis. First, BEH1–4 proteins, like BES1 and BZR1, accumulate in their dephosphorylated forms in the presence of BL, suggesting that all these proteins are similarly regulated by BR signaling. Second, reduction of BES1 protein and its closest homolog, BZR1, leads to a semidwarf phenotype similar to that of weak BR loss-of-function mutants. The results provide the first evidence that loss-of-function in *BES1* and related genes leads to a BR dwarf phenotype, which is an important confirmation of the idea that members of this gene family are necessary components of the BR signal transduction pathway.

While the central P domain of BES1 is the target of the BIN2 kinase and the PEST motif is implicated in BES1 protein degradation, direct evidence for the function of the P domain was still missing. In addition, the functions of the N and C domains of BES1 were unknown. Deletion of the BIN2 phosphorylation sites (P) stabilizes the protein and leads to a *bes1-D* phenotype (Figures 2C and 2D), providing direct evidence that BIN2 phosphorylation negatively regulates BES1 protein levels. It is somewhat surprising that deletion of either N or C domains also stabilizes the protein, suggesting that these two domains are also involved in BES1 degradation. How the BES1-N domain contributes to this process is not clear. The BES1-C domain is involved in the interaction with BIN2 (our unpublished data) and therefore may be responsible for bringing BIN2 kinase in proximity to the P domain.

In addition to recruiting BIN2, BES1-C domain also plays a positive role in BES1 function, as deletion of the C domain in the BES1-D protein abolishes the *bes1-D* mutant phenotype, although the truncated protein accumulates to high levels in plants (Figures 2B–2D). Two pieces of evidence suggest that the BES1-C domain serves as a transcriptional activation domain. First, full-length BES1 but not BES1-DΔC can activate transcription of a BR-induced reporter gene in a transient expression system (Figure 6C and Supplemental Figure S4) as well as in plants (Figure 6D). Second, BES1-C but not BES1-N fused to the GAL-4 DNA binding domain can activate transcription in yeast (our unpublished data).

The BES1-N domain is also essential for BES1 function since deletion of BES1-N domain in BES1-D largely abolishes the *bes1-D* phenotype (Figures 2C and 2D). In addition, BES1, without its C domain, acts as a dominant-negative form (Figure 2C), which is in agreement with the observation that the N domain of BES1 is involved in DNA binding (see below) and the C domain is probably involved in transcriptional activation. Truncated proteins with the DNA binding domain (N) but without the activation domain (C) are likely to compete with wild-type protein to bind DNA and therefore serve as a dominant-negative form (Lloyd et al., 1991; Nakabeppu and Nathans, 1991; Spinner et al., 2002). Consistent with this hypothesis, expression of BES1-DΔC reduces BES1-D-mediated activation of the *SAUR-AC1* gene promoter (Supplemental Figure S4).

We discovered that BES1 is a transcription factor and binds to BR target gene promoters both in vitro and in vivo (Figures 5 and 6A). The amino-terminal part of BES1, including the NLS and a stretch of highly conserved amino acids (N domain), is sufficient for DNA binding. While the BES1-NLS, which is highly enriched with basic amino acid residues, is likely responsible for contacting DNA like many other transcription factors (Boulikas, 1994), the N domain may serve as a dimerization domain. Although the proposed BES1 DNA binding domain does not have obvious homology to known bHLH factors, it can form a basic helix-loop-helix structure with several key residues conserved (Figure 5B and data not shown). Of particular interest is the conserved Glu-13Arg-16 motif in the BES1 basic region. Crystallography studies of several bHLH proteins indicate that the Glu-13Arg-16 pair in the basic region of bHLH proteins determines the DNA binding specificity, since both residues are involved in contacting the CA sequence in the E box (CANNTG) (Toledo-Ortiz et al., 2003). Consistent with the idea, BES1 binds to an E box sequence but not to a mutant E box with the CA motif mutated (Figure 5). Analysis of BL-induced genes indicates that E box sequences are enriched in many BR-induced gene promoters (Nemhauser et al., 2004), suggesting that BES1 may directly bind to some of these gene promoters. The BES1 DNA binding domain is also highly conserved between BES1 and its homologs, sharing 96%, 79%, 79%, 76%, and 81% similarities to BZR1, BEH1, BEH2, BEH3, and BEH4, respectively (Figure 5B). A database search revealed homologs of BES1 family members in other plant species but not in nonplant systems (our unpublished data). Our results therefore suggest that BES1 is a founding member of a new family of plant-specific, bHLH transcription factors that are used in plant steroid hormone signaling.

BES1 by itself appears to bind DNA weakly in vitro and interacts with BIM1 to synergistically bind to target gene promoters (Figure 5). Several lines of evidence support the notion that BES1 and BIM1 form heterodimers. First, although BIM1-C domain was identified using BES1-C domain as bait, these two proteins also interact through their DNA binding domains (Figures 3C

and 3D). Second, BES1, BIM1, and a BES1/BIM1 complex bind to wild-type but not to a mutant E box (Figure 5A). Third, both BES1 and BIM1 were found to associate with *SAUR-AC1* and *SAUR-AC1*-like gene promoters in vivo (Figure 6). Heterodimerization between transcription factors, including bHLH proteins, is widely used to generate diversity as well as specificity for different biological processes. For example, the bHLH proteins, Max and Myc, form a heterodimer to activate gene expression and cellular growth while a Mad-Max heterodimer inhibits transcription and promotes cell differentiation (Nair and Burley, 2003). Myogenic and neurogenic bHLH proteins interact with ubiquitous bHLH proteins to promote muscle and neuron differentiation, respectively (Lee, 1997; Molkentin and Olson, 1996). The BES1/BIM1 interaction provides a unique paradigm to study how these proteins form heterodimers to regulate plant steroid hormone-regulated gene expression.

A BES1/BIM1 heterodimer appears to activate the *SAUR-AC1* promoter both in a transient expression system (Figure 6C) and in plants (Figure 6D). While BES1 is a weak transcriptional activator, BIM1 by itself does not activate transcription but enhances BES1-mediated target gene activation (Figure 6C). BIM1, therefore, may function in BR signaling by facilitating BES1 binding to target gene promoters. This hypothesis is supported by the observation that plants overexpressing BIM1 enhance *SAUR-AC1* expression in the presence of BL, conditions in which BES1 accumulates (Figure 6D). Both the loss- and gain-of-function mutant phenotypes of *BIM1* and its paralogs support our conclusion that BIM1 interacts with BES1 and plays a positive role in BR-regulated gene expression (Figure 4).

Several possibilities can be proposed to explain the relatively weak phenotype of the *bim1 bim2 bim3* triple mutant. First, other transcription factors with no significant homology to BIM1 may compensate for the loss of *BIM1*/*BIM2*/*BIM3* function in helping BES1 bind to BR target gene promoters. There are 147 bHLH proteins found in *Arabidopsis* (Toledo-Ortiz et al., 2003), and some of them could functionally compensate for the loss-of-function in *BIM1*, *BIM2*, and *BIM3* genes. Second, it is possible that BES1 interacts with BIM1/BIM2/BIM3 to activate some target genes, while other non-bHLH factors are required to activate other target genes. Third, it is possible that BES1 can function as a homodimer in the absence of BIM1 family proteins to activate target gene expression to a certain extent. Consistent with these possibilities, we previously observed that BES1 seems to differentially activate BR-induced gene expression (Yin et al., 2002), likely by interacting with different partners on different promoters. This proposed mode of action for BES1 is somewhat similar to SMAD proteins in TGFβ signaling (Shi and Masssague, 2003). In response to the hormone, SMAD proteins are phosphorylated and translocated to the nucleus and interact with different transcription factors to activate different target genes for different responses. The SMAD partners include many different families of DNA binding proteins, including forkhead, homedomain, E box protein, Jun/Fos, Runx, CREBP, and E2F (Shi and Masssague, 2003).

Our results establish that BES1 is a BR-regulated transcriptional activator that binds directly to BR target gene promoters through a novel DNA binding domain to activate target gene expression. We also provide evidence that BES1 cooperates with the bHLH transcription factor BIM1 to bind target gene promoters more efficiently and to regulate BR target gene expression. These discoveries provide a framework to further understand how plant steroids differentially regulate gene expression and consequently regulate different responses such as cell elongation, vascular development, senescence, and stress responses. It will be crucial to determine the target genes for both BES1 and BIM1 as well as their homologs to identify different target genes for the distinct biological responses. In addition, the dissection of BES1 target gene promoters will reveal other promoter elements and transcription factors that contribute to the specificity of BR-induced gene expression mediated by BES1 and BIM1.

Experimental Procedures

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype *Columbia* (*Col-0*) was the wild-type in all the experiments described. Seeds were germinated on 1/2 MS medium (GIBCO-BRL) plus 1% sucrose. Plants were grown either in long day (15 hr light/9 hr dark) or short day (9 hr light/15 hr dark) conditions at 22°C.

Constructs

BEH1–4 and *BIM1–3* full-length cDNAs were amplified by RT-PCR from *Col-0* RNA and cloned into pCHF3-GFP (Yin et al., 2002) or pCHF3-3FLAG, and the GFP or FLAG were fused in frame to the C termini of these proteins. For *BES1*RNAi, a *BES1* cDNA containing the whole coding region was amplified and cloned into KpnI/EcoRI and ClaI/XbaI sites of pHANNIBAL (Wesley et al., 2001) in antisense/sense direction. The DNA fragment containing a CaMV35S promoter:1SEB-intron-*BES1* was excised by NotI and cloned into the same site of the binary vector pART27 (Wesley et al., 2001). BES1 deletions were generated with *pBES1*::BES1-GFP or *pBES1*:BES1-D::GFP (Yin et al., 2002) by PCR amplification of different BES1 or BES1-D fragments (NLS, NLS-N, NLS-N-P, P-C, C, N). *BES1*Δ*N*/*BES1-D*Δ*N*, *BES1*Δ*C*/*BES1-D*Δ*C*, or *BES1*Δ*P*/*BES1-D*Δ*P* constructs were obtained by ligations of NLS/P-C, NLS-N-P, or NLS-N/C fragments into the *BES1* promoter GFP cassette. For recombinant protein expression, cDNA fragments were cloned into pGEX5X-1 (GST tag, Amersham Pharmacia), pET28a (6xHis tag, Novagen), or pMAL2c (MBP tag, NEB). *SAUR-AC1* promoter region was amplified by PCR and cloned into a luciferase expression binary vector (Friedrichsen et al., 2002). All three E boxes in the SAUR-AC promoter were mutated by PCR-based mutagenesis. All constructs were confirmed by DNA sequencing. The details of cloning are available upon request.

Plant Transformation and Analyses of Transgenic Plants

Agrobacterium tumefaciens (stain GV3101) containing plasmid constructs were used to transform plants by the floral dip method (Clough and Bent, 1998). Transgenic lines were identified by selection in 1/2 MS medium plus 50 mg/l kanamycin. At least ten transgenic lines were generated for each construct. The expression of transgenes was analyzed by Northern and Western blots with appropriate probes or antibodies for BES1 (Mora-Garcia et al., 2004), MBP (NEB), GFP (Molecular Probes), and FLAG (Sigma). Phenotypes of representative transgenic lines were analyzed and documented either with an inverted microscope (Leica) or a digital camera. At least 15 seedlings were measured for each genotype for hypocotyl elongation assays and averages, and standard errors were calculated.

Yeast Two-Hybrid Screen

The Matchmaker System 3 (Clontech) was used to identify interacting proteins of BES1-C domain (amino acid residues 268–335) using an *Arabidopsis* cDNA library constructed with 3-day-old etiolated seedlings (Kim et al., 1997).

BIM1, *BIM2*, and *BIM3* T-DNA Knockouts

T-DNA knockout lines for *BIM1* (SALK-85924), *BIM2* (SALK-074689), and *BIM3* (SALK-79683) were identified from the SIGnAL *Arabidopsis* T-DNA collection (Alonso et al., 2003). The homozygous lines were identified by PCR with primers from T-DNA vector and *BIM1–3* genes. The primers used were the following: *bim1* (TCGATTCCAC GAATTTGGTGACTTCTGCCG; CCATGAAGCTCTGGCTCTTAAGCA CAGACC), *bim2* (CCGCTTGTGAGAATGTTCAGATTCTTCCTC; AG AAAGAGACCACGTGCATCACGTGACCAC), and *bim3* (TGGCTG CTTCACTGAGTTGCATATGATGAG; TCATCTCCTTCAGCAAACAAT CAGCCTGG).

Transient Expression by Particle Bombardment

A particle bombardment instrument (Bio-Rad) was used to deliver plasmids into plant tissues according to the manufacturer's instructions. For each treatment, 2 μg each of a *35S:GUS* construct (internal control), *SAUR-AC1::LUC* or mutant *SAUR-AC1::LUC*, *35S:BES1-GFP* or *35S:BES1-D-GFP* (Yin et al., 2002), and *BES1::BES1-DΔC* and *35S::BIM1–3FLAG* plasmids were coated onto gold particles and used to bombard three 1/2 MS plates containing approximately 300 mg of 2-week-old wild-type seedlings. The bombarded tissues were incubated in the dark for 24 hr and used to prepare protein extracts with 500 μl extraction buffer containing 100 mM KPO_4 (pH 8), 1 mM EDTA, 5% glycerol, and 10 mM DTT. The luciferase activities were measured by a luminometer with luciferin and 50 μl extract using an EG&G Berthold Microplate Luminometer LB96V (Wellesley, MA). The GUS activities were measured using 50 μl extract as described (Yin and Beachy, 1995). Reporter gene expression was determined by normalizing the luciferase activities with the GUS activities. The experiments were repeated twice with three duplicates each; representative results are presented.

Protein:Protein and Protein:DNA Interaction Assays

GST pull-down experiments were carried out as described previously (Yin et al., 2002). Protein:DNA electrophoretic mobility shift assays (EMSAs) were performed as described (Yin and Beachy, 1995). Briefly, oligonucleotide probes were synthesized, annealed, and labeled with P32-γ-ATP using T4 nucleotide kinase. 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 about 0.1 ng probe (20,000 cpm) and indicated amount of 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, 28.6g/l glycine, 0.78 g/l EDTA [pH 8.7]).

Chromatin Immunoprecipitation

Wild-type, *BES1-GFP*, *BES1-D-GFP*, or *BIM1–3XFLAG* transgenic seedlings were used in ChIP experiments mostly as described (Johnson et al., 2002). For each experiment, about 5 g of tissues from 2–3-week-old seedlings were used for ChIP with anti-GFP (Molecular Probes) or anti-FLAG (Sigma) antibodies. The ChIP products and input controls were used to detect specific promoters by PCR analyses. The primers used were the following: SAUR-AC1 (GCAG AAAGGAGTCAGTGATG; AAGACAGGACCACATGATTTG) and SAUR-AC-like (TGGCAAGTCTCTGCAACATC; TTGGAGCACCTAAACCA GAC).

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References

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science *301*, 653–657.

Asami, T., Min, Y.K., Nagata, N., Yamagishi, K., Takatsuto, S., Fujioka, S., Murofushi, N., Yamaguchi, I., and Yoshida, S. (2000). Characterization of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor. Plant Physiol. *123*, 93–100.

Beato, M., and Klug, J. (2000). Steroid hormone receptors: an update. Hum. Reprod. Update *6*, 225–236.

Boulikas, T. (1994). Putative nuclear localization signals (NLS) in protein transcription factors. J. Cell. Biochem. *55*, 32–58.

Caño-Delgado, A., Yin, Y., Yu, C., Vafeados, D., Mora-García, S., Cheng, J.-C., Nam, K.H., Li, J., and Chory, J. (2004). BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. Development *131*, 5341–5351.

Choe, S., Schmitz, R.J., Fujioka, S., Takatsuto, S., Lee, M.O., Yoshida, S., Feldmann, K.A., and Tax, F.E. (2002). *Arabidopsis* brassinosteroid-insensitive *dwarf12* mutants are semidominant and defective in a glycogen synthase kinase 3beta-like kinase. Plant Physiol. *130*, 1506–1515.

Chou, P.Y., and Fasman, G.D. (1978). Empirical predictions of protein conformation. Annu. Rev. Biochem. *47*, 251–276.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. *16*, 735–743.

Clouse, S.D. (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant J. *10*, 1–8.

Clouse, S.D. (2002). Brassinosteroid signal transduction: clarifying the pathway from ligand perception to gene expression. Mol. Cell *10*, 973–982.

Clouse, S., and Sasse, J. (1998). Brassinosteroids: essential regulators of plant growth and development. Annu. Rev. Plant Physiol. Plant Mol. Biol. *49*, 427–451.

Coll-Garcia, D., Mazuch, J., Altmann, T., and Mussig, C. (2004). EXORDIUM regulates brassinosteroid-responsive genes. FEBS Lett. *563*, 82–86.

Friedrichsen, D.M., Joazeiro, C.A., Li, J., Hunter, T., and Chory, J. (2000). Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. Plant Physiol. *123*, 1247–1256.

Friedrichsen, D.M., Nemhauser, J., Muramitsu, T., Maloof, J.N., Alonso, J., Ecker, J.R., Furuya, M., and Chory, J. (2002). Three redundant brassinosteroid early response genes encode putative bHLH transcription factors required for normal growth. Genetics *162*, 144–156.

Goda, H., Shimada, Y., Asami, T., Fujioka, S., and Yoshida, S. (2002). Microarray analysis of brassinosteroid-regulated genes in *Arabidopsis*. Plant Physiol. *130*, 1319–1334.

Goda, H., Sawa, S., Asami, T., Fujioka, S., Shimada, Y., and Yoshida, S. (2004). Comprehensive comparison of auxin-regulated and brassinosteroid regulated genes in *Arabidopsis*. Plant Physiol. *134*, 1555–1573.

He, Z., Wang, Z.Y., Li, J., Zhu, Q., Lamb, C., Ronald, P., and Chory, J. (2000). Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. Science *288*, 2360–2363.

He, J.X., Gendron, J.M., Yang, Y., Li, J., and Wang, Z.Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *99*, 10185–10190.

Iliev, E., Xu, W., Polisensky, D.H., Oh, M.-H., Torisky, R.S., Clouse, S.D., and Braam, J. (2002). Transcriptional and posttranscriptional regulation of *Arabidopsis TCH4* expression by diverse stimuli: roles of cis regions and brassinosteroids. Plant Physiol. *130*, 770–783.

Johnson, L., Cao, X., and Jacobsen, S. (2002). Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. *12*, 1360–1367.

Kim, J., Harter, K., and Theologis, A. (1997). Protein-protein interactions among the Aux/IAA proteins. Proc. Natl. Acad. Sci. USA *94*, 11786–11791.

Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. Bioinformatics *17*, 1244–1245.

Lee, J.E. (1997). Basic helix-loop-helix genes in neural development. Curr. Opin. Neurobiol. *7*, 13–20.

Li, J., and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell *90*, 929–938.

Li, J., and Chory, J. (1999). Brassinosteroid actions in plants. J. Exp. Bot. *50*, 332–340.

Li, J., and Nam, K.H. (2002). Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. Science *295*, 1299–1301.

Li, J., Nam, K.H., Vafeados, D., and Chory, J. (2001). *BIN2*, a new brassinosteroid-insensitive locus in *Arabidopsis*. Plant Physiol. *127*, 14–22.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell *110*, 213–222.

Lloyd, A., Yancheva, N., and Wasylyk, B. (1991). Transformation suppressor activity of a Jun transcription factor lacking its activation domain. Nature *352*, 635–638.

Mandava, N.B. (1988). Plant growth-promoting brassinosteroids. Annu. Rev. Plant Physiol. Plant Mol. Biol. *39*, 23–52.

Mangelsdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841–850.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835–839.

Molkentin, J.D., and Olson, E.N. (1996). Defining the regulatory networks for muscle development. Curr. Opin. Genet. Dev. *6*, 445–453.

Mora-Garcia, S., Vert, G., Yin, Y., Cano-Delgado, A., Cheong, H., and Chory, J. (2004). Nuclear protein phosphotases with Kelch-repeat domains modulate the response to bassinosteroids in *Arabidopsis*. Genes Dev. *18*, 448–460.

Mussig, C., Fischer, S., and Altmann, T. (2002). Brassinosteroid-regulated gene expression. Plant Physiol. *129*, 1241–1251.

Nair, S.K., and Burley, S.K. (2003). X-ray structures of Myc-Max and Mad-Max recognizing DNA: molecular bases of regulation by proto-oncogenic transcription factors. Cell *112*, 193–205.

Nakabeppu, Y., and Nathans, D. (1991). A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. Cell *64*, 751–759.

Nam, K.H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell *110*, 203–212.

Nemhauser, J.L., Mockler, T.C., and Chory, J. (2004). Interdependency of brassinosteroid and auxin signaling in *Arabidopsis*. PLoS Biol 2(9): e258 DOI: 10.1371/journal.pbio.0020258.

Pérez-Pérez, J.M., Ponce, M.R., and Micol, J.L. (2002). The *UCU1 Arabidopsis* gene encodes a SHAGGY/GSK3-like kinase required for cell expansion along the proximodistal axis. Dev. Biol. *242*, 161–173.

Shi, Y., and Masssague, J. (2003). Mechanisms of TGFβ signaling from cell membrane to the nucleus. Cell *113*, 685–700.

Spinner, D.S., Liu, S., Wang, S.W., and Schmidt, J. (2002). Interaction of the myogenic determination factor myogenin with E12 and a DNA target: mechanism and kinetics. J. Mol. Biol. *317*, 431–445.

Toledo-Ortiz, G., Huq, E., and Quail, P.H. (2003). The *Arabidopsis* basic/helix-loop-helix transcription factor family. Plant Cell *15*, 1749–1770.

Wang, Z.Y., Seto, H., Fujioka, S., Yoshida, S., and Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature *410*, 380–383.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., and Chory, J. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev. Cell *2*, 505–513.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., et al. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. *27*, 581–590.

Yin, Y., and Beachy, R.N. (1995). The regulatory regions of the rice tungro bacilliform virus promoter and interacting nuclear factors in rice (*Oryza sativa* L.). Plant J. *7*, 969–980.

Yin, Y., Wang, Z.Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell *109*, 181–191.