Three Redundant Brassinosteroid Early Response Genes Encode Putative bHLH Transcription Factors Required for Normal Growth

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ABSTRACT

Brassinosteroids (BRs) are a class of polyhydroxylated steroids that are important regulators of plant growth and development. We have identified three closely related basic helix-loop-helix (bHLH) transcription factors, BEE1, BEE2, and BEE3, as products of early response genes required for full BR response. Comparison of the phenotypes of plants that overexpress *BEE1* with *bee1 bee2 bee3* triple-knockout mutant plants suggests that BEE1, BEE2, and BEE3 are functionally redundant positive regulators of BR signaling. Expression of *BEE1*, *BEE2*, and *BEE3* is also regulated by other hormones, notably abscisic acid (ABA), a known antagonist of BR signaling. Reduced ABA response in plants overexpressing *BEE1* suggests that BEE proteins may function as signaling intermediates in multiple pathways.

BRASSINOSTEROIDS (BRs) are a class of polyhydroxylated steroids found throughout the plant kingdom. Analyses of mutants impaired in BR synthesis or perception reveal a key role for BRs in normal plant growth and development. BR mutants of Arabidopsis are short in the dark with open and developed cotyledons in contrast to wild-type seedlings, which have elongated hypocotyls and closed cotyledons. When grown in the light, Arabidopsis BR biosynthetic and response mutants are severe dwarfs with dark green epinastic leaves, shortened stems, and delayed senescence and have reduced fertility and apical dominance (Clouse *et al.* 1996; Kauschmann *et al.* 1996; Li *et al.* 1996; Szekeres *et al.* 1996; Azpiroz *et al.* 1998; Choe *et al.* 1999, 2000; Noguchi *et al.* 1999). BRs affect multiple processes, including promotion of cell expansion and xylem differentiation. Some BR-regulated genes have been identified in Arabidopsis, several of which are cell wall modification enzymes that have been proposed to play a role in cell expansion (Kauschmann *et al.* 1996; Xu *et al.* 1996; Nicol *et al.* 1998; Yin *et al.* 2002). BR signaling can also repress the expression of some BR biosynthesis genes, creating a negative feedback signal to dampen BR biosynthesis (Mathur *et al.* 1998; Noguchi *et al.* 2000; Mussig *et al.* 2002). Neither of these gene classes is likely to represent the primary molecular targets of BR signaling; rather, these targets are largely unknown.

Despite extensive genetic screening, only one loss-of-function BR-insensitive mutant, *bri1*, has been identified (Clouse *et al.* 1996; Kauschmann *et al.* 1996; Li and Chory 1997). BRI1, a leucine-rich repeat (LRR) receptor serine/threonine kinase, is a critical component of the BR receptor complex (Li and Chory 1997; Friedrichsen *et al.* 2000; Oh *et al.* 2000; Wang *et al.* 2001). Brassinolide (BL), the most biologically active BR, binds with high affinity to membrane fractions in a BRI1-dependent manner, and this binding is abolished by mutations in the extracellular domain of BRI1 (Wang *et al.* 2001).

Other components of the BR signal transduction pathway have been identified by their gain-of-function phenotypes. Overexpression of *BAK1*, a gene encoding another leucine-rich repeat receptor kinase, partially suppresses the phenotype of a weak *bri1* allele (Li *et al.* 2002). BAK1 was also identified by its interaction with BRI1 and shown to modulate BR signaling (Nam and Li 2002). A semidominant BR response mutant, *bin2*, has a phenotype similar to *bri1* mutants. The *bin2* phenotype results from a hypermorphic mutation in a glycogen synthase kinase-3, suggesting that wild-type BIN2 is a negative regulator of BR signaling (Li *et al.* 2001b; Li and Nam 2002). Two mutants, *bes1* and *bzr1*, were identified as suppressing *bri1* phenotypes, as well as being resistant to brassinazole, a BR biosynthesis inhibitor. *BES1* and *BZR1* encode closely related novel proteins that accumulate in the nucleus following BR treatment, where they regulate gene expression (Wang *et al.* 2002; Yin *et al.* 2002). Identical dominant mutations identified in both genes stabilize the respective proteins and increase their accumulation in the nucleus in the absence

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of BRs. Moreover, BES1 and BZR1 can be phosphorylated by the negative regulator BIN2, resulting in their turnover (He *et al.* 2002; Yin *et al.* 2002). Finally, overexpression of a putative serine carboxypeptidase, encoded by *BRS1*, partially suppresses a weak *bri1* allele; however, the *brs1* loss-of-function phenotype has not been reported and the role of BRS1 in BR signaling is not yet known (Li *et al.* 2001a).

Unlike *bri1* loss-of-function mutations, mutants in components of the BR signaling pathway do not mimic the phenotypes of steroid-deficient mutants. Functional redundancy resulting from extensive gene duplications in Arabidopsis is one probable explanation. Loss-of-function mutations in *BAK1* produce only weak phenotypes, perhaps due to the residual action of other LRR-type kinases (Li *et al.* 2002; Nam and Li 2002). BIN2 is 1 of 10 GSK3/Shaggy-like kinases in Arabidopsis and cosuppression studies indicate that reduced BIN2 levels have only a weak effect on plant growth (Li and Nam 2002). BES1 and BZR1 are part of a six-member family, and their loss-of-function phenotypes have not been reported.

In a screen for BR early response genes downstream of the BRI1 receptor complex, we identified three genes encoding closely related putative basic helix-loop-helix (bHLH) proteins named *BR Enhanced Expression* (*BEE1*, *BEE2*, and *BEE3*). Induction of these genes occurs within 30 min of treatment with BL, the most active BR, and does not require new protein synthesis. Auxin, cytokinin, and ethylene also induce expression of a subset of *BEE* genes, suggesting that BEE proteins may function in multiple signaling pathways affecting growth. While *bee1*, *bee2*, and *bee3* single or double mutants do not have any notable phenotype, a triple mutant carrying null alleles of all three genes has a reduced response to BL in several assays and has both seedling and floral phenotypes similar to known BR mutants. Transcription of *BEE1*, *BEE2*, and *BEE3* is repressed by another plant hormone, abscisic acid (ABA). ABA is a known antagonist of BR signaling, and analysis of seedlings overexpressing *BEE1* indicates that BEE1 may act in this antagonism.

MATERIALS AND METHODS

Plant growth and hormone treatment: The Arabidopsis wild type was Columbia-0. Growth conditions and seed sterilization methods were described previously (Friedrichsen *et al.* 2000). For the mRNA analysis in various hormone or cycloheximide treatments, 10-day-old wild-type or *bri1-116* mutant seedlings were incubated with the hormone diluted in 0.5× Murashige-Skoog (MS) salts, 1% sucrose for 2–2.5 hr (Friedrichsen *et al.* 2000). The concentrations for the treatments were: 1 μM BL (CIDtech Research), 100 μM ABA, 100 μM 1-aminocyclopropane-1-carboxylate, 5 μM benzoamino purine (BA), 100 μM gibberellic acid (GA_3), 50 μM 1-naphthaleneacetic acid (NAA), 50 μM cycloheximide alone or with 1 μM BL. Stocks were in 80% ethanol with identical dilutions except for NAA, for which the stock was dissolved in sodium hydroxide, and mock treatment was an equal amount of solvent alone. For hypocotyl length measurements, the same concentrations of hormones were used as for mRNA analysis. Seeds were sown on replicate plates containing hormone and grown in continuous light for 3 days.

Seedlings for root assays were grown on vertical plates with various concentrations of either BL or ABA. For the BL assay, seeds were sown directly on the hormone plates and measured after 7 days ($n = 10$; two repeats). The seedlings for ABA root assays were germinated on MS plates, transplanted to vertical plates after 2–3 days, and then measured when 7 days old ($n = 10$; two repeats). For BL dose response curves in the light, seedlings were grown in continuous light and hypocotyls were measured on day 4 ($n = 10$; two repeats). For BL dose response curves on dark-grown seedlings, seeds were given 4 hr of light to induce germination before being placed in the dark and were measured after 5 days. Two independently isolated triple-mutant lines were used ($n = 20$; three repeats). In the *era1* assays, hypocotyls of light-grown seedlings were measured after 7 days ($n = 20$; three repeats). For all assays, plates were kept at 4° for 4 days before light treatment.

Statistical analysis was performed in R (Ihaka and Gentleman 1996), using the lme function from the nlme package (Pinheiro and Bates 2000). For each analysis of the effects of different genotypes on hormone response, a linear mixed-effects model was used, treating genotype and hormone concentration as fixed effects and experimental replicate as a random effect, similar to a two-way ANOVA with an error term. Because the response to hormone was nonlinear in most cases and not easily modeled, different concentrations were treated as unordered levels of the hormone variable. In addition to the genotype and hormone main effects, a hormone-by-genotype interaction term was tested. When the interaction term was found to be significant, we concluded that genotype affected hormone response. *P* values associated with particular levels of hormone treatment were adjusted for multiple comparisons using the Holm method.

Fluorescent differential display and cDNA isolation: The samples for fluorescent differential display (FDD) were 10-day-old seedlings grown in liquid 0.5× MS 1% sucrose culture under long-day conditions (16 hr light, 8 hr dark). Both *det2-1* and *bri1-104* mutants (Li *et al.* 1996; Li and Chory 1997), which are strong alleles, were treated with either the mock treatment (ethanol) or 1 μM BL for 30 min. FDD analysis and cloning of the desired cDNAs was performed as described previously (Kuno *et al.* 2000).

Northern analysis: Total RNA was isolated using Trizol reagent (GIBCO BRL Life Technologies, Rockville, MD). For RNA gel blots, 20 μg total RNA was run on a formaldehyde gel and blotted. The blots were hybridized using the SuperHyb kit (Molecular Research Center, Cincinnati). The *BEE1*, *BEE2*, and *BEE3* probes for Northern analysis were PCR products made using gene-specific primers to amplify unique regions within each transcript: *BEE1* (5′-GTGGCTCTCCCTTTATTTCTCTC-3′; 5′-CTCTCTTTCTCGTCTTCTTCTTCC-3′), *BEE2* (5′-ATGGTTTCACCTGAGTTTCTTC-3′; 5′-TGGTTTCTGAATTTCTGAAGA-3′), and *BEE3* (5′-CTCAGAAATGGCGAATCTCTCT-3′; 5′-TATTCTTCGACCTCTTCCCTCTC-3′).

cDNA cloning and construction of *BEE1*-Ox: Full-length *BEE1* and *BEE2* cDNA clones were obtained from an Arabidopsis cDNA library (Kieber *et al.* 1993). The cDNA for *BEE2* is predicted to encode a 288-amino-acid protein and 5′-RACE was performed using a GIBCO BRL kit. The *BEE1*-Ox lines were created using the *Kpn*I-*Bam*HI fragment from the cDNA clone and inserting it in a plant pPZP221 expression vector under the control of the 35S promoter (Hajdukiewicz *et al.* 1994; Neff *et al.* 1999).

***BEE1* and *BEE2* promoter luciferase assays:** Transgenic Ara-

bidopsis carrying transcriptional fusions for either the −127 to −8 *BEE1* or the −897 to −12 *BEE2* full-length promoter to luciferase were grown on 0.5× MS, 1% sucrose plates under long-day conditions. Ten-day-old seedlings were used for these assays. The morning before the assay began, the seedlings were presprayed with luciferin solution [2.5 mM beetle luciferin (Promega, Madison, WI), 0.01% Triton X-100, 0.5× MS, 1% sucrose] to remove background luminescence. Sixteen hours before the start of the assay, the seedlings were gently removed from the plate and put individually into wells containing 100 μl of luciferin solution in 96-well plates. The assay started 1 hr after dawn with several prereadings to select rows of five seedlings with equal basal expression. Seedlings were treated with mock or hormone treatment: BL (1 μM) or ABA (100 μM). Measurements were made using a MicroLumat Plus from EG&G Berthold (Wellesley, MA).

Phylogenetic analysis of bHLH sequences: Sequences were trimmed to include only the bHLH domain and aligned with CLUSTALW (Thompson *et al.* 1994), with manual adjustment. For tree reconstruction, two optimality criteria, distance and maximum parsimony, were employed. The PROTDIST and FITCH programs from the PHYLIP 3.5 package (Felsentein 1993) were used to construct distance trees. Maximum parsimony trees were evaluated in PAUP* (Swofford 1998), using a codon-based substitution matrix and a heuristic search with TBR branch swapping. Both methods gave similar topologies.

Isolation of T-DNA insertion alleles in *BEE1*, *BEE2*, and *BEE3*: A total of 60,000 T-DNA Arabidopsis insertion lines (J. Alonso and J. Ecker, unpublished data) were screened for *bee1*, *bee2*, or *bee3* mutants by PCR. Primers specific for the left border of the T-DNA were used with gene-specific primers to identify mutant lines: *BEE1* (5′-CCCGGAAACTCTCCAGAC AGTAGTAACAA-3′; 5′-CCTTATAACATCCGGGCACCATATC TTGCA-3′), *BEE2* (5′-GCAGAGGATGAAACAGAGCCAAGCAT GAA-3′; 5′-GGAGGACCTGTGAAGTAAGCCTGAAACTAG-3′), and *BEE3* (5′-CTCTACCTCTTCTGCTCAAGTTTCCATAAA-3′; 5′-AATCATAGCAAACATCACCAGTCTTACGAG-3′).

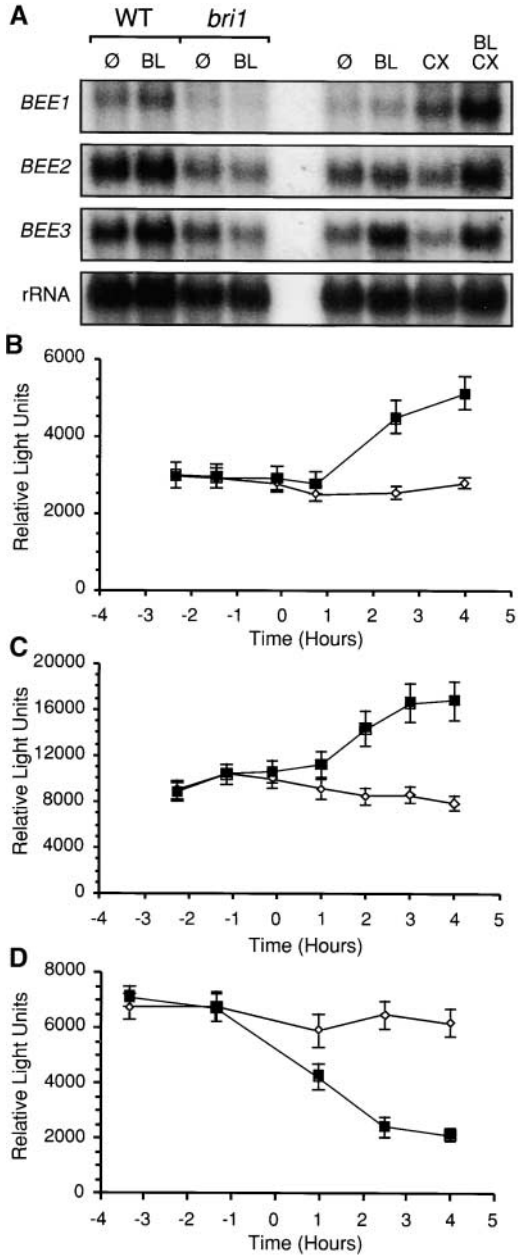


Figure 1.—Induction of *BEE1*, *BEE2*, and *BEE3* by BL treatment requires functional BRI1 but not *de novo* protein synthesis. (A) Expression of *BEE1*, *BEE2*, and *BEE3* in response to either mock (Ø) or BL treatment in either wild-type or *bri1* mutant seedlings and (right) the effect of cycloheximide on *BEE1*, *BEE2*, and *BEE3* induction in wild-type seedlings with mock (Ø), BL, cycloheximide (CX), or both BL and cycloheximide (BL CX) treatment. The rRNA probe is shown as a control for loading. (B) BL significantly induces transcription of the luciferase reporter gene under control of the *BEE1* promoter. (C) BL significantly induces transcription of the luciferase reporter gene under control of the *BEE2* promoter. Seedlings underwent (◇) mock (ethanol) or (■) BL treatment. (D) ABA significantly represses transcription of *BEE2::LUC* construct shown in C. Seedlings underwent (◇) mock (NaOH) or (■) ABA treatment. Error bars represent SE.

RESULTS

Three closely related putative bHLH transcription factors are induced by BL treatment: BR growth promotion requires *de novo* synthesis of both RNA and proteins (Mandava 1988). To identify novel BR-regulated genes, fluorescent differential display was performed with RNA from a BR biosynthetic mutant, *det2*, and a strong receptor mutant, *bri1* (Li *et al.* 1996; Li and Chory 1997). One of the genes induced by BL within 30 min of treatment in *det2*, but not in *bri1*, was named *BEE1* (***B****R* ***E****nhanced* ***E****xpression* **1**). *BEE1* (At1g18400) is predicted to encode a 245-amino-acid protein with sequence similarity to bHLH transcription factors. Northern analysis confirmed *BEE1* is induced two- to threefold by BL, with a maximum around 2 hr (Figure 1A). This induction, although small, was seen in six independent Northern blots and is consistent with microarray chip analysis indicating that subtle changes in expression may be the nature of the BR response, as most BR-regulated genes are induced only two- to threefold by BL treatment (Mussig *et al.* 2002; Yin *et al.* 2002).

Several predicted proteins from the Arabidopsis sequencing project are similar to BEE1 within the bHLH domain, and two of these were also induced twofold by

BL treatment and thus are named BEE2 (At4g36540) and BEE3 (At1g73830; Figure 1A). BEE1 and BEE2 contain 77% sequence identity within the bHLH domain. BEE1 and BEE3 have 94% sequence identity in the bHLH domain, with the sequence homology continuing from the bHLH domain through the C terminus of both proteins. Another predicted bHLH, At1g25330, with high similarity to BEE1 and BEE3 (Figure 2, A and B), shows extremely low expression in seedlings and is not BL induced (data not shown).

BEE1, BEE2, and BEE3 share high sequence identity and group together when compared with other known or predicted bHLH proteins in Arabidopsis (Figure 2, A and B). A search of the complete Arabidopsis genome for the Interpro (IPR001092) helix-loop-helix DNA-binding domain results in 145 putative bHLH proteins (Arabidopsis Genome Initiative 2000). Alignment of the bHLH domain, by either distance or maximum parsimony criteria, groups BEE1, BEE2, and BEE3 in a subfamily with 16 total members. Although BEE2 shares sequence homology outside the bHLH domain with another predicted protein, At2g18300, this gene was not regulated by BL in seedlings (data not shown). Several other members of this subfamily were tested and no additional BL-induced genes were identified (Figure 2A).

***BEE1*, *BEE2*, and *BEE3* are early response genes in BR signaling:** The induction of all three genes, *BEE1*, *BEE2*, and *BEE3* by BL requires functional BRI1 and does not require *de novo* protein synthesis (Figure 1A), making them the first early response genes characterized in the BR response pathway. Moreover, the addition of cycloheximide enhances BL induction, possibly indicating a short-lived negative regulator of *BEE1*, *BEE2*, and *BEE3* expression analogous to repressors of the auxin-regulated AUX/IAA genes (Abel *et al.* 1995). To determine the duration of induction, transgenic plants containing either the *BEE1* or the *BEE2* promoter controlling the expression of a luciferase reporter gene were tested for BL responsiveness. Induction of either the *BEE1* ($n = 12$; Figure 1B) or the *BEE2* promoter luciferase fusion ($n = 15$; Figure 1C) was maintained even after 6 hr of BL treatment. The luciferase assays confirm that although the change in *BEE1* and *BEE2* expression is relatively small, it is significant, highly reproducible, and continues for several hours.

To further examine the possible roles of *BEE1*, *BEE2*, and *BEE3* in BR signaling, we used a reverse genetics approach to identify a T-DNA insertion in each gene (Figure 2C). Each of these insertions is predicted to eliminate the expression of the RNA, and this was confirmed by either Northern blot or reverse transcription-PCR (data not shown). Single (*bee1*, *bee2*, or *bee3*) or double (*bee1 bee2* or *bee1 bee3* or *bee2 bee3*) knockout mutants did not have any obvious developmental or hormone response phenotypes (data not shown). However, the *bee1 bee2 bee3* triple mutant had a light-grown developmental phenotype that resembled a weak BR response mutant (Figure 3A).

***BEE1*, *BEE2*, and *BEE3* are not dedicated to BR response:** Cross-talk between primary effect genes of other plant hormone pathways has been observed previously, including the SAUR genes originally identified as downstream of auxin signaling and now shown to be upregulated in the presence of brassinosteroids (Yin *et al.* 2002). To determine whether the *BEE* genes were also acting in other hormone pathways, expression of *BEE1*, *BEE2*, and *BEE3* in the presence of five additional plant hormones was tested (Figure 4A). The hormones tested were auxin, ethylene, GA, cytokinin, and ABA. Auxin and BRs are known to act synergistically in the promotion of stem elongation (Mandava 1988; Yi *et al.* 1999); consistent with this synergism, auxin induced expression of both *BEE1* and *BEE3*. Ethylene also induced expression of *BEE1* and *BEE3*, while cytokinin promoted expression of *BEE1* and *BEE2*. Although BRs can act additively with GAs in some bioassays (Mandava 1988), GAs did not affect *BEE1*, *BEE2*, and *BEE3* mRNA accumulation. ABA and BRs have been shown to act antagonistically (Mandava 1988; Steber and McCourt 2001), and we observed that ABA repressed the expression of *BEE1*, *BEE2*, and *BEE3*. Strikingly, the only regulation common to all three genes was induction by BR and repression by ABA.

Triple mutants were found to be significantly shorter than wild-type plants in the absence of added hormone (Figures 3A and 4C). To determine whether the phenotype of the triple mutant resulted from reduced response to specific hormones or a general defect in growth, hypocotyl lengths were measured from seedlings grown in the presence of the same panel of hormones described above (Figure 4B). Three outcomes are possible when a dwarf mutant is treated with growth-promoting hormones. If the defect in the mutant is unrelated to a given treatment, the response should parallel that of wild type. In other words, the difference between wild type and the mutant should diminish with treatment, as is observed when *det1* mutants are treated with BL (Li *et al.* 1996). If dwarfism is caused by a severely diminished response to the hormone, treatment should result in little change in phenotype, as is the case with BL treatment of *bri1* mutants (*e.g.*, Kauschmann *et al.* 1996). Finally, if dwarfism is due to a partial loss of response, mutants should show a decreased slope of response relative to wild type. Analysis of the slopes of response to various hormone treatments revealed that only cytokinin, NAA, and BL treatments differed between wild type and triple mutants (Figure 4C). The increased sensitivity of the triple mutants to cytokinin is difficult to interpret since cytokinin had no effect on wild-type hypocotyl length, and the triple mutant shows none of the characteristics expected in a cytokinin biosynthetic mutant. Hypersensitivity to cytokinin in the triple mutants may result from antagonism of the cytoki-

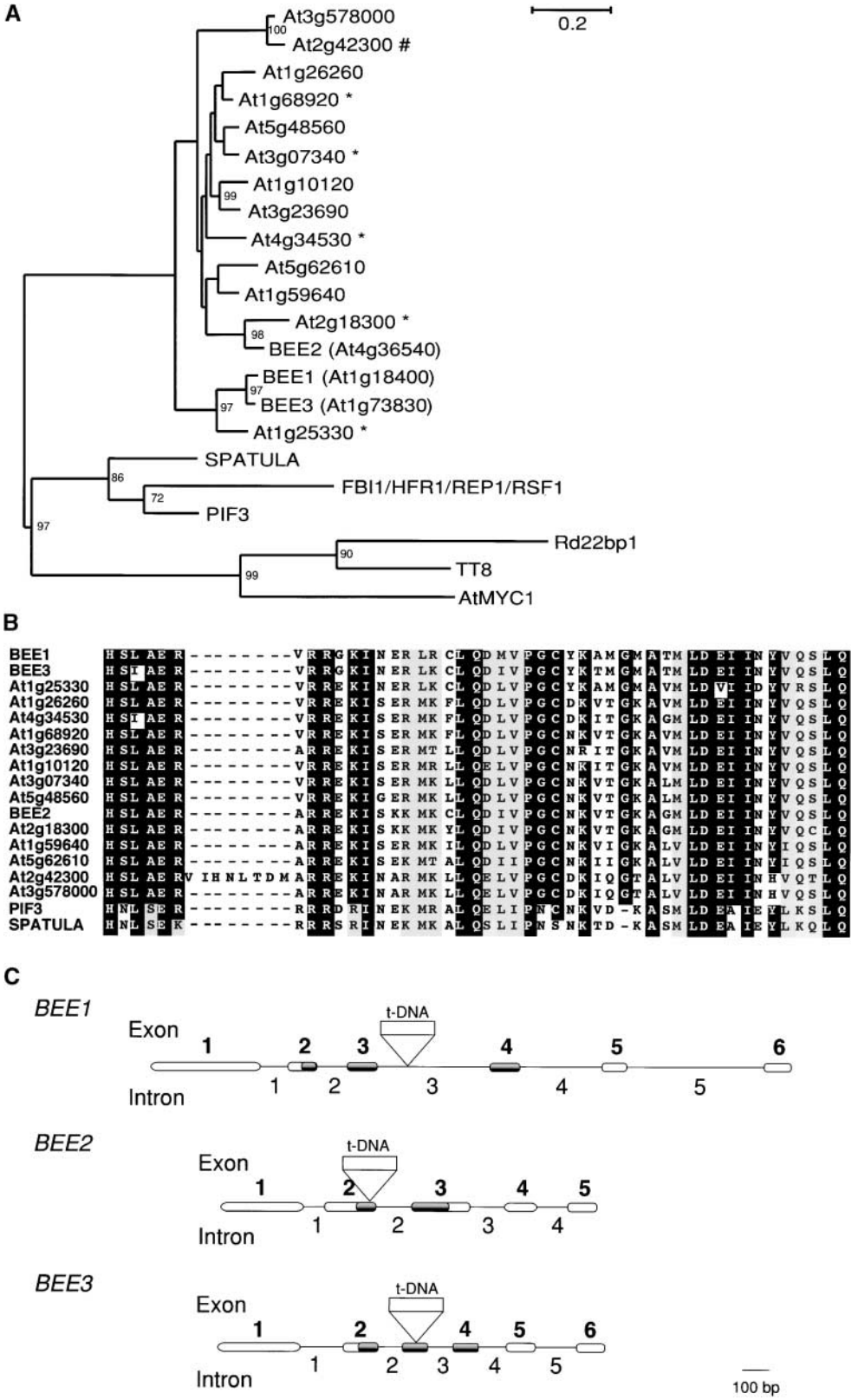


FIGURE 2.—BEE1, BEE2, and BEE3 belong to a subfamily of putative bHLH proteins. (A) Distance-based phylogenetic tree of BEE1, BEE2, and BEE3 with other members of the subfamily plus other bHLHs from Arabidopsis: SPATULA (AAG33640); PIF3 (AAC95156); HFR1 (AAG40617), RSF1 (SPIEGELMAN *et al.* 2000), REP1 (AAG45733), FBI1 (AAK-15282); Rd22bp1 (BAA25078); TT8 (CAC14865); and Atmyc1 (BAA11933). *, those not regulated by BL treatment; #, the transcript is not expressed in seedlings. Bootstrap values >70% are as indicated. (B) Alignment of bHLH domains for members of the BEE subfamily with Arabidopsis PIF3 and SPATULA for reference. Solid boxes indicate regions of >90% identity. (C) Schematic representation of T-DNA insertions into *BEE1*, *BEE2*, and *BEE3* genomic sequences. bHLH domain is shaded and T-DNA insertion sites are where indicated.

nin and BR signaling pathways, as observed in other systems (MANDAVA 1988). While the triple mutant appeared to be less responsive than wild type to NAA, this may result from the physical limitations of the hypocotyl, as growth of both wild-type and mutant hypocotyls is extremely inhibited by NAA treatment used in these experiments (Figure 4B). In contrast to the above cases, BR was the only treatment that enhanced the growth

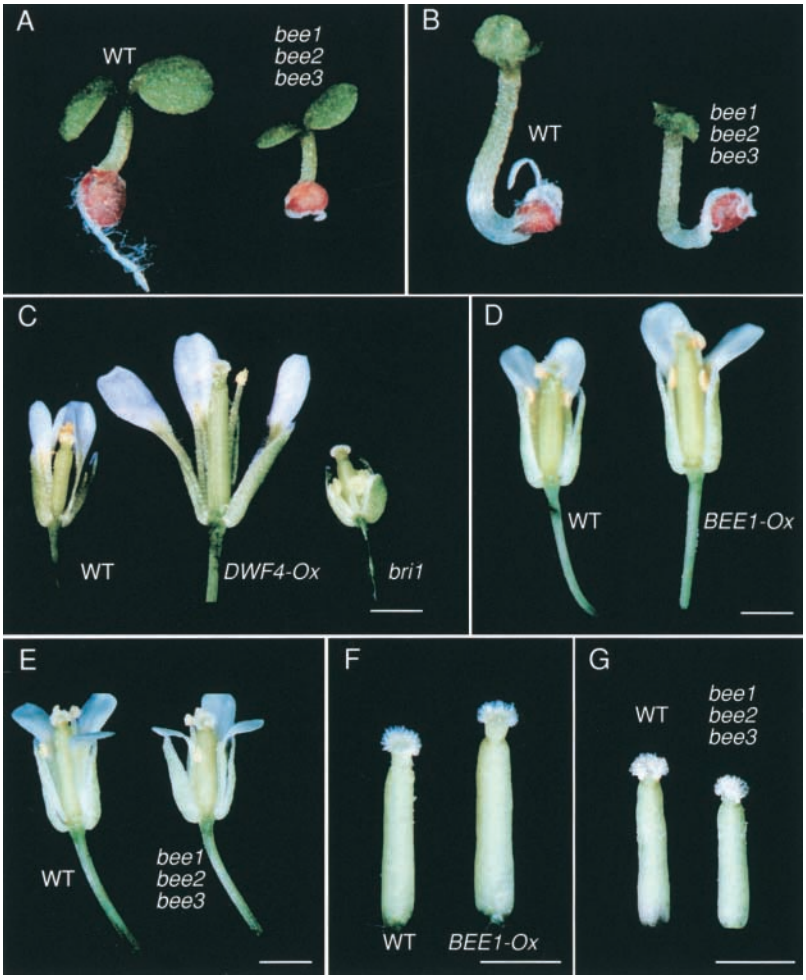


FIGURE 3.—The *BEE1*-Ox and triple mutants have seedling and floral phenotypes similar to known BR mutants. (A) *bee1 bee2 bee3* plants (right) have shorter hypocotyls than those of wild type (left). (B) The difference in hypocotyl length between *bee1 bee2 bee3* plants (right) and wild type (left) is enhanced with the addition of BL. (C) BR mutants have floral phenotypes. Wild-type, *DWF4*-Ox, and *bri1* flowers are pictured as labeled. (D) The flowers of *BEE1*-Ox plants are larger than those of wild type (left). (E) *bee1 bee2 bee3* triple mutants (right) have smaller floral organs than those of wild type (left). (F) Wild-type (left) and *BEE1*-Ox (right) gynoecia. (G) The gynoecium of the triple mutants is smaller than that of wild type (left). In all pictures, some organs of the flowers were removed to better view the remaining organs. Bars, 1 mm.

of wild-type seedlings and whose response was impaired in triple mutants. This result, coupled with the findings that none of the single or double *bee* mutant combinations show any obvious phenotype and that only BR and ABA regulate all three transcripts, strongly suggests that although the *BEE* genes likely function in multiple hormone response pathways, the growth defect observed in the triple mutant is caused by diminished BR response.

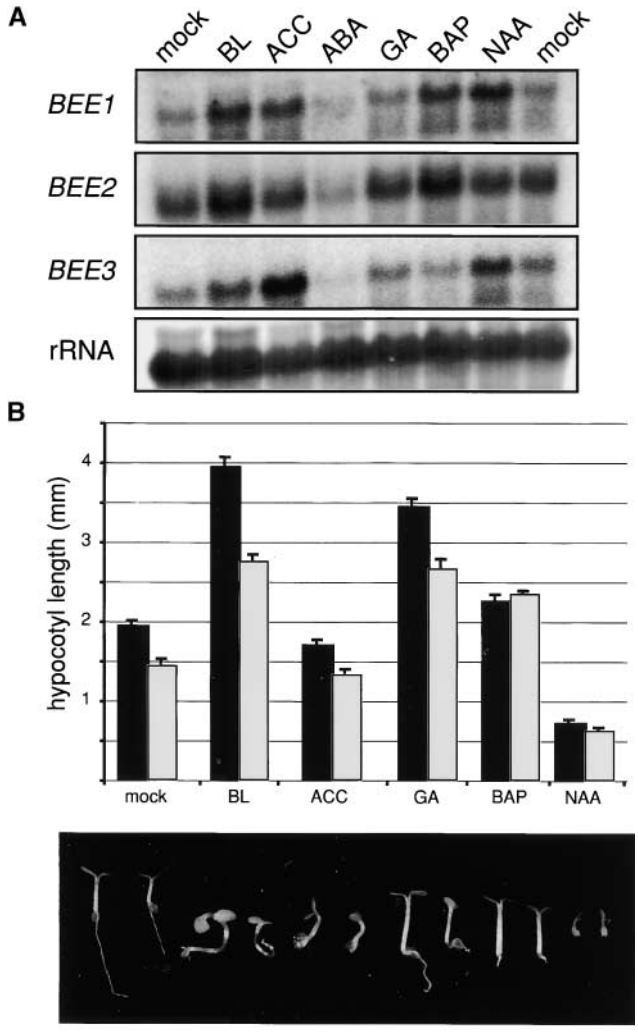
***bee1 bee2 bee3* triple mutants are less responsive to BRs:** To examine the moderate reduction in BR response of the triple mutant more carefully, seedlings were germinated in both light and dark in the presence of varying concentrations of BL. When germinated in the light, the triple-mutant seedlings had hypocotyls shorter than those of wild-type seedlings and a reduced response to BL growth promotion of the hypocotyl ($\sim$50–70% of wild type, $P < 1.24 \times 10^{-7}$, Figures 3, A and B and 5A). Exogenous BL inhibits hypocotyl elongation of wild-type seedlings grown in the dark (EPHRITIKHINE *et al.* 1999). We found that the triple mutant showed only $\sim$25–50% of the wild-type response to BL ($P < 1 \times 10^{-10}$, Figure 5B). Our finding that the loss of function of *BEE1*, *BEE2*, and *BEE3* results in a reduced responsiveness to BRs shows that BEE1, BEE2, and BEE3 act as positive regulators of BR signaling.

It has been shown previously that exogenous BRs inhibit root growth (CLOUSE *et al.* 1996; EPHRITIKHINE *et al.* 1999; LI and NAM 2002). No effect on BR perception could be detected in roots of the triple mutant. While *BEE* genes are expressed in most aerial tissues, *BEE1* is the only *BEE* gene expressed in the root (data not shown). Interestingly, several independent lines overexpressing *BEE1* mRNA (*BEE1*-Ox) showed no defects in hypocotyl perception of BRs, but had roots that were more sensitive to exogenously added BL than those of wild type ($P < 8.17 \times 10^{-6}$, Figure 5C). The different phenotypes observed in *bee1 bee2 bee3* and *BEE1*-Ox may result from subtle differences in pattern or level of expression between the cauliflower mosaic virus 35S promoter used for overexpression and native promoters, or it may reflect true organ-specific differences in interacting factors.

In addition to the well-characterized role of BRs in hypocotyl cell elongation, previous studies have also indicated that BRs are required for cell elongation in floral organs (CHOE *et al.* 1999, 2000). Flowers of BR biosynthesis mutants are reduced in size with shorter gynoecia and stamens than those of wild type. Overexpression of *DWF4*, a BR biosynthetic enzyme, results in enhancement of BR-regulated cell elongation (CHOE *et*

al. 2001; Wang *et al.* 2001). We found that floral organs in all whorls of *DWF4*-Ox flowers were larger than those in comparable wild-type flowers (Figure 3C). Similarly, all floral organs of transgenic lines that overexpress *BEE1* were larger than those of comparable wild-type flowers, particularly the gynoecium, although this effect was subtler than that seen in the *DWF4*-Ox flowers (Figure 3, D and F). Thus, increases in either the hormone itself or a downstream regulator result in similar growth effects in the flower. In spite of the similarity in floral phenotypes, *BEE1*-Ox plants did not have an obvious leaf phenotype. In contrast to increased signaling mutants, *bri1* flowers were smaller overall, with dramatically reduced stamens and gynoecia (Figure 3C). *bee1 bee2 bee3* flowers were also smaller than those of wild type (Figure 3, E and G). The clearly reduced size of triple-mutant gynoecia as compared to wild type suggests a role for BEE1, BEE2, and BEE3 in normal gynoecium development. Moreover, the floral phenotypes of transgenic lines that overexpress *BEE1* and *bee1 bee2 bee3* triple mutants show that BEE1, BEE2, and BEE3 play a significant role in promoting growth outside of the seedling.

***BEE1*-Ox connects BR and ABA response pathways:** In addition to upregulation by BL, all three *BEE* genes were also repressed by ABA (Figures 1D and 4A). The repression of *BEE1* and *BEE2* by ABA was confirmed by treating seedlings expressing the respective promoter-luciferase fusions with ABA (Figure 1D). BRs and ABA have been shown previously to act antagonistically (Mandava 1988). ABA promotes dormancy in seeds while BRs can promote germination (Steber and McCourt 2001). In wild-type plants, ABA at low concentrations promotes the growth of roots and at high concentrations inhibits their growth (Ghassemian *et al.* 2000). Roots of either *bri1* or *sax1*, a BR biosynthetic mutant, are hypersensitive to ABA (Clouse *et al.* 1996; Ephritikhine *et al.* 1999). *BEE1*-Ox roots, which are hypersensitive to BRs, were partially insensitive to ABA ($P < 1.6 \times 10^{-4}$, Figure 6A). *BEE1*-Ox roots failed to respond to the growth-promoting effects of low concentrations of ABA, although root growth was sensitive to high doses of ABA. Interestingly, the high-dose ABA response has recently been shown to require functional ethylene signaling components (Ghassemian *et al.* 2000), suggesting that transgenic lines overexpressing *BEE1* are insensitive specifically to the ethylene-independent phase of ABA-regulated root growth. As was the case for the BL root response, the triple mutant shows no change in ABA response, perhaps indicating additional redundant factors yet to be identified that act in the root.



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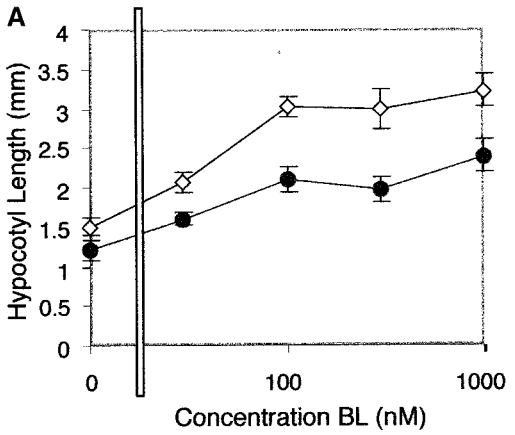
ANOVA table	numDF	denDF	F-value	p-value
(Intercept)	1	222	1191.84	< 1E-10
genotype	1	222	118.70	< 1E-10
treatment	5	6	44.50	1.15E-04
genotype:treatment	5	222	18.66	< 1E-10

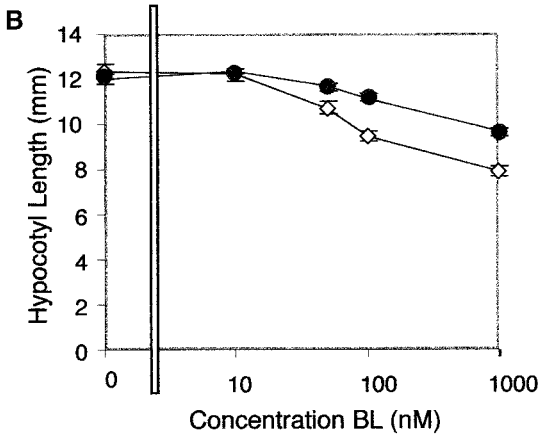
Sig Interact[a]	Value[b]	Std Err	p-value[c]
gtbbb:BL	-0.67	0.15	5.11E-05
gtbbb:BAP	0.59	0.15	5.01E-04
gtbbb:NAA	0.40	0.15	0.027

Figure 4.—*BEE1*, *BEE2*, and *BEE3* are regulated by other plant hormones. (A) The expression of *BEE1*, *BEE2*, and *BEE3* is responsive to different hormone treatments. Detailed description of treatments can be found in materials and methods. The rRNA probe is shown as a control for loading. (B) Hypocotyl lengths of wild-type and triple-mutant seedlings grown in the presence of the same panel of hormones shown in A. Triple mutants show reduced response to BR and an increased response to cytokinin. Wild type, solid bars; triple mutants, shaded bars. Error bars give 95% confidence intervals. (C) ANOVA table for experiment in B. A significant genotype term ($P < 0.05$) indicates that the strains were different in the mock treatment. (a) Significant Interactions shows the particular treatments where the mutants differ from wild type. (b) Value indicates the difference in treatment response between triple mutants and wild type relative to mock treatments. (c) Corrected for multiple comparisons. numDF, numerator degrees of freedom; DenDF, denominator degrees of freedom.



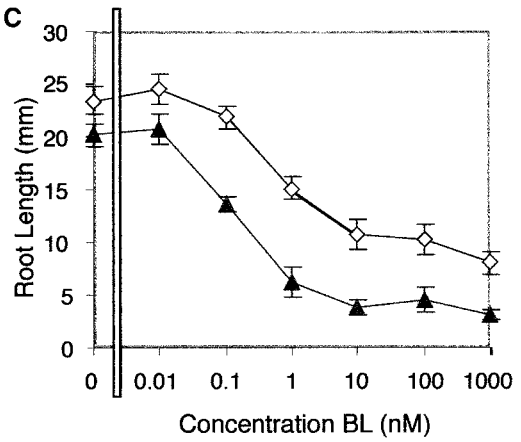
ANOVA table	numDF	denDF	F-value	p-value
(Intercept)	1	287	10271.90	$< 1*10^{-10}$
genotype	2	287	102.74	$< 1*10^{-10}$
[BL]	4	287	179.26	$< 1*10^{-10}$
genotype:[BL]	8	287	6.38	$1.24*10^{-07}$

Sig Interact[a]	Value[b]	Std Err	% of WT[c]	p-value[d]
triple:[BL] 100	-0.63	0.17	58.7	0.003
triple:[BL] 300	-0.71	0.18	51.7	0.001
triple:[BL] 1000	-0.54	0.18	68.7	0.014



ANOVA table	numDF	denDF	F-value	p-value
(Intercept)	1	1127	1288.05	$< 1*10^{-10}$
genotype	2	1127	79.10	$< 1*10^{-10}$
[BL]	4	1127	252.02	$< 1*10^{-10}$
genotype:[BL]	8	1127	14.56	$< 1*10^{-10}$

Sig Interact[a]	Value[b]	Std Err	% of WT[c]	p-value[d]
triple:[BL] 50	1.23	0.31	24.50	0.0003
triple:[BL] 100	2.00	0.30	30.90	$5.10*10^{-10}$
triple:[BL] 1000	2.10	0.29	53.16	$< 1*10^{-10}$



ANOVA table	numDF	denDF	F-value	p-value
(Intercept)	1	254	251.46	$< 1*10^{-10}$
genotype	1	254	298.80	$< 1*10^{-10}$
[BL]	6	254	253.43	$< 1*10^{-10}$
genotype:[BL]	6	254	5.93	$8.17*10^{-06}$

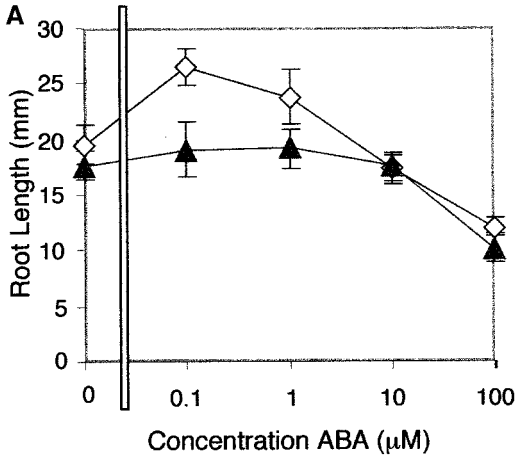
Sig Interact[a]	Value[b]	Std Err	% of WT[c]	p-value[d]
BEE1Ox:[BL] 0.1	-4.91	1.32	398.95	0.001
BEE1Ox:[BL] 1	-5.57	1.14	166.88	$1.13*10^{-05}$
BEE1Ox:[BL] 10	-3.63	1.17	128.53	0.009

FIGURE 5.—*BEE* genes play a role in BL-regulated growth responses. (A and B) *bee1 bee2 bee3* hypocotyls are less responsive to BL than are wild type. (A) Seedlings were germinated in continuous light on increasing concentrations of BL and their hypocotyls were measured on day 4 for (◇) wild type and (●) *bee1 bee2 bee3*. (B) Seedlings were germinated in the dark on increasing concentrations of BL and their hypocotyls were measured after 5 days for (◇) wild type and (●) *bee1 bee2 bee3*. (C) Roots from lines overexpressing *BEE1* (*BEE1*-Ox) are more responsive to exogenous BL than are wild type. Seedlings were grown on vertical plates with increasing concentrations of BL and their roots were measured after 7 days for (◇) wild type and (▲) *BEE1-Ox*. In the ANOVA tables to the right of each graph, a significant genotype:[BL] interaction term ($P < 0.05$) indicates that the strains had different responses to BL. (a) Significant Interactions shows the particular BL concentrations [BL] where the mutants differ from wild type. (b) Value indicates the difference in BL response between the mutants and wild type at the indicated [BL]. (c) Column expresses the mutant responses as a percentage of the wild-type response at a given [BL]. (d) Corrected for multiple comparisons. Error bars give 95% confidence intervals. numDF, numerator degrees of freedom. DenDF, denominator degrees of freedom.

Since BR mutants have an ABA response phenotype, we wanted to determine if ABA mutants have BR phenotypes. An ABA-hypersensitive mutant, *era1*, was tested for altered BR response (CUTLER *et al.* 1996). When compared to wild type, *era1* hypocotyls were less responsive to BL ($P < 1 \times 10^{-10}$, Figure 6B). *era1* hypocotyls were taller than those of wild type in the absence of hormone, potentially indicating that *era1* is involved in multiple developmental signals. Thus, ABA and BL, which act as phenotypic antagonists in several assays, coregulate in opposing fashion the expression of *BEE1*, *BEE2*, and *BEE3* signaling intermediates, suggesting a new mechanism for plant hormone cross-talk.

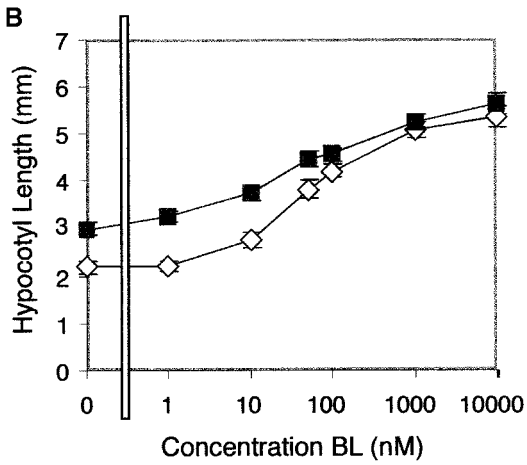
DISCUSSION

Previous genetic screens had limited success in identifying BR response loci, suggesting that BR signal components may be essential for viability or act redundantly. In this article, we identify three closely related putative transcription factors, BEE1, BEE2, and BEE3, that are functionally redundant. BEE1, BEE2, and BEE3 play a



ANOVA table	numDF	denDF	F-value	p-value
(Intercept)	1	183	79.59	$4.44*10^{-16}$
genotype	1	183	29.95	$1.44*10^{-07}$
[ABA]	4	183	53.35	$<1*10^{-10}$
genotype:[ABA]	4	183	5.95	$1.60*10^{-04}$

Sig Interact[a]	Value[b]	Std Err	% of WT[c]	p-value[d]
BEE1Ox:[ABA] 0.1-5.47	1.71	20.8	0.006	



ANOVA table	numDF	denDF	F-value	p-value
(Intercept)	1	859	172.23	$<1*10^{-10}$
genotype	1	859	145.82	$<1*10^{-10}$
[BL]	6	859	384.92	$<1*10^{-10}$
genotype:[BL]	6	859	10.64	$<1*10^{-10}$

Sig Interact[a]	Value[b]	Std Err	% of WT[c]	p-value[d]
era1:[BL] 100	-0.48	0.16	76.2	0.013
era1:[BL] 1000	-0.62	0.16	78.4	0.001
era1:[BL] 10000	-0.54	0.18	83.0	0.013

FIGURE 6.—Plants overexpressing *BEE1* have ABA response phenotypes and an ABA mutant has a BR response phenotype. (A) *BEE1*-overexpressing (*BEE1*-Oxs) roots are partially insensitive to ABA. Roots of 7-day-old seedlings were measured from (◇) wild type and (▲) *BEE1-Ox*. (B) ABA-hypersensitive mutant, *era1*, has decreased sensitivity to BL. Hypocotyls of 7-day-old seedlings were measured from (◇) wild type and (■) *era1*. In the ANOVA tables beside each graph, a significant genotype:[hormone] interaction term ($P < 0.05$) indicates that the strains had different responses to hormone. (a) Significant Interactions shows the particular hormone concentrations where the mutants differ from wild type. (b) Value indicates the difference in hormone response between the mutants and wild type at the indicated concentration. (c) Column expresses the mutant responses as a percentage of the wild-type response at a given hormone concentration. (d) Corrected for multiple comparisons. Error bars give 95% confidence intervals. numDF, numerator degrees of freedom; DenDF, denominator degrees of freedom.

significant role in growth throughout the plant. Mutants lacking all three proteins are less responsive to BRs, displaying both seedling and floral phenotypes characteristic of known BR mutants.

While the genomic sequence of Arabidopsis contains a large amount of structural redundancy, similar to other sequenced eukaryotic genomes, Arabidopsis is unique in its predicted number of large multigene families (ARABIDOPSIS GENOME INITIATIVE 2000; BOUCHE and BOUCHEZ 2001). Specifically, 35.0% of Arabidopsis genes are unique, which is less than that of either *Caenorhabditis elegans* (55.2%) or Drosophila (72.5%), and Arabidopsis has an unusually high proportion of gene families with five or more members [37.4%; *C. elegans* (24.0%) and Drosophila (12.1%); ARABIDOPSIS GENOME INITIATIVE 2000]. This raises the question of whether structurally related genes have partially or completely redundant functions. One of the earliest and best-studied examples is the genes encoding phytochromes (CLACK *et al.* 1994; REED *et al.* 1994; CASAL 2000). The five Arabidopsis phytochromes (PHYA–E) are red/far-red light receptors with both distinct and overlapping functions. For example, PHYA is unique as the critical receptor for de-etiolation in far-red light. On the other hand, *phyD* or *phyE* mutants have no visible phenotypes, except when combined with *phyB* (DEVLIN *et al.* 1998, 1999). Several other examples of functional redundancy have been reported (PELAZ *et al.* 2000; BOUCHE and BOUCHEZ 2001; LEE and SCHIEFELBEIN 2001; ZHAO *et al.* 2001), and it is likely that this phenomenon is widespread in plants. Still, it should be noted that while the apparent lack of phenotype for mutants in members of multigene families may be due to complete functional redundancy, another possibility is that unique functions are apparent only in specific physiological conditions or cell types, which may be difficult to identify.

Here, we present an analysis of three putative bHLH transcription factors. There are ~145 bHLH genes in Arabidopsis. *BEE1*, *BEE2*, and *BEE3* belong to a subfamily of 16 predicted genes. Although triple mutants show significant BR response phenotypes consistent with being positive regulators of BR signaling, they do not have the traditional BR dwarf adult phenotype. The subtlety of the triple knockout mutant phenotype compared with that of *bri1* mutants implies that the BR signaling pathway does not absolutely require the function of BEE1, BEE2, and BEE3. One possibility is that BR signaling branches soon after BRI1 and BEE1, BEE2, and BEE3 are involved in only one branch of the BR re-

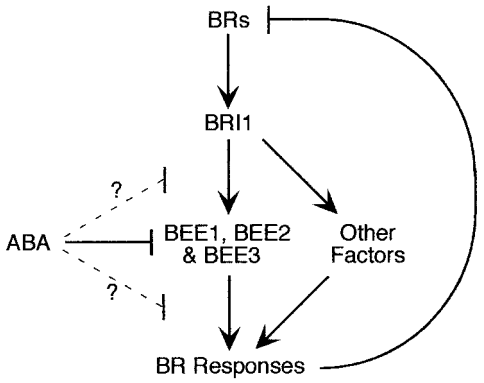


Figure 7.—BR and ABA regulation of *BEE1*, *BEE2*, and *BEE3*. *BEE1*, *BEE2*, and *BEE3* expression is induced by BRs through BRI1. This induction may be dampened by a negative feedback loop, which represses the expression of BR biosynthetic enzymes after treatment with the hormone. *BEE1*, *BEE2*, and *BEE3* are required for full perception of the BR signal. The BR antagonist, ABA, decreases the expression of *BEE1*, *BEE2*, and *BEE3* in a new example of hormone cross-talk affecting signaling intermediates. ABA may also negatively regulate other BR signaling components. The interaction of the signaling pathways of these two hormones may be important for modulating growth responses during development.

sponse (Figure 7). Recent work on BZR1 and BES1 indicates that this branched pathway model may be true, as expression of *CPD*, a gene encoding a key BR biosynthetic enzyme, is strongly repressed in *bzr1* mutants but is largely unchanged in *bes1* mutants (Wang *et al.* 2002; Yin *et al.* 2002). Another not mutually exclusive model is that there are additional redundant factors for BEE1, BEE2, and BEE3. Functional redundancy does not require that the products share sequence identity or BR inducibility, leaving a large list of potential BEE1, BEE2, and BEE3 partners. *bee1 bee2 bee3* triple mutants provide a sensitized background for identifying these additional components.

In addition to redundant factors, another challenge in identifying BR signaling components is the relatively small changes in gene expression, which appear to be the hallmark of BR response. BR induction of published BR-regulated genes, including cell wall modification enzymes BRU1 and TCH4, is only two- to fourfold (Zurek and Clouse 1994; Xu *et al.* 1996; Muñoz *et al.* 1998; Yoshizumi *et al.* 1999; Hu *et al.* 2000). This magnitude of BR regulation was confirmed by microarray chip analysis in which we found ~30 Arabidopsis genes (of ~5500) whose expression was induced by a short BR treatment and was not induced in the *bri1* background; of these, most were induced by only two- to threefold (Yin *et al.* 2002). Similar results were obtained from microarray experiments comparing wild-type and BR biosynthesis mutants (Mussig *et al.* 2002). The BR response pathway contains a negative feedback loop potentially important for tightly controlled regulation of BR signaling (Figure 7). Repression of *CPD* following BR treatment requires both functional BRI1 and *de novo* protein synthesis (Mathur *et al.* 1998). This negative feedback mechanism may be responsible for quenching BR-induced gene expression. Thus, although the effects of BRs on gene expression are small, analysis of *bee1 bee2 bee3* triple mutants shows that this gene regulation is physiologically significant.

In animals, bHLH proteins are important for a variety of developmental processes, including myogenesis and neurogenesis, where they function as either homo- or heterodimers to activate or repress transcription through E-box-elements (Massari and Murre 2000). In Arabidopsis, bHLH proteins are involved in several developmental programs, including floral organogenesis, hormone responses, and light signaling through phytochrome (Abe *et al.* 1997; Ni *et al.* 1998; Fairchild *et al.* 2000; Soh *et al.* 2000; Spiegelman *et al.* 2000; Heisler *et al.* 2001). While currently we do not know the mechanistic role of BEE1, BEE2, and BEE3, we might anticipate that they are regulators of downstream target genes important for hormone growth responses, perhaps by either homo- or heterodimerization, acting through E-box-related elements. Our preliminary data suggest that overexpression of *BEE1* positively affects expression of *TCH4*, a BR-induced gene whose promoter contains predicted E-box elements (D. Friedrichsen and J. Chory, data not shown).

Cross-talk between plant hormones is important for normal development, and these interactions appear to play a key role in determining BR response. Several physiological studies have documented cross-talk between BRs and other plant hormones, although no mechanism for this phenomenon has been proposed. For instance, auxin, BRs, and GAs all promote cell elongation although interaction among these hormones has different effects. BRs can act synergistically with auxin and additively with GAs (Mandava 1988). In addition to promotive effects, BRs can act antagonistically with other hormones, *e.g.*, ABA (Mandava 1988). Seed germination is one example where BRs and GAs promote one response, germination, while ABA inhibits it (Steber and McCourt 2001). BRs are able to rescue GA-deficient mutants, which normally fail to germinate. Additionally, BR biosynthetic and perception mutants are hypersensitive to ABA in either germination or root responses (Clouse *et al.* 1996; Ephritikhine *et al.* 1999). We show here that *era1*, a recessive ABA-hypersensitive mutant, has a reduced response to BRs and that overexpression of BEE1 confers partial resistance to ABA.

Hormone cross-talk has mostly been found to alter regulation of hormone biosynthetic enzymes. Multiple hormones, including auxin, BRs, and cytokinin, have been found to regulate ethylene levels (Mandava 1988; Yi *et al.* 1999). While BRs increase ethylene production only in etiolated seedlings, auxin induces production of ethylene under both light- and dark-grown conditions (Mandava 1988; Yi *et al.* 1999). Our data indicate a

mechanism for BR and ABA antagonism via regulated expression of *BEE1*, *BEE2*, and *BEE3* (Figure 7). In this model, *BEE1*, *BEE2*, and *BEE3* are early response genes induced by BRs through the BRI1 receptor complex. The expression of *BEE1*, *BEE2*, and *BEE3* is repressed by ABA through an unknown ABA receptor. Opposing regulation of *BEE1*, *BEE2*, and *BEE3* by BRs and ABA is a new example of hormone cross-talk where antagonists regulate expression of signaling intermediates instead of biosynthetic enzymes. Further analysis may reveal additional roles for the *BEE* genes in other pathways involved in regulating growth.

Increasingly, a primary focus of research in all organisms with sequenced genomes is to assign function to the large number of genes without loss-of-function phenotypes. By combining detailed physiological and statistical analysis with genetic approaches, we have demonstrated that three bHLH family members, BEE1, BEE2, and BEE3, are early response BR signaling components required for full BR response. Opposing regulation of *BEE1*, *BEE2*, and *BEE3* expression by ABA and BRs may be important in maintaining the balance between these hormones during Arabidopsis development. As such, *BEE1*, *BEE2*, and *BEE3* provide a promising starting point for characterizing regulatory elements necessary for integrating hormone signals. Finally, this work provides a clear example of the power of new reverse genetic tools in dissecting gene function in a complex, highly redundant genome.

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