Developmental Cell, Vol. 2, 505–513, April, 2002, Copyright ©2002 by Cell Press

# Nuclear-Localized BZR1 Mediates Brassinosteroid-Induced Growth and Feedback Suppression of Brassinosteroid Biosynthesis

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## Summary

**Plant steroid hormones, brassinosteroids (BRs), are perceived by a cell surface receptor kinase, BRI1, but how BR binding leads to regulation of gene expression in the nucleus is unknown. Here we describe the identification of BZR1 as a nuclear component of the BR signal transduction pathway. A dominant mutation *bzr1-1D* suppresses BR-deficient and BR-insensitive (*bri1*) phenotypes and enhances feedback inhibition of BR biosynthesis. BZR1 protein accumulates in the nucleus of elongating cells of dark-grown hypocotyls and is stabilized by BR signaling and the *bzr1-1D* mutation. Our results demonstrate that BZR1 is a positive regulator of the BR signaling pathway that mediates both downstream BR responses and feedback regulation of BR biosynthesis.**

## Introduction

Both plants and animals use steroids for regulation of growth and development, but they appear to use two different signaling mechanisms. Most animal steroid responses are mediated by members of the nuclear receptor family of ligand-dependent transcription factors. Steroid binding triggers translocation of the receptors into the nucleus where they regulate gene expression through interactions with promoters of steroid-regulated genes (Hall et al., 2001; Olefsky, 2001). In contrast, plant steroid hormones, brassinosteroids (BRs), are perceived by a plasma membrane-localized receptor kinase (Wang et al., 2001).

Brassinosteroids regulate growth, differentiation, and homeostasis in plants. Deficiencies of BR biosynthesis or signaling cause dramatic growth defects, including dwarfism, sterility, and photomorphogenesis in the dark (Li et al., 1996; Clouse et al., 1996; Li and Chory, 1997). Molecular genetic studies of BR-insensitive mutants in *Arabidopsis* have led to the identification of a receptor kinase, BRI1, as a key signaling component in the BR response (Clouse et al., 1996; Li and Chory, 1997). BRI1 is a leucine-rich repeat (LRR) receptor kinase, with an extracellular domain containing 25 LRRs and a 70 amino acid island motif between the 21$^{st}$ and 22$^{nd}$ LRRs, a transmembrane domain, and a cytoplasmic kinase domain (Li and Chory, 1997). BRI1 is localized to the plasma membrane of growing cells, where it functions as a critical component of the BR receptor (Friedrichsen et al., 2000; He et al., 2000; Wang et al., 2001). Biochemical studies have shown that brassinolide (BL), the most active BR, binds to BRI1 and activates its kinase activity in *Arabidopsis* plants (Wang et al., 2001). BL binding and subsequent kinase activation is abolished by a mutation in the 70 amino acid island motif of the extracellular domain. Thus, BRI1 perceives the BR signal through its extracellular domain and initiates a signal transduction cascade through its cytoplasmic kinase activity. How the BR signal is transduced from BRI1 to the nucleus remains unclear. Recent molecular genetic studies have implicated a glycogen synthase kinase 3-like protein (BIN2) as a negative regulator of BR responses (Li et al., 2001; Li and Nam, 2002). The precise role of BIN2 in the signaling pathway is still not known.

Here we identify a dominant *Arabidopsis* mutant, *bzr1-1D*, which is insensitive to the BR biosynthetic inhibitor brassinazole. *bzr1-1D* suppresses phenotypes of BR-deficient and -signaling mutants. Light-grown *bzr1-1D* plants have lower expression of a BR biosynthetic gene, decreased levels of BRs, and reduced stature when compared to wild-type. *BZR1* encodes a nuclear protein that is stabilized by BR signaling and by the *bzr1-1D* mutation. BZR1 protein accumulation correlates with growing regions of the embryonic stem, and overexpression of BZR1 partially suppresses *bri1*. Thus, BZR1 is a positive regulator of the BR signaling pathway, with roles in BR-induced growth response and in feedback regulation of BR biosynthesis.

## Results

### The *brassinazole-resistant 1-1D* (*bzr1-1D*) Mutant Has Increased BR Response and Feedback Inhibition of BR Biosynthesis

Brassinazole (BRZ) is a triazole compound that specifically blocks brassinolide biosynthesis by inhibiting the cytochrome P450 steroid C-22 hydroxylase encoded by the *DWF4* gene (Asami et al., 2001). BRZ causes deetiolation and dwarf phenotypes similar to those of BR-deficient mutants (Asami et al., 2000; Figure 1A). Mutants that are insensitive to BRZ and fail to deetiolate on medium containing BRZ may have reduced BRZ uptake, overproduce BRs, be hypersensitive to BRs, or have constitutive BR responses. We screened 200,000 EMS-mutagenized *Arabidopsis* M2 seeds and isolated

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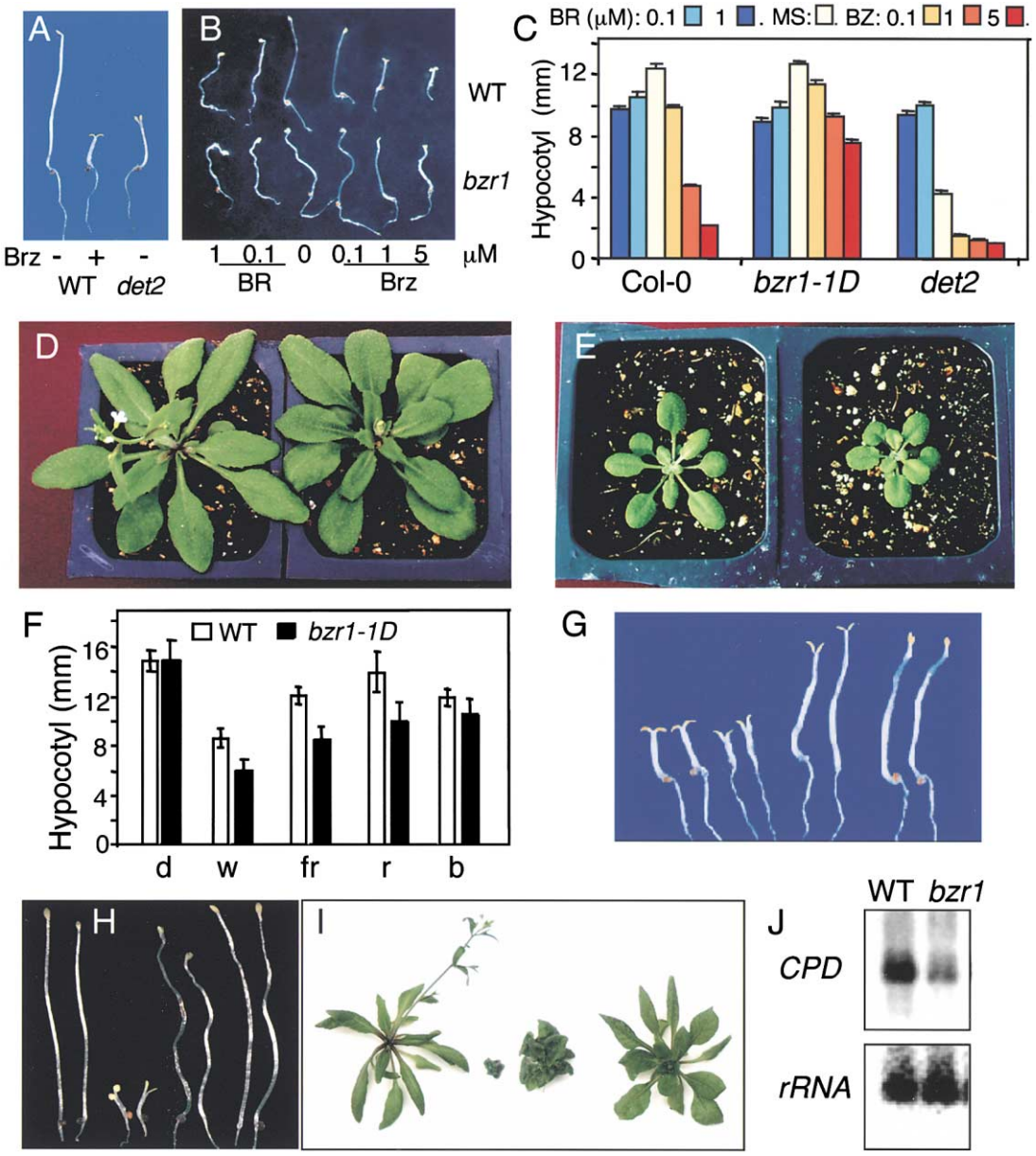



Figure 1. Phenotypes of the *bzr1-1D* Mutant

(A) BRZ causes a BR-deficient phenotype.

(B) Wild-type and *bzr1-1D* seedlings grown on medium containing BR or BRZ in the dark for 6 days.

(C) Average hypocotyl lengths of wild-type, *bzr1-1D*, and *det2* mutant plants grown as in (B).

(D) Wild-type and *bzr1-1D* mutant grown in long days (16 hr light, 8 hr dark) for 26 days.

(E) Wild-type and *bzr1-1D* plants grown in short days (9 hr light/15 hr dark) for 26 days.

(F) Hypocotyl length of wild-type (WT) and *bzr1-1D* seedlings grown for 7 days in the dark (d), under white (w; 41.5 $\mu E/m^2 s$), far red (fr; 0.4 $\mu E/m^2 s$), red (r; 23.4 $\mu E/m^2 s$), and blue light (b; 5 $\mu E/m^2 s$).

(G–I) The *bzr1-1D* mutation suppresses both *det2* and *bri1* mutants. Each panel shows from left to right (two seedlings/sample in [G] and [H]) wild-type, *det2* (G) or *bri1-116* (H and I) mutants, corresponding double mutant with *bzr1-1D*, and *bzr1-1D* single-mutant plants. Plants were grown on 2 μM BRZ (G) or on regular MS medium (H) in the dark for 6 days, or in continuous light for 4 weeks (I).

(J) RNA gel blot shows *CPD* RNA expression levels in the wild-type and *bzr1-1D* mutant. 25S rRNA is the loading control.

a dominant mutant that had a long hypocotyl when grown on BRZ in the dark. We named this mutant *brassinazole-resistant 1-1D* (*bzr1-1D*). The phenotypes of *bzr1-1D* mutant plants are shown in Figure 1. Compared with wild-type seedlings, dark-grown *bzr1-1D* mutant seedlings had normal hypocotyl length when grown on unsupplemented medium, slightly shorter hypocotyls on medium containing BL, and longer hypocotyls on medium containing BRZ (Figures 1B and 1C). The *bzr1-1D* seedlings grown on MS medium or on low concentration of BRZ had curly hypocotyls, similar to wild-type plants grown on BL medium (Figure 1B). These phenotypes

suggest that the *bzr1-1D* mutant either overaccumulates or is hypersensitive to BL.

When grown in light, *bzr1-1D* plants have reduced stature, with slightly darker green and wider leaves, and shorter petioles than wild-type (Figure 1D). The shorter petiole phenotype is more dramatic when plants are grown under short-day photoperiods (Figure 1E). The *bzr1-1D* mutant seedlings grown under various light conditions had shorter hypocotyls than wild-type plants (Figure 1F). *bzr1-1D* plants also had delayed flowering and produced more leaves than wild-type under both long- and short-day conditions (Figure 1D). The phenotypes of light-grown *bzr1-1D* mutants suggest reduced BR action, in contrast to the phenotypes of increased BR action of dark-grown seedlings.

Genetic interactions between *bzr1-1D* and other BR mutants were studied. The *bzr1-1D* mutant was crossed with the BR biosynthetic mutant *det2*, and the receptor mutant *bri1* (*bri1-116* is shown, but similar results were obtained with the weak allele *bri1-6*; Noguchi et al., 1999; Wang et al., 2001). In each case, the double mutants had nearly the same dark-grown phenotypes as the *bzr1-1D* single mutant (Figures 1G–1I), indicating that *bzr1-1D* is epistatic to both BR-deficient and BR receptor mutants. Phenotypes of light-grown *bzr1-1D*/*bri1* double mutants were intermediate between the single mutants in stature, leaf color (Figure 1I), and fertility (data not shown). *bzr1-1D* completely suppresses *bri1* mutants in the dark and partially suppresses all phenotypes of *bri1* in light, providing strong evidence that *BZR1* is a major component downstream of BRI1.

To determine whether the *bzr1-1D* mutation affected BR biosynthesis, we measured BR levels in the mutant plants and found that *bzr1-1D* plants had reduced levels (by 10- to 20-fold compared to wild-type) of the BR intermediates downstream of the step catalyzed by DWF4 (ng/g fresh weight, wild-type/*bzr1-1D*: 6-deoxocathasterone, 0.79/0.73; 6-deoxo-teasterone, 0.23/0.16; 6-deoxo-typhasterol, 1.59/0.11; 6-deoxo-castasterone, 2.31/0.14; castasterone, 0.24/0.01). Some BR biosynthetic genes are feedback suppressed by BR signaling (Mathur et al., 1998; Noguchi et al., 1999). An RNA gel blotting experiment showed that *bzr1-1D* mutant had reduced expression of one of these genes, *CPD* (Figure 1J). These results suggest that *bzr1-1D* had increased feedback inhibition of BR biosynthesis, which is consistent with enhanced BR signaling and the smaller stature of light-grown *bzr1-1D* plants.

### *BZR1* Defines a Plant-Specific Gene Family

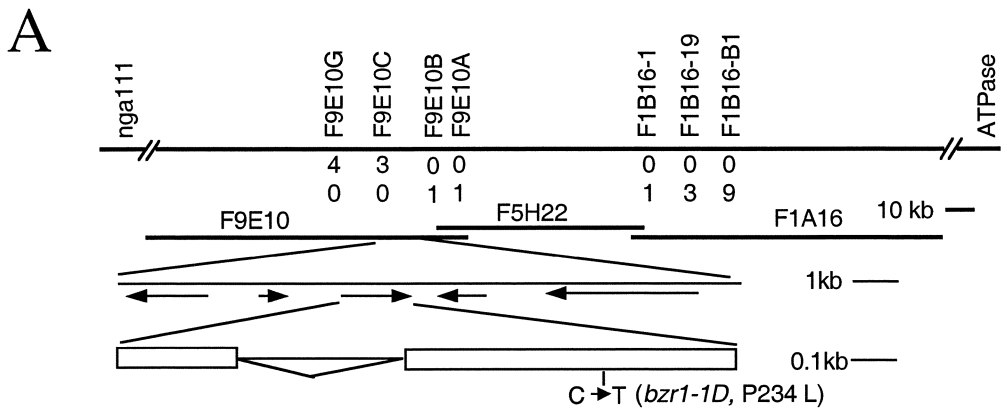
The *bzr1-1D* mutation was mapped to the bottom arm of chromosome I. After analyzing 4016 chromosomes, we mapped the *bzr1* mutation to a 14.5 kilobase region on the sequenced BAC clone F9E10 (Figure 2A). We sequenced the four annotated open reading frames in this region, and found a single base pair change in one hypothetical gene, which changed the predicted proline at position 234 to leucine. We named this gene *BZR1*. The *BZR1* gene encodes a putative protein of 336 amino acids, with a predicted molecular weight of 36.5 kDa (Figure 2B). Database searches revealed sequence homology between BZR1 and other plant proteins with unknown functions. These include five additional *Arabidopsis* genes (88%, 53%, 52%, 39%, and 37% amino acid sequence identity), the tomato *Lat61* gene previously identified as an anther specific gene (65% identity), and a rice gene (44% sequence identity; Figure 2B). The first 21 amino acids of BZR1 are hydrophobic, which could potentially mediate interaction with membranes. Immediately following the hydrophobic residues is a putative bipartite nuclear localization signal sequence (NLS). There are 25 serine/threonine residues that match the phosphorylation site sequence of GSK-3 kinase (S/TXXXS/T, where S/T is serine or threonine and X is any amino acid; Frame and Cohen, 2001), with 20 of them conserved in four of the six homologs (Figure 2B). In addition, a putative PEST sequence was identified from amino acids 232 to 252, a region that encompasses the *bzr1-1D* mutation (Rechsteiner and Rogers, 1996).

To confirm that the *BZR1* gene is responsible for the *bzr1-1D* phenotypes, we transformed wild-type and mutant *BZR1* genes into *Arabidopsis* (Figure 3). Consistent with the dominant nature of the *bzr1-1D* mutation, transformation of wild-type plants with the mutant *bzr1-1D* gene (data not shown) or a *bzr1-1D::*cyan fluorescent protein (CFP) fusion gene (mBZR1-CFP) caused *bzr1-1D*-like phenotypes (Figures 3A and 3F). Expression of the wild-type BZR1-CFP fusion protein using either the *BZR1* promoter or the constitutive cauliflower mosaic virus 35S promoter did not cause obvious phenotypes in either the wild-type or *bzr1-1D* background (Figure 3A), but overexpression of BZR1-CFP partially suppressed a weak allele of *bri1* (Figures 3C–3E). Overexpression of the mutant *bzr1-1D* gene increased cell elongation in various organs (Figures 3B and 3E) and nearly completely rescued the *bri1*-5 phenotype (Figures 3C–3E). In contrast to the *bzr1-1D* mutant itself, plants overexpressing the mutant *bzr1-1D* gene had a long-petiole phenotype similar to transgenic plants overexpressing the BR biosynthetic enzyme DWF4 and the receptor fusion protein BRI1-GFP (Figure 3F; Wang et al., 2001). These results demonstrate that BZR1 is a positive regulator of BR responses.

### BZR1 Protein Accumulates in the Nucleus of Elongating Hypocotyl Cells of Dark-Grown Seedlings and Is Stabilized by BR and *bzr1-1D* Mutation

The tissue-specific expression and subcellular localization of BZR1 protein were determined using a chimeric BZR1-cyan fluorescent protein (CFP) fusion expressed in transgenic *Arabidopsis* plants from the endogenous *BZR1* promoter (BZR1-CFP). Expression of the fusion protein containing the *bzr1-1D* mutation (mBZR1-CFP) caused a *bzr1-1D*-like phenotype (Figure 3A), suggesting that the BZR1-CFP fusion proteins were functional. All transgenic lines showed nuclear fluorescence (Figure 4), indicating that BZR1 is a nuclear protein.

Since BRs are known to regulate cell expansion, we were interested to determine whether BZR1 expression correlated with elongating cells. Dark-grown hypocotyls are particularly good tissues to examine cell expansion, because hypocotyl cells expand rapidly in the absence of cell divisions and the precise growing region during development is well-defined (Gendreau et al., 1997). For

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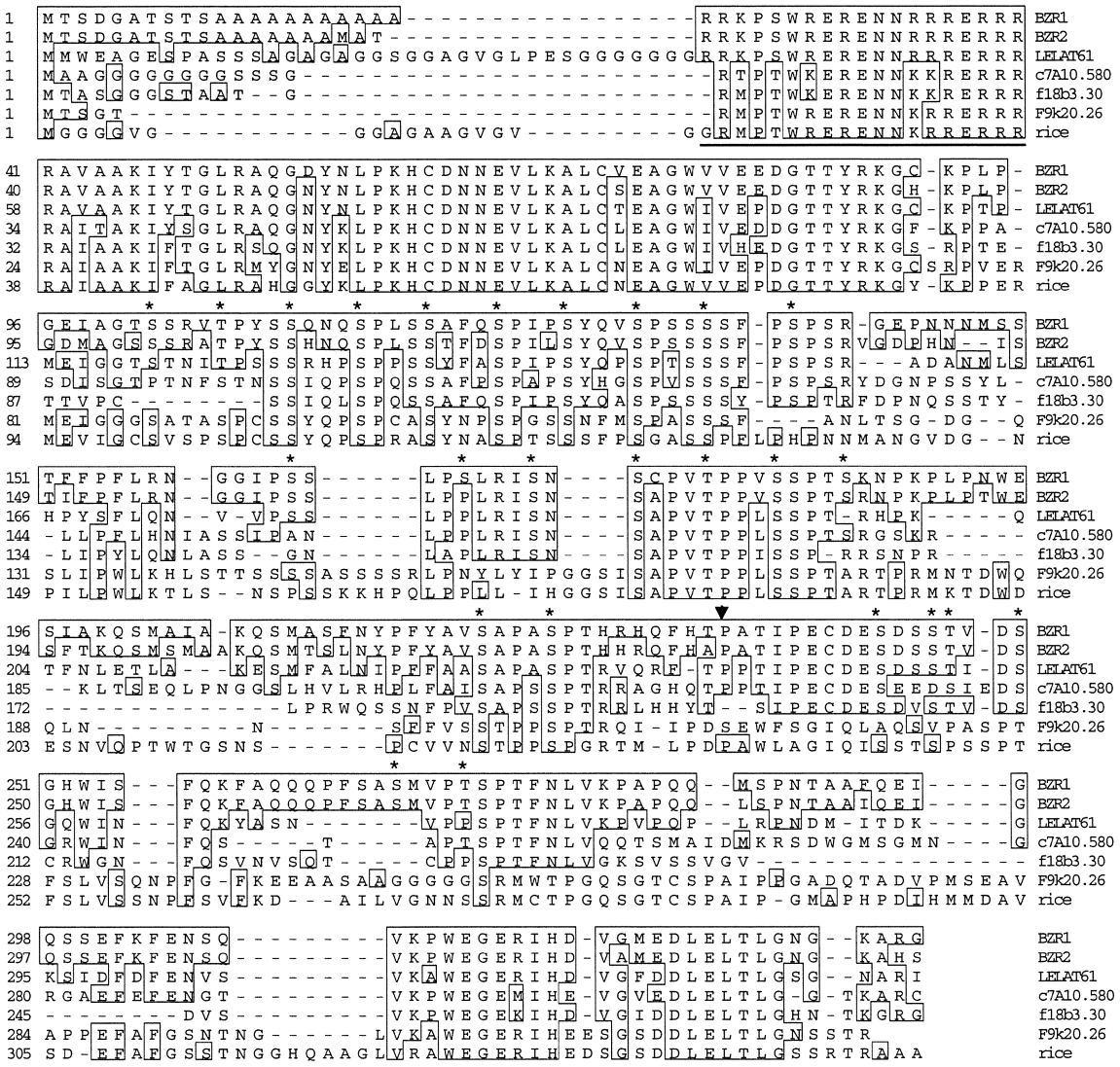


Figure 2. Molecular Characterization of *BZR1*

(A) The chromosomal region containing *BZR1* is diagrammed as the top line with molecular markers shown above the line. The number below the corresponding markers indicates the numbers of recombination breakpoints between the markers and *bzr1-1D* in 4016 chromosomes.

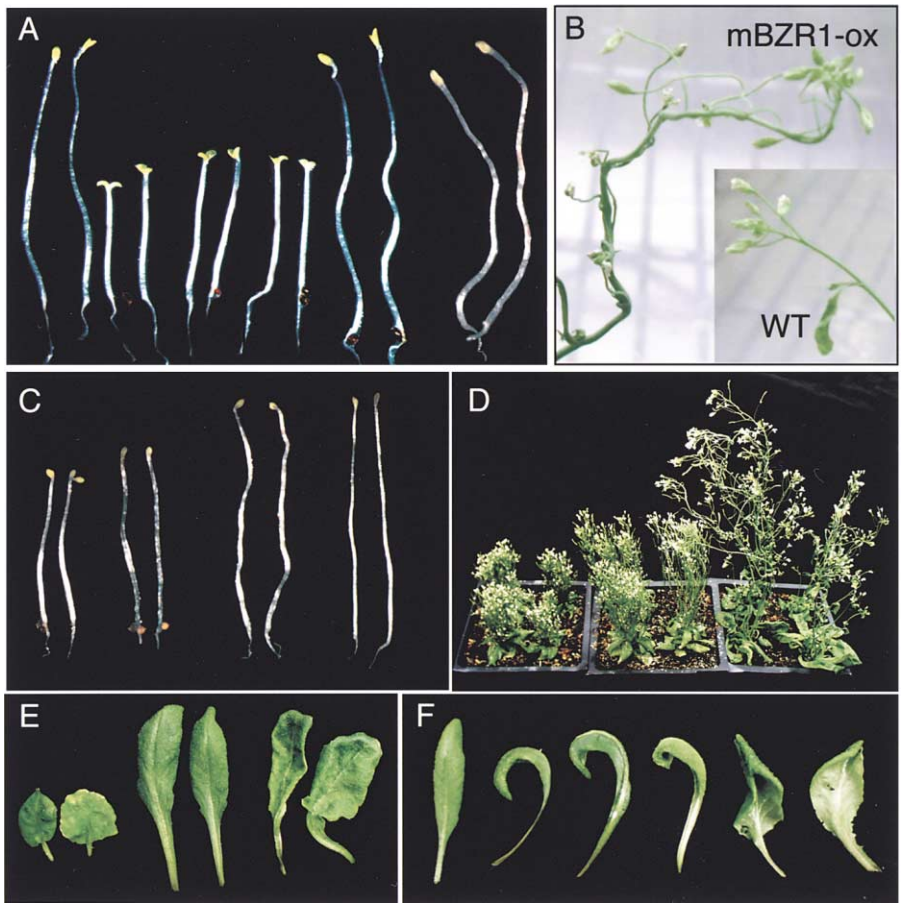


Figure 3. Phenotypes of Plants Transformed with Wild-Type and Mutant *BZR1* Genes

(A) Seedlings grown in the dark on 2 μM BRZ for 6 days. Two seedlings are shown for each sample. From left to right: *bzr1-1D*, wild-type, 35S-BZR1, BZR1-CFP, mBZR1-CFP, and 35S-BZR1/*bzr1-1D*.

(B) Overexpression of mutant form of BZR1 (mBZR1-ox) causes increased stem and pedicel elongation.

(C and D) Overexpression of wild-type and mutant BZR1 partially and completely suppresses the phenotypes of *bri1-5*.

(C) Six-day-old dark-grown seedlings. From left to right: *bri1-5*, 35S-BZR1/*bri1-5*, 35S-mBZR1/*bri1-5*, and wild-type.

(D) From left to right: *bri1-5*, 35S-BZR1/*bri1-5*, and 35S-mBZR1/*bri1-5* plants grown in light for 5 weeks.

(E) From left to right in pairs: Rosette leaves of *bri1-5*, wild-type, and *bri1-5*/35S-mBZR1 plants.

(F) From left to right: rosette leaves of wild-type, *DWF4* overexpresser, BRI1-GFP overexpresser, 35S-mBZR1-CFP, mBZR1-CFP, and *bzr1-1D* plants.

the first 2 days postgermination, the growth of the hypocotyl is due mainly to the expansion of the eight cells at the base. Beginning on days 3 and 4, cells at the top of the hypocotyl start to expand, whereas the basal five cells stop growing (Gendreau et al., 1997). We used *BZR1* native promoter-driven BZR1-CFP transgenic lines to observe the pattern of BZR1-CFP protein accumulation along hypocotyl epidermal cells at different developmental stages (Figure 4A). At 36 hr postgermination, the strongest BZR1-CFP levels were observed in the lower half of the hypocotyls (Figure 4A, a1-d1). After 48 hr, the most intensive nuclear BZR1-CFP was found in the mid-upper region of the hypocotyls (Figure 4A, a2-d2). On the third day, BZR1-CFP fluorescence was no longer visible in the basal region and began to fade out in the mid-lower zone of the hypocotyls (Figure 4A, a3-d3). On day 4, only the cells just below the apical hook had strong nuclear BZR1-CFP signals, whereas cells at the lower half of the hypocotyl had no BZR1-CFP fluorescence (Figure 4A, a4-d4). Thus, BZR1 protein accumulation correlates with the growing region of the hypocotyl, suggesting a role for BZR1 in cell expansion in dark-grown *Arabidopsis* seedlings.

We also followed the expression pattern of mutant

The BAC clones are shown as overlapping lines. *BZR1* was mapped between markers F9E10B and F9E10C. Arrows show the annotated genes in this region. The *bzr1-1D* mutation was found in gene F9E10.7. The exons of *BZR1* are shown as boxes, and the intron as a triangle. (B) Sequence alignment of BZR1 and its homologs in *Arabidopsis* (BZR2, c7A10.580, f18b3.30, and F9k20.26), tomato (LELAT61), and rice. Sequences identical to BZR1 are boxed. The putative nuclear localization signal, putative sites of phosphorylation by GSK-3 kinase, and the proline residue mutated to leucine in *bzr1-1D* are marked by underlining, asterisks, and an arrow, respectively.

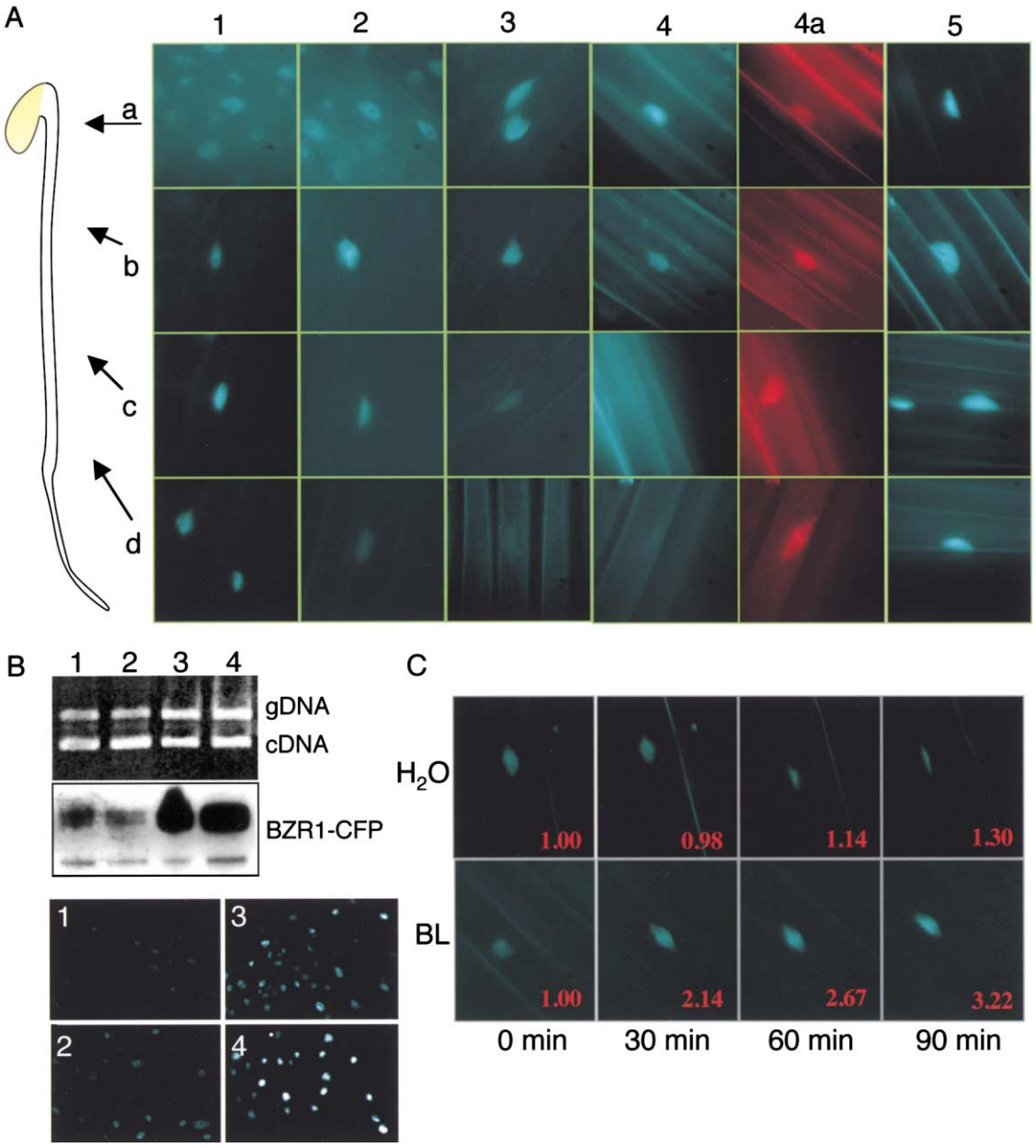


Figure 4. BZR1-CFP Accumulates in the Nucleus of Elongating Cells of Dark-Grown Hypocotyls

(A) Using *BZR1* native promoter-driven BZR1-CFP transgenic lines, BZR1 levels were monitored in different regions of elongating hypocotyls (a, just below the apical hook region; b, mid-upper hypocotyl region; c, mid-lower hypocotyl region; and d, basal cells) at various developmental stages of dark-grown seedlings (1, 36 hr after germination; 2, 48 hr after germination; 3, 72 hr after germination; and 4, 96 hr after germination). Accumulation patterns of native promoter-driven mutant mBZR1-CFP in a 4-day-old etiolated seedling are shown in 5. 4a, DAPI staining of the nuclei.

(B) *bzr1-1D* mutation increases BZR1-CFP fusion protein accumulation. RT-PCR quantitation of *BZR1-CFP* transgene RNA expression (top panel), Western blot quantitation of BZR1-CFP proteins (middle panel), and microscopic analysis of nuclear accumulation of BZR1-CFP proteins (lower four panels) in two BZR1-CFP (1, 2) and two mBZR1-CFP (3, 4) transgenic lines. Upper and lower bands in top panel are PCR products from genomic DNA as an internal control and from RNA (cDNA), respectively.

(C) BL treatment increases BZR1-CFP protein nuclear accumulation. Micrographs show BZR1-CFP fluorescence in nuclei of cells at the middle of hypocotyl, before (0 min), and at various times after treatment of dark-grown seedlings with 1 μM BL or water. Numbers inside the panels show the fold of change of nuclear fluorescence compared to that before the treatment.

BZR1-CFP (mBZR1-CFP) driven by *BZR1*'s native promoter. In this case, the pattern of mBZR1-CFP expression was uniform along the length of the hypocotyls at different developmental stages (Figure 4A, a5-d5), and there was increased fluorescence in all the lines examined.

To determine whether the *bzr1-1D* mutation affected the accumulation of the BZR1 protein, we compared

the protein levels between BZR1-CFP and mBZR1-CFP transgenic lines. The RNA level of the BZR1-CFP transgene was measured by RT-PCR, and transgenic lines expressing similar levels of *BZR1-CFP* RNA were selected for analysis by Western blotting (Figure 4B). We found that plants transformed with the mBZR1-CFP construct accumulated about 10-fold higher levels of the CFP fusion protein than those transformed with wild-type BZR1-CFP (Figure 4B), indicating that the *bzr1-1D* mutation caused increased accumulation of BZR1 protein.

We also tested the effect of BL treatments on BZR1-CFP protein accumulation. When seedlings expressing the BZR1-CFP fusion protein were grown in the dark for 5 days and then treated with BL, we observed an increase of the CFP signal in the nucleus (Figure 4C). This increase was detectable 30 min after BL treatment, and the signal level peaked after 1 hr (Figure 4C). The fact that both the *bzr1-1D* mutation and BL treatment increased BZR1 protein levels indicates that BL-induced accumulation of BZR1 protein is an important event in BR signaling.

## Discussion

Brassinosteroids are perceived by the cell surface receptor kinase BRI1. How the signal is transduced to regulate nuclear gene expression remains unknown. We present identification of BZR1 as a nuclear component of the BR signaling pathway that mediates both BR-induced growth responses and feedback regulation of BR biosynthesis. The tissue-specific expression of BZR1 protein in elongating cells and the stabilization of BZR1 protein by either the *bzr1-1D* mutation or BR signaling demonstrate that BZR1 is a positive regulator of the response pathway and BR-induced BZR1 protein accumulation is an important event in BR signaling.

The tissue-specific expression pattern of *BZR1* correlates temporally and spatially with the pattern of cell elongation in dark-grown seedlings. The establishment of the gradient of cell elongation and BZR1 protein accumulation along the hypocotyl temporally correlates with the onset of *CPD* gene expression in the cotyledons at this stage (Mathur et al., 1998), and thus may be due to a gradient of BR concentration along the hypocotyl. During seedling development, the *CPD* gene starts to be expressed in cotyledons 3 days after germination, and the expression level continues to increase up to 8 days. Since *CPD* encodes an essential enzyme for BR biosynthesis and is feedback suppressed by BRs, the expression of *CPD* is expected to correlate with the sites of BR biosynthesis, but not with either BR accumulation or BR responsiveness (Mathur et al., 1998). The mutually exclusive expression patterns of CPD and BZR1 in cotyledons and flanking hypocotyl cells, respectively, are consistent with BZR1's roles in BR-regulated cell elongation and feedback regulation of BR biosynthesis. The low-level expression of BZR1 in cotyledons presumably allows CPD expression and BR biosynthesis in this tissue, which would establish a gradient of BRs along the hypocotyl with the highest BR level in cotyledons and adjacent hypocotyl cells. Such a BR gradient may be responsible for the gradient of BZR1-CFP accumulation in hypocotyls. Indeed, we have observed that BZR1-CFP transgenic seedlings grown on medium containing BL show nuclear fluorescence throughout the hypocotyls (data not shown). Thus, the tissue- and cell-specific expression patterns of BZR1 protein and the *CPD* gene are consistent with BR stabilization of BZR1 protein during seedling development.

The opposite effects of *bzr1-1D* on cell elongation in light and dark are likely due to its actions on downstream growth response and on feedback suppression of BR biosynthesis, rather than BZR1 itself having opposite activities in the dark versus light. In the dark, *bzr1-1D* predominantly affects growth response and either does not affect BR biosynthesis or its effect on BR synthesis is masked by its direct activation of downstream BR responses. When the *bzr1-1D* gene is overexpressed in transgenic plants, the plants show increased cell elongation in both dark and light; this is presumably because when BZR1's activities on both downstream BR responses and BR biosynthesis are saturated, the former becomes dominant. This observation also suggests that sufficient activation of BZR1 can compensate for the BR deficiency caused by feedback inhibition of BR biosynthesis. The different effects of *bzr1-1D* mutation on growth response and BR biosynthesis in light and dark suggest that BZR1 is a point of crosstalk between BR and light signaling pathways. Light may regulate BZR1 or its interacting partners to modulate BZR1's activities on growth and feedback regulation of BR biosynthesis.

BZR1 and its homologs represent a small family of plant proteins unrelated to any gene outside the plant kingdom. These genes have not been previously identified in genetic screens. This may be due to lethality caused by loss-of-function mutations, or functional redundancy among the homologous genes. The fact that the *bzr1-1D* mutation partially suppresses every aspect of the *bri1* phenotype, including cell elongation and leaf color and fertility, suggests that BZR1 plays a central role in BR response and may be essential for plant growth and development. On the other hand, the *BZR2* gene shares 88% sequence identity with *BZR1* and may have overlapping or redundant functions. A *bri1* suppressor mutant (*bes1*; Yin et al., 2002) contains a mutation of *BZR2* that changes the same corresponding amino acid residue (proline 233 to leucine in BZR2) as *bzr1-1D*. BZR1 and BES1 may have redundant functions, and knockout of both genes may be required to obtain an obvious phenotype. However, *bzr1-1D* and *bes1-D* plants have different phenotypes when grown in light, suggesting that their biological functions in plant development are distinct rather than completely overlapping.

When grown in the dark, the *bes1-D* mutant shows the same phenotypes of BRZ insensitivity and *bri1* suppression as *bzr1-1D*. However, light-grown *bes1-D* mutant plants show phenotypes of increased petiole elongation, opposite to that of *bzr1-1D* (Yin et al., 2002). Such different light-grown phenotypes between *bzr1-1D* and *bes1-1D* are consistent with their different effects on feedback regulation of BR biosynthetic genes. While the *bzr1-1D* mutant has reduced BR levels and *CPD* gene expression, the *bes1* mutation has only a small effect on *CPD* gene expression (Yin et al., 2002). BES1 thus

mediates downstream growth responses, but not feedback regulation of BR biosynthesis. Such two overlapping pathways may allow the plant to fine-tune the levels of BR biosynthesis and sensitivity in different tissues and cells. BZR1 and BES1 may interact with different downstream components, have different target genes, or be regulated differently by developmental or environmental cues. Identification of loss-of-function alleles of *BZR1*, *BES1*, and other *BZR1* homologous genes will elucidate their precise functions.

As a nuclear protein, BZR1 is likely to mediate BR regulation of gene expression. BZR1 may function as a transcription factor itself, as a cofactor that interacts with transcription factors, or may modulate chromosomal structures in the nucleus. BZR1 is likely to be regulated by BR signals through protein phosphorylation, and this regulation could affect the accumulation of BZR1 protein in the nucleus. BR regulation of BZR1 accumulation may involve the BIN2 kinase (Li and Nam, 2002; Yin et al., 2002). Identification of BZR1-interacting proteins and genes directly regulated by BZR1 will further elucidate the mechanism of its function in BR responses.

## Experimental Procedures

### Plant Materials and Growth Conditions

*A. thaliana* ecotype Columbia (Col-0) was used as the wild-type. Landsberg *erecta* was used for genetic mapping of *bzr1-1D*. The *bri1-5, bri1-6*, and *bri1-116* alleles are in the Ws2, En, and Col-0 ecotypes, respectively (Noguchi et al., 1999). The plant growth media contains 1/2× MS nutrient agar (GIBCO-BRL), with 1% sucrose and trace elements as stated. Plants grown in light were exposed to 250 $\mu E/m^2 s$ light.

### Isolation of the *bzr1-1D* Mutant

EMS-mutagenized Col-0 seeds were purchased from Lehle Seeds. Approximately 200,000 M2 seeds from 18 independent parental groups (1250 M1/group) were screened on 0.5× MS medium containing 1% sucrose and 2 μM BRZ (Asami et al., 2000). After growth for 6 days in the dark, seedlings with longer hypocotyls than background were identified, and transferred to soil. The *bzr1-1D* mutant was backcrossed to wild-type Col-0 for four generations before other experiments were performed.

### BR Measurements

Plants were grown in short days (9 hr light/15 hr dark) for 40 days. Lyophilized materials (from 50 g fresh weight) were extracted twice with 500 ml of MeOH:$CHCl_3$ (4:1), and deuterium-labeled internal standards (1 ng/g fresh weight) were added. Quantitation was performed as described (Noguchi et al., 1999).

### Cloning of *BZR1*

*BZR1* was identified by map-based cloning using procedures similar to those described (Li and Chory, 1997; Lukowitz et al., 2000). The *bzr1-1D* mutant was crossed with the *Arabidopsis* La-*er* ecotype, and the F1 plants were self-pollinated. F2 plants were grown on BRZ medium in the dark for 6 days, and seedlings with wild-type phenotypes were selected for analysis using molecular markers (Lukowitz et al., 2000). After scoring 4016 chromosomes, *BZR1* was mapped to a 14.5 kilobase region on the sequenced BAC clone F9E10 (GenBank accession number AC013258) on the bottom of chromosome I. The sequences of annotated open reading frames within this region were amplified by polymerase chain reaction (PCR) from the genomic DNA of the *bzr1-1D* mutant; the PCR products were sequenced, and the sequences were compared with the GenBank sequence of BAC clone F9E10.

### Molecular Methods

Details of the methods used in these studies are available from the authors (zywang@andrew2.stanford.edu or chory@salk.edu). All binary vector constructs were transformed into *Agrobacterium* strain GV3101 and then transformed into plants using the floral dip method (Clough and Bent, 1998).

### Fluorescence Microscopy

Seedlings expressing BZR1-CFP were mounted on glass slides using PBS as a mounting medium. All images were collected on a DeltaVision deconvolution microscope (Applied Precision). Specific filter sets were used for ECFP (426 ± 5 nm excitation, 470 ± 20 nm emission) and DAPI (360 ± 20 nm excitation, 457 ± 25 nm emission). The color of the images was artificially added in Photoshop 6.0 (Adobe Systems). In the BL feeding experiments, after the first cell image was taken, 1 μM of BL was applied to the slide and the same cell was followed for 2 hr. For colocalization of BZR1-CFP and the nucleus, plant material was treated with 50 ng/ml of DAPI for 30 min before imaging.

### Acknowledgments

We thank Dr. Suguru Takatsuto (Joetsu University of Education) for deuterated BRs, Dr. Yukihisa Shimada for the *CPD* clone, and Ms. Masayo Sekimoto for excellent technical assistance. This work was supported by grants from the USDA (9935301-7903) and the Howard Hughes Medical Institute to J.C., by a postdoctoral fellowship from NSF and start-up funds from the Carnegie Institution to Z.-Y.W., and by the Bioarchitect Research Program at RIKEN from the Ministry of Education, Science, and Culture (Japan) to T.N.

Received: February 4, 2002
Revised: March 19, 2002

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## Accession Numbers

The GenBank accession numbers for the *BZR1* and *BZR2* sequences reported in this paper are AF494338 and AY093500, respectively.