# *BIN2*, a New Brassinosteroid-Insensitive Locus in Arabidopsis[1]

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Brassinosteroids (BRs) play important roles throughout plant development. Although many genes have been identified that are involved in BR biosynthesis, genetic approaches in Arabidopsis have led to the identification of only one gene, *BRI1*, that encodes a membrane receptor for BRs. To expand our knowledge of the molecular mechanism(s) of plant steroid signaling, we analyzed many dwarf and semidwarf mutants collected from our previous genetic screens and identified a semidwarf mutant that showed little response to exogenous BR treatments. Genetic analysis of the *bin2* (*BR-INSENSITIVE 2*) mutant indicated that the BR-insensitive dwarf phenotype was due to a semidominant mutation in the *BIN2* gene that mapped to the middle of chromosome IV between the markers CH42 and AG. A direct screening for similar semidwarf mutants resulted in the identification of a second allele of the *BIN2* gene. Despite some novel phenotypes observed with the *bin2*/+ mutants, the homozygous *bin2* mutants were almost identical to the well-characterized *bri1* mutants that are defective in BR perception. In addition to the BR-insensitive dwarf phenotype, *bin2* mutants exhibited BR insensitivity when assayed for root growth inhibition and feedback inhibition of *CPD* gene expression. Furthermore, *bin2* mutants displayed an abscisic acid-hypersensitive phenotype that is shared by the *bri1* and BR-deficient mutants. A gene dosage experiment using triploid plants suggested that the *bin2* phenotypes were likely caused by either neomorphic or hypermorphic gain-of-function mutations in the *BIN2* gene. Thus, the two *bin2* mutations define a novel genetic locus whose gene product might play a role in BR signaling.

Brassinosteroids (BRs) are a special class of plant steroids that have wide distribution throughout the plant kingdom and are essential for normal plant growth and development (Clouse and Sasse, 1998). Plants that are defective in BR biosynthesis display characteristic mutant phenotypes that include a dark-green dwarf stature, epinastic round leaves, delayed flowering and senescence, reduced apical dominance and male fertility, and abnormal skotomorphogenesis in the dark (Li et al., 1996; Szekeres et al., 1996). Despite rapid progress in the studies of BR biosynthesis (Mussig and Altmann, 1999), little is known about how plants recognize these steroids and transduce their signals to regulate a wide variety of physiological processes (Schumacher and Chory, 2000).

To understand the molecular mechanism(s) by which BRs regulate plant growth and development, we have taken a genetic approach to screen for mutants that display characteristic BR-deficient phenotypes but cannot be rescued by BR feeding (Li and Chory, 1997). It is surprising that all 18 BR-insensitive mutants (*bin*) that we recovered in our original screen were found to be new alleles of a previously characterized genetic locus, *BRASSINOSTEROID-INSENSITIVE 1* (*BRI1*; Clouse et al., 1996; Kauschmann et al., 1996). *bri1* mutants are almost identical in appearance to *cpd*, the most severe BR-deficient mutant identified so far (Szekeres et al., 1996), and are unresponsive specifically to BRs while retaining responsivity to other plant hormones (Clouse et al., 1996). Moreover, the BR-specific insensitivity was also observed at the molecular level. BR-induced expression of two xyloglucan endotransglycosylase (*XET*) genes, *TCH4* and *meri5*, was missing in the *bri1-2* mutant, although gibberellin (GA)-induced *meri5* expression was normal (Kauschmann et al., 1996). *BRI1* mutations also cause an increased accumulation of brassinolide (BL) and its immediate biosynthetic precursors (Noguchi et al., 1999). Taken together, these results suggest that *BRI1* encodes an essential component of a BR signaling pathway.

*BRI1* encodes a Leu-rich repeat (LRR) receptor-like kinase that is composed of an extracellular domain containing 25 LRRs disrupted by a 70-amino acid island between the 21st and 22nd LRRs, a single-pass transmembrane domain, and a cytoplasmic kinase domain with Ser/Thr specificity (Li and Chory, 1997). Sequencing of mutant alleles identified the 70-amino acid island and the kinase domain as the two most important domains required for BRI1 function (Li and Chory, 1997; Noguchi et al., 1999; Friedrichsen et al., 2000). Recent studies have pro-

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vided additional evidence to argue strongly that BRI1 is a critical component of a membrane steroid receptor. It has been shown that BRI1 is a plasma membrane protein and can function as a Ser/Thr kinase in vitro (Friedrichsen et al., 2000; Oh et al., 2000). A chimeric receptor, containing the extracellular domain, the transmembrane region, and a short juxtamembrane segment of the BRI1 protein fused to the cytoplasmic kinase domain of the rice disease resistance receptor-kinase Xa21, responded to BR treatment to activate an Xa21-specific plant defense response pathway (He et al., 2000). In addition, a BL-binding activity, which requires a functional BRI1 extracellular domain, could be co-immunoprecipitated with the BRI1 protein from Arabidopsis membrane protein extracts (Wang et al., 2001). Furthermore, BL treatment of Arabidopsis seedlings could activate autophosporylation of the BRI1 protein (Wang et al., 2001). Based on these results and what we have learned from animal receptor kinase-mediated cellular signaling (Weiss and Schlessinger, 1998), we have hypothesized that BR binding, either directly or indirectly, to the BRI1's extracellular domain, can stimulate its cytoplasmic kinase activity, leading to recruitment of other BR signaling components to the receptor and activation of the BR signaling cascade.

To identify additional components involved in BR signaling, we have analyzed various dwarf and semidwarf mutants from our previous screen and identified an interesting semidwarf mutant. Here, we present genetic, molecular, and physiological data to show that this mutant defines a novel genetic locus, *BRASSINOSTEROID-INSENSITIVE 2* (*BIN2*) whose gain-of-function mutations block normal BR signal transduction. Thus, *BIN2* might encode a negative regulator of BR signaling in plant growth.

## RESULTS

### Identification of a New BR-Insensitive Locus, *BIN2*

Careful re-examination of a collection of dwarf and semidwarf mutants from a previous genetic screen for BR-insensitive mutants (Li and Chory, 1997) identified a semidwarf plant that is unresponsive to the exogenous application of BL. Compared with wild-type seedlings, this mutant was darker green, displayed reduced apical dominance, had reduced male fertility, and flowered late. At maturity, the main inflorescence stem was about one-half of the wild-type height. In addition, both rosette and cauline leaves were extremely curly (Fig. 1, A and B).

When selfed, this mutant segregated out three phenotypic classes of progeny (Fig. 1, A and B), including *bri1*-like extreme dwarfs, semidwarfs resembling the original mutant, and wild-type plants, with an approximately 1:2:1 ratio (Table I). We hypothesized that the original mutant was heterozygous for a semidominant mutation that causes the BR-insensitive dwarf phenotype and that the three phenotypic classes corresponded to plants containing a homozy-


**Figure 1.** Morphology of *bin2* mutants. Plants were grown on either synthetic medium (A) or in soil (B) as described in "Materials and Methods." C, Twelve-day-old normal-grown seedlings of *cpd*, *bri1*, wild-type, *bin2*/+, and *bin2-1* mutant were treated with 0.1 $\mu$M BL and photographed 2 d after the treatment. D, De-etiolation of known BR mutants and *bin2* mutants. Seedlings were germinated and grown for 10 d in complete darkness. Seedlings shown in A, B, and D are *cpd*, *bri1*, wild type, *bin2-1*/+, and *bin2-1* (from left to right)

**Table I.** *Genetic analysis of bin2 mutants*

| Plant(s)/Cross | No. of Plants | | | | Expected Ratio | $\chi^2$ |
|---|---|---|---|---|---|---|
| | Total | Wild type | Semidwarf | Extreme dwarf | | |
| The original mutant ($M_2$)/self | 252 | 58 | 129 | 65 | 1:2:1 | 0.532 |
| 10 Wild-type plants ($M_3$)/self | 1,254 | 1,254 | – | – | – | – |
| 20 Semidwarfs ($M_3$)/self | 3,629 | 894 | 1,857 | 878 | 1:2:1 | 2.132 |
| Extreme dwarf × wild type | | | | | | |
| $F_1$ | 16 | – | 16 | – | – | – |
| $F_2$ (15 plants) | 2,759 | 685 | 1,401 | 673 | 1:2:1 | 1.111 |
| Two $F_2$ populations | 252 | 62 | 132 | 58 | 1:2:1 | 0.841 |
| Grown in total darkness[a] | – | (Long) | (Intermediate) | (Short) | – | – |
| *bin2-1* × *bin2-2* | 28 | – | 15 | 13 | – | – |
| *bin2-1* × *bri1-101* | | | | | | |
| $F_1$ | 13 | – | 13 | – | – | – |
| $F_2$ (total 13 plants) | 798 | 139 | 309 | 350 | 1:2:2.33 | 1.074 |

[a] When grown in complete darkness, these two $F_2$ populations segregated out three classes of seedlings, represented by long, intermediate, and short hypocotyls, respectively.

gous, a heterozygous, or no mutation. Consistent with this hypothesis, upon self-pollination, all wild-type-looking plants produced only wild-type progeny, whereas all semidwarf plants segregated out all of the three phenotypic classes observed with the progeny of the original mutant (Table I). The extreme dwarfs were male sterile and did not yield any seeds, but when crossed with wild type, they produced only semidwarf $F_1$ progeny, each giving rise to an $F_2$ population displaying the three phenotypes with the expected 1:2:1 ratio (Table I). Upon exogenous application of BL, neither the semi- nor the extreme dwarfs of a segregating population showed any visible phenotypic change, whereas *cpd* mutants grown on the same medium were rescued to a wild-type appearance (Fig. 1C). The mutation in the original mutant was named *bin2* for *BR-INSENSITIVE 2* after *BIN1*, which is allelic to *BRI1* (Li and Chory, 1997).

Most BR-deficient mutants and *bri1* mutants exhibit a de-etiolated phenotype characterized by a short, thick hypocotyl and opened, expanded cotyledons when grown in the dark. To test whether *bin2* mutation has a similar effect on the dark growth of seedlings, a segregating population was germinated in the dark and their phenotypes were carefully examined. As expected, three phenotypes represented by short, intermediate, and long hypocotyls with the expected 1:2:1 ratio were observed in this population (Table I; Fig. 1D). It is interesting that the curly growth pattern found on the cauline leaves of heterozygous plants was also observed on their dark-grown hypocotyls. Curly growth of hypocotyl also occurs in the homozygous mutants; however, because of their short hypocotyls, only a small kink was observed.

Although the phenotype of the *bin2* heterozygotes suggested that this mutant was different from the previously identified *bri1* mutants, the close resemblance between the homozygous *bin2* mutants and *bri1* mutants prompted us to test whether this mutant carries a new allele of the *BRI1* gene. Homozygous *bin2* mutants were crossed to homozygous *bri1-101* mutants and all the resulting $F_1$ plants were identical to the *bin2*/+ heterozygous plants (data not shown). A total of 798 $F_2$ plants from four resulting $F_1$ plants were analyzed; among them were 350 strong dwarfs, 309 intermediate dwarfs, and 139 wild-type-looking plants with a ratio of 2.52:2.22:1 (Table I), indicating that *BIN2* is not allelic to *BRI1*, which was supported by sequencing the *BRI1* gene from homozygous *bin2* mutants.

To determine the map position of the *BIN2* locus, homozygous *bin2* mutants were crossed to wild-type plants of Landsberg *erecta*. One hundred twenty-five wild-type-looking plants from an $F_2$-segregating population were used for a PCR-based linkage analysis. Three cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) per chromosome were used to map the *BIN2* locus. No linkage was found between the $BIN2^+$ phenotype and any of the tested CAPS markers on chromosome I, II, III, or V; however, a significant linkage was found between wild-type phenotype and a CAPS marker in the middle of chromosome IV (AG) with a recombination frequency of 1.2%. A nearby CAPS marker (CH42) was then chosen for another PCR-based linkage analysis and two different recombinants were identified at this new position, thus placing the *BIN2* locus between the two markers. This mapping data further confirms that *BIN2* is a new BR-insensitive locus.

To identify additional *bin2* alleles, 50,000 ethyl methanesulfonate-mutagenized seeds of Arabidopsis ecotype Columbia (Col-0) containing the *gl-1* mutation were screened, and two mutants similar in appearance to the *bin2*/+ heterozygous mutant were identified. When crossed to the original *bin2* homozygous mutant, one of them produced an $F_1$ offspring of both *bin2*-like and *bin2*/+-like mutants with an approximately 1:1 ratio (Table I), indicating that a new *bin2* allele was identified. We named the original allele *bin2-1* and the new allele *bin2-2*.

### *bin2* Is a BR-Specific Hormone-Insensitive Mutant

Plant hormones interact with each other in very complex ways and it has been observed that hormone response mutants often exhibit insensitivity to multiple plant hormones. For example, an auxin mutant, *axr2*, is insensitive not only to auxin but also to ethylene and abscisic acid (ABA; Wilson et al., 1990), whereas an ethylene-insensitive mutant, *ein2*, is resistant to cytokinins (Cary et al., 1995; Alonso et al., 1999), ABA (Beaudoin et al., 2000; Ghassemian et al., 2000), and auxin transport inhibitors (Fujita and Syono, 1996). Sensitivity to hormone is often quantified by a root growth inhibition assay because most plant hormones when applied at high concentrations inhibit root growth. To determine whether the *bin2* mutation affects BR response specifically or results in insensitivity to multiple plant hormones, we analyzed root elongation of *bin2* mutants and wild-type seedlings grown in medium containing various plant hormones including ABA (0.5 $\mu$M ABA), auxin (1 $\mu$M 2,4-dichlorophenoxyacetic acid), BR (1 $\mu$M BL), cytokinin (1 $\mu$M kinetin), ethylene (1 $\mu$M 1-aminocyclopropane-1-carboxylic acid, a precursor for ethylene), and GA (1 $\mu$M or 50 $\mu$M $GA_3$). Like *bri1* mutants, *bin2* mutants are insensitive only to BL, but retain normal sensitivity toward other plant hormones (Fig. 2). It is interesting that *bin2* mutants display a hypersensitivity to ABA, which was also observed in both *bri1* mutants and BR-deficient mutants (Clouse et al., 1996; Ephritikhine et al., 1999a).

The BR insensitivity of the *bin2* mutation was observed over a wide range of BR concentrations. As indicated in Figure 3, a significant difference in BR insensitivity between *bin2* mutants and wild-type seedlings was observed between concentrations of 1 nM and 1 $\mu$M BL. It was quite interesting to note that BL, at a concentration of 0.01 pM, can dramatically stimulate root elongation (Fig. 3). Such a stimulated root elongation is not solely dependent on the BIN2-mediated signaling pathway because both *bin2* heterozygous and homozygous mutants exhibited BR-stimulated root elongation at such an ultra-low concentration of BL, despite the degrees of stimulation of the *bin2* mutants being lower than that of the wild-type seedlings. Similar root growth stimulation by picomolar concentrations of BRs was observed previously in the *bri1* mutants (Clouse et al., 1996) and several other plant species (Mandava, 1988).

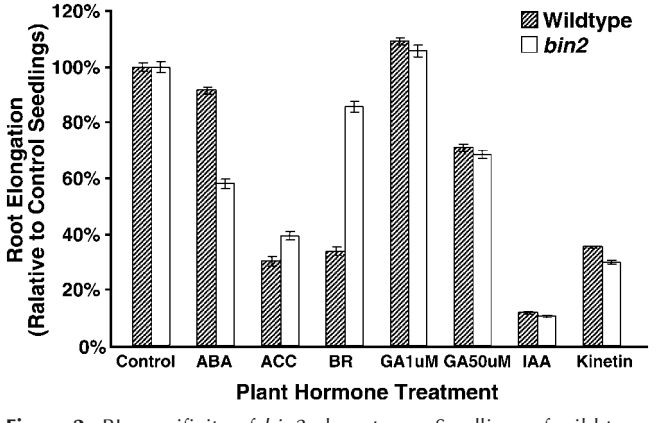


**Figure 2.** BL specificity of *bin2* phenotypes. Seedlings of wild-type and *bin2* mutants were geminated and grown on one-half-strength Murashige and Skoog medium containing various plant hormones. Their root lengths were measured on the 8th d after germination. Each bar represents the mean measurement of 60 to 80 seedlings from two duplicate experiments and is expressed as percentage growth compared with the mean root elongation of the same genotype grown on medium containing the same volume of 80% (v/v) ethanol used for dissolving various plant hormones. Error bars indicate SE.

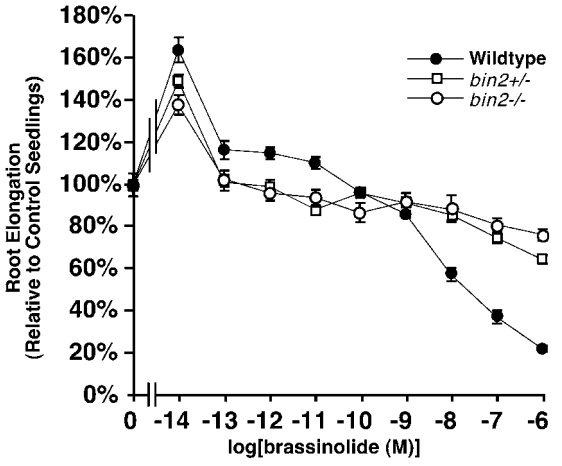


**Figure 3.** Quantitative analysis of BL sensitivity of *bin2* mutants. Seedlings of wild-type, *bin2-1/+*, and *bin2-1* mutants were germinated and grown on medium containing increasing concentrations of BL. Root elongation was measured 8 d after germination. Each data point represents the average root elongation of 60 to 80 seedlings of two duplicate experiments. Inhibition of root growth by BL is expressed relative to the root elongation of the same genotype grown on medium containing the same volume of 80% (v/v) ethanol used to dilute BL from a 2-mM stock solution. Error bar represents SE.

### The *bin2* Mutation Affects the Feedback Inhibition of *CPD* Gene Expression by BR

A recent study indicated that the transcription of the Arabidopsis *CPD* gene, encoding a steroid hydroxylase involved in BR biosynthesis, is down-regulated only by BRs but is not affected by other plant hormones (Mathur et al., 1998). To learn more about the effects of the *bin2* mutations on BR responses at the molecular level, we have examined the steady-state levels of *CPD* mRNAs in wild-type, *bri1*, *bin2/+*, and *bin2* mutants grown for 12 d in the presence or absence of 1 $\mu$M BL. As indicated by Figure 4, BL treatment inhibited the accumulation of *CPD* transcript by approximately 70% in wild-type plants, whereas the *CPD* transcript level was unaffected by the BL treatment in either *bri1* or *bin2* homozygous mutants. It is intriguing that an approximately 50% reduction in the amount of steady-state *CPD* mRNA

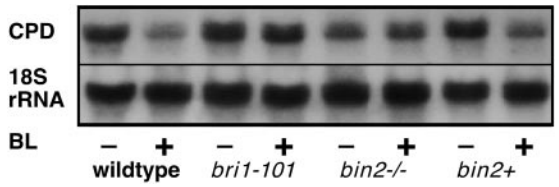


**Figure 4.** BL-regulated *CPD* gene expression in *bri1* and *bin2* mutants. Total RNAs were isolated from 12-d-old seedlings grown on medium with (+) or without (−) 1 $\mu$M BL, run on a denaturing agarose gel, and transferred to a nylon membrane. Hybridization was performed first with a probe derived from the *CPD* gene. To compare loading levels of different samples, the membranes were stripped and rehybridized with an 18S *rDNA* probe.

was observed in BL-treated *bin2*/+ plants in comparison with their untreated counterparts, although the heterozygotes behave very similarly to the homozygous *bin2* mutants with regard to BL-induced morphological change and root growth inhibition. It is possible that the BL-inhibited transcription of the *CPD* gene exhibits a higher BL sensitivity than the BL-mediated cell elongation. The remaining BR signaling activity in the heterozygotes is high enough to allow some degree of BL-mediated feedback inhibition on the *CPD* gene expression but might be too low to activate the cellular machinery to induce observable cell elongation. Another interesting observation is that the basal level of the *CPD* transcript is reduced in the *bin2* homozygous mutants but is slightly increased in the heterozygous mutants, suggesting that a wild-type level of BIN2 activity might be required for the basal expression of the Arabidopsis *CPD* gene.

### The Dominant Nature of the *bin2-1* Mutation

The semidominance of the *bin2* phenotype could be caused by a haplo-insufficient loss-of-function mutation or a gain-of-function mutation that creates a new function (neomorphic), increases wild-type gene function (hypermorphic), or antagonizes the action of the wild-type gene product (antimorphic; Muller, 1932). Whereas a loss-of-function mutation would clearly indicate a role for BIN2 in BR signaling, the gain-of-function mutations may not be good indicators of the endogenous function of the BIN2 protein. To determine whether the wild-type *BIN2* gene is involved in a BR signaling pathway, it is necessary to know whether the *bin2* mutation is a loss-of-function or gain-of-function mutation.

A common practice for determining the type of mutation for a given dominant or a semidominant phenotype is to conduct a gene dosage analysis. Such an experiment usually requires the availability of a chromosomal deletion and/or duplication for the gene and involves comparing the severity of the mutant phenotype associated with various genotypes in which the copy number of the mutant or wild-type gene is varied. Due to the lack of well-characterized Arabidopsis deletions or duplication stocks, it is not practical to alter specifically the gene copy number for the *BIN2* gene. Instead, we adopt a triploid strategy for the gene dosage experiment (Timpte et al., 1994).

We crossed the homozygous *bin2-1* mutants to a tetraploid line (CS3151, obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus) carrying a dominant yellow-green mutation to generate triploid $F_1$ plants that contain one copy of the mutated *bin2* gene and two copies of the wild-type *BIN2* gene. The phenotype of the resulting triploid plants was carefully examined to determine the effects of an additional copy of the wild-type *BIN2* gene on the *bin2*/+ heterozygous phenotype. If the *bin2-1* mutation causes haplo insufficiency or an antimorphic gain-of-function, we would expect that the addition of a wild-type *BIN2* gene would alleviate the *bin2*/+ phenotype and the resulting $F_1$ (*bin2*/+/+) plants would be similar morphologically to wild-type plants. Although many seeds failed to germinate on the Murashige and Skoog medium, a few dozen seeds did germinate and grew into adult plants producing $F_2$ seeds. Despite their yellow-green color that indicates successful crosses, the $F_1$ triploids displayed a phenotype that is similar to, if not more severe than, that of a typical *bin2*/+ heterozygous mutant (data not shown), suggesting that the *bin2-1* mutation is likely a neomorphic or hypermorphic gain-of-function mutation. Such a conclusion is in line with the fact that this allele was identified from 100,000 ethyl methanesulfonate-mutagenized seeds that were screened to identify at least 18 alleles of the *BRI1* gene because a dominant mutation occurs much more rarely than loss-of-function mutations.

## DISCUSSION

By re-examination of a collection of darker green dwarf and semidwarf mutants obtained from our previous genetic screens for BR-insensitive mutants, we have identified a semidominant *bin2* mutation that gives rise to a BR-insensitive dwarf phenotype. An additional screening for mutants that display the *bin2*/+ heterozygous phenotype resulted in the identification of a second *bin2* allele. Although the heterozygous *bin2*/+ mutants had some novel phenotypes (e.g. curly growth of cauline leaves and dark-grown hypocotyls), the homozygous *bin2* mutants displayed many phenotypes that mimic those of the *bri1* mutants. In addition to the BR-insensitive dwarf phenotypes, *bin2* mutants showed insensitivity to BR in root growth inhibition assay and feedback inhibition of *CPD* gene expression.

Although many genetic screens for BR response mutants have been conducted in several laboratories, only one Arabidopsis gene, *BRI1*, has been repeatedly identified (Clouse et al., 1996; Kauschmann et

al., 1996; Li and Chory, 1997; Noguchi et al., 1999). It was suggested that BRI1 might be the only component of a BR signaling pathway that can be identified by genetic approaches, whereas other components are either functionally redundant or essential for embryogenesis. All known *bri1* mutations are recessive loss-of-function mutations, whereas the two *bin2* mutations are semidominant. Thus, it is quite possible that the observed *bin2* phenotype might be caused by rare antimorphic gain-of-function mutations in the *BRI1* gene. However, there are three pieces of evidence that argue strongly against such a possibility. First, a cross between *bri1* and *bin2* mutants yielded plants that looked like the *bin2*/+ plants instead of *bri1* mutants. In addition, the $F_2$ plants segregated out wild-type plants. Second, although both *BRI1* and *BIN2* loci map to chromosome IV, *BIN2* is located in the middle of the chromosome, whereas *BRI1* maps to the lower arm of the chromosome. Third, sequence analysis of the *BRI1* gene in the *bin2* mutants did not reveal any mutation. Taken together, we concluded that the two *bin2* mutations define a new genetic locus whose gene product might play a role in BR signaling involved in plant growth.

The semidominance of the *bin2* phenotype can be caused by either haplo-insufficient loss-of-function mutations or gain-of-function mutations. Our phenotypic analysis of the *bin2*/+/+ triploid plants suggested that the *bin2* mutations were likely to be gain-of-function mutations. One potential problem for the triploid approach is that triploid plants often have larger cells than diploid plants and hence the cellular concentration of wild-type BIN2 protein may not increase proportionally with the copy number of the corresponding gene (Timpte et al., 1994). Thus, increasing the copy number of the wild-type *BIN2* gene may not be able to significantly ameliorate the *bin2*/+ phenotype even if the *bin2* mutations are loss-of-function or antimorphic gain-of-function mutations. Nevertheless, such an approach has been successfully employed to determine the genetic nature for several dominant mutations in Arabidopsis (Timpte et al., 1994; Rate et al., 1999).

There are three different types of gain-of-function mutations, namely neomorphic, antimorphic, and hypermorphic mutations (Muller, 1932). Our triploid analysis suggests that the *bin2* phenotype is likely caused by either neomorphic or hypermorphic mutations. A neomorphic mutation would result in the production of a gene product with a novel function not present in the wild-type BIN2 protein. For example, a mutation in the substrate recognition domain of a protein kinase could confer a broader substrate specificity to the kinase, allowing the mutated kinase to phosphorylate other proteins that cannot be phosphorylated by the wild-type kinase. Thus, a neomorphic mutation defines a gene product involved in a separate process. Nevertheless, the *bin2* mutations interfere with the normal BR signaling, possibly by compromising the function of a genuine BR signaling component, thus providing access for investigating the molecular mechanism(s) of BR signaling involved in plant growth. The *bin2* phenotype alternatively could be caused by a hypermorphic mutation in the *BIN2* locus. Because a hypermorphic gain-of-function mutation often causes the gene product to perform its normal function excessively, such hypermorphic *bin2* mutations might define a negative regulator in the BR signaling pathway involved in growth regulation.

To determine whether the endogenous BIN2 protein is involved in BR signal transduction, it is crucial to know the phenotypes of *bin2* loss-of-function mutation. A common characteristic of gain-of-function mutations of whatever kind is that they can be suppressed by a second mutation in the same gene that knocks out the deleterious gene product they produce. Thus, a common strategy to identify loss-of-function alleles of a given dominant mutation is to conduct a genetic screen to identify intragenic suppressors (Hua and Meyerowitz, 1998). Due to the male-sterile phenotype of the homozygous *bin2* mutations, a simple suppressor screen is not feasible. Although alternative strategies do exist for intragenic suppressor screens, such as screening for wild-type plants from the $F_1$ progeny derived from a cross between $\gamma$-ray irradiated *bin2* pollen and flowers of a male-sterile mutant with a $BIN2^+$ genotype, a quick strategy would be to use a PCR-based reverse genetic method (Winkler et al., 1998) to identify T-DNA "knockout" alleles once the *BIN2* gene is cloned. It is quite possible that the *BIN2* gene may be a member of a redundant gene family, and its loss-of-function mutations may not reveal any physiological or developmental defect. If that is the case, all members of the gene family with similar expression patterns have to be eliminated to confirm the suspected biological function for the BIN2 protein.

BRs are known to interact with other plant hormones in very complicated ways. For example, they interact additively with GAs and synergistically with auxins, but antagonistically with ABA (Mandava, 1988). The *bin2* mutants are insensitive only to BRs but retain full sensitivity to auxins, cytokinins, ethylene, and GAs. Consistent with the early physiological data (Sasse, 1990), the *bin2* mutants display an enhanced sensitivity to ABA. Such a result is also in line with the root growth inhibition analysis of other BR-related mutants. For example, *bri1* mutants are hypersensitive to ABA (Clouse et al., 1996). The *sax1* mutant, a recently identified BR-deficient mutant, exhibits a similar ABA-hypersensitive phenotype that can be rescued by exogenous BR application (Ephritikhine et al., 1999a, 1999b).

Although the *bin2* mutants show normal response to auxin by the root growth inhibition assay, some BR-related mutants are hypersensitive to auxins. The *sax1* mutant was in fact identified in a genetic screen for auxin-hypersensitive mutants (Ephritikhine et al.,

1999a). A tomato (*Lycopersicon pimpinellifolium*) BR-insensitive dwarf mutant (*cu-3*) that was thought to be defective in a tomato homolog of the BRI1 protein, displays an auxin hypersensitivity despite its normal ABA sensitivity (Koka et al., 2000). The curly growth phenotype of the *bin2*/+ mutant might indicate an interaction between BR and auxin. The curly growth of light-grown cauline leaves and dark-grown hypocotyls is most likely due to the differential cell growth in those tissues. Differential growth is a common growth response of plants to many environmental stimuli (e.g. light and gravity), and auxin is implicated in this process (Lehman et al., 1996). The identification of a novel BR-insensitive locus thus provides new opportunities to investigate the interaction between BRs and other plant hormones.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis Col-0 was the wild-type control. Arabidopsis ecotype Landsberg carrying the *erecta* mutation (L*er*) was used for mapping purposes. *bri1-101* (Li and Chory, 1997) and *cpd* (Szekeres et al., 1996) were used for morphological comparison with the newly identified *bin2* mutants. *bin2-1* was the mutant used for all the experiments conducted in this study except for testing allelism between *bin2-1* and *bin2-2*. Seeds were surfaced sterilized by washing for 15 min in 70% (v/v) ethanol containing 0.05% (v/v) Tween 20, followed by three 5-min washes with 95% (v/v) ethanol. Seeds were then dried on sterilized filter papers under sterile conditions and sown on one-half-strength Murashige and Skoog medium containing 1% (w/v) Suc and 0.8% (w/v) phytagar (both Murashige and Skoog salt and phytagar were purchased from Life Technologies, Inc., Rockville, MD). The plates were wrapped with two layers of aluminum foil and left at 4°C for 48 h to break dormancy. Seedlings were grown in growth chambers at 22°C under long-day growth conditions (16 h light/8 h dark). Two weeks after germination, seedlings were hand transferred into soil (Sunshine #5, Sun Gro Horticulture, Inc., Bellevue, WA) and grown in a growth room under the same controlled conditions.

### Isolation of *bin2* Mutants

For isolation of *bin2-1* mutant, seeds of the dwarf and semidwarf mutants that were collected from our previous screen for *bri1* mutants (Li and Chory, 1997) were germinated on one-half-strength Murashige and Skoog medium supplemented with 100 nM BL (CIDtech Research Inc., Ontario, Canada). Seven days after germination, seedlings were carefully examined by monitoring petiole elongation, leaf expansion, and root growth inhibition on a daily basis to identify mutants that showed no or reduced sensitivity to BL. For isolation of the *bin2-2* mutant, 50,000 ethyl methanesulfonate-mutagenized seeds of Col-0 carrying the *gl-1* mutation, derived from nine independent pools of 680 $M_1$ plants, were purchased from Lehle Seeds (Round Rock, TX). Approximately 1,000 seeds per petri plate (15 × 150 mm) were screened for seedlings resembling *bin2-1* heterozygous mutants.

### Genetic Analysis

*bin2* homozygous mutants derived from self-fertilization of the original semidwarf mutant were back-crossed three times to eliminate any unlinked second site mutation. To test for allelism between the *BRI1* and *BIN2* loci, pollen from homozygous *bin2* mutants was used to pollinate homozygous *bri1-101* mutants and the phenotype of the resulting $F_1$ plants was analyzed when grown on one-half-strength Murashige and Skoog plates and in soil. The *bri1* heterozygosity was confirmed by PCR-based genotyping using a CAPS marker derived from the *BRI1* gene (the *bri1-101* mutation destroys a *Xho*I site in the *BRI1*'s coding sequence; Li and Chory, 1997). The allelism test between the *bin2-1* mutant and the newly identified *bin2*/+-like mutants was performed by crossing pollen of the *bin2*-like mutants to *bin2-1* homozygous plants.

### Hormone Sensitivity Assays

Seeds were geminated and grown on vertically oriented one-half-strength Murashige and Skoog plates containing 1 μM 2,4-dichlorophenoxyacetic acid, 0.5 μM ABA, 1 μM kinetin, 1 μM 1-aminocyclopropane-1-carboxylic acid, 1 μM or 50 μM $GA_3$ (all plant hormones except BL were purchased from Sigma, St. Louis), or varying concentrations of BL (as indicated in Fig. 3). Root lengths of individual seedlings were measured after 8 d in the growth room. For each hormone or each concentration of BL, 60 to 80 seedlings were measured and the average length of their root growth was used to plot Figures 2 and 3.

### DNA and RNA Analysis

DNA was isolated from the homozygous *bin2* mutants as described by Li and Chory (1998) and sequence analysis of the *BRI1* gene was performed as described by Friedrichsen et al. (2000). Total RNAs were extracted from seedlings that were grown on one-half-strength Murashige and Skoog-phytagar medium supplemented with or without 1 μM BL (Carpenter and Simon, 1998), subjected to electrophoresis on MOPS [3-(*N*-morpholino)-propanesulfonic acid]-formaldehyde agarose gel, and transferred to filters (Hybond-XR, Amersham Pharmacia Biotech Inc., Piscataway, NJ) that were hybridized with *CPD*, or *18S rDNA*-derived probes. Prehybridization and hybridization were carried out at 42°C in a solution of 1 mM EDTA, 250 mM sodium-phosphate (pH 7.4), 7% (w/v) SDS, 1% (w/v) casein, and 50% (v/v) formamide.

### Mapping of *BIN2*

A homozygous *bin2-1* mutant was pollinated with L*er* pollen, and the resulting $F_1$ plants were selfed to generate

a mapping population. DNAs isolated from 125 individual wild-type-looking seedlings were used for CAPS analysis (Konieczny and Ausubel, 1993). Three CAPS markers (http://www.Arabidopsis.org/aboutcaps.html) per chromosome were selected.

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