Cell Research (2008) 18:472-478.

www.nature.com/cr

ORIGINAL ARTICLE

Is kinase activity essential for biological functions of BRI1?

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Brassinosteroids (BRs) are a major group of plant hormones that regulate plant growth and development. BRI1, a protein localized to the plasma membrane, functions as a BR receptor and it has been proposed that its kinase activity has an essential role in BR-regulated plant growth and development. Here we report the isolation and molecular characterization of a new allele of *bri1*, *bri1-301*, which shows moderate morphological phenotypes and a reduced response to BRs under normal growth conditions. Sequence analysis identified a two-base alteration from GG to AT, resulting in a conversion of 989G to 989I in the BRI1 kinase domain. An *in vitro* assay of kinase activity showed that bri1-301 has no detectable autophosphorylation activity or phosphorylation activity towards the BRI1 substrates TTL and BAK1. Furthermore, our results suggest that bri1-301, even with extremely impaired kinase activity, still retains partial function in regulating plant growth and development, which raises the question of whether BRI1 kinase activity is essential for BR-mediated growth and development in higher plants.

Keywords: brassinosteroid, *bri1*, kinase activity, *Arabidopsis thaliana*

Cell Research (2008) **18**:472-478. doi: 10.1038/cr.2008.36; published online 11 March 2008

Introduction

Brassinosteroids (BRs) are natural plant growth-promoting compounds with a structure similar to that of animal steroid hormones. Recent progress in plant molecular genetics has shown that, although BRs are distributed in plants at an extremely low level, they have significant effects on plant growth and development, including seed germination, stem and root elongation, seedling photomorphogenesis, vascular differentiation, leaf development, pollen tube growth, stress resistance, and senescence [1, 2].

Unlike animal steroid hormones that bind to nuclear receptors and directly activate target genes [3], BRs are perceived at the plasma membrane by BRI1, a leucine-rich repeat receptor-like kinase (LRR-RLK) in *Arabidopsis thaliana* [4-7]. Homologs of BRI1 have also been identified in rice, tomato, barley, pea, and cotton [8-12]. The BRI1 contains a leucine-rich repeat (LRR) extracellular domain, a transmembrane domain, and a cytoplasmic kinase domain with serine/threonine specificity [6, 13]. Studies have shown that the extracellular domain, especially the island and the neighboring C-terminal LRR repeat, is responsible for perceiving BRs [5]. It has been proposed that the kinase activity of BRI1 is essential for the biological functions that mediate plant growth and development [13].

BAK1, another LRR-RLK that is localized to the plasma membrane, was identified by searching for BRI1-interacting proteins using a yeast two-hybrid screen and by screening for *bri1* suppressors with an activation-tagging approach in *A. thaliana* [14, 15]. It has been shown that the interaction of BAK1 with BRI1 forms a heterodimer, which initiates the signal transduction cascade [16]. BAK1 is phosphorylated as a result of binding to BRI1 [14, 15, 17]. Furthermore, another two BRI1-interacting proteins, transthyretin-like (TTL) and BRI1 kinase inhibitor 1 (BKI1), have been identified by a yeast two-hybrid screen using the BRI1 kinase domain as bait [18, 19]. TTL and BKI1 are substrates of BRI1 and act as negative regulators in BR-mediated plant growth [18, 19].

In this paper, we report the isolation and characteriza-

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Received 26 June 2007; revised 29 September 2007; accepted 22 October 2007; published online 11 March 2008

tion of a new *bri1* allele, *bri1-301*, which shows a weak morphological phenotype and a reduced sensitivity to BRs. The mutation from GG to AT in *bri1-301* results in the conversion of 989G to 989I in the BRI1 kinase domain, which leads to undetectable kinase activity in bri1-301. Our findings from this new *bri1* allele raise questions about the role of BRI1 kinase activity in plant growth and development.

Results

Isolation and characterization of a new bri1 allele, bri1-301

By using a two-step screening approach (see Materials and Methods), we isolated a morphological mutant with a much shorter hypocotyl compared with the wild type when grown under long-wavelength light (> 460 nm) (Figure 1A). In addition, when grown under white light conditions, the mutant displayed weak morphological abnormalities, including round leaves, short petioles, prolonged life span, and slightly shorter plant height with nearly normal fertility (Figures 1B and 1C). However, the mutant exhibited hypocotyl elongation under white light conditions and etiolated phenotype in the dark, similar to the wild type (Figure 1A).

To understand the molecular basis of this mutant, we cloned the gene responsible for the mutant phenotype using a map-based approach. Briefly, genetic analysis showed that all the 41 F_1 progeny from a cross between the mutant and a wild-type plant had the wild-type phenotype. In the F_2 generation there were 858 wild-type to 289 mutant plants. These results indicate that the mutant possesses a recessive mutation at a single nuclear locus. Using an F_2 mapping population derived from a cross between the mutant and its polymorphic ecotype Landsberg *erecta* (L*er*), the mutant gene was found to be tightly linked to the *BRI1* locus [6] (data not shown). An allelic test in which the mutant was crossed with *bri1-101*, a strong allele of *bri1* [13], showed that the mutant is allelic to *bri1* (data not shown). Therefore, the mutant was designated *bri1-301*, which has been extensively used in the study of BR signaling pathways [14, 18, 20-22].

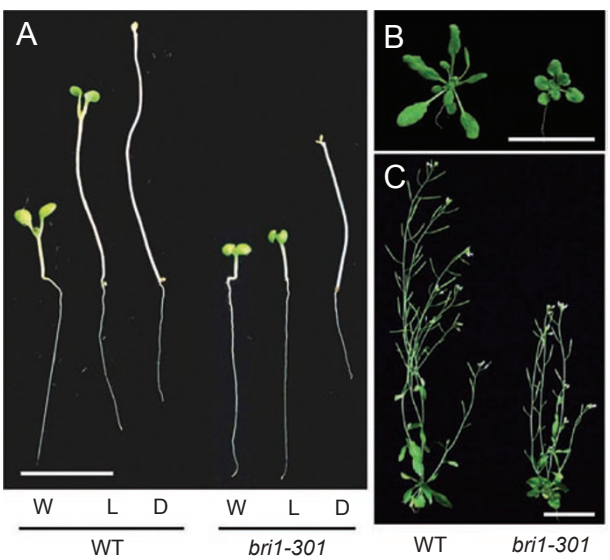

Figure 1 Phenotypes of *bri1-301* mutant plants. **(A)** Seven-day-old wild-type and *bri1-301* mutant seedlings grown on 0.5 × MS plates under normal (white) light (W), long-wavelength light (> 460 nm) (L) and in the dark (D). **(B)** One-month-old wild-type and *bri1-301* mutant plants grown under normal light at 23 °C. **(C)** Two-month-old wild-type and *bri1-301* mutant plants grown under normal light at 23 °C. Bars in **(A)** = 1 cm, in **(B)** and **(C)** = 5 cm.

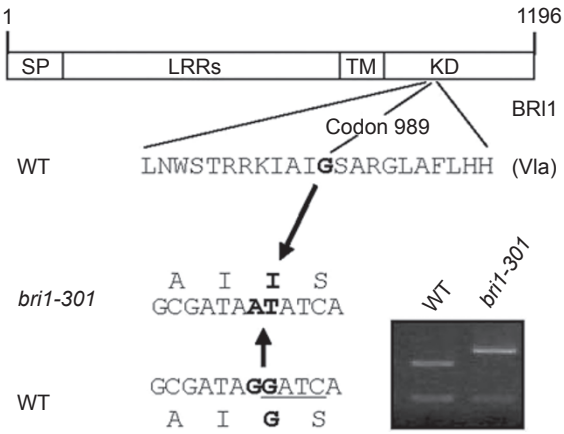

Figure 2 Molecular lesion of *bri1-301*. The diagram shows the full-length BRI1 protein with a defined signal peptide (SP) domain, leucine-rich repeat (LRR) domain, transmembrane (TM) domain and kinase domain (KD). The change of GG to AT in *bri1-301* results in the conversion of glycine (G) to isoleucine (I) at amino-acid residue 989 in the VIa subdomain of the BRI1 kinase domain. The mutation of GG to AT eliminates an *Mbo*I recognition site (underlined).

Molecular lesion in bri1-301

The molecular lesion in *bri1-301* was identified by comparing the genomic DNA sequences of the mutant and wild-type plants. A two-base alteration from GG to AT, which causes a conversion from Gly to Ile of codon 989 in the VIa subdomain of the kinase domain of BRI1, was identified (Figure 2). This two-base change in *bri1-301* resulted in the elimination of the *Mbo*I restriction enzyme site, which allowed us to develop a co-dominant cleaved amplified polymorphic sequences (CAPS) marker to distinguish between *bri1-301* and wild-type plants (Figure 2).

Altered expression patterns of light-regulated genes in bri1-301

The *bri1-301* mutant was isolated using a two-step screening approach and each step was under a different light condition, suggesting that *BRI1* may be involved in the light signaling pathway. To find out whether the altered response of *bri1-301* to light resulted from an interruption to the light signaling pathway, we examined the expression patterns of two light-inducible genes, *RbcS1A* and *Lhcb1.3*, using real-time PCR analysis [23-25]. As shown in Figure 3, the expression levels of *RbcS1A* and *Lhcb1.3* in the wild-type seedlings are greatest under white light, but are reduced to approximately 50% under long-wavelength light and to 20% in the dark. In *bri1-301*, the expression levels of *RbcS1A* and *Lhcb1.3* under long-wavelength light are almost the same as the levels under white light. Although the expression of *RbcS1A* and *Lhcb1.3* in *bri1-301* is reduced in the dark, the expression levels are >40% of the expression levels under white light. The expression patterns of *RbcS1A* and *Lhcb1.3* in *bri1-101* are similar to those of *bri1-301* (Figure 3). These results demonstrate that the mutation in BRI1 was able to enhance the expression of the light-inducible genes *RbcS1A* and *Lhcb1.3*, both under long-wavelength light and in the dark, which suggests that *BRI1* participates in the light signaling pathway.

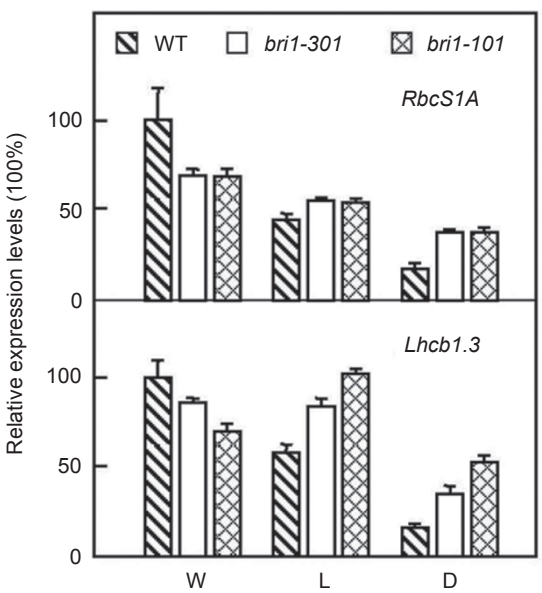

Figure 3 Effects of the *BRI1* mutation on the expression of light-inducible genes under different light conditions. The relative expression levels of the light-inducible genes *RbcS1A* and *Lhcb1.3* are shown for the wild-type, *bri1-301* and *bri1-101* seedlings grown under white light (W), long wavelength (> 460 nm) (L) and in the dark (D).

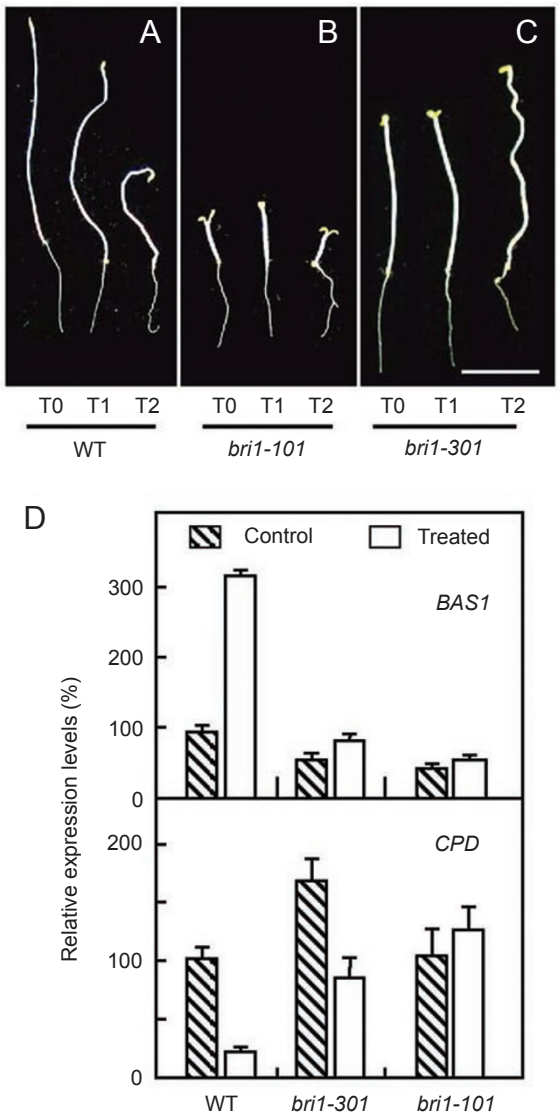

Figure 4 Responses of wild-type and *bri1-301* seedlings to treatment with different concentrations of 24-epi-BL. **(A–C)** Seven-day-old wild-type **(A)**, *bri1-101* **(B)** and *bri1-301* **(C)** seedlings grown on 0.5 × MS plates supplemented with 0 nM (T0), 10 nM (T1) or 1 000 nM (T2) 24-epi-BL in the dark. Bar = 1 cm. **(D)** The expression levels of *BAS1* and *CPD* in wild-type, *bri1-301* and *bri1-101* seedlings grown on 0.5 × MS plates supplemented with 0 μM (Control) and 1 μM (Treated) 24-epi-BL in the dark.

Response of bri1-301 to BRs

As an essential plant growth regulator, BRs can affect plant morphology at an extremely low concentration. As shown in Figure 4, the wild-type seedlings responded to exogenous 24-epi-brassinolide (24-epi-BL) in a concentration-dependent manner (Figure 4A). The strong allele *bri1-101* seedlings showed nearly undetectable additional phenotypic alterations at either low or high concentra-

tions of BL (Figure 4B), whereas the *bri1-301* seedlings responded to exogenous BL at a high concentration (1 μM) with a stimulated effect (Figure 4C). These results suggest that the response of *bri1-301* to BRs is altered.

To substantiate the altered response of *bri1-301* to exogenous BL, we compared the expression levels of *BAS1* and *CPD* among wild-type, *bri1-101*, and *bri1-301* plants. *BAS1* encodes a cytochrome P450 that is involved in BL degradation and is upregulated by BRs [26]. *CPD* encodes a steroid hydroxylase that is involved in BR biosynthesis and is downregulated by BRs [27]. In wild-type plants, the expression of *BAS1* was significantly induced by exogenous BL, but its expression was only slightly affected by BL in both *bri1-301* and *bri1-101* (Figure 4D). In wild-type plants, expression of *CPD* was strongly suppressed by BL (Figure 4D). However, in *bri1-301*, *CPD* expression was only moderately affected compared with the wild type (Figure 4D).

Kinase activity is undetectable in bri1-301

To understand whether the Gly-to-Ile missense replacement in the kinase domain of bri1-301 affects its kinase activity, we expressed and purified the bri1-301 kinase domain (bri1-301-KD), fused with glutathione *S*-transferase (GST), and assayed its kinase activity *in vitro* (Figure 5). The expressed kinase domain of the wild type, BRI1-KD, showed strong autophosphorylation activity as previously reported [13], whereas the kinase activity of the expressed bri1-301-KD or bri1-101-KD was undetectable (Figure 5A). In addition, we noticed that the electrophoretic mobility of BRI1-KD was slower than that of both bri1-301-KD and bri1-101-KD (Figure

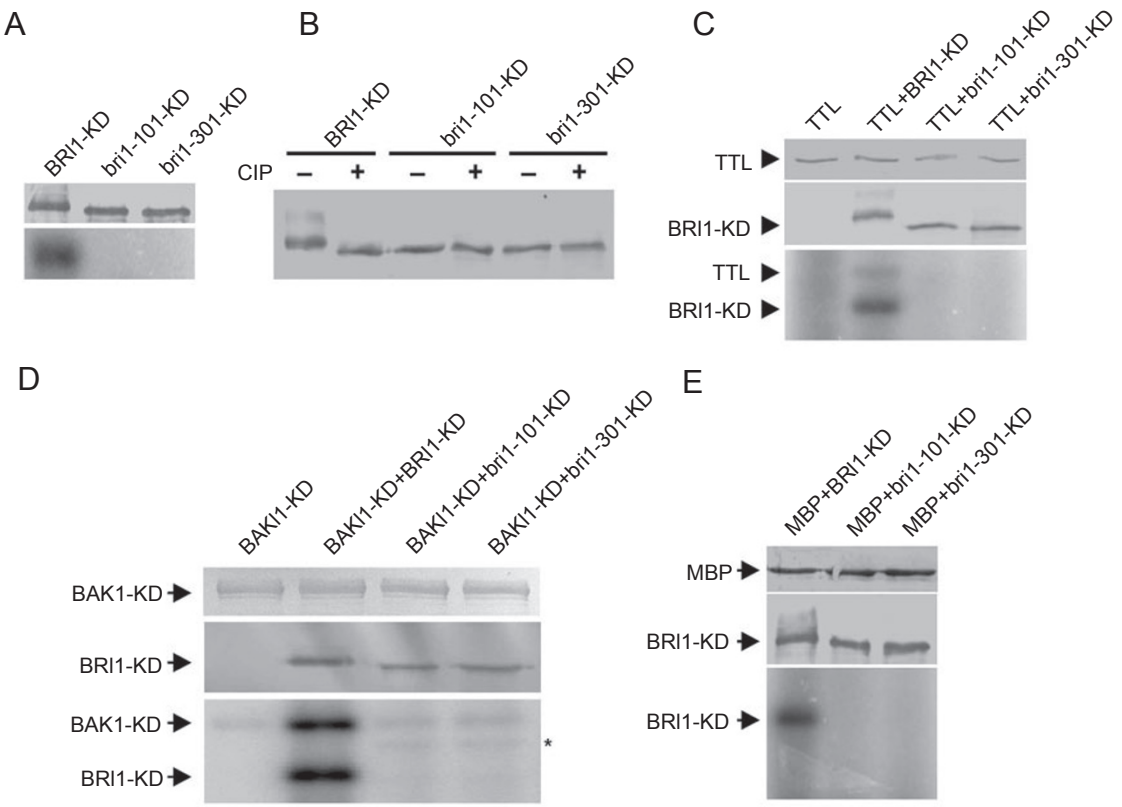

Figure 5 *In vitro* kinase activity assays of the BRI1, bri1-101 and bri1-301 kinase domains. **(A)** The expressed and purified BRI1 kinase domain (BRI1-KD) shows strong autophosphorylation activity, but the expressed bri1-301 or bri1-101 kinase domain (bri1-301-KD or bri1-101-KD) has undetectable autophosphorylation activity. The upper panel shows the purified GST fusion proteins detected by western blotting and the lower panel shows the level of protein phosphorylation detected by autoradiography. **(B)** The electrophoretic mobility of BRI1-KD is slower than that of bri1-301-KD and bri1-101-KD. However, after treatment with CIP, they show a similar mobility. **(C)** BRI1-KD is able to phosphorylate its interacting protein TTL, but bri1-301-KD cannot. **(D)** BRI1-KD is able to phosphorylate its natural substrate BAK1-KD, but bri1-301-KD cannot. * indicates unspecific bands. **(E)** The control experiment shows that MBP alone cannot be phosphorylated by BRI1-KD. In **(C–E)**, the upper two panels show the purified MBP and GST fusion proteins detected by Western blotting and the lower panel shows the level of protein phosphorylation detected by autoradiography.

5A), which suggests that the slower mobility may result from the autophosphorylation of BRI1-KD. In fact, after treatment with calf intestinal phosphatase (CIP), BRI1-KD showed the same electrophoretic mobility as bri1-301-KD and bri1-101-KD, which were not affected by the CIP treatment (Figure 5B). These results demonstrate that both bri1-301-KD and bri1-101-KD have defective autophosphorylation.

To test whether bri1-301 or bri1-101 has also lost its ability to phosphorylate its native substrates (an example is TTL [18]), we expressed and purified the full-length TTL protein, fused with maltose binding protein (MBP), and measured its phosphorylation using the expressed kinase domain of BRI1. As shown in Figure 5C, the wild-type BRI1-KD showed strong kinase activity towards its substrate TTL, but neither bri1-301-KD nor bri1-101-KD had detectable kinase activity.

A similar result was also observed with BAK1 as a native substrate of BRI1 [14, 15, 17]. Phosphorylated BAK1-KD was barely detectable when BAK1-KD was incubated with bri1-301-KD or bri1-101-KD, and this low phosphorylation activity appears to come from BAK1 autophosphorylation activity, as has been previously described [15, 17] (Figure 5D). By contrast, the phosphorylation of BAK1-KD was apparently enhanced when incubated with BRI1-KD (Figure 5D).

Taken together, we may conclude that the mutation in the bri1-301 kinase domain results in undetectable kinase activity, at least in an *in vitro* assay system.

Discussion

In this paper, we report the isolation of a weak *bri1* allele, *bri1-301*, using a two-step screening approach. A single amino-acid Gly-to-Ile change in the VIa subdomain of the BRI1 kinase domain produces a defective protein that has undetectable kinase activity. However, the *bri1-301* mutant plant shows a mild morphological phenotype when under normal growth conditions, indicating that the loss of kinase activity in bri1-301 protein may not abolish the involvement of BRI1 in regulating plant growth and development.

Many *bri1* alleles have been isolated to date and most of them are strong alleles that exhibit severe phenotypes, including extreme dwarfism, a prolonged life span, and complete male sterility [13, 28, 29]. Many of them, for example *bri1-1*, *bri1-3*, *bri1-101*, *bri1-103*, *bri1-105*, *bri1-115*, and *bri1-117*, have defects in the kinase domain [13, 28], and the kinase activity of bri1-101 has been proved to decrease greatly, suggesting that the kinase activity is crucial for the biological function of BRI1 [13]. However, our studies on *bri1-301* raise the question of whether kinase activity is essential for BRI1 in mediating plant growth and development. In fact, there have been some clues to indicate that BRI1 kinase activity might not be indispensable for its function. First, the BRI1 kinase domain is not involved in binding to its ligand BRs [5]. Second, the interaction between BRI1 and its interacting proteins BAK1 and BKI1 is independent of kinase activity [14, 19]. Third, the severity of the *bri1* mutant phenotype does not always correlate with a loss of bri1 kinase activity. For example, *bri1-104*, a strong *bri1* allele, has only 50% reduced kinase activity [18].

BRI1 has been identified to interact directly with several proteins, including the BR signaling positive regulator BAK1 [14, 15] and negative regulators TTL [18] and BKI1 [19]. A possible interpretation of the differences between the *bri1-301* and *bri1-101* phenotypes is that the Gly-989-Ile mutation in the VIa subdomain in bri1-301 may not only abolish the kinase activity of BRI1 but also alters its conformation, leading to the constitutive release of negative regulatory interactors from BRI1 and/or the recruitment of positive regulatory interactors to BRI1 and thus the activation of BRI1 signaling. However, bri1-101 may be defective in generating or maintaining a functional conformation that is required for BRI1 to interact with its interactors.

Interestingly, *bri1-8*, another weak *bri1* mutant, also carries a conserved amino-acid change from Arg to Gln in the Vla subdomain [29], implying that the Vla subdomain might be important for the BRI1 conformation. *bri1-8* and *bri1-301* are the only two as-yet-identified weak *bri1* alleles that harbor mutations in the BRI1 kinase domain [28]. Further investigation of the two weak alleles and characterization of more *bri1* mutants that have mutations in the kinase domain will help us to further understand the role of BRI1 kinase activity in its biological functions.

In plants, growth and development are the consequence of an interplay between environmental factors and intrinsic programs. Light is one of the most important environmental factors and regulates many developmental processes throughout the plant life cycle [30, 31]. Increasing studies have suggested that light signaling interacts with the BR signaling pathway [32-35]. The findings of undetectable kinase activity in bri1-301 and altered transcription patterns of the light-inducible *RbcS1A* and *Lhcb1.3* genes in the *bri1-301* seedlings grown under long-wavelength light and in the dark suggest that the BRI1 kinase domain might function in light signaling, providing an explanation for why BRs interact with light. Therefore, *bri1-301* may also serve as a valuable tool in studying the mechanisms underlying the crosstalk between light and BRs.

Materials and Methods

Plant materials

A. thaliana plants were grown on vermiculite saturated with 0.3 × B5 medium [36] under continuous light (80-120 μE/m^2 s) at 23 °C as described previously [37], or under light conditions as indicated in the text. For plants grown in petri dishes, seeds were surface-sterilized with 70% (v/v) ethanol for 3 min and 15% commercial bleach solution plus 0.025% Triton X-100 for 15 min, rinsed three times with sterile water, and suspended in 0.1% agar. The sterilized seeds were plated on 0.5 × MS medium [38] containing 0.8% agar and pre-incubated at 4 °C in the dark for 2 days before being cultured under the conditions as indicated.

Isolation and mapping of bri1-301

A. thaliana ecotype Columbia (Col-0) wild-type plants were mutagenized with EMS as described previously [39], and the *bri1-301* mutant was isolated using a two-step screening procedure. The mutagenized M_2 seeds were germinated and grown at 23 °C for 7 days under long-wavelength light (>460 nm) obtained using a yellow filter (Roscolux #10 Medium Yellow Filter, SSSL Syracuse Scenery & Stage Lighting Co., Inc), and dwarf seedlings were selected and grown under normal white light to produce M_3 seeds, which were subsequently subjected to the second-step screening for those showing a normal etiolated phenotype when grown in the dark.

To isolate the gene responsible for the mutant phenotype through a map-based cloning approach, the homozygous mutant plant was pollinated with L*er* pollens and an F_2 mapping population was constructed using F_1 hybrid self-pollination to generate a segregating F_2 mapping population. *Arabidopsis* DNA was isolated from individual mutant plants as described previously [40]. Linkage between *bri-301* and molecular markers was determined using CAPS and simple sequence length polymorphisms (SSLP) markers [41, 42]. To examine allelism, a complementation test was performed between *bri1-301* and *bri1-101*.

Light conditions and treatment with 24-epi-BL

Seedlings were vertically grown on 0.5 × MS medium under continuous white light, continuous long-wavelength light (> 460 nm) and in the dark. Seeds were germinated on 0.5 × MS medium supplemented with various concentrations of 24-epi-BL in the dark.

Real-time PCR analysis

Total RNAs were prepared using the guanidine thiocyanate extraction method as described previously [43]. Complementary DNA was reverse transcribed from total RNAs with oligo(dT) as the primer and real-time PCR analysis was carried out according to instructions from the manual of SYBR® GREEN PCR Master Mix using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers used in the experiments were Tubulin-rt-F and Tubulin-rt-R for *Tubulin* (5′-ACC TAC TGG TCT GAA GAT GGC AT-3′ and 5′-TTT CTC CTG AAC ATA GCT GTG AAC T-3′); rbcS1A-rt-F and rbcS1A-rt-R for *RbcS1A* (5′-ACA AGC AAC GGC GGA AGA-3′ and 5′-CGG AAT CGG TAA GGT CAG GA-3′); Lhcb1.3-rt-F and Lhcb1.3-rt-R for *Lhcb1.3* (5′-GGA GCT CAA GAA CGG AAG ATT G-3′ and 5′-TCT CTA TCG GTC CCT TAC CAG TG-3′); BAS1-rt-F and BAS1-rt-R for *BAS1* (5′-CCC GTT GGC TTC ATA CCG T-3′ and 5′-TTA CAG CGA GTG TCA ATT TGG C-3′); and CPD-rt-F and CPD-rt-R for *CPD* (5′-CTT ACC GCA AAG CCA TCC A-3′ and 5′-TCA TCA CCA CCA CCG TCA AC-3′).

Kinase assay

To produce the GST and MBP fusion proteins, the BRI1 kinase domain was cloned into the pGEX-6p-1 vector (Amersham Biosciences), and the full-length TTL ORF and the BAK1 kinase domain were cloned into the pMAL-p2X vector (New England Biolabs), respectively. Fusion protein induction and purification were performed according to the manufacturer's protocols (Amersham Biosciences for the GST-BRI1-KD fusion proteins and New England Biolabs for the MBP-TTL and MBP-BAK1-KD fusion proteins). Kinase assays were performed *in vitro* at 25 ºC for 1 h in a 20-μl reaction mixture containing 20 mM Tris, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$ and 1 μl of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences), with approximately the same amount of purified proteins determined by western blot analysis. The kinase reaction was terminated by adding 5 μl of 5 × SDS sample buffer and boiling for 10 min, and then proteins were separated using 8% SDS-PAGE. The gel was exposed to phosphor plates and scanned with a phosphorimager (Typhoon 8600, Amersham Biosciences) to visualize the phosphorylated bands.

Acknowledgments

We thank Prof Joanne Chory (The Salk Institute for Biological Studies, USA) for providing the *Arabidopsis bri1-101* mutant seeds. This work was supported by grants from the National Natural Science Foundation of China (grant numbers: 30070074, 30330040 and 30570161).

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