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Activation-Tagged Suppressors of a Weak Brassinosteroid Receptor Mutant

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ABSTRACT Brassinosteroids (BRs) are important plant hormones that act synergistically with auxin to regulate a variety of plant developmental and physiological processes. In the past decade, genetic and biochemical studies have revealed a linear signaling pathway that relies on protein phosphorylation to transmit the BR signal into the nucleus, altering expression of hundreds of genes to promote plant growth. We conducted an activation-tagging based suppressor screen to look for *Arabidopsis* genes that, when overexpressed by inserted *35S* enhancer elements, could suppress the dwarf phenotype of a weak BR receptor mutant *bri1-301*. This screen identified a total of six dominant *activation-tagged bri1 suppressors* (*atbs-Ds*). Using a plasmid rescue approach, we discovered that the *bri1-301* suppression effect in four *atbs-D* mutants (*atbs3-D* to *atbs6-D*) was caused by overexpression of a *YUCCA* gene thought to be involved in tryptophan-dependent auxin biosynthesis. Interestingly, the three activation-tagged *YUCCA* genes belong to the *YUCCA IIA* subfamily that includes two other members out of 11 known *Arabidopsis YUCCA* genes. In addition, our molecular studies revealed a T-DNA insertion near a basic helix-loop-helix gene in *atbs1-D* and a T-DNA insertion in a region carrying a BR biosynthetic gene in *atbs2-D*. Further studies of these *atbs-D* mutants could lead to better understanding of the BR signaling process and the BR–auxin interaction.

Key words: Brassinosteroid; BRI1; *bri1-301*; auxin; YUCCA.

INTRODUCTION

Brassinosteroids (BRs) are a class of plant-specific polyhydroxylated steroids that play important roles in plant growth and development (Clouse and Sasse, 1998). Mutants defective in either BR biosynthesis or signaling share similar morphological/developmental phenotypes including dwarfed stature, round and dark green rosette leaves, short petioles, delayed flowering and senescence, reduced male fertility, and de-etiolation in the dark (Clouse and Feldmann, 1999). Unlike the animal steroid hormone receptors that function as nuclear transcription factors, the major BR receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) is a cell surface-localized leucine-rich-repeat receptor-like kinase (Kinoshita et al., 2005; Li and Chory, 1997) that initiates a phosphorylation-mediated signaling cascade to regulate stability, nuclear localization, and/or DNA binding activity of several plant-specific transcription factors (Li, 2005; Li and Jin, 2007). BR binding to BRI1 leads to rapid dissociation of BRI1 Kinase Inhibitor 1 (BKI1) (Wang and Chory, 2006) to allow heterotetramerization and transphosphorylation of BRI1 and its co-receptor BRI1-Associated Receptor Kinase 1 (BAK1) (Li et al., 2002; Nam and Li, 2002; Wang et al., 2008). The activation of two receptor kinases leads to activation of a family of BR SIGNALING KINASES (BSKs) (Tang et al., 2008) and a protein phosphatase bri1 SUPPRESSOR 1 (BSU1) (Mora-Garcia et al., 2004) and inhibition of a GSK3-like kinase, BRASSINOSTEROID INSENSITIVE 2 (BIN2) (Choe et al., 2002; Li and Nam, 2002), likely via proteasome-mediated protein degradation or tyrosine dephosphorylation (Kim et al., 2009; Peng et al., 2008). As a result, two BIN2 substrates bri1 EMS SUPPRESSOR 1 (BES1) (Yin et al., 2002; Zhao et al., 2002) and BRASSINAZOLE RESISTANT 1 (BZR1) (Wang et al., 2002) become dephosphorylated and more stable (He et al., 2002), accumulate in the nucleus (Gampala et al., 2007; Ryu et al., 2007), and bind to their corresponding regulatory sequences (He et al., 2005; Yin et al., 2005), to influence expression of many known BR-responsive

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genes (Vert et al., 2005), thus promoting cellular elongation and plant growth.

Many strategies have been employed in the past decade to identify the aforementioned BR signaling components and proteins that regulate BR biosynthesis and signaling. These strategies include forward genetic screens for BR-insensitive dwarf mutants, which has so far identified over 30 loss-of-function alleles of *BRI1* (Bouquin et al., 2001; Clouse et al., 1996; Li and Chory, 1997; Noguchi et al., 1999; Tanaka et al., 2005; Xu et al., 2008) and eight gain-of-function alleles of *BIN2* (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), suppressor screens for mutations that revert the dwarf phenotype or resistant to a BR biosynthesis inhibitor (BAK1, BES1, BZR1, and BSU1) (Li et al., 2002; Mora-Garcia et al., 2004; Wang et al., 2002; Yin et al., 2002), and yeast two-hybrid screen coupled with reverse genetics (BAK1, BKI1, BES1, and BZR1) (Nam and Li, 2002, 2004; Wang and Chory, 2006; Zhao et al., 2002). Among them, the most successful approach is the suppressor screen using the activation-tagging approach (Weigel et al., 2000). The insertion of a T-DNA that carries four copies of an enhancer element derived from the cauliflower mosaic virus *35S* promoter into an *Arabidopsis* genome could lead to significant overexpression of a T-DNA flanking gene, resulting in a desirable morphological/developmental/physiological phenotype and easy identification of the responsible gene. The activation-tagging-based suppressor screens were responsible for the identification of BAK1 (Li et al., 2002), BSU1 (Mora-Garcia et al., 2004), bri1 SUPPRESSOR 1 (BRS1, a secreted carboxypeptidase) (Li et al., 2001a), BRI1-LIKE 1 (BRL1, a BRI1 homolog specifically expressed in vascular tissue) (Zhou et al., 2004), and bri1-5 ENHANCED 1 (BEN1, a dihydroflavonol 4-reductase-like protein regulating the levels of active BR) (Yuan et al., 2007).

One requirement for a successful activation tagging-based suppressor screen is that the primary mutant should produce many seeds from which to select T1 transgenic plants. The majority of known *bri1* mutants are male sterile, explaining why all the activation-tagging screens for potential BR signaling were carried out in *bri1-5*, a weak BR receptor mutant with bri1-5 retained in the endoplasmic reticulum (ER) (Hong et al., 2008). Interestingly, the other known weak allele of *BRI1*, *bri1-9*, is also caused by ER retention of a mutated BR receptor (Jin et al., 2009, 2007). Suppressor screens using ethylmethanesulfonate (EMS) mutagenized *bri1-9* seeds led to identification of three identical mutations in BES1 (Zhao et al., 2002) and mutations in key components of the bri1-9 ER retention mechanism (Jin et al., 2009, 2007). The weakest, possibly the most interesting, known *bri1* mutant so far is *bri1-301* that carries a two-nucleotide change (GG-AT) in the *BRI1* gene, causing conversion of Gly989 to Ile in the cytoplasmic kinase domain of BRI1 (Xu et al., 2008). Interestingly, while the Gly989Ile mutation completely inhibits the *in vitro* kinase activity of a BRI1 fusion kinase, *bri1-301* can grow as tall and produce as many seeds as the wild-type control yet exhibits many characteristic phenotypes of known BR mutants, including a compact rosette with rounder leaves and short petioles (Xu et al., 2008). We expected that an activation-tagging-based suppressor screen in this mutant background might be able to uncover additional components/regulators of the BR signaling pathway.

It is well known that BRs act synergistically with another plant hormone auxin to promote plant growth and regulate plant development (Hardtke, 2007). Auxin is synthesized from tryptophan or its precursor via multiple biosynthetic routes (Chandler, 2009). One of the major breakthroughs in studying auxin biosynthesis was the discovery of the *Arabidopsis YUCCA* gene by activation tagging, which encodes a flavin monooxygenase-like protein catalyzing the hydroxylation of tryptamine, a key step in the tryptamine route (Zhao et al., 2001). The *Arabidopsis* genome encodes 11 *YUCCA* genes that can be grouped into three subfamilies: *YUCCA I*, *YUCCA IIA*, and *YUCCA IIB* (Cheng et al., 2006; Xia et al., 2009), and overexpression of *YUCCA3*, *4*, *5*, or *6* results in similar morphological phenotypes and elevated auxin levels (Kim et al., 2007; Marsch-Martinez et al., 2002; Woodward et al., 2005; Zhao et al., 2001). A previous study suggested that the auxin-overproduction *yucca* phenotype requires a functional BR receptor, since a *yucca bri1* double mutant still exhibits a dwarf phenotype (Nemhauser et al., 2004). Interestingly, neither auxin nor BR mutant was identified by genetic screens for mutants defective in the biosynthesis or response of the other hormone.

Here, we report isolation of six dominant *activation-tagged bri1 suppressors* (*atbs-Ds*) from a collection of 25,000 activation-tagged transgenic *bri1-301* lines. DNA blot analysis revealed that four of them carry a single T-DNA, with the other two containing two T-DNA insertions. Using a plasmid rescue approach, we recovered seven T-DNA/genome junction fragments and discovered that the *bri1-301* suppression phenotype in four *atbs-D* mutants was caused by overproduction of one of the three closely related members of the YUCCA family. Our discoveries thus provided additional genetic support for the interaction between the plant steroid hormone and auxin. These *atbs-D* mutants will be excellent tools in studying the BR signaling process and the biochemical mechanisms of the BR/auxin interaction.

RESULTS AND DISCUSSION

The Full-Length bri1-301 Is a Kinase-Dead Protein

Among all studied alleles of the BR receptor gene *BRI1*, *bri1-301* is one of the weakest alleles (Figure 1A). A recent study, using an *E. coli*-expressed fusion protein between glutathione-*S*-transferase and the cytoplasmic kinase domain of BRI1, showed that the *bri1-301* mutation completely inactivates the kinase activity of the BR receptor *in vitro* (Xu et al., 2008). To test whether this was just an artifact of the *E. coli*-expressed fusion protein, we decided to use our previously established yeast expression system, which was used to show that activation of the full-length receptor kinases of BRI1 and

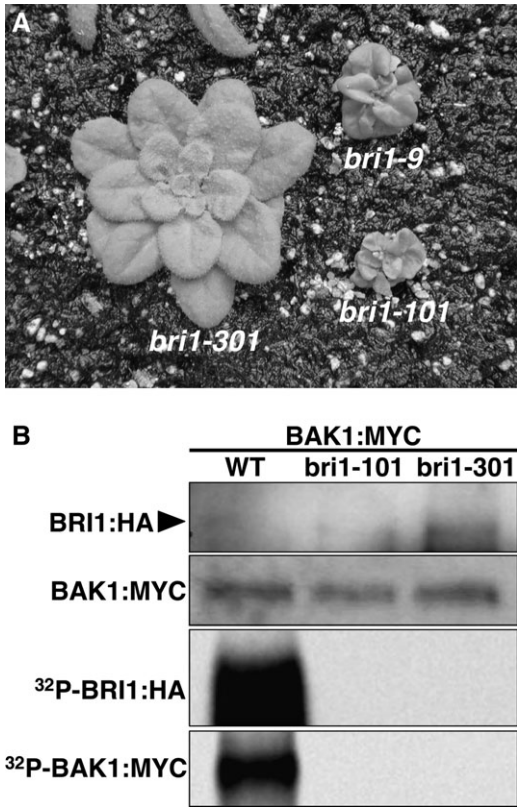

Figure 1. *bri1-301* Is a Weak *bri1* Allele but the Mutation Inhibits BRI1 Kinase Activity.

(A) Shown here are 4-week-old soil-grown plants of three allelic *bri1* mutants.

(B) *In vitro* phosphorylation assay of immunoprecipitated BRI1/BAK1 complex. Expression of full-length HA-tagged BRI1 and MYC-tagged BAK1 in yeast cells was monitored by immunoblot analysis with anti-HA and anti-MYC antibodies, respectively. The BRI1/BAK1 complex immunoprecipitated with anti-HA antibody was incubated in 50 μL kinase assay buffer with [γ-^{32}P]-ATP at 25°C for 30 min, separated by SDS-PAGE, and visualized by autoradiography.

its partner BAK1 requires their heterodimerization and transphosphorylation (Nam and Li, 2002). If one of the kinases was inactivated by single amino acid changes, no phosphorylation activity could be detected on either protein. As shown in Figure 1B, co-expression of wild-type BRI1 and BAK1 led to strong phosphorylation of the two full-length proteins. However, co-expression of the wild-type BAK1 with bri1-101 or bri1-301 resulted in no detectable phosphorylation on either receptor-like kinase. Our results thus confirmed that activation of BRI1 or BAK1 requires transphosphorylation by their partners and that the *bri1-301* mutation inhibits the kinase activity of BRI1. Although it remains to be tested whether bri1-301 is a kinase-dead BR receptor in *Arabidopsis*, *bri1-301* is certainly an interesting BR receptor mutant that can be used for an activation-tagging-based genetic screen to look for additional regulators of BR signaling.

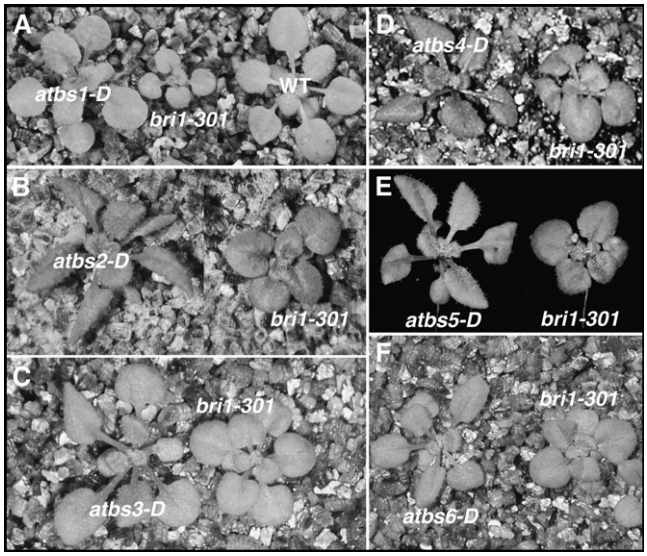

Figure 2. The Activation-Tagging Screen Discovered Six *atbs-D* Mutants.

(A–F) Shown here are 3-week-old soil-grown seedlings of *bri1-301* and 6 *atbs-D* mutants. Each *atbs-D*/*bri1-301* pair was grown under the exact same conditions.

Generation of 25 000 Activation-Tagged *bri1-301* Lines

We thus transformed a total of 10 trays (~150 plants/tray) of *bri1-301* mutants via a vacuum infiltration method with *Agrobacterium* strain GV3101 carrying the activation-tagging vector pSKI015 that contains the BASTA-resistance gene and the pUC19 plasmid sequence (Weigel et al., 2000). Seeds collected from infiltrated *bri1-301* plants were directly sown into soil and 2–4-week-old growing seedlings were sprayed with BASTA to select herbicide-resistant lines. A total of ~25,000 BASTA-resistant *bri1-301* plants were generated and screened for plants exhibiting wild-type-like rosette, leading to identification of six *activation-tagged bri1 suppressor-Dominant* (*atbs-D*) mutants (Figure 2A–2F).

DNA Blot Analysis of T-DNA Insertions in *atbs-D* Mutants

To determine how many copies of the activation-tagging T-DNA were inserted into each *atbs-D* mutant, total genomic DNAs isolated from these six *atbs-D* mutants and *bri1-301* were digested with either *Eco*RI or *Hind*III and hybridized with a ^{32}P-labeled pUC19 plasmid-derived DNA probe in several DNA blot experiments. Because both restriction enzymes cut the inserted T-DNA once near the left border while the pUC19 plasmid-derived fragment is near the T-DNA right border (Figure 3A), the hybridizing DNA fragments detected in each mutant must contain flanking genomic DNA of the *bri1-301*. As shown in Figure 3B–3E, four *atbs-D* mutants, *atbs1-D*, *2-D*, *5-D*, and *6-D*, contain a single hybridizing band, while both *atbs3-D* and *atbs4-D* contain at least two copies of the pUC19-carrying T-DNA inserts. Consistent with the DNA blot results, *atbs3-D* segregated out BASTA-resistant

bri1-301-looking plants in its T2 generation. However, no BASTA-resistant *bri1-301*-like seedlings were found in the T2 generation of *atbs4-D* (data not shown), suggesting that one of the inserted T-DNAs might be truncated and lack the intact BASTA-resistance gene that is located near the left border, since integration of a T-DNA into plant chromosomes is a polar process, with the right border integrating first.

Overexpression of *YUCCA8* in the *atbs3-D* Mutant

To understand the underlying biochemical mechanisms by which these *atbs-D* mutations suppress *bri1-301*, one has to know where the T-DNAs are inserted and which flanking gene(s) are overexpressed. Since each pUC19-hybridizing *Eco*RI or *Hind*III-digested fragment is smaller than 16 kb in size (Figure 3B–3E) and carries the pUC19 plasmid DNA with a bacterial replication origin and an ampicillin resistance gene (Weigel et al., 2000), we decided to use the plasmid rescue approach (Weigel et al., 2000) to recover the T-DNA flanking genomic DNA fragments. Consistent with the DNA blot result, two flanking genomic DNA fragments were rescued from *atbs3-D* (Figure 4A and 4B). Sequencing analysis of a rescued ~6.3-kb *Eco*RI fragment revealed that one T-DNA was inserted ~270 bp upstream of the predicted transcriptional initiation site of *At5g55050* that encodes a GDSL-motif lipase/hydrolase-like protein (Figure 4A). Based on the orientation of the inserted T-DNA, the 1.4-kb-long *35S* enhancer sequence was located ~80 bp upstream of the predicted TATA-box of *At5g55050* but was ~12 kb away from the predicted initiation site of its upstream neighbor *At5g55040* encoding a 145-amino-acid polypeptide of no known function. RNA blot analysis indicated that *At5g55050* was overexpressed in the *atbs3-D* mutant (Figure 4C); however, PCR-based genotyping analysis showed that this insertion was not co-segregated with the *atbs3-D* phenotype (Figure 4D), since the T-DNA-specific

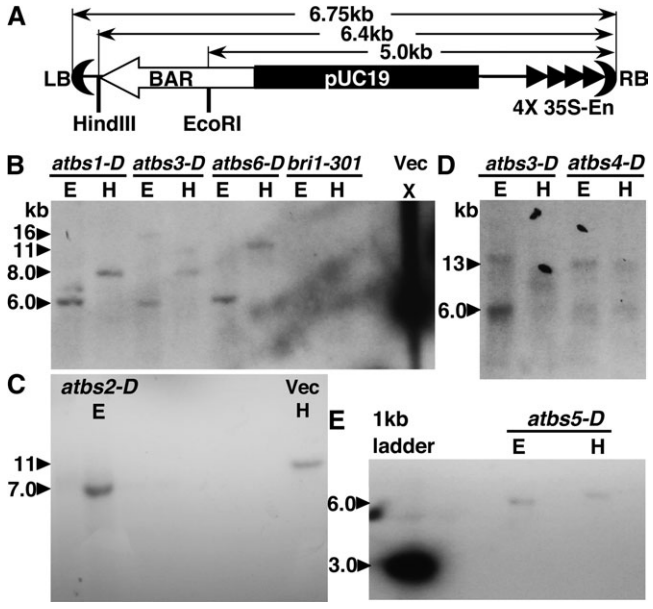

Figure 3. DNA Blot Analysis of T-DNA Insertions.

(A) Schematic presentation of the T-DNA being inserted into the *Arabidopsis* chromosomes. Black moon symbols the T-DNA borders, black bar represents the pUC19 plasmid sequence, open arrow indicates the BASTA-resistance gene, and 4 repeated arrows denote 4 copes of the *35S* enhancer element. Black vertical bars show the positions of EcoRI and HindIII restriction sites and the numbers indicate length of DNA fragments.

(B–E) Shown here are results of 4 independent DNA blot experiments. Total genomic DNAs were digested overnight with EcoRI or HindIII, separated by 0.8% agarose gel, transferred to nylon filter, hybridized with a ^{32}P-labeled probe of linearized pUC19 plasmid, and analyzed by autoradiography. Shown on the left of each panel are the positions and sizes of DNA molecular markers. E and H stand for EcoRI and HindIII, respectively, while Vec indicates vector control.

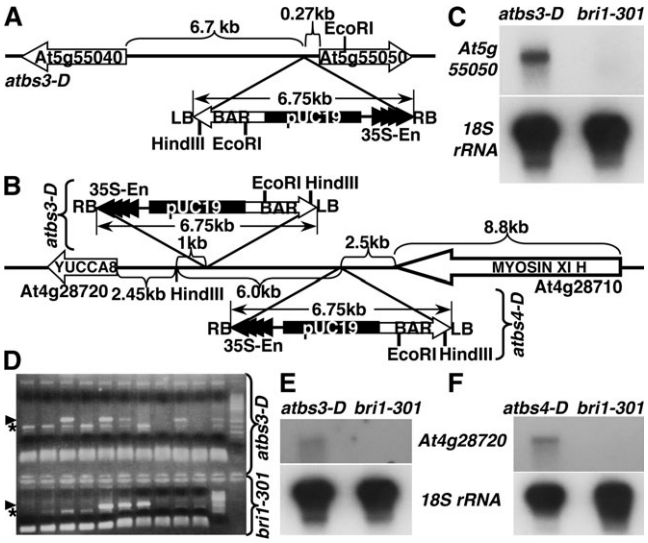

Figure 4. Molecular Analysis of T-DNA Insertions in *atbs3-D* and *atbs4-D* Mutants.

(A, B) Schematic presentation of T-DNA insertion sites on chromosomal DNAs represented by black lines. Open arrows indicate annotated genes with gene names shown inside and below the arrows. The big open triangle symbols the inserted T-DNA containing 4 copies of the *35S* enhancer (small black arrows), the pUC19 DNA (black bar) and the BASTA-resistance gene (open arrow). The numbers indicate the distances between T-DNA insertions and flanking genes or EcoRI/HindIII sites (indicated by short vertical bars).

(C, E, F) RNA blot analysis of T-DNA flanking genes. Total RNAs isolated from 4-week-old soil-grown plants were separated by agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-labeled probe of PCR-amplified fragment of a T-DNA flanking gene (upper panel) or a 18S rDNA fragment (lower panel), and analyzed by autoradiography.

(D) PCR-based segregation analysis of the T-DNA insertion near *At5g55050*. Total genomic DNAs isolated from 11 *atbs3-D*-like and 10 *bri1-301*-like T2 segregants of *atbs3-D* were used to amplify a T-DNA/genome junction fragment. The resulting PCR fragments were separated by agarose gel and visualized by ethidium bromide staining. Arrow indicates the amplified junction fragment while star denotes a nonspecific band.

junction fragment could be amplified from both *atbs3-D*-like and *bri1-301*-like T2 segregants of *atbs3-D*.

It is thus likely that the *atbs3-D* phenotype is caused by the other T-DNA insertion. Rescue and sequencing analysis of a ~8.0-kb *Hind*III fragment revealed that the second T-DNA was inserted ~3.5 kb upstream of the predicted initiation site of *At4g28720* and ~7.5 kb downstream of the predicted 3′-end of *At4g28710* (Figure 4B). The sequence data also indicated that the *35S* enhancer was located ~3.5 kb upstream of *At4g28720* but was ~22.6 kb downstream of the predicted initiation site of *At4g28710*. At4g28710 is annotated as the *Arabidopsis* MYOSIN XIH involved in actin-based movement (Avisar et al., 2009), while At4g28720 is known as a member (YUCCA8) of the *Arabidopsis* YUCCA family involved in tryptophan-dependent auxin biosynthesis (Cheng et al., 2006; Zhao et al., 2001). RNA blot analysis revealed that the *YUCCA8* gene was overexpressed in the *atbs3-D* mutant (Figure 4E), explaining its narrow and epinastic leaves also observed with other known *YUCCA*-overexpressing mutants (Kim et al., 2007; Marsch-Martinez et al., 2002; Woodward et al., 2005).

Identification of the Second T-DNA Insertion Near *YUCCA8* in *atbs4-D*

The effect of *YUCCA8* overexpression on *bri1-301* was confirmed by discovery of the T-DNA insertion site in *atbs4-D*. Sequencing analysis of a rescued ~12.5-kb *Hind*III fragment from *atbs4-D* revealed the presence of a T-DNA insertion between *At4g28710* and *At4g28720* with the *35S* enhancer sequence located ~8.5 kb upstream and ~16.6 kb downstream of the initiation sites of *YUCCA8* and *MYOSIN XIH* genes, respectively (Figure 4B). Consistent with our hypothesis of the second T-DNA being truncated, the two ~6-kb pUC19-hybridizing *Eco*RI/*Hind*III fragments seen on the DNA blot (Figure 3D) were not recovered in our repeated plasmid-rescue experiments (likely caused by truncation of the pUC19 plasmid) and both the ~12.5-kb *Hind*III and ~13-kb *Eco*RI fragments were derived from the first T-DNA insertion. As expected, RNA blot analysis showed that the *YUCCA8* gene was indeed overexpressed in *atbs4-D* (Figure 4F). The identification of two T-DNA insertions upstream of the *YUCCA8* gene in two independent *atbs-D* mutants argued strongly for the causal relationship between *YUCCA8* overexpression and the suppressed *bri1-301* phenotype.

Overexpression of Close Homologs of *YUCCA8* in Two Other *atbs* Mutants

The *bri1-301*-rescuing effect of an overexpressed *YUCCA* gene was further confirmed by recovery of flanking genomic DNA fragments from two additional *atbs-D* mutants, *atbs5-D* and *atbs6-D*. As shown in Figure 5A and 5B, sequencing analysis of a rescued ~7.7-kb *Eco*RI fragment from *atbs5-D* revealed that the *35S* enhancers were located ~3.3 kb upstream and ~18.6 kb downstream of the predicted transcription sites of *At5g43890* and *At5g43900* genes, respectively, while the sequence information of a rescued ~7.2-kb *Eco*RI fragment from *atbs6-D* predicted that the *35S* enhancers were located ~5.5 kb upstream and ~17 kb downstream of initiation sites of *At1g04610* and *At1g04600* genes, respectively. Interestingly, the genes closer to the *35S* sequence of the inserted T-DNAs encode close homologs of YUCCA8, YUCCA5 [also known as SUPER1 (Woodward et al., 2005)] in *atbs5-D*, and YUCCA3 in *atbs6-D*, while the other flanking genes far away from the *35S* enhancers encode homologs of MYOSIN XIH, MYOSIN XI 6 in *atbs5-D*, and MYOSIN XIA in *atbs6-D*. Consistent with locations of the *35S* enhancers, RNA blot analysis showed that *YUCCA5* and *YUCCA3* genes were overexpressed in *atbs5-D* and *atbs6-D*, respectively. Taken together, our study demonstrated that overexpression of a *YUCCA* gene was able to suppress the compact rosette phenotype of the weak BR receptor *bri1-301* and provided additional genetic support for the well known interaction between BR and auxin. Our discoveries coupled with a previous finding that *YUCCA* overexpression was not able to suppress a severe *bri1* dwarf mutant (Nemhauser et al., 2004) strongly support an earlier hypothesis that auxin response requires a functional BR signaling pathway.

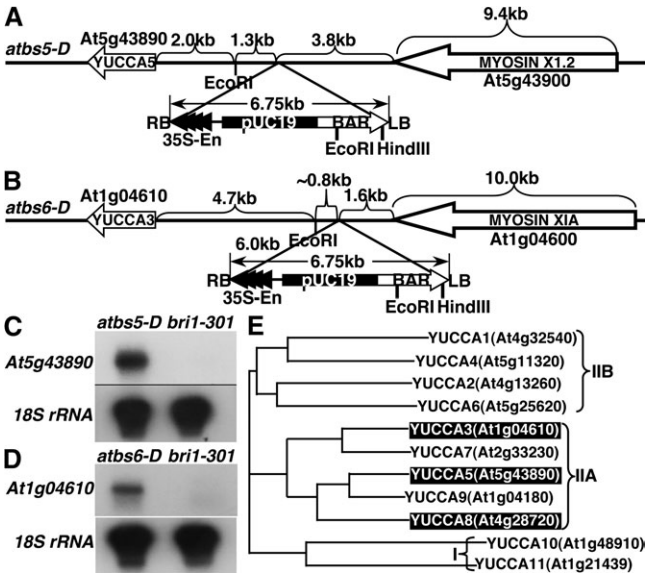

Figure 5. Molecular Analysis of T-DNA Insertions in *atbs5-D* and *atbs6-D* Mutants.

(A, B) Schematic presentation of T-DNA insertion sites on chromosomal DNAs in *atbs5-D* (A) and *atbs6-D* (B).

(C, D) RNA blot analysis of *YUCCA* gene expression. Total RNAs isolated from 4-week-old soil-grown *atbs5-D* (C) or *atbs6-D* (D) were separated by agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-labeled probe of PCR-amplified *YUCCA* fragment (upper panel) or a 18S rDNA fragment (lower panel), and analyzed by autoradiography.

(E) Phylogenetic tree of 11 *Arabidopsis* YUCCA proteins. Sequence alignment and phylogenetic analysis were conducted with the Clustal W2 program at http://www.ebi.ac.uk/Tools/clustalw2/index.html. The 3 YUCCAs identified in this study were shaded black.

It is interesting to note that the three *YUCCA* genes that were tagged in the four *atbs-D* mutants are clustered together with two other *YUCCA*s, *YUCCA7* (*At2g33230*) and *YUCCA9* (*At1g04180*), in phylogenetic analysis, forming the *YUCCA IIA* subfamily out of 11 *Arabidopsis YUCCA* genes that were previously classified into three groups: *YUCCA I*, *YUCCA IIA*, and *YUCCA IIB* (Cheng et al., 2006; Xia et al., 2009) (Figure 5E). Besides sequence similarity, the five *YUCCA IIA* genes all have a *MYOSIN XI*-type gene as their neighbors on chromosomes. One of the differences between the three tagged *YUCCA IIA* genes and two other *YUCCA IIA* genes is the presence of additional genes between *YUCCA* and *MYOSIN*: *At2g33233* between *YUCCA7* and *MYOSIN XID* and *At1g04170* plus *At1g04171* between *YUCCA9* and *MYOSIN XIB*. It is thus quite possible that an insertion of the activation-tagging T-DNA could produce other morphological phenotypes that would prevent their discovery as activation-tagged *bri1* suppressors. This likely explanation might also be responsible for our failure to tag other *YUCCA* genes in our genetic screen, although the activation-tagged *YUCCA6* (also known as HYPERTALL1) was shown to produce long, narrow leaves with elongated petiole (Kim et al., 2007)—a phenotype often associated with enhanced BR signaling (Nam and Li, 2002; Yan et al., 2009).

Molecular Analysis of T-DNA Insertions in *atbs1-D* and *atbs2-D* Mutants

Our plasmid rescue experiments also identified the T-DNA insertions in both *atbs1-D* and *atbs2-D* mutants. As shown in Figure 6A, the *35S* enhancer-carrying T-DNA was inserted 5.9 and 1.5 kb upstream of *At1g74500* and *At1g74510*, respectively, with four copies of the *35S* enhancer pointing towards the predicted start codon of *At1g74500*. RNA blot analysis using probes derived from the two flanking genes indicated that *At1g74500* was overexpressed in *atbs1-D* (Figure 6C and 6D), suggesting that the suppressor phenotype is likely caused by *At1g74500* overexpression. Interestingly, *At1g74500* encodes a 93-amino-acid basic helix-loop-helix (bHLH) protein that is similar in sequence to the rice BRASSINOSTEROID UPREGULATED 1 that was recently implicated in BR signaling (Tanaka et al., 2009). Further experiments are needed to confirm whether *At1g74500* is indeed the *ATBS1 (activation-tagged bri1 suppressor 1)* gene and, if so, to investigate how this small bHLH protein regulates BR signaling in *Arabidopsis*.

Figure 6B shows that the T-DNA in *atbs2-D* was inserted into a region on chromosome 2 that contains *DET2* encoding a steroid 5α-reductase catalyzing a critical step of BR biosynthesis (Li et al., 1997, 1996). However, the clustered *35S* enhancers are located very close to the TATA-box of the annotated *At2g38070* gene encoding a member of the DUF740 family, but is ~11.8 kb away from the *DET2* transcriptional initiation site. Besides, there is another coding sequence between the T-DNA and *DET2*. Therefore, it is unlikely that the *atbs2-D* phenotype was caused by *DET2* overexpression. Indeed, RNA blot analysis indicated that the *DET2* expression level was not affected by the T-DNA insertion (Figure 6E). Further experiments are underway to investigate the underlying mechanism by which the *atbs2-D* mutation suppresses the weak BR receptor mutant.

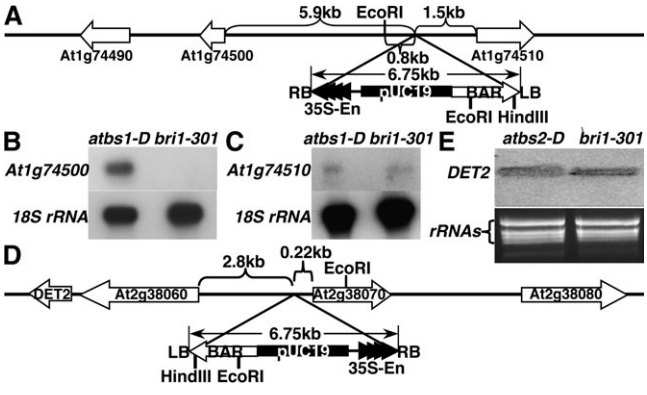

Figure 6. Molecular Analysis of T-DNA Insertions in *atbs1-D* and *atbs2-D*.

(A, B) Schematic presentation of the T-DNA insertion in *atbs1-D* (A) and *atbs2-D* (B).

(C–E) RNA blot analysis of *At1g74500* (C), *At1g74510* (D), and DET2 (E). Total RNAs isolated from 4-week-old soil-grown mutants of *bri1301*, *atbs1-D*, or *atbs2-D* were separated by agarose gel, stained with ethidium bromide for loading control (lower panel in E), transferred to nylon membrane, and hybridized with a ^{32}P-labeled cDNA fragment of *At1g74500*, *At1g74510*, *DET2* or 18S rDNA (used as a loading control in C and D), and analyzed by autoradiography.

METHODS

Plant Materials and Growth Condition

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control for phenotypic comparison, while *bri1-301* (Xu et al., 2008) was used as the starting genetic materials for the activation-tagging suppressor screen. Seed germination and plant growth conditions were described previously (Li et al., 2001b).

Generation of Activation-Tagged *bri1-301* Transgenic Plants

The activation-tagging plasmid pSKI015 was previously described (Weigel et al., 2000) and transformed into GV3101 *Agrobacterium tumefaciens* cells. Due to instability of the clustered *35S* enhancers in *Agrobacterial* cells when kept at 4°C, the pSKI015 plasmid was freshly transformed into competent GV3101 cells for each plant transformation experiment. *Agrobacterial* cells of stationary phase were collected by centrifugation at 5,000 *g* at 4°C, re-suspended in 3 vol. of liquid half-strength Murashige and Skoog (MS) medium containing 2% sucrose, 10 μg L^{-1} benzylaminopurine, and 0.15% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX), and used to transform 6–7-week-old flowering *bri1-301* mutants via the vacuum infiltration method (Bechtold and Pelletier, 1998). Seeds collected from infiltrated *bri1-301* plants were sterilized as

previously described (Li et al., 2001b), mixed with 0.08% (w/v) Phytagar (Invitrogen), stored at 4°C for 48 h, and sown directly into soil. Transgenic *bri1-301* mutants were selected by spraying Finale (AgrEvo, Montvale, NJ) at 1:1000 dilution once every 3 d for half a month and visually inspected for plants exhibiting wild-type-like morphology. A potential *atbs-D* mutant was PCR-genotyped to eliminate any pollen or seed contaminant using the following CAPS primers: 5′-CTGGCGATAGAACTGCTAAC-3′ and 5′-GCTGTTTCACCCATCCAAC-3′ (the restriction enzyme *Dpn*II cuts the PCR fragment from wild-type twice but cuts that from *bri1-301* only once).

Plasmid Rescue to Recover the Flanking Genomic DNA of T-DNA Insertions

Approximately 1 μg total genomic DNA was digested overnight at 37°C in a 40-μL reaction with *Eco*RI or *Hind*III. After denaturing enzymes by 10 min heating at 65°C and phenol/chloroform extraction, the digested DNAs were ethanol precipitated and ligated overnight in a 200-μL reaction volume with 80 U T4 DNA ligase (New England Biolabs) at 16°C. Ligated DNAs were extracted with phenol/chloroform, precipitated with ethanol, re-suspended in water, and transformed into *E. coli* competent cells by electroporation with a Micro-Pulser Electroporator (BIO-RAD) following the manufacturer's suggested protocol. Electroporated cells were selected on ampicillin-containing medium; and the rescued plasmids were purified using the Qiagen's Plasmid Mini Kit, sequenced at the University of Michigan's sequencing core facility, and compared with the published *Arabidopsis* genome to determine T-DNA/genome junctions.

Nucleic Acid Analyses

Total genomic DNAs were isolated either by the Qiagen's DNeasy Plant Mini Kit according to the manufacturer's recommended protocol (for DNA blot analysis and plasmid rescue) or by a previously described 'miniprep' method (Li and Chory, 1998) for genotyping analysis. Approximately 0.5-μg genomic DNAs of *bri1-301* or an *atbs-D* mutant was digested with *Eco*RI or *Hind*III overnight at 37°C and separated by 0.8% agarose gel. A previously described protocol (Lincoln et al., 1990) was used to transfer DNAs to Hybond-N membrane (Pharmacia) and hybridized with a ^{32}P-labed probe derived from the pUC19 plasmid. Total RNAs were isolated from shoots of 4-week-old soil-grown plants using the Qiagen's RNeasy Plant Mini Kit according to the manufacturer's recommended procedure and analyzed by Northern blot following a previously described protocol (Li et al., 2001b) and a ^{32}P-labeled DNA probe amplified from a gene of interest.

Yeast Expression and Phosphorylation Assay

The plasmids *pYES2-BRI1:HA* and *pESC-BAK1:MYC* and detailed methods for expressing the full-length BRI1 and its co-receptor BAK1 in yeast cells, immunoprecipitating the BRI1/BAK1 complex, extraction of total yeast proteins, and immunoblot analysis of BRI1 or BAK1 were previously described (Nam and Li, 2002). PCR-based site-directed mutagenesis was conducted to introduce *bri1-101* or *bri1-301* mutation into *pYES2-BRI1:HA* using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The *in vitro* phosphorylation assay using immunoprecipitated BRI1/BAK1 proteins was performed as previously described (Nam and Li, 2002).

Accession Number

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: NP_194980 (YUCCA1, At4g32540), NP_193062 (YUCCA2, At4g13260), NP_171955 (YUCCA3, At1g04610), NP_850808 (YUCCA4, At5g11320), NP_199202 (YUCCA5, At5g43890), NP_197944 (YUCCA6, At5g25620), NP_180881 (YUCCA7, At2g33230), NP_194601 (YUCCA8, At4g28720), NP_171914 (YUCCA9, At1g04180), NP_175321 (YUCCA10, At1g48910), and NP_173564 (YUCCA11, At1g21430).

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