Steroid hormones are conserved between animals and plants as signaling molecules to control growth and development. Plant steroid hormones, brassinosteroids (BRs), appear to play an important role in plant cell elongation. BRs bind to leucine-rich repeat kinase BRASSINOSTEROID-INSENSITIVE 1 (BRI1) localized to the plasma membrane, activate transcription factors in collaboration with cytosolic kinases and phosphatases, and regulate BR-responsive gene expression, but the details regarding the BR signaling pathway from perception to nuclear events remain unknown. In this study we used chemical genetics to identify an evolutionarily conserved transmembrane protein, Brz-insensitive-long hypocotyls 4 (BIL4), and demonstrated its role as a critical component of plant cell elongation occurring upon BR signaling. A dominant mutation, bil4-1D, showed cell elongation in the presence of the BR-specific inhibitor Brz. Brz suppresses expression of the BIL4 gene in wild-type plants, and overexpression of BIL4 in bil4-1D suppresses the BR deficiency caused by Brz. Our results indicate that BIL4 mediates cell elongation on BR signaling.

Key words: brassinosteroid; Brz; signaling; seven-transmembrane domain protein; cell elongation

Brassinosteroids (BRs) are plant steroid hormones that regulate plant growth and development. In dicots, deficiencies in BR biosynthesis or signal transduction cause de-etiolated hypocotyls and opened cotyledons upon germination in the dark, and dwarfism with shortened leaves and stems after growth under light. Moderate activation of BR biosynthesis or signal transduction promotes hypocotyl and stem elongation and outward curling of the leaf. These phenotypes due to BR up-regulation result due to a failure to regulate normal cell elongation of the stem and leaf sheaths.

In cell elongation, BRs are recognized by BRASSINOSTEROID INSENSITIVE 1 (BRI1), a leucine-rich repeat receptor-like serine/threonine kinase (LRR-RLK) that localizes to the plasma membrane. Activation of BRI1 kinase by BR binding induces dimerization with and activation of another LRR receptor kinase, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), and causes disassociation of BRI1 KINASE INHIBITOR 1 (BK1). BR activation of receptor kinases is assumed to transduce intracellular signals and to inhibit BRASSINOSTEROID INSENSITIVE 2 (BIN2), a glycogen synthase kinase 3-like kinase, or to activate phosphatase bril-5 SUPPRESSOR 1 (BSU1). BIN2 and BSU1 control the phosphorylation status of the transcription factors BRASSINAZOLE RESISTANT 1/Brz-INSENSITIVE-LONG HYPOCHOTYL 1 (BZR1/BIL1) and bril-EMS-SUPPRESSOR 1 (BES1), which regulate the various target genes. In BR signaling, the mechanism of action occurring between the receptor on the plasma membrane and the transcription factors has not previously been elucidated.

Here we report the isolation and characterization of an Arabidopsis mutant, Brz-insensitive-long hypocotyls 4.
1D (bil4-1D), from activation tagging mutant lines. This mutant was found to be insensitive to the BR biosynthesis inhibitor Brz during dark germination and it also displays a slender dwarf phenotype similar to the wild type exposed to excessive stimulation with BRs. We identified the BIL4 gene encoding a novel seven transmembrane domain protein, and found that overexpression of it caused cell elongation in the presence of Brz. The possible functions of BIL4 as a positive regulator of plant cell elongation via BR signaling are discussed.

Materials and Methods

Plant materials and growth conditions. Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type plant. Seeds were germinated on medium containing 1/2 Murashige and Skoog (MS) medium (Duchefa) and 0.8% phytoagar (Duchefa, Haarlem, The Netherlands) with 1.5% sucrose, and were transferred to soil. Plants were grown at 22 °C under white light (a 16 h light/8 h dark cycle for long-day conditions).

Screening for bil4-1D mutants. Approximately 10,000 of the RIKEN GSC Arabidopsis activation tagging lines39 were screened on 1/2 MS medium with 3 µM Brz.39 After growth for 7 d in the dark, seedlings with hypocotyls longer than the controls were identified and transferred to the soil. Heterozygous bil4 plants were used in all the experiments described in this paper. TAIL-PCR was used to amplify the flanking genomic sequences of the T-DNA of pPCVICE4HPT, as described previously.39 Total RNA was extracted from 8-d-old seedlings of wild-type and plants with an RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized with PrimeScript (Takara), and was used as the RT-PCR template. Quantitative real-time PCR was performed according to the instructions provided for the Thermal Cycler Dice (Takara) with the SYBR Premix ExTaq system (Takara). The sequences of gene-specific primers for RT-PCR were as follows: BIL4, 5′-CTCTACCCGATGTAGTGCGG-3′ and 5′-CTAATATCACAAGCCTTGA-3′; the constitutively expressed control gene ACT2 (At3g18780), 5′-AAGGCTGGATTTGCAGGA-3′ and 5′-TCACGTCCAGCAAGGT-3′.

Generating transgenic plants. In the recapitulation of the bil4-1D phenotype, BIL4 cDNA was amplified from Arabidopsis Col-0 cDNA with primers for BIL4-Forward 5′-CACCATGGAATCAGACGCGAAGCAG-GAG-3′ and BIL4-Reverse 5′-CTAATATCACAAGCCTTGAAG-3′, and cloned into pENTR/D-TOPO (Invitrogen). The pENTR-BIL4 obtained was further cloned into binary vector pGWB240 containing a CaMV 35S promoter by a Gateway strategy. The resulting p35S-BIL4 construct was transformed into Col-0 by the floral dipping method. Transgenic plants were screened on 1/2 MS medium with 25 mg/L of kanamycin.

Quantitative real-time PCR. Total RNA was extracted from wild-type plants with an RNaseasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized with PrimeScript (Takara), and was used as the RT-PCR template. Quantitative real-time PCR was performed according to the instructions provided for the Thermal Cycler Dice (Takara) with the SYBR Premix ExTaq system (Takara). The sequences of gene-specific primers for RT-PCR were as follows: BIL4, 5′-CGGCCATCCAAGCTGTTCTC-3′ and 5′-GCACGGACGGCGAATCTTT-3′, and for the constitutively expressed control gene ACT2, 5′-CCGATACCAAGCGTTCCTC-3′ and 5′-TCACGTCACCGACGTCAAAGCT-3′.

Results

Isolation and characterization of the bil4-1D mutant

Brz is a triazole compound that directly binds to the cytochrome P450 steroid C-22 hydroxylase encoded by the DWARF4 (DWF4) gene and specifically inhibits BR biosynthesis.19,22 Brz treatment reduces the BR content of plant cells and has allowed investigation of the functions of BRs in a variety of plant species.13 Because mutants that are insensitive to Brz can activate BR-signaling and BR-biosynthesis, we screened Brz-insensitive-long hypocotyl (bil) mutants in Arabidopsis. We have isolated the bzl1/bil1 mutant from EMS-mutation lines, and identified BZL1/BIL1 with a bHLH transcription factor that acts as a positive regulator in BR signaling.11,12 In the case of the bzl1/bil1 mutant, the mutation promoted stability of the BZL1/BIL1 protein, and the stabilized protein up-regulated BR-signaling, which allowed insensitivity to Brz. A technique called activation tagging can overcome genetic redundancy and the embryonic lethality that prevents genetic analysis based on loss-of-function mutants.18 In a study of BR signaling, a BSU1 phosphatase was identified from activation tagging of the BR receptor bril mutant.30 We exposed this activation tagged line to Brz in order to investigate a novel component of BR signaling.

In the dark, wild-type plants are typically etiolated with an elongated hypocotyl and a closed cotyledon. Treatment with Brz, which inhibits elongation of the hypocotyls and enhances opening of the cotyledon in the wild type, is termed de- etiolation in the dark. Under these growth conditions, we screened approximately 10,000 Arabidopsis activation tagged lines and isolated a semi-dominant mutant, Brz-insensitive-long hypocotyls 4-1D (bil4-1D). bil4-1D mutant seedlings showed longer hypocotyls and closed cotyledons when grown in the dark on a medium containing Brz (Fig. 1). In the dark without Brz, the hypocotyls of bil4-1D plants were elongated normally and were similar in size to the wild type. After growing for 2–3 weeks under light, bil4-1D displayed narrow, outward-curving leaves (Fig. 2A, B) that resembled plants overexpressing BR11 or BSU13 in which BR-signaling is up-regulated. The shape of the bil4-1D leaves was extremely constrained, and total leaf expansion was suppressed (Fig. 2B). In wild-type plants treated with Brz, moderate concentrations caused moderate leaf expansion, but excessive amounts (10–100 µM) caused inhibition of leaf expansion concomitantly with the outward curving and narrow shape (Fig. 3). The leaf shape of bil4-1D resembled that of the wild type exposed to excessive concentrations of BR. After growing for 42 d at termination of flowering, there were 2- to 2.5-fold more inflorescences and branches than in the wild type (Fig. 2C), similarly to an overexpressor of the BL biosynthesis gene, DWF4.23 At termination of flowering, the height of the bil4-1D plants was approximately 54% lower than that of the wild type (Fig. 2D). This phenotype was also observed in plants exposed to excessive BR (Fig. 3). In the bil4-1D plants, the additional branching can be associated with slight increases in the number of siliques per plant (Fig. 2E), but did not lead to increases in the number of seeds per plant (Fig. 2F). The decrease in the number of seeds was due mainly to a defect in seed development, since siliques length decreased less than in the wild type (Fig. 2G).

Identification and characterization of the BIL4 gene

Co-segregation of the Brz-insensitive phenotype with the selection marker and TAIL-PCR indicated a T-DNA
insertion in an intergenic region at the end of chromosome III in bil4-1D (Fig. 4A). The At3g63310 gene, located approximately 2.7 kbp upstream of the T-DNA insertion, was overexpressed (Fig. 4B). The expressions of At3g63320 and At3g63330 in bil4-1D were identical to the wild type (data not shown). To confirm that overexpression of At3g63310 caused the bil4-1D phenotype, the At3g63310 coding region was placed immediately downstream of the CaMV 35S promoter and transformed into wild-type Arabidopsis. The BIL4 overexpressor obtained (BIL4-OX) showed longer hypocotyls and closed cotyledons when grown in the dark on medium containing Brz (Fig. 4C, D). In plants grown in soil under light, BIL4-OX exhibited outwardly curving, narrow leaves and shorter, thinner inflorescences than the wild type, similarly to the bil4-1D mutant (Fig. 4E). Hence we referred to At3g63310 as BIL4, a possible positive regulator of BR responses.

BIL4 encodes a novel protein that is perhaps a membrane protein with seven transmembrane domains and an extramembrane N-terminal domain as determined by transmembrane structure predictions (Fig. 5A). Four homologs of BIL4 were present in Arabidopsis: At1g03070, At4g02690, At4g15470, and At4g14730 (Fig. 5B). BLAST searches of the BIL4 amino acid sequence identified similar genes in rice (Oryza sativa), tomato (Lycopersicon esculentum), red pepper (Capsicum chinense), barley (Hordeum vulgare), maize (Zea mays), sitka spruce (Picea sitchensis), poplar (Populus trichocarpa), and moss (Physcomitrella patens; Fig. 5C). These BIL4 homologs were also predicted to be seven transmembrane domain proteins, suggesting
evolutionary conservation of BIL4 in most plants (Fig. 5B). These genes had not previously been analyzed or described in detail.

**Brz decreased expression of BIL4 mRNA**

To examine the possible functions of BIL4 in plant development, the spatial and temporal expression patterns of **BIL4** were analyzed by real-time PCR for each organ. **BIL4** was expressed in many organs, including leaf, root, inflorescence, flower, and light- and dark-grown seedlings (Fig. 6A). Each organ of the **bil4-1D** mutant and **BIL4-OX** showed an abnormal phenotype in comparison to the wild type, indicating that **BIL4** plays important roles in many plant organs.

To examine the molecular function of **BIL4** in brassinosteroid signaling, we analyzed the effect of **Brz** treatment on **BIL4** expression. When wild-type **Arabidopsis** germinated in the dark for 7 d were treated with 3 μM **Brz** for 3 h, the expression level of **BIL4** was the same as in the wild type without **Brz** (Fig. 6B). When wild-type **Arabidopsis** were grown in the dark and continuously treated with 3 μM **Brz** for 14 d, the plants clearly demonstrated a de-etiolated-in-the-dark phenotype, and the expression of **BIL4** decreased.

**Discussion**

In the first decade of molecular biological **BR** research, **BR**-deficient mutants of **Arabidopsis** played an important role in the exploration of unknown **BR** mechanisms of action. The identification and characterization of **Arabidopsis** **BR**-biosynthesis mutants such as **de-etiolated 2** (**det2**) and **dow4** has revealed the importance of **BRs** in regulation of plant growth. These **BR** biosynthesis-deficient mutants have a pleiotropic dwarf phenotype that can be recovered to a wild-type phenotype by feeding of **BR**. The **Arabidopsis** **bri1** mutant displays a pleiotropic dwarf phenotype, including root elongation, that is insensitive to the inhibition caused by excessive treatment with **BR**. **BR11** is a member of the LRR receptor kinase family, and it plays an important role in **BR**-signaling via its direct binding with **BR**. Although these findings were very important to **BR** research, the **BR**-related genes identified by analysis of **BR**-deficient mutants were limited to **BR**-biosynthesis genes, the **BR**-receptor **BR11**, and only two other **BR**-insensitive mutants. Factors downstream of **BR** signaling after binding with **BR11** were not revealed by the **BR**-deficient-mutants. The next step in **BR** signaling research was to find and analyze suppressor mutants that suppress **BR**-deficiency, as these suppressors can be activated during **BR** signaling.

**Brz** is a specific inhibitor of **BR** biosynthesis. It can bind directly to the **DWF4** enzyme, a cytochrome P450 monooxygenase that catalyzes the 22-hydroxylation of the **BR** side chains, through the triazole base of **Brz**. Finally, **Brz** treatment reduces **BR** content in plant cells. We thought that using **Brz** to screen genetically for mutants showing resistance against these inhibitors might identify new aspects of **BR** signaling transduction. It is possible to alter the **BR**-deficient condition from low to high for fine screening, sometimes without **Brz**. Furthermore, it is possible to use the mutant normal condition in cases of backcrossing and for mapping. Hence this method has advantages over mutant screening using **BR**-deficient mutants as a background.

We applied this chemical biology approach using **Brz** to isolate new mutants that show activation of **BR**-signaling. Compared to the wild type, the **bil4-1D** mutant plants isolated from activation tagging lines and grown in the dark displayed the longer hypocotyls characteristic of cell elongation on medium containing...
Brz. *bil4-1D* presented as a slender dwarf plant with outward curving, narrow leaves similar to those of plants treated with excessive amounts of BL. These results suggest that BIL4 is involved in cell elongation through BR signaling. In addition, increases in the number of inflorescences and branches were observed in the *bil4-1D* mutant. Consistently with this, the BR-biosynthesis enzyme *DWF4* overexpressed transformant AOD4 (*Arabidopsis* plant ectopically overexpressing *DWF4*) also exhibited increased branches. Root elongation in the *bil4-1D* was also extremely inhibited. This result suggests that BIL4 is also involved in aspects of the downstream BR signaling pathway.

Expression of the *BIL4* gene occurs at comparatively high levels in the inflorescence and root, but expression was also found in other organs, including the leaf, flower, and hypocotyl, when the plant was grown in the dark and under light. Since all organs of the *bil4-1D*...
mutant were abnormal in comparison to the wild type, these results indicate that BIL4 plays an important and ubiquitous role in many plant organs. When wild-type hypocotyls germinated in the dark for 7 d were treated with Brz for a short period, 3 h, BIL4 expression followed the same pattern as the wild type without Brz. When wild-type plants were grown and stimulated by Brz continuously in the dark for 14 d, the expression of BIL4 was clearly lower in the wild type exposed to Brz than in the wild type without Brz exposure. This result indicates that BIL4 involvement in BR signaling is a comparatively slow process. In addition, the result can indicate that the molecular mechanism of action of bil4-1D and BIL4-OX is similar to a Brz–insensitive plant, since overexpressed BIL4 and the associated downstream events can resist the suppression of BIL4 by Brz. These results indicate the advantage of a chemical biology approach using Brz. Chemical biological analysis with Brz can be compared to wild-type plants in a BR-deficient condition for a few hours or a few weeks in parallel. Thus this approach successfully revealed the unknown molecular mechanism of BIL4 action to be a slow effective factor in BR-signaling. These analyses could not have been performed by double-cross analysis between a BR-signaling mutant and a BR-deficient mutant.

BIL4 encodes a seven-transmembrane-domain protein. BIL4 homologs are highly conserved among diverse plant groups, including dicots such as tomato (Lycopersicon esculentum) and red pepper (Capsicum chinense), monocots such as rice, barley, and maize (Zea mays), trees, including the Sitka spruce (Picea sitchensis) and poplars (Populus trichocarpa), and mosses (Physcomitrella patens). This suggests an important role for the BIL4 gene family in the plant kingdom, although functional analyses of all BIL4 homologs have not yet been reported. Arabidopsis BIL4 and the homologous sequences At1g03070 and At4g02690 were predicted to localize to the plasma membrane, ER, or vacuolar membrane by subcellular localization predicting programs such as WoLF PSORT (http://wolfpsort.seq.cbrc.jp/). The BR receptors BRI1 and BAK1 are well known as transmembrane proteins on the plasma membrane. Another transmembrane protein thought to be involved in BR-signaling is DET3. det3 is a dwarf mutant with a de-etiolated phenotype when grown in the dark that is also insensitive to BL applications and shows organ-specific defects in cell elongation. DET3 encodes the large C subunit of V-ATPase. These results suggest that not only the plasma membrane, but also vacuolar membranes, are linked to and regulated by cell elongation through unknown mechanisms involving BR-signaling. BIL4 can have a critical function in BR signaling in this system. The evolutionary conservation of BIL4 homologs in the plant kingdom indicates that BIL4 plays an important role as a component of BR signaling in cell elongation, which is required for plant development.

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