Evolutionarily conserved BIL4 suppresses the degradation of brassinosteroid receptor BRI1 and regulates cell elongation

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Brassinosteroids (BRs), plant steroid hormones, play important roles in plant cell elongation and differentiation. To investigate the mechanisms of BR signaling, we previously used the BR biosynthesis inhibitor Brz as a chemical biology tool and identified the Brz-insensitive-long hypocotyl4 mutant (bil4). Although the BIL4 gene encodes a seven-transmembrane-domain protein that is evolutionarily conserved in plants and animals, the molecular function of BIL4 in BR signaling has not been elucidated. Here, we demonstrate that BIL4 is expressed in early elongating cells and regulates cell elongation in Arabidopsis. BIL4 also activates BR signaling and interacts with the BR receptor brassinosteroid insensitive 1 (BRI1) in endosomes. BIL4 deficiency increases the localization of BRI1 in the vacuoles. Our results demonstrate that BIL4 regulates cell elongation and BR signaling via the regulation of BRI1 localization.

Steroid hormones are bioactive substances that are widely conserved in eukaryotes1 and serve as signaling molecules to control growth and development. The plant steroid hormones known as brassinosteroids (BRs) play important roles in plant development and in responses to environmental cues, such as cell elongation, cell division, xylem development, stresses and pathogen resistance2-6. BRs are recognized by the BR receptor brassinosteroid insensitive 1 (BRI1), a Ser/Thr kinase, which has a leucine-rich repeat (LRR) domain and resides on the plasma membrane and endosomes. The dwarf phenotype of BRI1-deficient mutants (bri1)7 suggests that BRI1 plays an important role in plant growth. The plasma membrane proteins BRI1-associated receptor kinase 1 (BAK1)8, 9, BR-signaling kinase 1 (BSK1)10, and BRI1 kinase inhibitor 1 (BKI1)11 are involved in BR signaling by associating with BRI1.

BRI1 is localized not only to plasma membranes but also to endosomes12. The requirement that ligand-bound receptors, including innate immunity-related Toll-like receptors (TLRs), be localized to endosomes has been widely observed in animals13. BRI1 endocytosis is regulated by the guanine nucleotide exchange factor for ADP-ribosylation factor (ARF-GEF) GNOM14, adaptor protein complex-2 (AP-2)15, and plant-specific TPLATE adaptor complex (TPC), which is associated with key components of clathrin-mediated endocytosis (CME)16. BRI1 is considered to translocate to the vacuoles through processes that are regulated by ubiquitination17 and

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ESCRT protein, which mediates cargo protein transport to intraluminal vesicles (ILVs) of late endosome/multivesicular body (LE/MVB). However, the detailed molecular mechanisms of BRI1 endocytic trafficking remain to be fully elucidated.

We have previously synthesized Brz (brassinazole), a specific inhibitor of BR biosynthesis. Through its triazole base, Brz directly binds to the DWF4 enzyme, a cytochrome P450 monoxygenase that catalyzes the 22-hydroxylation of the BR sidechains. Brz treatment decreases the BR content and causes a dwarf phenotype in plants, which is similar to the phenotype of BR-deficient mutants. These responses to Brz were used to search in a chemical biology screen for gain-of-function mutants that are resistant to Brz. The first gene identified using this approach was BZR1. BIL1 has been identified to be identical to BZR1, and BES1 is homologous to BZR1. The BIL1/BZR1 and BES1 genes encode master transcription factors that regulate the expression of thousands of genes.

In our recent study, we have identified Brz-insensitive-long hypocotyl 4–1D (bil4-1D) from activation-tagging lines of Arabidopsis thaliana (hereafter, Arabidopsis) with a chemical biology approach using Brz. The bil4-1D phenotype is caused by the overexpression of a novel gene. BIL4 has homologous genes in other plant species (e.g., tomato, rice, and maize). Hydropathy plot analysis suggests that BIL4 contains seven transmembrane domains. The hypocotyl phenotype of bil4-1D may suggest that BIL4 acts as a positive regulator in BR signaling, but the mechanism of action of BIL4 in BR signaling and plant development has not been predicted. In this study, we identified BIL4 to be a regulatory factor affecting both endocytosis of the BR receptor BRI1 and BR-mediated plant cell elongation.

**Results**

**BIL4 positively regulates BR signaling.** In the presence of Brz, dark-grown wild-type Arabidopsis seedlings exhibit de-etiolation traits, such as short hypocotyls and open cotyledons, as if the plants were grown under light. Both bil4-1D and BILA-OX plants exhibit an elongated hypocotyl when grown on Brz in the dark. In this study, both bil4-1D and BILA overexpressor (BILA-OX) plants, as compared with the wild-type, in the adult stage showed slender dwarf leaves and an increased number of branches, whereas the petioles and leaf blades were slightly smaller than those of the wild-type (Supplementary Fig. S1). We have previously found that the rosette leaves of plants exposed to excessive concentrations of BR are smaller than those of mock-treated plants. The hypocotyl elongation of bil4-1D and BILA-OX reflects the increased BR signaling and suggests that BIL4 may be a positive regulator of BR signaling. However, the detailed role and importance of BIL4 in plant development and BR signaling have not yet been analyzed.

BIL4 encodes a seven-transmembrane-domain protein with four homologs in Arabidopsis (Supplementary Fig. S2a) and homologs in many other plants, such as rice, tomato, maize, poplar, and moss. G protein-coupled receptors (GPCRs) are also seven-transmembrane-domain proteins, although BIL4 does not have a functional GPCR domain. A detailed BLAST search indicated that BIL4 is a member of a gene family that is evolutionarily conserved in plants and animals, including humans and mice, although this protein family has not been well characterized (Fig. 1a,b; Supplementary Fig. S2c; Supplementary Table S1).

We next studied the genetic interaction between a BILA-OX and the BR receptor mutant bri1-5, which is a weak allele and displays a semi-dwarf phenotype. We found that BILA overexpression partially rescues the short hypocotyl phenotype of bri1-5 when germinated in the dark (Fig. 1c,d) and the short inflorescences phenotype of bri1-5 when grown in the light (Fig. 1e,f). These results further suggest that BIL4 is involved in BR signaling.

To determine the effects of BILA overexpression, we analyzed the expression of BR-responsive genes. Using qRT-PCR, in bil4-1D and BILA-OX plants, we detected increased transcript levels of two BR-regulated genes, TCH4 and SAUR-AC1, which are upregulated during BR-regulated cell elongation (Fig. 1g). In addition, the levels of the BR biosynthesis intermediates typhasterol and castasterone were lower in the BILA-OX plant than in the wild-type plants (Supplementary Table S2). Certain BR biosynthesis genes are regulated by feedback inhibition, and the BR levels are suppressed in the BR signaling-activated mutant bil1/bzr1-2 and upregulated in the BR receptor-deficient mutant bri1-5. These results indicate that BILA acts as a positive factor in BR signaling.

To investigate the regulatory growth effects caused by BILA overexpression in BR signaling, we used immunoblotting analyses to characterize the phosphorylation status of the BR signaling transcription factor BIL1/BZR1, which has been used as a downstream biochemical marker of BR signaling. Without BR treatment, phosphorylated BIL1/BZR1 was detected in the wild-type plants, and dephosphorylated BIL1/BZR1 was detected by BR treatment. BR treatment, the amount of dephosphorylated BIL1/BZR1 that was detected in the BILA-OX and bil1-1/bzr1-1D plants was higher than that in the BR-treated wild-type plants (Fig. 1h). These results indicate that BILA acts as a positive regulator in BR signaling.

**BILA regulates cell elongation.** To investigate the roles of BILA in plant development in vivo, we examined plants that had been transformed with an RNAi targeting BILA (Fig. 2a). At the adult stage, the BILA-RNAi plants showed shorter inflorescences and smaller leaves than those of the wild-type plants (Fig. 2b,c). The hypocotyls of the BILA-RNAi plants were shorter than those of the wild-type plants grown with and without Brz in the dark (Fig. 2d,e). We also obtained a T-DNA insertion mutant (BILA-KO) (Supplementary Fig. S3a). The T-DNA was inserted approximately 60 bp upstream of the ATG start codon of BILA. The BILA-KO mutant had a phenotype similar to that of wild-type plants grown in control medium, but in the presence of Brz, the BILA-KO mutants had slightly shorter hypocotyls and slightly smaller leaves, thus suggesting that BILA-KO mutants display relatively weak BR-deficient phenotypes (Supplementary Fig. S3b,c). In the BILA-RNAi lines, the expression of BILA homologs BILA-H1, BILA-H3, and BILA-H4 was suppressed (Supplementary Fig. S4a). The suppression of BILA and homologous genes in BILA-RNAi-2 plants was stronger than that in BILA-RNAi-1 plants and was proportional to the strength of the dwarf phenotype in each line. In the BILA-RNAi lines, the expression levels of the BR-upregulated genes TCH4 and SAUR-AC1 was decreased (Supplementary Fig. S4b). The BL-stimulated
Figure 1. BIL4 is a positive regulator of BR signaling. (a,b) Hydrophobicity profiles of BIL4 (a) and the human homolog Golgi anti-apoptotic protein (hGAAP) (b). (c,d) Phenotype (c) and hypocotyl length (d) of wild-type (WT), bri1-5 and BIL4-OX bri1-5 double mutant seedlings grown in the dark for 7 days. The results are presented as the mean ± s.d. (n > 30 seedlings). (e,f) Phenotype (e) and inflorescence length (f) of WT, bri1-5 and BIL4-OX bri1-5 plants grown in soil for 6 weeks. The results are presented as the mean ± s.d. (n > 20 plants). (g) qRT-PCR analyses of TCH4 and SAUR-AC1 expression levels in wild-type, bil4-1D, and BIL4-OX plants grown in the dark for 8 days. The results are presented as the mean ± s.d. (h) BIL1/BZR1 phosphorylation status in wild-type, BIL4-OX, BIL4-RNAi-1 and bil1-1D/bzr1-1D plants (pBIL1/BZR1, phospho-BIL1/BZR1). Plants that were grown on medium containing 3 μM Brz in the dark for 7 days were submerged for 3 hr in medium containing 100 nM BL. Western blot analyses were performed using the anti-BIL1/BZR1 antibody (upper panel). The protein levels were detected using Ponceau S (lower panel). Numbers indicate the relative BIL1/BZR1 signal levels normalized to the Ponceau S-stained protein band.
Figure 2. BIL4 is important for cell elongation. (a) qRT-PCR analysis of BIL4 expression in wild-type (WT), BIL4-RNAi-1 (RNAi-1) and BIL4-RNAi-2 (RNAi-2) seedlings grown in the dark for 3 days. The results are presented as the mean ± s.d. (b, c) Three-week-old (b) and 5-week-old (c) wild-type and BIL4-RNAi plants. (d, e) Phenotype (d) and hypocotyl length (e) of wild-type and BIL4-RNAi seedlings grown on medium containing 3µM Brz in the dark for 6 days. The results are presented as the mean ± s.d., n > 30 seedlings. Asterisks indicate a significant difference from the wild-type plant (P < 0.01 by Student's t-test). (f to i) SEM images (f, h) and length (g, i) of the hypocotyl cells. The typical cell phenotype is marked by a white frame. Scale bar, 100µm. The results are presented as the mean ± s.e.m. Asterisks indicate a significant difference relative to wild-type plants (P < 0.01 by Student's t-test). RNAi seedlings were germinated on 1µM Brz (n > 17 cells; f, g), and overexpressing seedlings were germinated on 3µM Brz (n > 35 cells; h and i) in the dark for 7 days.
dephosphorylation of BIL1/BZR1 was decreased in BIL4-RNAi plants (Fig. 1h). The phenotypes, gene expression patterns, and BIL1/BZR1 phosphorylation statuses of BIL4-RNAi plants were similar to those of BR signaling-deficient mutants11, 32.

To investigate whether BIL4 plays a role in plant cell elongation or division in vivo, we first performed scanning electron microscopy (SEM) to analyze the hypocotyl cell elongation of BIL4-OX and BIL4-RNAi plants. The epidermal cells in the hypocotyl regions of the BIL4-RNAi plants were shorter than those cells in the wild-type plants when the plants were germinated on 1 μM Brz (Fig. 2f,g). In contrast, BIL4-OX exhibited enhanced cell elongation of the epidermal cells in the hypocotyls when the plants were germinated on 3 μM Brz (Fig. 2h,i). These results suggested that BIL4 positively regulates cell elongation in hypocotyls. We next observed the epidermal cell size in the rosette leaf of BIL4-OX and BIL4-RNAi. The leaf area of BIL4-RNAi plants was smaller than that of wild-type plants, and the leaf shape of BIL4-RNAi plants was distorted. The size of the leaf area was not different between BIL4-OX and wild-type plants (Fig. 3a,b). Nevertheless, the cell area of epidermal cells in the rosette leaf was wider in BIL4-OX plants than in wild-type plants. The epidermal cell size in BIL4-RNAi was smaller than that in wild-type plants and was proportional to the leaf area (Fig. 3c,d). The cell number in BIL4-OX and BIL4-RNAi plants was not significantly different from that of wild-type plants (Fig. 3e). These results suggested that BIL4 positively regulates cell elongation on the leaf surface.

**BIL4 is specifically expressed during the activation of cell elongation.** To gain further insight into the role of BIL4 in plant development and plant cell elongation, we examined BIL4 expression in each organ and during each growth stage. The BIL4 promoter region was fused to β-glucuronidase (GUS), and the expression pattern was assessed at different developmental stages. During the germination stage, BIL4 expression was observed in the juvenile root on day 1 after germination in both the light and dark (Fig. 4a,b). BIL4 expression in the root was greater near the root apical meristem on day 2 after germination (Fig. 4c,d). BIL4 expression in the root decreased on day 3 after germination, although the expression near the root apical meristem remained high (Fig. 4e,f). BIL4 was still highly expressed in the root apical meristem at sites in which both root cell division and

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**Figure 3.** BIL4 positively affects the size of leaf epidermal cells. (a) Rosette leaves of wild-type, BIL4-OX and BIL4-RNAi-2 (RNAi-2) plants 6 weeks after sowing. (b) Leaf area of wild-type, BIL4-OX and RNAi-2 plants. The results are presented as the mean ± s.d., n = 5 leaves. Asterisks indicate a significant difference from the wild-type plants (P < 0.01 by Student’s t-test). (c) Confocal images of the epidermis of 6-week-old wild-type, BIL4-OX and RNAi-2 rosette leaves stained with PI. Scale bar, 50 μm. (d) Cell size in the epidermis of wild-type, BIL4-OX and RNAi-2 rosette leaves. The results are presented as the mean ± s.d., n = 9 areas. Asterisks indicate a significant difference from the wild-type plant (P < 0.01 by Student’s t-test). (e) Epidermal cell number of wild-type, BIL4-OX and RNAi-2 rosette leaves. The results are presented as the mean ± s.d., n = 5 leaves; n.s. indicates no significant difference from the wild-type plants (by Student’s t-test).
expansion occur during the later growth stage (Fig. 4g). BIL4 expression was observed in the hypocotyl on day 2 after germination in the dark, a stage in which the cells were actively elongating (Fig. 4d); in contrast, BIL4 was not expressed in the short hypocotyls that grew in the light (Fig. 4a,c,e). BIL4 expression in the hypocotyl was significantly decreased on day 3 after germination in the dark (Fig. 4f). During the adult growth stage, the expression of BIL4 was detected in young rosette leaves and short inflorescences immediately after the initiation of their development (Fig. 4h,i). These results indicated that BIL4 is expressed in the very early developmental stages during the activation of cell division and elongation.

**BIL4 localizes to the TGN/EE, LE/MVB, and vacuolar membrane.** To analyze the functions of BIL4 in the cell, we generated transgenic plants harboring a BIL4 promoter::BIL4–green fluorescent protein (GFP) construct and examined the subcellular localization of BIL4. BIL4–GFP colocalized with the vacuolar membrane marker monomeric red fluorescent protein (mRFP) VAM3 (Fig. 5a) and γ-tonoplast intrinsic protein (γ-TIP)–mRFP (Supplementary Fig. S5), as indicated by a yellow signal present in the merged image in seedling roots.
BIL4–GFP was also associated with mobile and punctate structures that did not colocalize with mRFP–VAM3 of the root cells (Fig. 5a).

The punctate structures visualized with BIL4–GFP colocalized with the endocytic tracer FM4-64 (Fig. 5b). Brefeldin A (BFA) is widely used as a vesicle-trafficking inhibitor and leads to the aggregation of endosomal compartments. BFA treatment showed that the inhibited trafficking of the BIL4–GFP- and FM4-64-containing vesicles may be BFA dependent (Fig. 5c). BIL4–GFP and the trans-Golgi network/early endosome (TGN/EE) marker mRFP–SYP41 (d) were partially colocalized with the TGN/EE marker mRFP–SYP41 (d). Merged structures in b–f are marked by yellow arrows. The white arrowheads indicate characteristic interactions. (h) Co-immunoprecipitation of BRI1 and BIL4. Wild-type (Col-0) and transgenic plants coexpressing BRI1pro::BRI1–GFP and 35S::FLAG–BIL4 were grown for 7 days. FLAG–BIL4 was immunoprecipitated by anti-FLAG antibody, and the immunoblots were probed with anti-GFP or anti-FLAG antibody.

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to vacuoles and induces to form ring-like structures of LE/MVB\(^{13}\) (Supplementary Fig. S6e). BIL4–GFP co-labeled approximately 58% of the compartments with the TGN/EE marker mRFP–SYFP41 and approximately 66% of the compartments with the LE/MVB marker mRFP–ARA7 (Supplementary Fig. S7). BIL4–GFP-labeled punctate structures were often observed adjacent to the Golgi makers Arabidopsis 3,1,2-xyllosyltransferase\(^{22}\) (XylT)–mRFP and rat sialyltransferase\(^{24}\) (ST)–mRFP (Supplementary Fig. S8). BIL4–GFP was also localized to the punctate signals and vacuolar membrane in the 35 S promoter.::BIL4–GFP transformant, similarly to its localization in the 35 S promoter.::BIL4–GFP transformant (Supplementary Fig. S9a–e). The 35 S promoter.::BIL4–GFP transgenic plant seedlings showed longer hypocotyls than wild-type seedlings when grown in the dark on medium containing Brz, thus indicating that the fusion protein was functional (Supplementary Fig. S9f). These results suggested that the BIL4 protein localizes to and plays roles in the TGN/EE, LE/MVB, and vacuolar membranes.

**BIL4 interacts with BRI1 in the endosome.** In the BR signaling pathway, the BR receptor BRI1 localizes to the plasma membrane and punctate structures\(^{12}\). The BRI1–GFP-containing punctate structures were TGN/EE and LE/MVB\(^{43}\), which were similar to BIL4–GFP (Fig. 5b–e). To analyze the subcellular localization of BIL4 and BRI1, we generated transgenic plants harboring a BIL4 promoter.::BIL4–GFP construct and a BRI1 promoter.::BRI1–Venus construct. BIL4–GFP colocalized with BRI1–Venus in punctate structures (Fig. 5f).

To investigate a possible interaction between BIL4 and BRI1 in plant cells, we introduced plasmids for bimolecular fluorescence complementation (BiFC) into cultured Arabidopsis cells. We fused the full-length BIL4 to the N-terminal half of enhanced yellow fluorescence protein (EYFP) (BIL4–nEYFP) and full-length BRI1 to the C-terminal half of EYFP (cEYFP). Both of these constructs were introduced into cultured Arabidopsis cells, and the BIL4–BRI1 interaction was monitored by BiFC (Fig. 5g). Because BIL4 and BRI1 were localized in TGN/EE and LE/MVB\(^{43}\) (Fig. S5d,e), the detected fluorescence signal may represent TGN/EE and LE/MVB (Fig. 5g). As a negative control, the CERK1–cEYFP vector was transformed with the BIL4–nEYFP vector in cultured Arabidopsis cells. Although CERK1–cEYFP was expressed, the fluorescence signal of YFP was not detected (Supplementary Fig. S10).

To determine the in vivo interaction between BIL4 and BRI1 by using biochemical analyses, we attached full-length BIL4 to the C-terminus of FLAG under the control of the CaMV 35 S promoter and expressed this construct in an Arabidopsis BRI1–GFP transformant\(^{44}\). The BIL4–FLAG protein was immunoprecipitated by anti-FLAG antibodies in transgenic Arabidopsis plants expressing both BRI1–GFP and BIL4–FLAG, and the resulting immunoprecipitates were analyzed by western blotting with anti-FLAG and anti-GFP antibodies. The anti-FLAG antibody immunoprecipitated BIL4–FLAG and coimmunoprecipitated BRI1–GFP; whereas no BRI1–GFP signal was detected using the immunoprecipitates of the transformants expressing BRI1–GFP alone or wild-type Arabidopsis (Fig. 5h). These results suggested that the BIL4 protein interacts with the BRI1 protein.

**BIL4 regulates BRI1 trafficking in the plant cell.** Recent reports have suggested that BRI1 activates BR signaling when BRI1 is localized to plasma membranes; however, BRI1 undergoes endocytosis to attenuate signaling, thus suggesting that the subcellular localization of BRI1 is associated with the regulation of BR signaling\(^{14}\). BRI1 can be internalized and transported to TGN/EE and LE/MVB that are regulated by GNOM\(^{14,45}\). AP-2\(^{15}\) and TPLATE\(^{16}\) regulate the endocytosis of BRI1 through CME at the plasma membrane. BRI1 is similarly considered to translocate to the vacuoles through processes that are regulated by ubiquitination\(^{17}\) and the ESCRT protein\(^{18}\), which mediates cargo protein transport to ILVs of LE/MVB. Given that BIL4 interacts with BRI1 in the TGN/EE and/or LE/MVB, BIL4 is predicted to affect the trafficking of BRI1. We subsequently compared the localization of the BRI1–GFP fusion protein in the wild-type, BIL4-OX, and BIL4-RNAi plants. BIL4-OX background increased the number of BRI1–GFP-labeled endosome in epidermal cells of Arabidopsis roots (Supplementary Fig. S11). BFA caused the aggregation of BRI1–GFP from the TGN/EE into BFA bodies. The signal intensity and size of these BFA bodies increased in the BIL4-OX plants as compared to the wild-type plant background (Fig. 6a–d). In the wild-type plants, BRI1–GFP localized to endosomes and the wild-type membranes in the root tips (Fig. 6c). In the BIL4-RNAi plants, BRI1–GFP strongly localized to the vacuolar lumen in the root tips (Fig. 6f). In an immunoblot analysis, we observed increased BRI1 protein levels in BIL4-OX plants compared with those in wild-type plants. In contrast, BRI1 protein levels were decreased in BIL4-RNAi plants compared with those in wild-type plants (Fig. 6g). These results suggested that BIL4 inhibits BRI1 trafficking to the vacuoles from the TGN/EE and/or LE/MVB.

**Discussion**

Because the BR receptor BRI1 is important in plant development, the detailed mechanism of BR1-mediated signaling was investigated. BR binds to the extracellular ligand-binding domain of BRI1, which localizes to the plasma membrane\(^{46}\), thereby leading to the ligand-dependent dimerization and phosphorylation of BRI1\(^{47}\). BRI1 activation induces the dissociation of its inhibitor BKII from the plasma membrane and the phosphorylation of BSK1, a positive regulator of the pathway\(^{10,11}\). The BR signals are then transduced to the cytosolic positive phosphatase BSU1\(^{48}\) and the negative kinase BIN2\(^{31}\), thus leading to the activation of the transcription factors BIL1/BRZ1 and BES1, which regulate more than one thousand different target genes\(^{25,26}\). These results suggest that BRI1 plays important roles in the initiation of BR signaling.

BRI1 localizes not only to plasma membranes but also to endosomes\(^{12}\). The inhibition of the ARF-GEF GNOM decreases the endocytosis of BRI1\(^{14}\). The knockdown of Arabidopsis AP-2 or TPLATE-MUNISCIN-LIKE (TML) protein, a member of the TPC, inhibits the endocytosis of BRI1\(^{15,16}\). The inhibition of GNOM and AP-2 inhibits BRI1 endocytosis, which enhances its localization at the plasma membrane thereby promotes BR signaling\(^{18,19}\). Furthermore, BR treatment does not alter the endocytosis of BRI1\(^{41}\). These results suggest that the endocytosis of BRI1 does not promote BR signaling and that the maintenance of BRI1 at the plasma membranes without...
proceeding to endocytosis or quickly returning BRI1 to the plasma membranes after endocytosis is important for the promotion of BR signaling by BRI1.

In our recent study using a chemical biology approach with Brz27, we have identified BIL4, a seven-transmembrane-domain protein, as a positive regulator of BR signaling. BIL4 overexpression in the bil4-1D mutant and BIL4-OX transformant activates the transacting factor BIL1/BZR1 and increases the expression of the BR-induced genes TCH4 and SAUR-AC1. Similarly to the BR receptor BRI1, BIL4 localizes to the TGN/EE and LE/MVB. Our co-immunoprecipitation and BiFC analyses showed that BIL4 interacts with BRI1 in the TGN/EE and LE/MVB.

In BIL4-RNAi transformants, the vacuole lumen signals of BRI1–GFP, which were difficult to detect in wild-type plants under light conditions, were increased. In BIL4-OX transformants, the endosomal localization of BRI1–GFP visualized by BFA treatment increased relative to that of wild-type plants. These results suggested that BIL4 inhibits the translocation of BRI1 from the TGN/EE to the vacuoles. BIL4 might play important roles for BRI1 trafficking from the TGN/EE and LE/MVB to the vacuoles (Supplementary Fig. S12). The vacuolar transport of BRI1 is considered to be regulated by ubiquitination 17 and the ESCRT protein 18. It will be interesting to examine the relationship between these factors and BIL4 in future studies.

BIL4 is expressed very early in young elongating cells. The overexpression of BIL4 in the bil4-1D mutants and the BIL4-OX transformants promotes cell elongation, whereas the deficiency of BIL4 in the BIL4-RNAi transformants inhibits cell elongation. Although the expression of BIL4 in the hypocotyl was limited to 2 days after germination in the dark, its effect on cell elongation persisted until 7 days after germination. These results were similar to those on the nuclear import of BIL1/BZR1 from the cytosol in the hypocotyl for 96 hr after germination, whose effects persisted until 7 days after germination21.
In previous studies, loss-of-function mutants in BR signaling and BR biosynthesis have been reported to exhibit decreased cell size and number of leaf epidermal cells and mesophyll cells. Transformants overexpressing the BR receptor BRI1 show an increased size of leaf epidermal and mesophyll cells. Here, we found that the leaf epidermal cell size increased in BIL4-OX plants and decreased in BIL4-RNAi plants compared with wild-type plants. These results suggested that BIL4 plays important roles in cell elongation control in BR signaling.

BIL4 is evolutionarily conserved in eukaryotes including plants and animals. The most related protein in humans is the Golgi antiapoptotic protein (hGAAAP), which shares 49% amino acid similarity and 27% identity with Arabidopsis BIL4. Human hGAAAP exhibits 73% identity with the camelpox virus protein vGAAAP. hGAAAP localizes in the Golgi and is involved in cell death regulation via the modulation of intracellular Ca\(^{2+}\) flux. An increase in intracellular calcium is known to trigger vesicle fusion that system relates to SNARE, calmodulin and so on. It will be interesting to see if BIL4 could control calcium flux thereby contributing to BRI1 intracellular trafficking.

**Methods**

**Plant materials and growth conditions.** Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type plant. The bri1-5 mutant was in the Arabidopsis thaliana WS ecotype background. BIL4-KO mutant plants (CS378201) were obtained from the ABRC. The methods for seed sterilization and the conditions for plant growth have been previously described.

**Quantitative real-time PCR.** The methods for total RNA isolation, cDNA synthesis, and real-time PCR have been previously described. The sequences of the gene-specific primers for real-time PCR were as follows: for TCH4, 5′-CGAGTCTTTGGAACGCTGAT-3′ and 5′-CTTCCTGTTGAAAGCCACGG-3′; for SAUR-AC1, 5′-GAGATAGTGTCGCCGTTT-3′ and 5′-GTATTGTGTAAGCGCCCATTT-3′; for BIL4, 5′-CACTCTTTAAGGCTTGTGATA-3′ and 5′-TGAACTAGATAACAAATGGCAGCT-3′; for BIL4-H1, 5′-TGATTCTGAGGACGAGGATT-3′ and 5′-GGCAAAGACCATGAGAACGA-3′; for BIL4-H3, 5′-ACGAGCTGTAGCAGGAAATG-3′ and 5′-GGAGACTCCGAGCGTACA-3′; for BIL4-H4, 5′-TTCGCGATGGAACCGCTA-3′ and 5′-GAGCTGATTCGCCTGATA-3′; and for UBQ2, 5′-CCAAGATCCAGGACAAGAGA-3′ and 5′-TGGAGACAGCAGCATACATGCG-3′.

**BR measurements.** The plants were grown under long-day conditions (16 hr light/8 hr dark) for 30 days. The plants (20 g fresh weight) were extracted twice with 200 ml of MeOH, and deuterium-labeled internal standards (1 ng/g fresh weight) were added to the extract. BR purification and quantification were performed as previously described.

**Transgenic plant generation.** The BIL4-OX, ST–mRFP, mRFP–VAM3 and mRFP–SYP41 transgenic plants were generated as previously described. To knock down BIL4 by RNAi, BIL4 cDNA was amplified from Col-0 CDNA and cloned into the binary vector pGWB80 using a Gateway strategy. To observe BIL4 sub-cellular localization under the BIL4 promoter, a 1920-bp genomic fragment from Col-0 cDNA and cloned using Gateway technology into the binary vector pGWB5, which contains a 35S promoter and the GFP protein-coding sequence. For the observation of BIL4 sub-cellular localization, but no promoter. To observe BIL4 sub-cellular localization, the coding sequence of BIL4 without the stop codon was amplified from Col-0 CDNA and cloned using Gateway technology into the binary vector pGWB5, which contains a 35S promoter and the GFP protein-coding sequence. For the observation of BIL4 expression in plant organs, a 1460-bp genomic fragment from the BIL4 promoter region was amplified from Col-0 genomic DNA and cloned using Gateway technology into the binary vector pGWB3 containing the GUS coding sequence. The resulting constructs, pBIL4–GFP, pBIL4pro–GUS and pBIL4–RNAi, were cloned into pCol-0 using the floral dip method. The XylT–mRFP construct was generated by modifying XylT–GFP and then cloned into pBGW vectors. The plant organelle marker–mRFP constructs were transformed into the BIL4–GFP transfectants by using the floral dip method.

**GUS staining.** The plants expressing the BIL4 promoter::GUS reporter gene fusion were histochemically stained according to the method of Ito and Fukuda, with minor modifications. Digital images were captured using a Leica MZ FLIII stereomicroscope (Leica, Wetzlar, Germany).

**Scanning electron microscopy.** The samples were transferred to a low-vacuum scanning electron microscope, and the analysis was performed using a JSM-5600 LV microscope (JEOL, Tokyo, Japan).

**Statistical analysis of the root apical meristem.** The meristem size in Arabidopsis roots was measured as previously described.

**Statistical analysis of the leaf area and epidermal cell size.** The largest leaf per plant was measured for 5 plants of each line. Leaves were scanned, and the leaf area was measured with ImageJ software (http://rsb.info.nih.gov/ij/). Leaf regions at 75% of the leaf blade length were stained in 45 μg/ml PI for 30 min, rinsed and mounted in dH₂O, and cells at the abaxial epidermis were imaged using an LSM700 laser-scanning microscope (Zeiss). One or 2 areas per leaf were measured for 5 of the analyzed plants. The number of cells (150 cells on average) per drawn image area was counted in ImageJ. The average cell size was calculated from the ratio of the image area/cell number. The total cell number in the leaves was calculated from the multiplication of cell number per mm² by total leaf area.
Inhibitor treatments and FM4-64 staining. FM4-64 (Molecular Probes, Carlsbad, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) and applied to Arabidopsis plants at a final concentration of 4 µM for 3 min. The plants were washed with water to remove the excess dye and subsequently examined. BFA (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO, and the solution was applied to plants at a final concentration of 50 µM for 30 min. Four-day-old seedlings were incubated in distilled water containing 2 µM ConA, 50 µM CHX, or 33 µM Wm. The following stock solutions were used: 1 mM ConA in DMSO, 50 mM CHX in DMSO, and 33 mM Wm in DMSO.

Confocal laser scanning microscopy. The stable double transformants were observed using a Zeiss LSM700 or LSM780 confocal laser-scanning microscope (Zeiss, Jena, Germany).

Transformation of the Arabidopsis suspension culture line. An Arabidopsis suspension culture was transformed as previously described with minor modifications. Transformed Agrobacterium that was cultured in YEP medium containing appropriate antibiotics was suspended in modified MS medium containing acetosyringone. The Agrobacterium suspensions were inoculated into 10 ml of 2-day-old cultured Alex cells of Arabidopsis. To remove the Agrobacterium, 50 µl of 100 mg/ml chloramphenicol was added to these cultures 3 days after inoculation. Microscopy was performed 4 days after Agrobacterium inoculation. To construct the BiFC vector, the BIL4 coding region without stop codon was cloned into the Gateway binary vector pB4GWmY (from Dr. T. Mano, National Institute for Basic Biology) to generate BIL4-NEYFP, and the BRI1 and CERK1 coding regions without a stop codon were cloned into the Gateway binary vector pB4GWcY (from Dr. T. Mano, National Institute for Basic Biology) to generate BRI1-cEFYFP and CERK1-cEFYFP.

Co-immunoprecipitation. The proteins were extracted by grinding 5 g of 7-day-old seedlings in liquid N2, and further grinding in 14 ml of cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% CHAPS, and protease inhibitor cocktail tablets (Roche Diagnostics). The extract was centrifuged twice at 10,000 × g for 5 min (4 °C), and the resulting supernatant was cleared by filtration through a 0.8-µm Millex-AA filter (Millipore, Billerica, MA). The proteins in the supernatant were quantified using the Bio-Rad Protein Assay reagent and stored in liquid N2, and protease inhibitor cocktail tablets (Roche Diagnostics). The extract was centrifuged twice at 10,000 × g for 30 min, washed extensively, and eluted with 70 µl of 2 × LDS sample loading buffer (3% LDS, 60 mM Tris-HCl, pH 6.8, 6% [w/v] sucrose, and 0.003% [w/v] BPB). After incubation at 25 °C for 30 min, the proteins were resolved by SDS-PAGE. The immunoprecipitated proteins were detected by immunoblot analysis on Hybond ECL nitrocellulose membranes (GE Healthcare) using a monoclonal anti-Flag M2 antibody (Sigma-Aldrich) at a 1:1000 dilution and an anti-GFP antibody (Molecular Probes) at a 1:1200 dilution. The blots were developed using horseradish peroxidase (HRP)-linked secondary antibodies and the Immobilon Western Chemiluminescent HRP substrate (Millipore).

Immunoblot analysis. Proteins were extracted by grinding 100 mg of plants in liquid N2, and further grinding in 1x SDS buffer. The proteins were detected by immunoblot analysis on Hybond ECL nitrocellulose membranes (GE Healthcare) with different antibodies using Western Blot Immuno Booster Solution (Takara). The antibodies used for immunodetections were anti-BIL1/BZR1 (1:5000) and anti-BRI1 (1:5000, Agrisera). The secondary antibody was anti-rabbit-HRP (1:15000, Promega).

Imaging and image analysis of BFA bodies. The imaging zone was maintained to be consistent with that showing the epidermal cells of the root tip meristematic zone, 10–15 cells above the quiescent center. To measure the fluorescence signal in BFA bodies, we obtained 2–4 slices of epidermal cells. Image analysis and signal quantification were performed using the measurement function of the LSM software ZEN 2009 (Zeiss). The signal intensity ratio of a BFA-body region was quantified, normalized to the area, and divided by the signal intensity of a nearby plasma membrane.

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Author Contributions

T.N. and A.Y. conceived, designed and directed the research. A.Y. performed most of the experiments, A.Y., T.U. and C.S. performed the confocal laser scanning microscopy analysis, T.U. generated mRFP–VAM3, GFP–VAM3, and mRFP–SYP41, M.N. and M.M. generated the activation-tagging line, S.F. performed the BR measurement analysis. M.S., A.N., H.O., K.S. and T.A. directed and supervised the project. T.N. and A.Y. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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