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RESEARCH PAPER

Arabidopsis heterotrimeric G protein β subunit, AGB1, regulates brassinosteroid signalling independently of BZR1

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Abstract

The *Arabidopsis thaliana* heterotrimeric G protein β subunit, AGB1, is involved in both abscisic acid (ABA) signalling and brassinosteroid (BR) signalling, but it is unclear how AGB1 regulates these signalling pathways. A key transcription factor downstream of BR, BZR1, and its gain-of-function mutant, bzr1-1, were overexpressed in an AGB1-null mutant, *agb1-1*, to examine their effects on the BR hyposensitivity and the ABA hypersensitivity of *agb1-1*, and to examine whether AGB1 regulates the functions of BZR1. Because the amino acid sequence of AGB1 contains 17 putative modification motifs of glycogen synthase kinase 3/SHAGGY-like protein kinases (GSKs), which are known components of BR signalling, the interaction between AGB1 and one of the *Arabidopsis* GSKs, BIN2, was examined. Expression of bzr1-1 alleviated the effects of a BR biosynthesis inhibitor, brassinazole, in both the wild type and *agb1-1*, and overexpression of BZR1 alleviated the effects of ABA in both the wild type and *agb1-1*. AGB1 did not affect the phosphorylation state of BZR1 *in vivo*. AGB1 interacted with BIN2 *in vitro*, but did not affect the phosphorylation state of BIN2. The results suggest that AGB1 interacts with BIN2, but regulates the BR signalling in a BZR1-independent manner.

Key words: Abscisic acid, *Arabidopsis thaliana*, brassinosteroid, glycogen synthase kinase 3/SHAGGY-like protein kinase, heterotrimeric G protein β subunit, protein–protein interaction.

Introduction

Heterotrimeric GTP-binding proteins (G proteins, consisting of subunits Gα, Gβ, and Gγ) are signalling molecules found in a variety of eukaryotic organisms. They mediate ligand-binding signals from G protein-coupled receptors (GPCRs) to downstream pathways, and thus are involved in diverse cellular processes. In contrast to humans, which have 21 Gα genes, five Gβ genes, and 12 Gγ genes, *Arabidopsis thaliana* has only one Gα gene (*GPA1*), one Gβ gene (*AGB1*), and three Gγ genes (*AGG1–AGG3*) (for a review, see Jones and Assmann, 2004; Chakravorty *et al*., 2011). The physiological functions of plant G proteins can be evaluated by using G protein-deficient mutants. Such studies have suggested that plant G proteins have roles in signal transduction of various stimuli such as a stress-related phytohormone, abscisic acid (ABA) (for a review, see Perfus-Barbeoch *et al*., 2004). GPCRs on the plasma membrane in *A. thaliana* were shown to bind ABA (Liu *et al*., 2007; Pandey *et al*., 2009), supporting the importance of the G proteins in ABA signal transduction, although the validity of these studies is still in dispute (Gao *et al*., 2007; Jaffé *et al*., 2012).

G proteins are thought to mediate signal transduction via interacting with effector proteins and regulating their activities (for a review, see Pierce *et al*., 2002). Many G protein effectors have been identified in animals, but only some of them exist in *Arabidopsis* or other plant species (for a review, see Jones and Assmann, 2004), suggesting that plants have plant-specific mechanisms for G protein signalling. In fact, some putative plant-specific G protein effectors have been

identified. For example, THYLAKOID FORMATION 1 (THF1) physically interacts with GPA1 and is involved in GPA1-mediated sugar signalling and chloroplast development (Huang *et al.*, 2006; Zhang *et al.*, 2009). A cupin domain-containing protein, AtPirin1, also physically interacts with GPA1 and mediates ABA signalling (Lapik and Kaufman, 2003). NDL1 (N-MYC DOWNREGULATED-LIKE1) physically interacts with AGB1 and regulates auxin distribution in plants (Mudgil *et al.*, 2009). An acireductone dioxygenase-like protein, ARD1, also physically interacts with AGB1, and its enzyme activity is enhanced by Gβγ dimer (Friedman *et al.*, 2011). Overexpression of a Golgi-localized hexose transporter, SGB1, partially suppresses the phenotype of the AGB1-null mutant, *agb1-2* (H.X. Wang *et al.*, 2006). A comprehensive analysis of the G-protein interactome suggested that G proteins interact with cell wall-related proteins and thereby regulate the cell wall composition (Klopffleisch *et al.*, 2011). However, the molecular mechanisms underlying G protein-mediated signalling remain to be elucidated.

G proteins are suggested to play roles in signal transduction of a phytohormone, brassinosteroid (BR). For example, Gα deficiency causes BR hyposensitivities in both *Arabidopsis* (Ullah *et al.*, 2001, 2002) and rice (L. Wang *et al.*, 2006), and Gα deficiency in *Arabidopsis* enhances the dwarf phenotypes of *bri1-5* and *det2-1*, which have defects in BR biosynthesis and BR perception, respectively (Gao *et al.*, 2008). An AGB1-null mutant, *agb1-2*, is hyposensitive to BR in seed germination (Chen *et al.*, 2004). In addition, *agb1-2* has rounder leaves, more highly branched root systems, shorter siliques (Ullah *et al.*, 2003), and higher sensitivities to ABA (Pandey *et al.*, 2006). All these phenotypes of *agb1-2* are similar to the phenotypes of mutants that have defects in BR biosynthesis or BR signalling (for a review, see Clouse, 2011).

On the other hand, an established model of the BR signalling consists of specific types of protein kinases, protein phosphatases, and transcription factors. When a receptor kinase, BRI1 (BR INSENSITIVE1), binds BR, it phosphorylates BSK (BR signalling kinase) family protein kinases, which activate Kelch repeat-containing protein phosphatases, such as BSU1 (BRI1 SUPPRESSOR1) and BSL1 (BSU1-LIKE1). When activated, these protein phosphatases dephosphorylate and deactivate GSKs (glycogen synthase kinase 3/SHAGGY-like protein kinases). As a result, two GSK-substrate transcription factors, BZR1 (BRASSINAZOLE RESISTANT1, also known as BES2: BRI1 EMS SUPPRESSOR2) and BZR2 (also known as BES1) are dephosphorylated, and regulate their target gene expression, which leads to BR responses (Kim and Wang, 2010; for a review, see Sun *et al.*, 2010). Type 2A protein phosphatases (PP2As) are involved in dephosphorylating BZR1 and BZR2 (Tang *et al.*, 2011). BZR1 and BZR2 share 88% amino acid sequence identities, and redundantly function in BR signalling (Kim and Wang, 2010; for a review, see Sun *et al.*, 2010). The phosphorylation states of BZR1 and/or BZR2 and their target gene expression have often been examined to characterize mutant and transgenic plants that have different BR responses (He *et al.*, 2002; Yin *et al.*, 2002; Vert and Chory, 2006; Peng *et al.*, 2008; Kim *et al.*, 2009; Yan *et al.*, 2009; Rozhon *et al.*, 2010; Tang *et al.*, 2011).

It is still unclear how G proteins participate in the BR signalling, or whether the well-studied components of BR signalling interact with G proteins. Here, to gain further insights into the interaction between G proteins and BR signalling, the functions of BZR1 in *agb1-1* were examined. In addition, because AGB1 has many putative GSK modification sites, the interaction between AGB1 and one of the GSKs, BIN2, was examined.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used throughout the experiments. Seeds of *gpa1-4* (CS6534), *agb1-1* (CS3976), and *agb1-2* (CS6536) were obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org). Surface-sterilized seeds were sown on 0.5× MS medium (0.8% w/v agar, 0.5× MS salts, 1% w/v sucrose, 0.5 g l^{-1} MES, pH 5.8) with or without ABA (Wako, Japan), the brassinosteroid brassinolide (BR) (Brassino Co., Ltd., Japan), brassinazole (BRZ) (Tokyo Kasei, Japan), or bikinin (Calbiochem) (concentrations are shown in the figures), and then incubated for 3 d at 4 °C (stratification). After stratification, plants were grown at 22 °C under a 16 h light/8 h dark photoperiod or in the dark. To generate transgenic plants expressing BZR1–green fluorescent protein (GFP), the genomic region corresponding to the open reading frame (ORF) of *BZR1* (*AT1G75080*) was amplified by genomic PCR as previously described (Tsugama *et al.*, 2011) using the following primer pair: 5′-GGGTCTAGAA TGACTTCGGATGGAGCTACG-3′ and 5′-TCCTCTAGAACC ACGAGCCTTCCCATTTCC-3′ (*Xba*I sites are underlined). The PCR products were digested by *Xba*I, and inserted into the *Spe*I site of pBI121-35SMCS-GFP (Tsugama *et al.*, 2012*a*), generating pBI121-35S-BZR1-GFP. To generate transgenic plants expressing bzr1-1–GFP, PCR was performed using pBI121-35S-BZR1-GFP as template and either of the following two primer pairs: (i) 5′-GT TTCATACCCTGGCTACTATACCTGAATGTGATG-3′ and 5′-T CCTCTAGAACCACGAGCCTTCCCATTTCC-3′; or (ii) 5′-GG GTCTAGAATGACTTCGGATGGAGCTACG-3′ and 5′-GGTA TAGTAGCCAGGGTATGAAACTGGTGGCGATG-3′. The two kinds of PCR products obtained with (i) and (ii) were mixed and used as template for PCR using the following primer pair: 5′-GG GTCTAGAATGACTTCGGATGGAGCTACG-3′ and 5′-TCC TCTAGAACCACGAGCCTTCCCATTTCC-3′ (*Xba*I sites are underlined). The resultant PCR products, which correspond to the *bzr1-1* DNA fragment, were digested by *Xba*I, and inserted into the *Spe*I site of pBI121-35SMCS-GFP, generating pBI121-35S-bzr1-1-GFP. The wild type (WT) and *agb1-1* were transformed with either pBI121-35S-BZR1-GFP or pBI121-35S-bzr1-1-GFP by the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). GFP expression in T_2 plants was checked by fluorescence microscopy as previously described (Zhang *et al.*, 2008), and only GFP-positive plants were used for measuring hypocotyl lengths, scoring green cotyledons, and western blotting.

Western blot analysis of BZR1 phosphorylation states

Transgenic plants expressing BZR1–GFP were grown in the presence or absence of BR, BRZ, or bikinin as described above, and sampled at the time points indicated in the figures. Cell extracts were prepared as previously described (Tsugama *et al.*, 2011), and used for western blotting using anti-GFP antibody (MBL, Japan). Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and an LAS-1000 plus image analyzer (Fuji Film, Japan). Images were processed with Canvas X software (ACD Systems).

Reverse transcription–PCR (RT–PCR)

Plants were grown with 0.5 μM ABA for 20 d or without ABA for 10 d, and sampled. Total RNA was prepared as previously described (Chomczynski and Sacchi, 1987), and cDNA was synthesized from 2 μg of the total RNA with PrimeScript Reverse Transcriptase (TakaraBio, Japan) using an oligo(dT) primer. The reaction mixtures were diluted 25 times with distilled water and used as templates for PCR. GoTaq Green Master Mix (Promega) was used for semi-quantitative RT–PCR, and GoTaq qPCR Master Mix (Promega) for quantitative RT–PCR. Primers used for RT–PCR are given in Supplementary Table S1 available at *JXB* online. In quantitative RT–PCR, the PCR was run using a StepOne Real-Time PCR System (Applied Biosystems), and relative expression levels were calculated by the comparative C_T method using *UBQ5* as an internal control gene.

Prediction of three-dimensional structure and GSK modification sites of AGB1

The three-dimensional structure of AGB1 (AT4G34460.1) was predicted using SWISS-MODEL (http://swissmodel.expasy.org). The output pdb file was read by RasMol (http://www.openrasmol.org) to visualize the structure. GSK modification sites in AGB1 were predicted by the Eukaryotic Linear Motif database (ELM, http://elm.eu.org). The predicted 17 GSK modification sites in AGB1 (amino acid positions 46–53, 67–74, 93–100, 109–116, 132–139, 142–149, 146–153, 173–180, 185–192, 206–213, 208–215, 221–228, 231–238, 264–271, 296–303, 347–354, and 351–358) were highlighted using RasMol.

In vitro GST pull-down assay

Hexahistidine-tagged AGB1 (His-AGB1) was expressed in *Escherichia coli* and purified as previously described (Tsugama *et al*., 2012*b*). Expression of His-AGB1 in *E. coli* was confirmed by western blotting using HisProbe-HRP (Thermo Fisher Scientific). To express glutathionine *S*-transferase (GST)-fused BIN2 (GST–BIN2), the ORF of *BIN2* (*AT4G18710*) was amplified by RT–PCR using the following primer pair: 5′-GAGGATCCATGGCTGATGATAAGGAGATGCC-3′ and 5′-CCCACTAGTTCCAGATTGATTGATTCAAGAAGC-3′ (*Bam*HI site is underlined). The PCR products were digested by *Bam*HI and inserted into the *Bam*HI–*Sma*I site of pGEX-6P-3 (GE Healthcare). This construct was transformed into the *E. coli* strain, BL21 (DE3). Transformed cells were cultured at 37 °C in LB medium until the OD_{600} reached 0.5, and was then incubated at 28 °C for 2 h after addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM to express GST–BIN2. Expression of GST–BIN2 was confirmed by western blotting using an anti-GST antibody (GE Healthcare). Crude *E. coli* extracts were prepared as previously described (Tsugama *et al*., 2012*b*). GST–BIN2 in the *E. coli* extracts was bound to glutathione–Sepharose 4 Fast Flow (GE Healthcare) following the manufacturer's instructions, and washed four times with 1× Tris-buffered saline (TBS). A solution containing purified His-AGB1 was added to the GST–BIN2-bound resin, and the mixture was incubated at room temperature for 30 min with gentle shaking. The resin was then washed four times by TBS, resuspended in 20 mM reduced glutathione in 50 mM TRIS-HCl, pH 8.0, and incubated at room temperature for 10 min to elute GST–BIN2. His-AGB1 in the elutant was analysed by western blotting using HisProbe-HRP. For negative controls, 250 mM imidazole was used instead of the solution containing His-AGB1, and GST alone instead of GST–BIN2. After detecting His-AGB1, the blot was washed three times by Tween–phosphate-buffered saline (PBS)–EDTA, which was made by adding 0.5 M EDTA, pH 8.0, to Tween–PBS (0.1% v/v Tween-20 in PBS) to 10 mM final concentration of EDTA, for deprobing the HisProbe-HRP. The blot was then washed twice by Tween–TBS and used for a western blot analysis of phosphoproteins using Phos-tag Biotin BTL-104 (Wako, Japan) (Kinoshita *et al*., 2006). Signal detection and image processing were performed as described above.

Yeast three-hybrid (Y3H) assays

pGBK-AGB1 (Tsugama *et al*., 2012*b*) was digested by *Hpa*I and *Sal*I, and the resultant DNA fragment containing the full-length ORF of *AGB1* and a partial coding sequence (CDS) of the GAL4 DNA-binding domain (GAL4BD) was inserted into pBridge (Clontech), generating pBridge-AGB1. The ORF fragment of *AGG1* (*AT3G63420*) was obtained by PCR using pGAD-AGG1 (Tsugama *et al*., 2012*b*) as template and the following primer pair: 5′-GAGAGATCTATGCGAGAGGAAACTGTGGT-3′ and 5′-CCTAGATCTAAGTATTAAGCATCTGCAGCC-3′ (*Bgl*II sites are underlined). The PCR products were digested by *Bgl*II, and inserted into the *Bgl*II site of pBridge-AGB1, generating pBridge-AGB1-AGG1. The ORF of *AGG1* was obtained by digesting pGAD-AGG1 by *Nde*I and *Xho*I, and inserted into the *Nde*I–*Sal*I site of pGBKT7 (Clontech), generating pGBK-AGG1. The ORF of *AGG1* was obtained by PCR using pGAD-AGG1 as template and the following primer pair: 5′-GAGGAATTCATGCGAGAGGAAACTGTGGT-3′ and 5′-CCTAGATCTAAGTATTAAGCATCTGCAGCC-3′ (*Eco*RI and *Bgl*II sites are underlined). The PCR products were digested by *Eco*RI and *Bgl*II, and inserted into the *Eco*RI–*Bam*HI site of pBridge, generating pBridge-AGG1. The ORF of *AGB1* was obtained by PCR using pGBK-AGB1 as template and the following primer pair: 5′-GAGGATCCATGTCTGTCTCCGAGCTCAAAG-3′ and 5′-CCCGGATCCTCAAATCACTCTCCTGGTCC-3′ (*Bam*HI sites are underlined). The PCR products were digested by *Bam*HI, and inserted into the *Bgl*II site of either pBridge or pBridge-AGG1, generating pBridge-2-AGB1 or pBridge-AGG1-AGB1, respectively. The ORFs of *BIN2* were obtained by digesting pGEX-6P-BIN2 by *Nco*I and *Spe*I, and were inserted into the *Nco*I–*Xba*I site of pGADT7-Rec (Clontech), generating pGAD-BIN2. The ORF fragments of *BIN2* were obtained again by digesting pGAD-BIN2 by *Nco*I and *Bam*HI, and were inserted into the *Nco*I–*Bam*HI site of pGBKT7 (Clontech), generating pGBK-BIN2. pGBK-BIN2 was digested by *Hpa*I and *Pst*I, and the resultant fragment containing the full-length ORF of *BIN2* and a partial CDS of GAL4BD was inserted into the *Hpa*I–*Pst*I site of pBridge-2-AGB1, generating pBridge-BIN2-AGB1. The ORF of *BZR1* was amplified by PCR using a cDNA clone of *BZR1* (RAFL04-20-E20), which was obtained from RIKEN BRC Experimental Plant Division (Seki *et al*., 2002), as template and the following primer pair: 5′-GAGGAATTCATGACTTCGGATGGAGCTACG-3′ and 5′-TCCTCTAGAACCACGAGCCTTCCCATTTCC-3′ (*Eco*RI and *Xba*I sites are underlined). The PCR products were digested by *Eco*RI and *Xba*I, and inserted into the *Eco*RI–*Xba*I site of pGADT7-Rec, generating pGAD-BZR1. The *Saccharomyces cerevisiae* strain AH109 was transformed with combinations of pGAD and pBridge constructs. After transformation, at least four colonies grown on synthetic dextrose (SD) medium lacking leucine and tryptophan (SD/–Leu/–Trp), were streaked on SD/–Leu/–Trp and SD/–Leu/–Trp lacking histidine or lacking both histidine and adenine. Reporter gene activation was quantified by a β-galactosidase assay as described in the Yeast Protocols Handbook (Clontech).

Bimolecular fluorescence complementation (BiFC)

The ORF of *BIN2* was amplified using pGBK-BIN2 as template and the following primer pair: 5′-GAGTCTAGAATGGCTGATGATAAGGAGATGCC-3′ and 5′-CCCACTAGTTCCAGATTGATTGATTCAAGAAGC-3′ (*Xba*I and *Spe*I sites are underlined). The PCR products were digested by *Xba*I and *Spe*I, and inserted into the *Spe*I site of pBS-35SMCS-cYFP (Tsugama *et al*., 2012*b*), generating pBS-35S-BIN2-cYFP. The ORF of AGG1 was amplified by PCR using pGBK-AGG1 as template and the following primer pair: 5′-GGGACTAGTATGCGAGAGGAAACTGTGG-3′ and 5′-CCACTAGTAAGTATTAAGCATCTGCAGCC-3′

(*Spe*I sites are underlined). The PCR products were digested by *Spe*I and inserted into the *Spe*I site of pBS-35SMCS-cYFP, generating pBS-35S-AGG1-cYFP. These cYFP (C-terminal yellow fluorescent protein) constructs and pBS-35S-nYFP-AGB1 (Tsugama *et al.*, 2012*b*) was used to co-express cYFP-fused protein and nYFP-fused AGB1 in *Arabidopsis* mesophyll protoplasts. *Arabidopsis* mesophyll protoplasts were prepared and transformed as previously described (Yoo *et al.*, 2007; Wu *et al.*, 2009). Recovered YFP fluorescence was observed by fluorescence microscopy 12 h after transformation.

Results

ABA enhances the BR hyposensitive phenotype of agb1

The leaves of *agb1-1* and *agb1-2* are rounder and their petioles are shorter than those of the WT. It was discovered that in the presence of ABA, leaves of *agb1-1* and *agb1-2* become even rounder and their petioles become even shorter. These ABA-induced phenotypes of *agb1-1* and *agb1-2* were similar to the phenotypes of mutants which have severe defects in BR biosynthesis or BR signalling (for a review, see Clouse, 2011). To examine whether the *agb1* phenotypes are due to impaired BR signalling, a BR biosynthesis inhibitor, BRZ, was tested for its effects on the phenotypes of *agb1-1* and *agb1-2*. BRZ made leaves rounder and petioles shorter in all the genotypes including the WT and *gpa1-4*. However, the leaf morphologies of *agb1-1* and *agb1-2* appeared to be more strongly affected by BRZ than those of the WT and *gpa1-4* (Fig. 1A; Supplementary Fig. S1 at *JXB* online). BRZ also inhibited hypocotyl elongation in the dark more severely in *agb1-1* and *agb1-2* than in the WT and *gpa1-4* (Fig. 1B; Supplementary Fig. S2), suggesting that *agb1* is hypersensitive to BRZ. Exogenously added BR induced hypocotyl elongation in *agb1-1* and *agb1-2*, but the elongation was less than that in the WT (Supplementary Fig. S3). Together, these results suggest that *agb1-1* and *agb1-2* are hyposensitive to BR and that their BR-hyposensitive phenotypes are enhanced by ABA. The BR hyposensitivities of *agb1* in seed germination were previously reported (Chen *et al.*, 2004), but AGB1 was not well characterized as a regulator of BR signalling. In previous studies, BR signalling mutants showed ABA hypersensitivities (for a review, see Clouse, 2011), supporting the idea that the ABA hypersensitivities of *agb1* are at least partly dependent on its BR hyposensitivity. Based on these results, it was hypothesized that AGB1 regulates BR signalling, and the interactions between AGB1 and BR signalling were further examined.

Expression of bzr1-1 alleviates effects of BRZ in both the wild type and agb1

BZR1 is a transcription factor downstream of BR signalling (for a review, see Kim and Wang, 2010). To examine BZR1 functions in *agb1*, BZR1 and its point-mutated (P→L at amino acid position 234) version, bzr1-1, were expressed as GFP-fused proteins (BZR1–GFP and bzr1-1–GFP) in *agb1-1* as well as in the WT (Table 1). Expression of *BZR1-GFP* and *bzr1-1-GFP* in transgenic plants was confirmed by RT–PCR (Supplementary Fig. S4 at *JXB* online). Expression of bzr1-1 is known to alleviate BRZ-induced inhibition of hypocotyl elongation in the dark (Wang *et al.*, 2002; Ryu *et al.*, 2007). In the presence of BRZ, the hypocotyl length of *bzr1-1/a* was greater than that of *agb1-1* but smaller than the hypocotyl lengths of the WT and *bzr1-1/WT*. In the absence of BRZ, the hypocotyl length of *bzr1-1/a* was comparable with that of *agb1-1* and slightly smaller than the hypocotyl lengths of the WT and *bzr1-1/WT* (Fig. 2A; Supplementary Fig. S5). These results suggest that expression of bzr1-1 partially suppresses the BRZ hypersensitivity of *agb1* but does not fully suppress the *agb1* phenotypes.

BR is known to induce dephosphorylation and activation of BZR1 (He *et al.*, 2002). However, neither BR nor BRZ

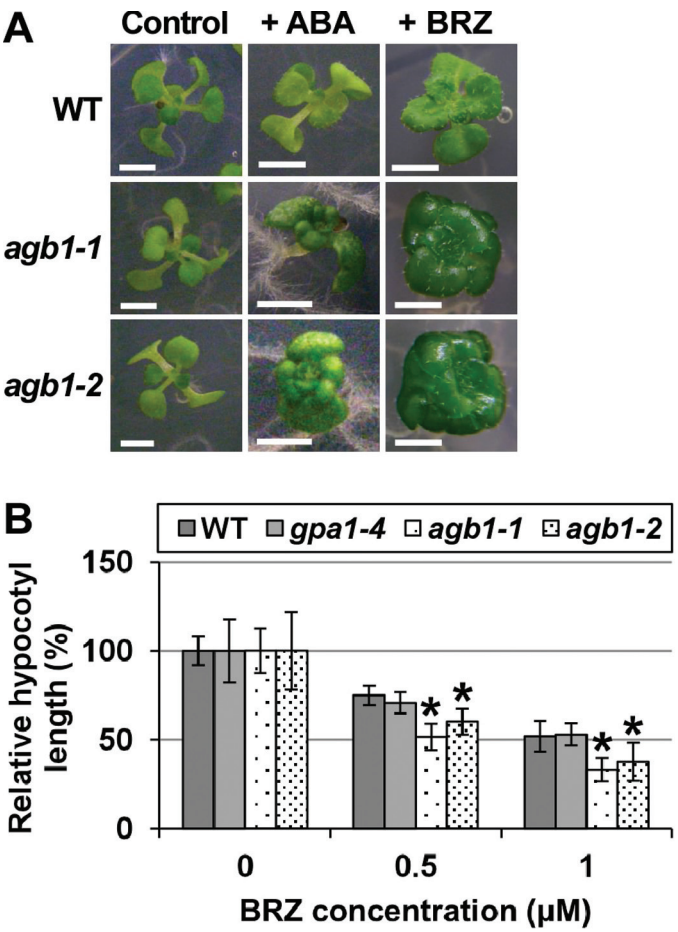

Fig. 1. BRZ hypersensitivity of *agb1*. (A) ABA- and BRZ-hypersensitive phenotypes of *agb1*. Plants were grown under a 16 h light/8 h dark photoperiod in the presence of either 0.5 μM ABA or 5 μM BRZ for 25 d (+ ABA or + BRZ, respectively), or in the absence of ABA or BRZ for 10 d (Control). A representative plant is shown for each genotype. Scale bars=3 mm. (B) BRZ hypersensitivity of *agb1* in hypocotyl elongation in the dark. Plants were grown for 5 d in the dark in the presence of 0, 0.5, or 1 μM BRZ. Relative hypocotyl lengths are shown (for absolute lengths, see Supplementary Fig. S2 at *JXB* online). Values are means ±SE (n=11–18). *P < 0.05 vs. the WT by Student's t-test.

Table 1. *Transgenic plants used in this study*

Abbreviation	Genotype	Definition
BZR1/WT	*BZR1-GFPox/WT*	BZR1–GFP-expressing WT
bzr1-1/WT	*bzr1-1-GFPox/WT*	bzr1-1–GFP-expressing WT
BZR1/a	*BZR1-GFPox/agb1-1*	BZR1–GFP-expressing *agb1-1*
bzr1-1/a	*bzr1-1-GFPox/agb1-1*	bzr1-1–GFP-expressing *agb1-1*

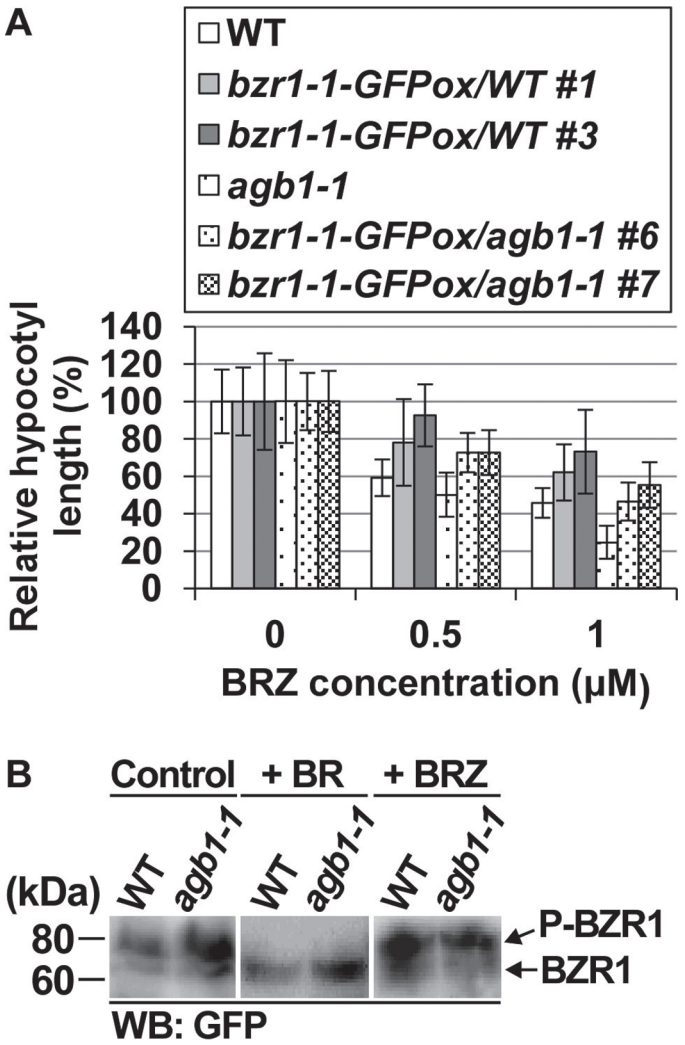

Fig. 2. Functions of BZR1 in *agb1*. (A) Expression of bzr1-1 partially suppresses BRZ hypersensitivity of *agb1-1*. Plants were grown in the dark in the presence of 0, 0.5, or 1 μM BRZ for 4 d, and their hypocotyl lengths were measured. Relative hypocotyl lengths are shown (for absolute lengths, see Supplementary Fig. S5 at *JXB* online). Values are means ±SE (n=15–24). *$P < 0.05$ vs. non-transgenic lines by Student's t-test. (B) AGB1 is not involved in BR-dependent regulation of BZR1 phosphorylation states. *BZR1-GFPox/WT* #9 and *BZR1-GFPox/agb1-1* #9 (only genetic backgrounds, WT and *agb1-1*, are shown) were grown in the presence of 20 nM BR for 15 d (+ BR), in the presence of 2.5 μM BRZ for 25 d (+ BRZ), or in the absence of BR and BRZ (Control), and used for western blotting using an anti-GFP antibody (WB: GFP). Experiments were performed in triplicate, and representative results are shown.

caused differences in BZR1–GFP phosphorylation states between *BZR1/WT* and *BZR1/a* (Fig. 2B), suggesting that AGB1 is not involved in BR-dependent changes of BZR1 phosphorylation states.

Overexpression of BZR1 suppresses effects of ABA in both the wild type and agb1

BZR1/WT, *bzr1-1/WT*, *BZR1/a*, and *bzr1-1/a* were grown in the presence of ABA. Interestingly, *BZR1/WT* but not *bzr1-1/WT* was larger in size than the WT. Similarly, *BZR1/a* but not *bzr1-1/a* was larger in size than *agb1-1*. The leaf morphology of *BZR1/a* was similar to that of *agb1-1* rather than that of the WT (Fig. 3A). The expresson level of *bzr1-1-GFP* in *bzr1-1/a* #6 was higher than the expression level of *BZR1-GFP* in *BZR1/a* #9 (Supplementary Fig. S6 at *JXB* online). These results suggest that BZR1 rather than bzr1-1 alleviates the ABA responses. ABA responses of *BZR1/WT* and *BZR1/a* were further evaluated by scoring their green cotyledons in the presence of ABA. The cotyledon greening rate in the presence of ABA was in the order of *BZR1/WT* > WT > *BZR1/a* > *agb1-1* (Fig. 3B), suggesting that overexpression of BZR1 alleviates ABA-induced growth retardation in both the WT and *agb1*, and that overexpression of BZR1 cannot fully suppress the ABA hypersensitivity of *agb1*. Among BZR1–GFP-overexpressing lines, *BZR1* expression levels and cotyledon greening rates in the presence of ABA were not correlated (Supplementary Fig. S7), which suggests that BZR1-dependent responses are saturated in the transgenic lines studied.

The expression levels of ABA-responsive genes, *RAB18* and *RD29A* (Umezawa *et al.*, 2006), were lower in *BZR1/WT* and *BZR1/a* than in the WT and *agb1-1* in the presence of ABA (Fig. 3C, upper panels), which is in agreement with the finding that overexpression of BZR1 alleviates the effects of ABA on cotyledon greening and subsequent growth (Fig. 3A, B). Under control conditions, the expression of BZR1 target (thereby BR-responsive) genes, *CPD* and *DWF4* (He *et al.*, 2005), was higher in *agb1-1* than in the WT, which is consistent with the finding that *agb1* is hyposensitive to BR. The *CPD* expresson level was lower in *BZR1/a* than in *agb1-1*, but no significant difference was observed in the *DWF4* expression levels between *agb1-1* and *BZR1/a* (Fig. 3C, lower panels). ABA increased the *DWF4* expression levels in both the WT and *agb1-1*, but both *BZR1/WT* and *BZR1/a* showed lower expression levels of *DWF4* than either the WT or *agb1-1* in the presence of ABA (Fig. 3C, lower right panel), again supporting the idea that BZR1 overexpression alleviates the effects of ABA. ABA significantly decreased the *CPD* expression levels and, in the presence of ABA, no difference was observed in the *CPD* expression levels among all the genotypes studied (Fig. 3C, lower left panel).

AGB1 interacts with BIN2

A motif-scanning program (ELM, http://elm.eu.org) identified 17 possible GSK modification sites in the amino acid sequence of AGB1. GSKs are known to regulate BR signalling

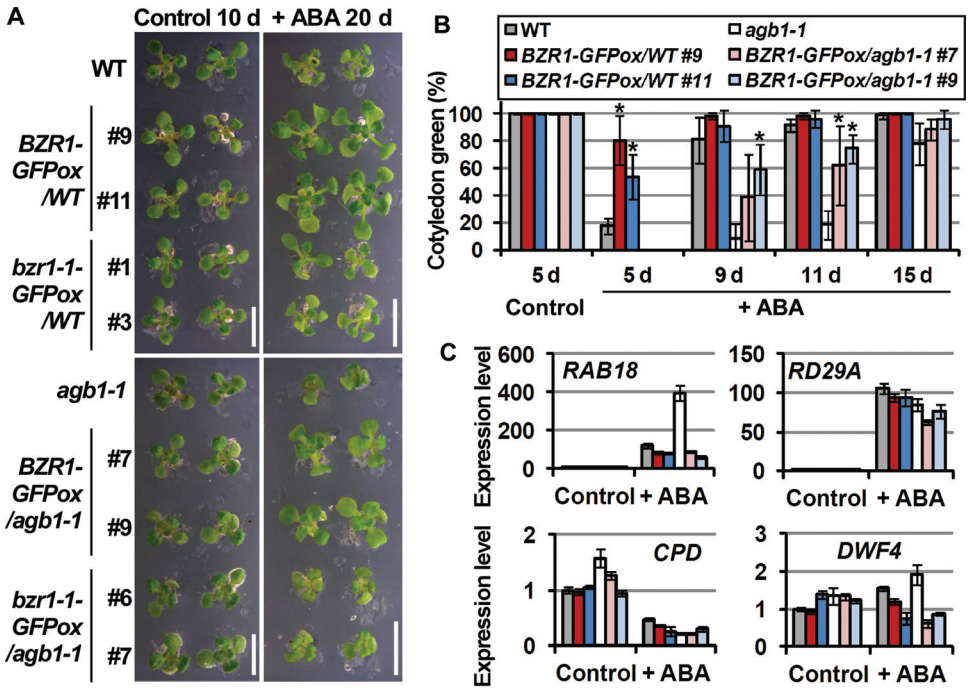

Fig. 3. Overexpression of BZR1 alleviates effects of ABA. (A) Faster growth of BZR1-overexpressing plants in the presence of ABA. Plants were grown in the presence of 0.5 μM ABA for 20 d (+ ABA 20 d) or in the absence of ABA for 10 d (Control 10 d). Two representative plant are shown for each genotype. Scale bars=1 cm. (B) Faster cotyledon greening of BZR1-overexpressing plants in the presence of ABA. Plants were grown in the presence of 0 (Control) or 1 μM ABA (+ ABA). Green cotyledons were scored at the indicated time points. More than 30 plants were used for scoring green cotyledons in each genotype. Experiments were performed in triplicate. Values are means ±SE. *$P < 0.05$ vs. non-transgenic lines by Student's *t*-test. (C) Quantitative RT–PCR analysis of ABA- and BR-responsive gene expression in BZR1-overexpressing plants. Plants were grown in the presence of 0.5 μM ABA for 20 d (+ ABA) or in the absence of ABA for 10 d (Control), and sampled. Relative expression levels of ABA-responsive genes (*RAB18* and *RD29A*) and BR-responsive genes (*CPD* and *DWF4*) were calculated by the comparative C_T method using *UBQ5* as an internal control gene and the WT sample as a reference sample. Experiments were performed in triplicate. Values are means ±SE.

negatively in *Arabidopsis* (He *et al*., 2002; Rozhon *et al*., 2010). Some of the putative GSK modification sites are located on the surface of the predicted three-dimensional structure of AGB1 (Fig. 4A), raising the possibility that AGB1 interacts with GSKs and is phosphorylated by them. To test this idea, the interaction between AGB1 and BIN2, the best-characterized plant GSK, was examined by an *in vitro* GST pull-down assay using His-AGB1 and GST–BIN2. His-AGB1 and GST–BIN2 were expressed in *E. coli* (Supplementary Fig. S8 at *JXB* online). GST–BIN2 was bound to resin and mixed with purified His-AGB1. After incubation, GST–BIN2 was eluted from the resin and His-AGB1 in the elutant was analysed by western blotting. His-AGB1 was detected only when both His-AGB1 and GST–BIN2 were present in the reaction mixture (Supplementary Fig. S9), suggesting that AGB1 and BIN2 interact *in vitro*. Neither ATP, which is required for phosphorylation by protein kinases, nor a GSK inhibitor, bikinin (De Rybel *et al*., 2009), affected the level of His-AGB1 detected in the pull-down assay (Fig. 4B, upper panel), suggesting that the kinase activity of BIN2 does not affect the interaction between AGB1 and BIN2. After the pull-down assay, phosphorylated proteins on the same blot were detected using a phosphoprotein probe, Phos-tag (Kinoshita *et al*., 2006). However, no clear difference was observed in the banding patterns of phosphoproteins in the presence or absence of His-AGB1 (Fig. 4B, lower panel), suggesting that AGB1 is not phosphorylated by BIN2, and that AGB1 does not affect BIN2 autophosphorylation.

The AGB1–BIN2 interaction was further examined by a yeast three-hybrid (Y3H) assay. In the Y3H system, three kinds of protein of interest were co-expressed as GAL4 activation domain (AD)-fused, GAL4 DNA-binding domain (BD)-fused, and haemagglutinin (HA)-tagged forms, respectively, in yeast cells, and subsequent reporter gene activation was checked by culturing the transformed cells on a selection medium. Yeast cells could grow on the selection medium when transformed with AD-fused BIN2 (AD-BIN2), BD-fused AGG1 (BD-AGG1), and HA-tagged AGB1 (HA-AGB1), whereas yeast cells could not grow when one of AD-BIN2, BD-AGG1, or HA-AGB1 was not expressed (Fig. 4C, upper four panels), suggesting that these three proteins can form a complex in yeast cells. Yeast cells also did not grow when the combination AD-BIN2, BD-AGB1, and HA-AGG1 was used (i.e. AGB1 and AGG1 for BD/HA fusions were swapped) (Fig. 4C, bottom). In this case, the BD may have interrupted the interaction between BIN2, AGB1, and AGG1.

A BiFC assay was also performed. The ORF of *AGB1* was fused downstream of the ORF of *nYFP* and the ORF of *BIN2* was fused upstream of the ORF of *cYFP*. When nYFP-fused

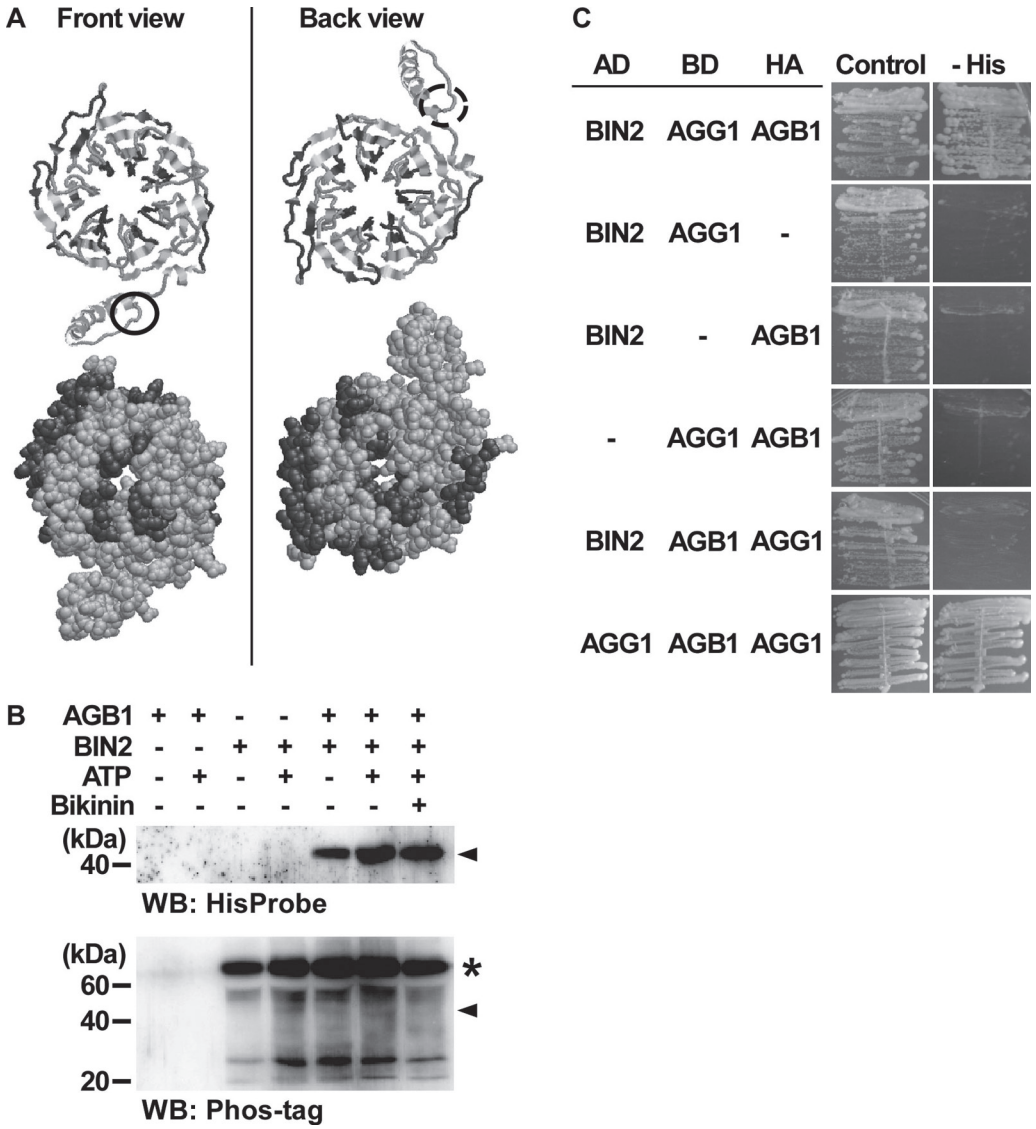

Fig. 4. AGB1 interacts with BIN2 *in vitro*. (A) GSK modification sites are present on the surface of the three-dimensional structure of AGB1. The three-dimensional structure of AGB1 is shown as cartoon models (upper) and space-filling models (lower). Putative GSK modification sites are shown in dark grey and the other amino acids in light grey. Circles indicate the N-termini. (B) *In vitro* GST pull-down assay. Hexahistidine-tagged AGB1 (His-AGB1) and GST-fused BIN2 (GST–BIN2) were expressed in *E. coli* and used for the analysis. For AGB1–, 250 mM imidazole was used instead of a solution containing purified His-AGB1. For BIN2–, GST alone was used instead of GST–BIN2. ATP was used in 0 (ATP –) or 1 mM (ATP +) final concentration. Bikinin was used in 0 (Bikinin –) or 100 μM (Bikinin +) final concentration. His-AGB1 was analysed by western blotting using a polyhistidine probe, HisProbe-HRP (WB: His). After detecting His-AGB1, the same blot was deprobed, and phosphoproteins on the blot were analysed using a Phos-tag (WB: Phos-tag). The positions of His-AGB1 (in both the upper and lower panels) are indicated by arrowheads. Putative GST–BIN2 signals are indicated by *. Experiments were performed in triplicate, and a representative result is shown. (C) Y3H interaction between BIN2, AGB1, and AGG1. The yeast reporter strain AH109 was transformed with pGADT7-Rec containing *BIN2* to express the GAL4 activation domain (AD)-fused BIN2, and pBridge containing either or both of *AGB1* and *AGG1* to express them as GAL4 DNA-binding domain (BD)-fused and HA-tagged forms. The combinations of the expressed proteins are shown at the left. Transformed cells were grown on SD medium (Control) and SD medium lacking histidine (– His). For each transformed cell line, four individual colonies that had appeared after transformation were tested, and representative results are shown. The combination of AD-fused AGG1, BD-fused AGB1, and HA-tagged AGG1 (bottom) is shown as a positive control.

AGB1 (nYFP–AGB1) and cYFP-fused BIN2 (BIN2–cYFP) were expressed together in *Arabidopsis* mesophyll protoplasts, speckled YFP signals were detected. The speckled signals were small in number and large in size (Supplementary Fig. S10 at *JXB* online), and might have resulted from aggregation of nYFP–AGB1 and BIN2–cYFP. It is unclear whether the fluorescence pattern reflects the interaction between AGB1 and BIN2 under physiological conditions.

Effects of AGB1 on the interaction between BIN2 and BZR1

BZR1 is a substrate of GSKs (He *et al.*, 2002; Rozhon *et al.*, 2010). The Y3H system was used to evaluate the effect of HA-AGB1 on the reporter gene activations, which are dependent on co-expressed AD-BZR1 and BD-BIN2. In either the presence or absence of HA-AGB1, yeast cells transformed with AD-BZR1 and BD-BIN2 could grow on high-stringency selection medium, but the activity of β-galactosidase, one of the reporter gene products, was lower in the presence of HA-AGB1 than in its absence (Supplementary Fig. S11 at *JXB* online), suggesting that HA-AGB1 can interfere to some extent with the interaction between BZR1 and BIN2 in yeast.

To examine the effects of AGB1 on the functions of GSKs *in vivo*, *BZR1/WT*, *bzr1-1/WT*, *BZR1/a*, and *bzr1-1/a* were grown in the presence of BR or bikinin, and their phenotypes were compared. BR- or bikinin-induced hypocotyl elongation was greater in *BZR1/WT* and *bzr1-1/WT* than in the WT. Similarly, BR- or bikinin-induced hypocotyl elongation was greater in *BZR1/a* and *bzr1-1/a* than in *agb1-1*. The effect of bzr1-1 overexpression on the BR- or bikinin-induced hypocotyl elongation was similar to the effect of BZR1 overexpression (Fig. 5A). In a previous study, bzr1-1 overexpression caused greater BR-induced hypocotyl elongation than BZR1 overexpression (Ryu *et al.*, 2007), which conflicts with the present result. Although the expression level of *bzr1-1-GFP* in *bzr1-1/a* #6 was higher than the expression level of *BZR1-GFP* in *BZR1/a* #9 (Supplementary Fig. S6 at *JXB* online), the expression levels of *bzr1-1-GFP* still might have been insufficient to cause the greater hypocotyl elongation. In the presence of bikinin, BZR1–GFP was detected as a single, lower sized band in both *BZR1/WT* and *BZR1/a* (Fig. 5B). In the presence of BR, BRZ, or bikinin, no clear difference was observed in BZR1–GFP localization between the WT and *agb1-1* (Supplementary Fig. S12). These results suggest that AGB1 is not involved in GSK-dependent BZR1 phosphorylation *in vivo*.

Discussion

This study has shown that the ABA hypersensitivity of *agb1* is partially suppressed by overexpressing BZR1 (Fig. 3), suggesting that the ABA hypersensitivity of *agb1* is at least partly dependent on the BR hyposensitivity of *agb1*. Although AGB1 is not phosphorylated by BIN2 (Fig. 4B) and does not affect the phosphorylation states of BZR1 or BIN2 (Figs 2, 4B, 5B), AGB1 has putative GSK modification sites on its surface (Fig. 4A) and physically interacts with BIN2 (Fig. 4B, C), supporting the idea that AGB1 regulates BR responses via interaction with GSKs. A model proposed by this study is shown in Fig. 5C.

Overexpression of bzr1-1 suppressed BRZ-hypersensitive phenotypes of *agb1-1*, but the hypocotyl lengths of *bzr1-1/a* were still lower than those of the WT and *bzr1-1/WT* (Fig. 2A). Similarly, BR- or bikinin-induced hypocotyl

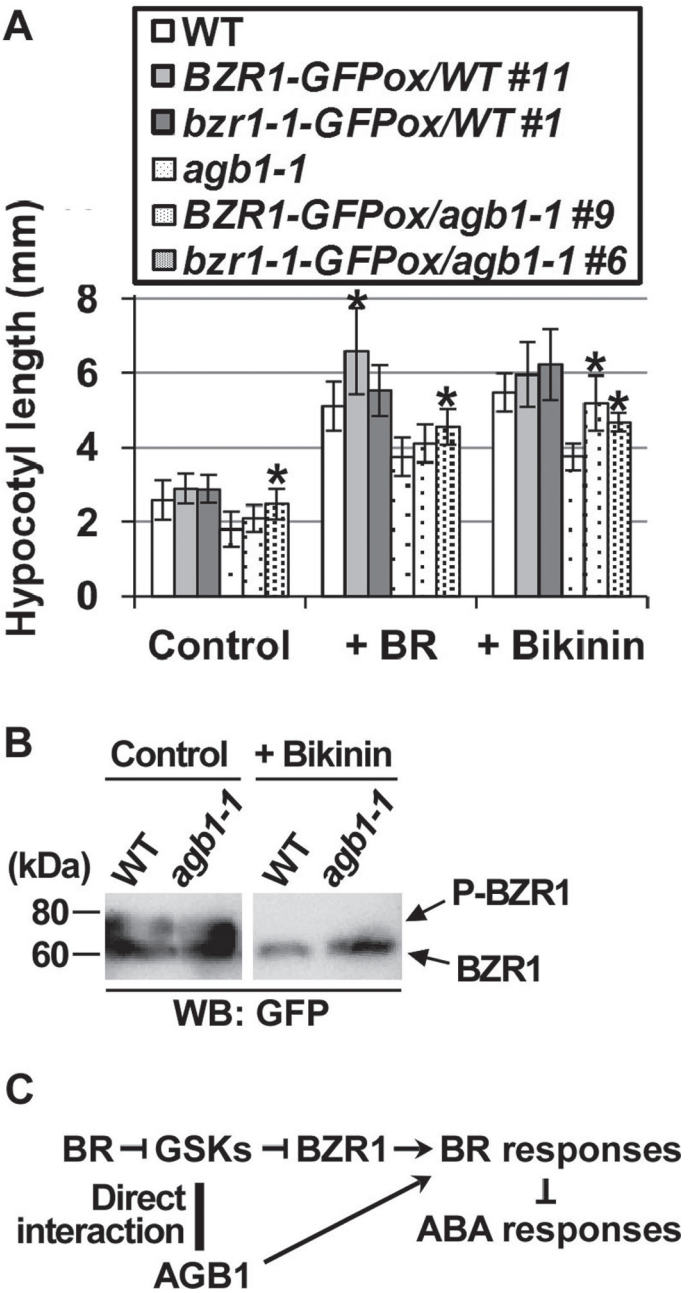

Fig. 5. AGB1 is not involved in GSK-dependent regulation of BZR1 functions. (A) Overexpression of BZR1 and bzr1-1 partially suppresses the BR hyposensitivity of *agb1-1*. Plants were grown under a 16 h light/8 h dark photoperiod for 15 d in the presence of either 20 nM BR (+ BR) or 40 μM bikinin (+ Bikinin), or in the absence of BR or bikinin (Control), and their hypocotyl lengths were measured. Values are means ±SE (n=15–22). *$P < 0.05$ vs. non-transgenic lines by Student's t-test. (B) AGB1 is not involved in GSK-dependent phosphorylation of BZR1. *BZR1-GFPox/WT* #9 and *BZR1-GFPox/agb1-1* #9 (only genetic backgrounds, WT and *agb1-1*, are shown) were grown for 15 d in the presence of 0 (Control) or 40 μM bikinin (+ Bikinin), and used for western blotting using an anti-GFP antibody (WB: GFP). Experiments were performed in triplicate and a representative result is shown. (C) A model proposed by this study.

elongation was not as great in *BZR1/a* and *bzr1-1/a* as it was in the WT, *BZR1/WT*, and *bzr1-1/WT* (Fig. 5A). These results indicate that BZR1 can enhance BR responses even in *agb1*, although AGB1 is required to activate BR responses fully. Because AGB1 is not necessary for regulating either BZR1 phosphorylation states or the subcellular localization of BZR1 (Figs 2B, 5B; Supplementary Fig. S10 at *JXB* online), AGB1 does not seem to be involved in regulating the functions of BZR1. BZR2 is another key transcription factor regulating the BR responses. It is also unlikely that AGB1 regulates the functions of BZR2 because BZR2 is similar to BZR1 in several respects, including amino acid sequence (88% identity), physiological function (Wang *et al*., 2002; Yin *et al*., 2002; for a review, see Kim and Wang, 2010), BR- and GSK-dependent phosphorylation states (He *et al*., 2002; Yin *et al*., 2002; Vert and Chory, 2006; Yan *et al*., 2009; Rozhon *et al*., 2010), and target genes (He *et al*., 2005; Vert and Chory, 2006; Sun *et al*., 2010).

AGB1 was originally identified as *ELK4* (*ERECTA-LIKE4*), a gene responsible for the *erecta* (*er*) phenotype. *agb1/elk4* shows several *er* phenotypes (e.g. rounder leaves with shorter petioles, shorter stems, more highly clustered flower buds, and shorter and wider siliques) (Lease *et al*., 2001). The well-studied BR-related mutants have these phenotypes as well (for a review, see Clouse, 2011), although the phenotypes of the BR-related mutants seem much more severe than those of *er* and *agb1*. *ER* encodes a receptor-like kinase (RLK) which has 20 extracellular leucine-rich repeats (LRRs), a single transmembrane domain, and an intracellular serine/threonine protein kinase domain (Lease *et al*., 2001). Interestingly, a BR receptor, BRI1, and its co-receptor, BAK1, are both LRR-RLKs. BR-mediated heterodimerization of BRI1 and BAK1 is thought to promote BR signalling (for a review, see Kim and Wang, 2010). Further studies are required to determine whether AGB1 and ER interact or whether ER is involved in BR signalling.

Overexpression of BZR1 alleviated the effects of ABA on cotyledon greening and subsequent growth in both the WT and *agb1-1* (Fig. 3). This is consistent with previous studies suggesting that mutants which have impaired BR responses are hypersensitive to ABA (for a review, see Clouse, 2011). *BZR1-GFPox/agb1-1* showed lower cotyledon greening rates in the presence of ABA than the WT (Fig. 3B). It is unclear how BR signalling decreases the ABA responses, but BZR1-mediated BR responses are thought to be insufficient to suppress the ABA hypersensitivity of *agb1* fully. It is also interesting that BZR1 overexpression rather than bzr1-1 overexpression alleviated the ABA effects (Fig. 3A). BZR1 and bzr1-1 have been suggested to differ in their protein stabilities (He *et al*., 2002; Wang *et al*., 2002; Tang *et al*., 2011), phosphorylation states (He *et al*., 2002; Tang *et al*., 2011), and subcellular localizations (Ryu *et al*., 2007; this study, Supplementary Fig. S12 at *JXB* online). In addition, bzr1-1 has a higher affinity for a subunit of PP2A than does BZR1 (Tang *et al*., 2011). BZR1 and bzr1-1 may have different affinities for other interaction partners that affect the expression of BZR1 target genes. These differences might lead to the different effects of BZR1 and bzr1-1 on the gene expression involved in the ABA responses. Further studies are required to elucidate the molecular mechanisms underlying BZR1-mediated ABA signalling.

AGB1 was shown to interact with BIN2 *in vitro* (Fig. 4B). However, AGB1 did not affect the kinase activity of BIN2 (Figs 4B, 5B) and was not phoshorylated by BIN2 (Fig. 4B). Thus the physiological relevance of the interaction between AGB1 and BIN2 is unclear. One possibility is that AGB1 regulates the phosphorylation of BIN2 substrates other than BZR1 and BZR2. ARF2 and YODA are two examples of BIN2 substrates other than BZR1. ARF2 is a transcription factor regulating auxin-responsive gene expression (Vert *et al*., 2008). YODA is a mitogen-activated protein kinase kinase kinase (MAPKKK) regulating stomatal development, and, interestingly, YODA has been suggested to be under the control of ER signalling as well as BIN2-dependent signalling (Kim *et al*., 2012). AGB1 is also involved in auxin signalling in *Arabidopsis* (Ullah *et al*., 2003), and *Pisum sativum* Gβ interacts with a MAPK (Bhardwaj *et al*., 2011). Therefore, it will be interesting to examine whether AGB1 regulates the BIN2-dependent phosphorylation of ARF2 and YODA.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Phenotypes of *agb1* grown in different concentrations of BRZ.

Figure S2. Absolute hypocotyl lengths of *agb1* grown in the presence of BRZ.

Figure S3. BR-induced hypocotyl elongation in *agb1*.

Figure S4. Semi-quantitative RT–PCR analysis of *BZR1-GFP* and *bzr1-1-GFP* expression.

Figure S5. Absolute hypocotyl lengths of bzr1-1–GFP-overexpressing plants grown in the presence of BRZ.

Figure S6. Expression levels of *BZR1-GFP* and *bzr1-1-GFP* in transgenic plants.

Figure S7. *BZR1* expression levels in *BZR1-GFP*-overexpressing lines and their responses to ABA.

Figure S8. Expression of His-AGB1 and GST–BIN2 in *E. coli*.

Figure S9. *In vitro* GST pull-down assay.

Figure S10. BiFC between AGB1 and BIN2.

Figure S11. Effects of AGB1 on the interaction between BIN2 and BZR1 in a Y3H system.

Figure S12. Subcellular localizations of BZR1–GFP in *agb1*.

Table S1. Primer pairs used for RT–PCR analyses.

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