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Arabidopsis heterotrimeric G protein β subunit interacts with a plasma membrane 2C-type protein phosphatase, PP2C52

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ABSTRACT

Heterotrimeric G proteins (Gα, Gβ, Gγ) play important roles in signal transduction among various eukaryotic species. G proteins transmit signals by regulating the activities of effector proteins, but only a few Gβ-interacting effectors have been identified in plants. Here we show by a yeast two-hybrid screen that a putative myristoylated 2C-type protein phosphatase, PP2C52, is an *Arabidopsis* Gβ (AGB1)-interacting partner. The interaction between AGB1 and PP2C52 was confirmed by an in vitro pull-down assay and a bimolecular fluorescence complementation assay. *PP2C52* transcripts were detected in many tissues. PP2C52 was localized to the plasma membrane and a mutation in the putative myristoylation site of PP2C52 disrupted its plasma membrane localization. Our results suggest that PP2C52 interacts with AGB1 on the plasma membrane and transmits signals via dephosphorylation of other proteins.

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1. Introduction

Heterotrimeric GTP-binding proteins (G proteins, consisting of subunits Gα, Gβ, and Gγ) are signaling molecules found in a variety of eukaryotic organisms. They mediate ligand-binding signals from G protein-coupled receptors (GPCR) to downstream pathways, and thus are involved in diverse cellular processes. In plants, studies of loss-of-function mutants have suggested that G proteins have roles in signal transduction of phytohormones, such as auxin, brassinosteroid, and abscisic acid (ABA) ([1], for a review). In particular, ABA was shown to be bound by Gα-interacting receptors on the plasma membrane [2,3], confirming the importance of the G proteins in ABA signal transduction.

In animals, GPCRs are activated upon binding of their ligands and promote the exchange of GDP to GTP on Gα, which leads to a conformational change of Gα and dissociation of the heterotrimer into Gα and a Gβγ dimer. Gα and Gβγ are active and independently move on the plasma membrane where they regulate the activities of effector molecules, such as adenylate cyclase, phospholipase C, and ion channels. This causes a rapid change in the local production of second messengers, which transmit the ligand-binding signals from GPCRs to downstream pathways ([4], for a review).

Similar regulatory mechanisms are thought to operate in plants. In agreement with the animal GPCR signaling system, Gα and Gβ in *Arabidopsis thaliana* (GPA1 and AGB1, respectively) are involved in ABA-dependent regulation of ion channels [5,6]. However, the numbers and kinds of GPCR signaling components in plants differ somewhat from those in animals. For example, humans have 21 Gα genes, five Gβ genes, and 12 Gγ genes while *Arabidopsis* has only one Gα gene (*GPA1*), one Gβ gene (*AGB1*) ([4], for a review) and three Gγ genes (*AGG1*, *AGG2*, *AGG3*) [7–9], and no human-like adenylate cyclase exists in *Arabidopsis* ([4], for a review). This raises the possibility of the existence of plant-specific mechanisms for G protein signaling.

In *Arabidopsis*, Gβ deficiency causes changes in morphology and sensitivities to various stimuli ([1], for a review, [10–15]), suggesting that effectors regulated by Gβγ have important physiological roles. So far some genetic and/or physical AGB1-interaction partners have been identified and characterized. For example, overexpression of a Golgi-localized hexose transporter, SGB1, partially suppresses the *agb1* phenotype [16]. NDL1 (N-MYC downregulated-like1) physically interacts with AGB1 and regulates auxin distribution in plants [17]. An acireductone dioxygenase-like protein, ARD1, also physically interacts with AGB1, and its enzyme activity is enhanced by Gβγ [18]. However, the molecular mechanisms underlying the AGB1-mediated signaling remain to be elucidated.

Abbreviations: ABA, abscisic acid; BiFC, bimolecular fluorescence complementation; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; ORF, open reading frame; PLC, phospholipase C; PP2C, 2C-type protein phosphatase; RT-PCR, reverse transcription-PCR; SE, standard error; TBS, Tris-buffered saline; YFP, yellow fluorescent protein; Y2H, yeast two-hybrid

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In this study, we used a yeast two-hybrid (Y2H) screen to identify AGB1-interacting factors and identified a putative myristoylated 2C-type protein phosphatase (PP2C), PP2C52, as a novel AGB1-interacting protein. A pull-down assay and a bimolecular fluorescence complementation (BiFC) assay also suggested that AGB1 and PP2C52 interact with each other. We also determined the expression pattern of *PP2C52* and attempted to determine the functions of PP2C52.

2. Materials and methods

2.1. Yeast two-hybrid (Y2H) analysis

Y2H experiments were performed using a Matchmaker Two-Hybrid System (Clontech). A cDNA library for the Y2H screen was prepared from mRNA samples from *Arabidopsis* mature leaves, using a Matchmaker Library Construction & Screening Kit (Clontech). Full-length cDNA clones of *AGB1* (*AT4G34460*), *AGG1* (*AT3G63420*) and *PP2C52* (*AT4G03415*) (RAFL05-19-B08, RAFL22-41-E11 and RAFL18-04-O14, respectively) were obtained from RIKEN BRC Experimental Plant Division [19]. The open reading frame (ORF) of *AGB1* was amplified by PCR using the cDNA clone as template and the following primer pair: 5′-CGCCATATGTCTGTCTCCGAGCTC-3′ and 5′-CCCGTCGACAATCACTCTCCTGGTCCTCC-3′ (*Nde*I and *Sal*I sites are underlined). The PCR products were digested by *Nde*I and *Sal*I, and cloned into the *Nde*I-*Sal*I site of pGBKT7, generating pGBK-AGB1. This plasmid was used as bait for the Y2H screen. The ORF of *AGG1* was amplified by PCR using the cDNA cloned as template and the following primer pair: 5′-GGGCATATGCGAGAGGAAACTGTGG-3′ and 5′-CCACTAGTAAGTATTAAGCATCTGCAGCC-3′ (*Nde*I and *Spe*I sites are underlined). The PCR products were digested by *Nde*I and *Spe*I, and cloned into the *Nde*I-*Xba*I site of pGADT7-rec, generating pGAD-AGG1. The ORF of *PP2C52* was amplified by PCR using the cDNA cloned as template and the following primer pair: 5′-CCCGAATTCTCTAGAATGGGGGGTTGTGTGTCGAC-3′ (*Eco*RI and *Xba*I sites are underlined) and 5′-GGGCTCGAGGAGTCTTCGATTTCTCTTCAGAG-3′ (the *Xho*I site is underlined). The PCR products were digested by *Eco*RI and *Xho*I, and cloned into the *Eco*RI-*Xho*I site of pGADT7-rec, generating pGAD-PP2C52. The ORF of *PP2C1* (*AT1G03590*) was amplified by reverse transcription-PCR (RT-PCR) using cDNA synthesized from total RNA from 2-week-old *Arabidopsis* seedlings as template and the following primer pair: 5′-GGGCATATGGGAGGTTGTATCTCTAAG-3′ and 5′-GAGGTCGACAAGTCTTTGGTTCCTCTCCAGGG-3′ (*Nde*I and *Sal*I sites are underlined). The PCR products were digested by *Nde*I and *Sal*I, and cloned into the *Nde*I-*Xho*I site of pGADT7-rec, generating pGAD-PP2C1. The ORF of *PP2C74* (*AT5G36250*) was amplified by RT-PCR using the following primer pair: 5′-CGCCATATGGGGTCCTGCTTATCATC-3′ and 5′-CCCGTCGACACTCTTTGGTTGGGACATATAC-3′ (*Nde*I and *Sal*I sites are underlined). The PCR products were digested by *Nde*I and *Sal*I, and cloned into the *Nde*I-*Xho*I site of pGADT7-rec, generating pGAD-PP2C74. To confirm the result of Y2H screen, pGBK-AGB1 and one of pGAD constructs above were co-introduced into the *Saccharomyces cerevisiae* strain AH109. After transformation, at least 4 colonies grown on the SD media lacking leucine and tryptophan (SD/-Leu/-Trp), were streaked on the SD/-Leu/-Trp and the SD media lacking leucine, tryptophan, adenine, and histidine.

2.2. In vitro pull-down assay

For the in vitro pull-down assay, pGAD-PP2C52 was digested by *Eco*RI and *Xho*I, and the resultant ORF fragments of *PP2C52* were inserted into the *Eco*RI-*Xho*I site of pGEX-5X-1 (GE Healthcare) in-frame to the coding sequence of glutathione S-transferase (GST), generating pGEX-5X-PP2C52. The coding sequence of *Myc*-tagged *AGB1* was amplified using pGBK-AGB1 as template and the following primer pair: 5′-CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGAGGAGCAGAAGCTGATC-3′ and 5′-CCCGTCGACAATCACTCTCCTGGTCCTCC-3′ (*Xba*I and *Sal*I sites are underlined). The PCR products were digested by *Xba*I and *Sal*I, and inserted into the *Xba*I-*Sal*I site of pET32b(+) (Novagen), generating pET-Myc-AGB1. To induce GST-fused PP2C52 (GST-PP2C52) and Myc-tagged AGB1, pGEX-5X-PP2C52 and pET-Myc-AGB1 were transformed into the *Escherichia coli* strain, BL21 (DE3). Transformed *E. coli* cells were cultured at 37 °C in LB medium until OD_{600} reached 0.5, and incubated at 28 °C for 2 h after an addition of IPTG to a final concentration of 0.2 mM. The cells were then harvested by centrifugation and resuspended in 1× TBS (Tris-buffered saline: 150 mM NaCl in 20 mM Tris–HCl, pH 7.5) with 2 mg/ml lysozyme (Wako, Japan). The cell suspension was frozen at −80 °C and thawed at room temperature. Freezing and thawing were repeated two more times to lyse the cells, and 2 units of recombinant DNase I (Takara Bio) was added to the solution. The solution was incubated at room temperature until the solution became fluid due to DNA degradation. The solution was then centrifuged at 12000 ×*g* for 5 min and the supernatant was used as crude protein extracts.

GST-PP2C52 in the crude extracts was bound to Glutathione Sepharose 4 Fast Flow (GE Healthcare) following the manufacturer's instructions and the resin was washed 4 times by 1× TBS. After removing 1× TBS, the resin was resuspended in the crude extracts containing Myc-AGB1 and incubated at room temperature for 60 min with a gentle shaking. The resin was then washed 4 times by 1× TBS and resuspended in 20 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. The suspension was incubated at room temperature for 15 min to elute GST-PP2C52. The slurry of the resin was centrifuged for a few min at 12000 ×*g*, and GST-PP2C52 and Myc-AGB1 in the supernatant were analyzed by immunoblotting using an anti-GST antibody (GE Healthcare) and an anti-Myc antibody (Medical & Biological Laboratories Co. Ltd. (MBL), Japan). To detect signals of Myc-AGB1 in the GST pull-down assay, a SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) was used. For the other immunoblot experiments, a SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used.

2.3. Bimolecular fluorescence complementation (BiFC)

The coding sequence of the N-terminal region of yellow fluorescent protein (nYFP, amino acids 1–154 of YFP) was amplified by PCR using pEYFP-N1 (Clontech) as template and the following primer pair: 5′-AAACTAGTATGGTGAGCAAGGGCGAGGAGC-3′ (the *Spe*I site is underlined) and 5′-CCCGAGCTCTCTAGAGGTGATATAGACGTTGTGGC-3′ (*Sac*I and *Xba*I sites are underlined). The PCR products were digested by *Spe*I and *Sac*I and inserted into the *Xba*I-*Sac*I site of pBS-35SMCS-GFP [20], generating pBS-35S-nYFP-1. The digested PCR products were also inserted into the *Spe*I-*Sac*I site of pBS-35SMCS-GFP [20], generating pBS-35S-nYFP-2. The ORF of *AGB1* was amplified by PCR using pGBK-AGB1 as template and the following primer pair: 5′-CGCTCTAGAATGTCTGTCTCCGAGCTC-3′ and 5′-CCCACTAGTAATCACTCTCCTGGTCCTCC-3′ (*Xba*I and *Spe*I sites are underlined). The PCR products were digested by *Xba*I and *Spe*I, and cloned into the *Xba*I site of pBS-35S-nYFP-1, generating pBS-35S-nYFP-AGB1. The ORF of *GPA1* was amplified by PCR using a cDNA clone of *GPA1*, which was obtained from RIKEN BRC Experimental Plant Division [19], as template and the following primer pair: 5′-GGGGTACCATGGGCTTACTCTGCAGTAG-3′ and 5′-GGACTAGTTAAAAGGCCAGCCTCCAG-3′ (*Kpn*I and *Spe*I sites are underlined). The PCR products were digested by *Kpn*I and *Spe*I and inserted into the *Kpn*I-*Spe*I site of pBS-35S-nYFP-2. The coding sequence of the C-terminal region of YFP (cYFP, amino acids 155–239 of YFP) was amplified by PCR using pEYFP-C1 as template and the following primer pair: 5′-CCACTAGTATGACCGCCGACAAGCAGAAGAAC-3′ and 5′-AAGAGCTCTTACTTGTACAGCTCGTCCATG-3′ (*Spe*I and *Sac*I sites are underlined). The PCR products were digested by *Spe*I and *Sac*I and inserted into the *Spe*I-*Sac*I site of pBS-35SMCS-GFP, generating pBS-35SMCS-cYFP. pGAD-PP2C52 was digested by *Xba*I and *Xho*I,

and the resultant ORF fragments of *PP2C52* were inserted into the *Xba*I-*Sal*I site of pBS-35SMCS-cYFP, generating pBS-35S-PP2C52-cYFP. pBS-35S-PP2C74-GFP [20] was digested by *Sal*I, and the resultant ORF fragments of *PP2C74* were inserted into the *Sal*I site of pBS-35SMCS-cYFP. A mixture of an nYFP construct and a cYFP construct (500 ng each) was used for particle bombardment to co-express proteins of interest in onion epidermal cells. Particle bombardment and fluorescence microscopy were performed as previously described [21]. Images were processed using Canvas X software (ACD Systems).

2.4. RT-PCR

A. thaliana ecotype Columbia-0 (Col-0) was used as the plant material. Seeds were surface sterilized and sown on 0.8% agar (Wako) containing 0.5× MS salts (Wako), 1%w/v sucrose and 0.5 g/l MES, pH 5.8, chilled at 4 °C in the dark for 48 h (stratified), and germinated at 22 °C. Plants were grown at 22 °C under 16-h light/8-h dark condition (light intensity 120 μmol m^{-2} s^{-1}). After 3 weeks of growth, plants were transferred onto rockwool cubes and grown further with 0.2× MS solution regularly supplied. For RNA extraction, leaves, roots, flowers and flower stalks from mature plants and 2-week-old seedlings were sampled.

Total RNA was prepared using RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized from 3 μg of the total RNA with PrimeScript Reverse Transcriptase (Takara Bio) using an oligo (dT) primer. The reaction mixtures were diluted 20 times with distilled water and used as a template for PCR. *PP2C52* expression was measured by real-time quantitative RT-PCR (qRT-PCR) using primers 5′-CTCGGCTGCGCGTGAATGGA-3′ and 5′-TCACGCGTGTGACGCCTTGT-3′, GoTaq qPCR Master Mix (Promega) and a StepOne Real-Time PCR System (Applied Biosystems). Relative expression levels were calculated by the comparative C_T method using *UBQ5* as an internal control gene.

2.5. Subcellular localizations of GFP- and mCherry-fused proteins

pGAD-PP2C52 was digested by *Xba*I and *Xho*I, and the resultant ORF fragments of *PP2C52* were inserted into the *Xba*I-*Sal*I site of pBS-35SMCS-GFP, generating pBS-35S-PP2C52-GFP. To introduce the glycine→alanine mutation at position 2 (G2A mutation) of PP2C52, the ORF of *PP2C52* was amplified by PCR using pBS-35S-PP2C52-GFP as template and the following primer pair: 5′-GGGTCTAGAATGGCGGGTTGTGTGTCGACTAGTAG-3′ and 5′- GGGCTCGAGGAGTCTTCGATTTCTCTTCAGAG-3′ (*Xba*I and *Xho*I sites are underlined). The PCR products were digested by *Xba*I and *Xho*I, and inserted into the *Xba*I-*Xho*I site of pBS-35SMCS-GFP. The constructs were introduced into onion epidermal cells as previously described [21]. pBS-35S-PP2C52-GFP was introduced into mesophyll protoplasts from wild type (Col-0) or *agb1-2* [22] as previously described [23,24]. Seeds of *agb1-2* (CS6536) were obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org).

To express GFP-fused AGB1 (GFP-AGB1), DNA fragments containing 35S promoter-GFP region were obtained by PCR using pBS-35SMCS-GFP as template and the following primer pair: 5′-AGATTAGCCTTTTCAATTTCAG-3′ and 5′-GGGACTAGTCTTGTACAGCTCGTCCATGCCG-3′ (*Spe*I site is underlined), digested by *Nco*I and *Spe*I, and inserted into the *Nco*I-*Xba*I site of pBS-35S-nYFP-AGB1, generating pBS-35S-GFP-AGB1. To express mCherry-fused PP2C52 (PP2C52-mCherry), the ORF of *mCherry* was amplified by PCR using pmCherry-N1 (Clontech) as template and the following primer pair: 5′-GGATCTAGAATGGTGAGCAAGGGCGAGGAGG-3′ and 5′-TTACTTGTACAGCTCGTCCATG-3′ (*Xba*I and *Bsr*GI sites are underlined). The PCR fragments were digested by *Xba*I and *Bsr*GI and inserted into the *Spe*I-*Bsr*GI site of pBS-35S-PP2C52-GFP, generating pBS-35S-PP2C52-mCherry. A mixture of pBS-35S-GFP-AGB1 and pBS-35S-PP2C52-mCherry (500 ng each) was used for particle bombardment to co-express GFP-AGB1 and PP2C52-mCherry in onion epidermal cells. Particle bombardment and fluorescence microscopy were performed as previously described [21]. Images were processed using Canvas X software (ACD Systems).

2.6. Phosphatase assay

GST-PP2C52 was expressed in *E. coli* and bound to Glutathione Sepharose 4 Fast Flow as described above. The resin was washed 4 times by 1× TBS and resuspended in 20 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. to elute GST-PP2C52. The slurry was centrifuged at 6000 ×*g* for 2 min and the supernatant was used as purified GST-PP2C52 solution for the phosphatase assay. To express polyhistidine-tagged AGB1 (His-AGB1), the ORF of *AGB1* was amplified by PCR using pGBK-AGB1 as template and the following primer pair: 5′-CGCTCTAGAATGTCTGTCTCCGAGCTC-3′ and 5′-CCCGTCGACAATCACTCTCCTGGTCCTCC-3′ (*Xba*I and *Sal*I sites are underlined). The PCR products were digested by *Xba*I and *Sal*I, and cloned into the *Nhe*I-*Xho*I site of $pRSET_B$ (Invitrogen), generating pRSET-AGB1. To express polyhistidine-tagged AGG1 (His-AGG1), the ORF of *AGG1* was amplified by PCR using the cDNA cloned as template and the following primer pair: 5′-GGGTCTAGAATGCGAGAGGAAACTGTGG-3′ and 5′-CCGTCGACAAGTATTAAGCATCTGCAGCC-3′ (*Xba*I and *Sal*I sites are underlined). The PCR products were digested by *Xba*I and *Sal*I, and cloned into the *Nhe*I-*Xho*I site of $pRSET_B$, generating pRSET-AGG1. pRSET-AGB1 or pRSET-AGG1 was introduced into BL21 (DE3). Transformed cells were cultured overnight at 37 °C, collected by centrifugation, and lysed as described above to prepare crude extracts containing His-AGB1 or His-AGG1. Addition of IPTG was not necessary to express His-AGB1 or His-AGG1. His-AGB1 or His-AGG1 in the crude extracts was bound to Ni-NTA His-Bind resin (Novagen) following the manufacturer's instructions. The resin was washed 4 times by 1× TBS and resuspended in 250 mM imidazole to elute His-AGB1 or His-AGG1. The slurry was centrifuged at 6000 ×*g* for 2 min and the supernatant was used as purified His-AGB1 or His-AGG1 solution for the phosphatase assay. For a control, mVenus/$pRSET_B$ [25], which was obtained from the RIKEN Brain Science Institute, was introduced into BL21 (DE3) to express polyhistidine-tagged mVenus (His-mVenus). His-mVenus was expressed and purified as described above, and used for the phosphatase assay.

The phosphatase assay was performed using a Non-Radioactive Serine–Threonine Phosphatase Assay System (Promega). To confirm the phosphatase activity of PP2C52, phosphatase reaction was performed in a volume of 50 μl containing 20 mM $MgCl_2$, 0.1 mM phosphopeptide (RRApTVA), 50 mM Tris–HCl, pH 7.5, 1/20 (v/v) purified GST-PP2C52 with or without 20 mM EDTA. After 20 min incubation at room temperature, 50 μl of molybdate dye/additive solution was added to the solution, and the solution was incubated for another 20 min at room temperature to fully develop the color. A_{600} of the solution was measured and the amounts of the released phosphate were calculated by a standard curve obtained from known concentrations of phosphate standard solutions. For a negative control, GST alone was expressed as GST-PP2C52 and used for the assay instead of GST-PP2C52. To examine the effects of AGB1 and AGG1, the reaction was performed in a volume of 50 μl containing 20 mM $MgCl_2$, 0.1 mM phosphopeptide (RRApTVA), 1/20 (v/v) purified GST-PP2C52, 1/10 (v/v) purified His-AGB1, and 1/10 (v/v) His-AGG1. When one or both of His-AGB1 and His-AGG1 were not added, imidazole was added to make a final concentration of 50 mM. The amounts of the released phosphate were calculated as above.

3. Results

3.1. PP2C52 interacts with AGB1

Using full-length AGB1 as bait, a Y2H screen of the *Arabidopsis* leaf library was performed to identify AGB1-interacting proteins. Even on

high-stringency selection media, which lack histidine and adenine, more than 3600 positive clones were obtained. Yeast colony PCR with an *AGG1*-specific primer revealed that 60–70% of these clones expressed AGG1. Plasmid inserts from non-*AGG1* clones were amplified by yeast colony PCR using a vector-specific primer pair and sequenced. Approximately 400 clones were examined and two of them were *PP2C52* (data not shown). PP2C52 was a putative protein phosphatase and thought to catalyze protein dephosphorylation. Protein dephosphorylation plays a role in many signaling pathways. Thus *PP2C52* was chosen for further analyses.

PP2C52 is classified into group E plant PP2Cs [26]. To examine the specificity of the AGB1-PP2C52 interaction, two other group E plant PP2Cs, PP2C1 and PP2C74, as well as PP2C52, were subjected to a Y2H assay. PP2C52 enabled yeast cells to grow on the selection media when it was co-expressed with AGB1, but neither PP2C1 nor PP2C74 had the same effect (Fig. 1A), suggesting that AGB1 specifically interacts with PP2C52 in yeast cells.

To examine whether PP2C52 interacts with AGB1 in vitro, a GST pull-down assay was performed. GST-fused PP2C52 (GST-PP2C52) was bound to resin and mixed with a solution containing Myc-tagged AGB1 (Myc-AGB1). After incubation, GST-PP2C52 was eluted from the resin and Myc-AGB1 in the elutant was analyzed by immunoblotting. Specific signals of Myc-AGB1 were detected only when both PP2C52 and AGB1 were present (Fig. 1B), indicating that PP2C52 interacts with AGB1 in vitro.

The AGB1-PP2C52 interaction in plant cells was examined with a BiFC assay. The ORF of *AGB1* was fused downstream of the ORF of *nYFP* and the ORF of *PP2C52* was fused upstream of the ORF of *cYFP*. When nYFP-fused AGB1 (nYFP-AGB1) and cYFP-fused PP2C52 (PP2C52-cYFP) were expressed together in *Arabidopsis* mesophyll protoplasts, YFP signals were detected in the peripheral region. Neither cYFP alone nor cYFP-fused PP2C74 (PP2C74-cYFP), which is a known plasma membrane-localized PP2C [20], recovered YFP fluorescence when expressed with nYFP-AGB1. In addition, neither nYFP alone nor nYFP-fused GPA1 (GPA1-nYFP) recovered YFP fluorescence when expressed with PP2C52-cYFP (Fig. 1C). These results suggest that AGB1 and PP2C52 specifically interact with each other on the plasma membrane, although it might be possible that expression levels of the proteins

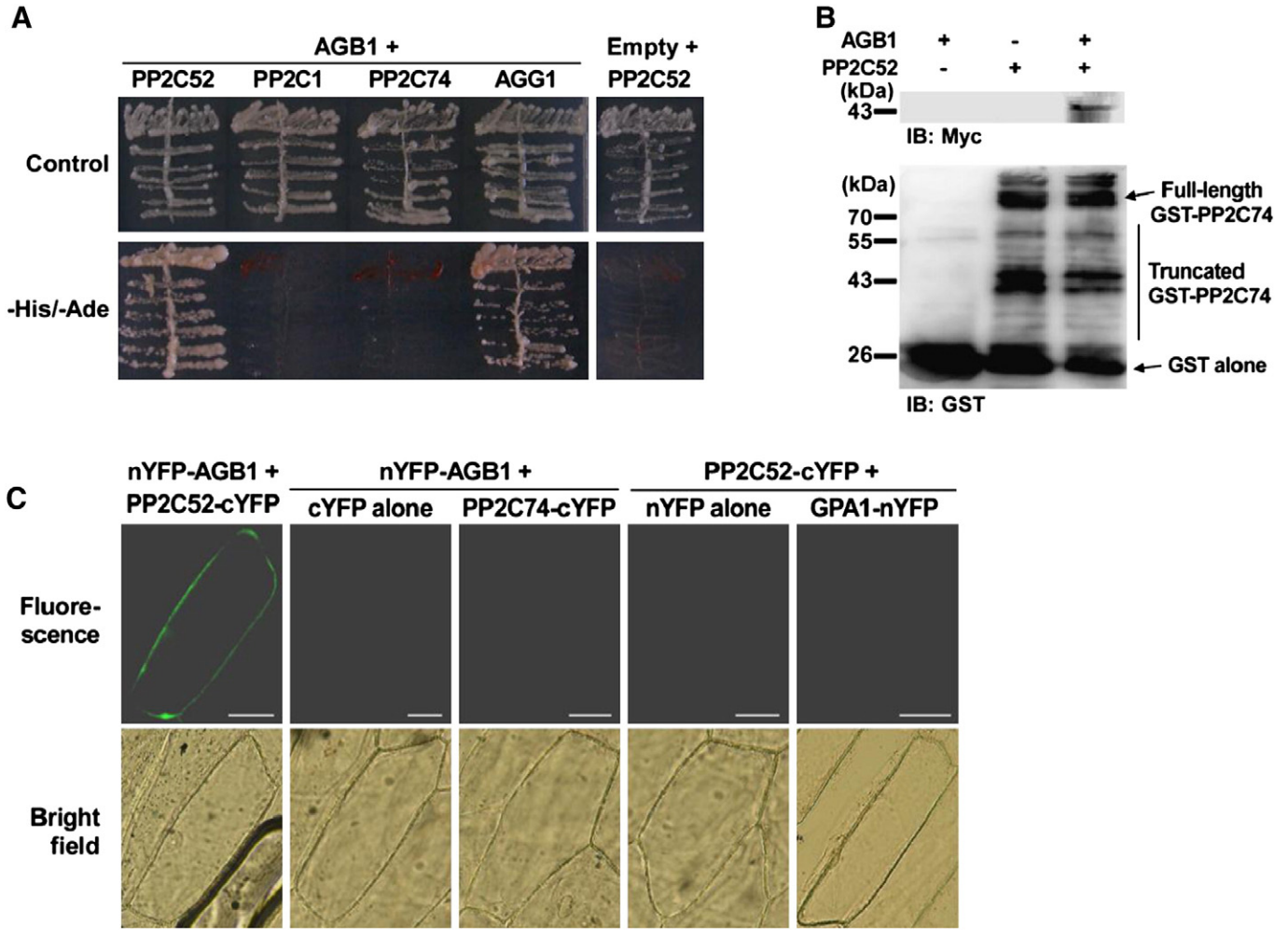

Fig. 1. Interactions between AGB1 and PP2C52. (A) Y2H interactions between AGB1 and PP2C52 homologs. The ORF of *AGB1* was cloned into pGBKT7, and the ORFs of *PP2C52*, *PP2C1*, *PP2C74* and *AGG1* were cloned into pGADT7-rec. The pGBKT7-AGB1 and one of the pGADT7-rec constructs were co-transformed into the yeast strain AH109. Cells were grown on control media and media lacking adenine and histidine (-His/-Ade). The combination of AGB1 + AGG1 is shown as a positive control. The combination of pGBKT7 empty vector (Empty) + PP2C52 is shown as a negative control. (B) In vitro GST pull-down assay. GST-fused PP2C52 and Myc-tagged AGB1 were expressed in *Escherichia coli* and used for the analysis. The presence or absence of each protein in the reaction mixture is shown as + or −, respectively. Experiments were performed 4 times and a representative result is shown. Antibodies used for immunoblotting are shown as IB: Myc and IB: GST. (C) BiFC in onion epidermal cells. The ORF of *AGB1* was cloned in frame behind the coding sequence of the N-terminal region of YFP (nYFP) to express nYFP-fused AGB1 (nYFP-AGB1), and the ORF of *PP2C52* was cloned in frame in front of the coding sequence of the C-terminal region of YFP (cYFP) to express cYFP-fused PP2C52 (PP2C52-cYFP). Both constructs were introduced into onion epidermal cells. nYFP-fused GPA1 (GPA1-nYFP) and cYFP-fused PP2C74 (PP2C74-cYFP) were used as controls. More than 20 cells were observed and a representative cell is shown. Scale bar = 50 μm.

which gave the negative results (cYFP alone, PP2C74-cYFP, nYFP alone, and GPA1-nYFP) were not high enough to recover YFP fluorescence.

3.2. Stage and tissue specificity of PP2C52 expression

The transcript levels of *PP2C52* in various tissues were examined by real-time quantitative RT-PCR. *PP2C52* mRNA expression was observed in all the tissues studied, being highest in flower stalks and lowest in seedlings (Fig. 2).

3.3. PP2C52 is localized to the plasma membrane via myristoylation

Subcellular localization of PP2C52 was examined using GFP-fused PP2C52 (PP2C52-GFP). When expressed in onion epidermal cells, the fluorescence of PP2C52-GFP was detected in the peripheral region of the cells (Fig. 3, left panel), suggesting that PP2C52 is localized to the plasma membrane. A motif scanning program (Myristoylator, http://web.expasy.org/myristoylator/) predicted that the N terminus of PP2C52 is myristoylated. Myristoylation is the process of adding a C: 14 fatty acid, myristate, to the N-terminal glycine of a subset of proteins and thus enhances their plasma membrane localization ([27], for a review). To examine whether myristoylation is responsible for the plasma membrane localization of PP2C52, the effects of substitution of the glycine residue at position 2, which is the putative myristoylation site of PP2C52, with an alanine residue were examined. The fluorescence of mutated PP2C52-GFP was detected in the nucleus but not in the plasma membrane (Fig. 3, right panel), suggesting that myristoylation is necessary for the plasma membrane localization of PP2C52. A motif-scanning program (cNLS Mapper, http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi [28]) predicted that the N-terminal region (position 34–63) of PP2C52 acts as a nuclear localization signal, supporting the idea that PP2C52 is localized to the nucleus when it is not myristoylated. PP2C52 also has eight potential palmitoylation sites (positions 4, 13, 32, 33, 136, 365, 369 and 405) (predicted by CSS Palm, http://csspalm.biocuckoo.org/prediction.php [29]). Palmitoylation reversibly attaches a C: 16 fatty acid, palmitate, to specific cysteine residues of target proteins. Because the plasma membrane localization of myristoylated proteins can be stabilized by palmitoylation ([27], for a review), the localization of PP2C52 may be controlled by palmitoylation as well as by myristoylation. To examine whether AGB1 affects the subcellular localization of PP2C52, PP2C52-GFP was expressed in mesophyll protoplasts from an AGB1-null mutant, *agb1-2* [22]. The fluorescence of PP2C52-GFP was detected in the cell periphery in the *agb1-2* protoplasts as well as in wild-type protoplasts (Supplementary Fig. S1), suggesting that AGB1 does not regulate the subcellular localization of PP2C52.

AGB1 is known to be localized to the plasma membrane and the nucleus [30]. We examined the co-localization of AGB1 and PP2C52 using GFP-fused AGB1 (GFP-AGB1) and mCherry-fused PP2C52 (PP2C52-mCherry). When GFP-AGB1 and PP2C52-mCherry were co-expressed in onion cells, GFP fluorescence was detected throughout the cell, while mCherry fluorescence was limited to the cell periphery (Supplementary Fig. S2), suggesting that AGB1 alone can be localized in the cytoplasm as well as to the plasma membrane and the nucleus, but the co-localization of AGB1 and PP2C52 is limited to the plasma membrane.

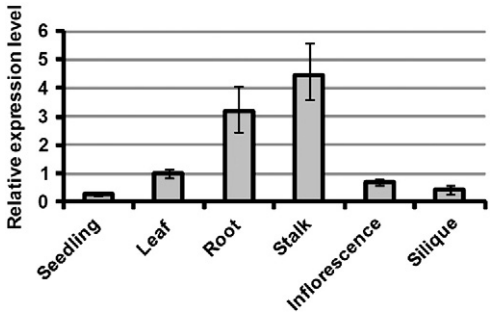

Fig. 2. Real-time quantitative RT-PCR analysis of *PP2C52* transcripts in various *Arabidopsis* tissues. Relative expression levels were calculated by the comparative C_T method using *UBQ5* as an internal control gene and leaf sample as a reference sample. Experiments were performed in triplicate. Values are means ± SE.

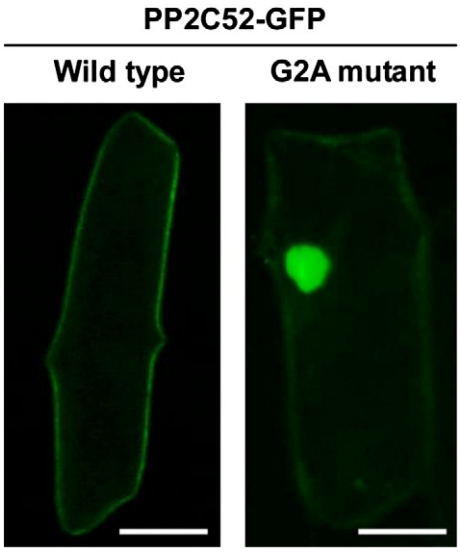

Fig. 3. Subcellular localization of PP2C52-GFP in onion epidermal cells. Wild-type PP2C52 or its G2A mutant, which has a glycine→alanine substitution at position 2, was expressed as a GFP-fusion protein. More than 10 transformed cells were observed and a representative cell is shown for each construct. Scale bars = 100 μm.

3.4. PP2C52 has protein phosphatase activity

To determine whether PP2C52 functions as a protein phosphatase, an in vitro phosphatase assay was performed using purified GST-PP2C52. Phosphate was released from a phosphorylated substrate when GST-PP2C52 was present in the reaction solution. Mg^{2+} is a co-factor required for PP2C activity. As expected, removal of Mg^{2+} by EDTA inhibited the phosphate release (Fig. 4A). These results suggest that PP2C52 has protein phosphatase activity in vitro. Because PP2C52 physically interacts with AGB1 and is a possible effector regulated by Gβγ, the effects of AGB1 and AGG1 on the activity of PP2C52 were examined using purified polyhistidine-tagged AGB1 and AGG1 (His-AGB1 and His-AGG1, respectively). However, a combination of AGB1 and AGG1 did not significantly change the activity of PP2C52 (Fig. 4B).

4. Discussion

This study has shown a specific interaction between AGB1 and PP2C52 (Fig. 1). Although AGB1 was localized to the cytoplasm (Supplementary Fig. S2), the site of the interaction between AGB1 and PP2C52 was limited to the plasma membrane (Fig. 1C). This was probably because the subcellular localization of PP2C52 was limited to the plasma membrane (Fig. 3, Supplementary Fig. S2). AGB1 and AGG1 did not affect the activity of PP2C52 (Fig. 4B), but it is possible that AGB1 is a substrate of PP2C52 or that AGB1 serves as a scaffold to broaden the substrate range of PP2C52. Not only AGB1 but also other components of plant G protein signaling are candidates for the substrates of PP2C52. Although none of the plant G proteins and their interaction partners has been shown to be regulated by phosphorylation, a human GPCR and yeast Gβ are known to be phosphorylated ([31,32], for a review, [33]). Further studies on the post-translational

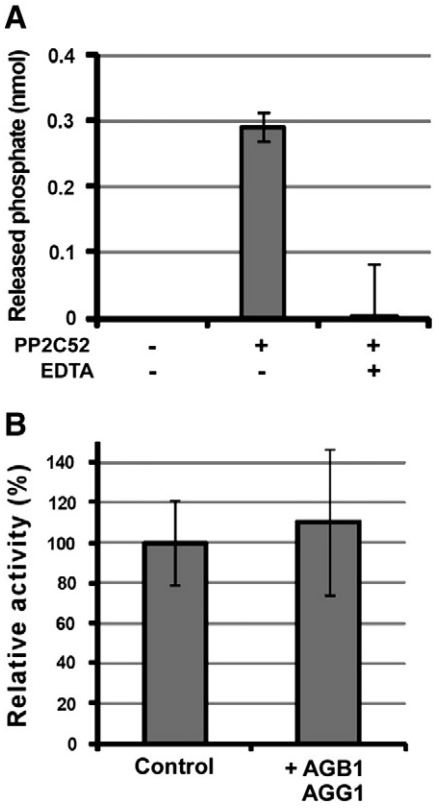

Fig. 4. In vitro phosphatase assay. (A) Confirmation of the phosphatase activity of PP2C52. GST-fused PP2C52 was expressed in *Escherichia coli*, purified and used for analysis. Phosphatase reactions were performed in the presence or absence of 20 mM EDTA (EDTA+ or EDTA−, respectively). For PP2C52-, GST alone was used for the reaction. Experiments were performed in triplicate. Values are means ± SE. (B) Effects of AGB1 and AGG1 on the phosphatase activity of PP2C52. AGB1 and AGG1 were expressed as polyhistidine-tagged proteins in *E. coli*, purified and used for analysis. Both of them were added to the phosphatase reaction mixture (shown as +AGB1 AGG1). For control, polyhistidine-tagged mVenus (a variant of GFP) was added to the reaction mixture. Experiments were performed in triplicate. Values are means ± SE.

modification and functional regulation of signaling components such as AGB1 and AGG1 are needed to elucidate the role of PP2C52 in plant G protein signaling.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.10.001.

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