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Arabidopsis G-protein β subunit AGB1 interacts with NPH3 and is involved in phototropism

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ABSTRACT

Heterotrimeric G proteins (Gα, Gβ and Gγ) have pleiotropic roles in plants, but molecular mechanisms underlying them remain to be elucidated. Here we show that Arabidopsis Gβ (AGB1) interacts with NPH3, a regulator of phototropism. Yeast two-hybrid assays, *in vitro* pull-down assays and bimolecular fluorescence complementation assays showed that AGB1 and NPH3 physically interact. NPH3-null mutation (*nph3*) is known to completely abolish hypocotyl phototropism. Loss-of-function mutants of AGB1 (*agb1-1* and *agb1-2*) showed decreased hypocotyl phototropism, and *agb1*/*nph3* double mutants showed no hypocotyl phototropism. These results suggest that AGB1 is involved in the NPH3-mediated regulation of phototropism.

1. Introduction

Heterotrimeric G-proteins (Gα, Gβ, and Gγ) are signaling molecules found in a variety of eukaryotic organisms. They mediate extracellular signals perceived by G-protein-coupled receptors (GPCRs) to intracellular effectors, and are involved in diverse cellular processes. Gβ of Arabidopsis (AGB1) is involved in regulating morphology and sensitivities to various stimuli ([1], for a review). Previous studies showed that AGB1 interacts with a Golgi-localized hexose transporter, SGB1 [2], a regulator of auxin transport, NDL1 [3], an ethylene biosynthesis-related protein, ARD1 [4], and many proteins involved in cell wall modification [5]. However, the molecular mechanisms underlying AGB1-mediated signaling are still unclear.

We previously performed a yeast two-hybrid screen using AGB1 as bait [6], and identified several putative AGB1-interacting proteins [6–9]. One of these proteins is NPH3 (Nonphototropic hypocotyl 3), which interacts with a blue light receptor, phot1 [10,11], and regulates phototropic responses [10,12] and leaf blue light responses [13]. *NPH3* has two transcript variants, *NPH3.1* and *NPH3.2*. NPH3.2 corresponds to the C-terminal region-truncated version of NPH3.1. NPH3.1 consists of N-terminal BTB domain, central NPH3 domain and C-terminal coiled-coil domain [14]. The coiled-coil domain of NPH3.1 interacts with phot1 [10], and the BTB domain interacts with CULLIN3 (CUL3), a scaffold protein for ubiquitin ligase complex [15]. Both of these interactions are involved in regulating the NPH3-mediated blue light responses [10,15].

Arabidopsis G proteins have been suggested to play roles in light responses. For example, blue light-induced phenylalanine production in etiolated seedlings is impaired in an Arabidopsis Gα (GPA1)-null mutant *gpa1-3* [16]. Another GPA1-null mutant, *gpa1-4*, and an AGB1-null mutant, *agb1-2*, are both less sensitive to blue light and far-red light in seed germination [17]. *gpa1-4* is less sensitive to the cell death mediated by a red light receptor, phyA, while *agb1-2* is more sensitive to the phyA-mediated cell death [18]. However, it is unclear whether G proteins are involved in the phototropic responses mediated by phot1 and NPH3.

Here we show that AGB1 physically interacts with NPH3 and is involved in the NPH3-dependent phototropic responses.

2. Materials and methods

2.1. Plant material, growth conditions and analysis of phototropism

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used throughout the experiments. Seeds of *nph3-7* (SALK_110039), *nph3-8* (CS322676), *agb1-1* (CS3976) [19] and *agb1-2* (CS6536)

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[20] were obtained from the Arabidopsis Biological Research Center (ABRC). For SALK_110039 and CS322676, T-DNA insertion was confirmed by genomic PCR analysis (Supplementary Fig. S1A and B). Primers used for the genomic PCR analysis are shown in Supplementary Table S1.

Seeds were surface sterilized and sown on 0.8% agar medium containing 0.5× Murashige and Skoog (MS) salts (Wako), 1% (w/v) sucrose and 0.5 g/L MES, pH 5.8, chilled at 4 °C in the dark for 3 d (stratified), and germinated at 22 °C. Plants were grown at 22 °C under 16-h-light/8-h-dark photoperiod for RT-PCR and genomic PCR analyses. To measure phototropic curvature, plants were grown for three days in the dark and were irradiated with unilateral blue light for 20 h [21].

2.2. Yeast two-hybrid (Y2H) analysis

pBluescript II SK⁻ NPH3.1, which contains full-length *NPH3.1* (see Supplementary Method S1), was digested by *Eco*RV and *Sac*I, and the resultant fragment containing the 3′ region (the position 1546-2241 from the start codon) of the open reading frame (ORF) of *NPH3.1* was inserted into the *Sma*I-*Sac*I site of pGADT7-Rec, generating pGAD-NPH3.1C. pBluescript II SK⁻ NPH3.1 (see Supplementary Method S1) was digested by *Eco*RI and *Xba*I, and the resultant fragment containing the full-length ORF of *NPH3.1* was inserted into the *Eco*RI-*Xba*I site of pGADT7-Rec, generating pGAD-NPH3.1. pGBK-AGB1 [6] and each pGAD construct were co-introduced into the *Saccharomyces cerevisiae* strain AH109. Reporter gene activation in the transformed yeast cells was examined by growing them on the SD (synthetic dextrose) medium lacking histidine and adenine as previously reported [6].

2.3. In vitro pull-down assay

To express GST-fused NPH3 variants in *Escherichia coli*, the ORFs of *NPH3.1*, *NPH3.2* and their truncated versions were inserted into pGEX-5X-1, generating pGEX-5X-NPH3.1, pGEX-5X-NPH3.2, pGEX-5X-BTB+I+II and pGEX-5X-III+IV, as described in Supplementary Method S1. These constructs were transformed into the *E. coli* strain BL21 (DE3), and transformed cells were cultured at 37 °C in LB medium until OD_{600} reached 0.5. IPTG was then added to the medium at 0.5 mM final concentration (for pGEX-5X-NPH3.1 and pGEX-5X-NPH3.2) or 0.3 mM final concentration (for pGEX-5X-BTB+I+II and pGEX-5X-III+IV), and the cells were further cultured at 28 °C for 4 h (for pGEX-5X-NPH3.1 and pGEX-5X-NPH3.2) or 3 h (for pGEX-5X-BTB+I+II and pGEX-5X-III+IV). The cells were then harvested by centrifugation and resuspended in 1× PBS (phosphate-buffered saline: 137 mM NaCl, 8.10 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) with 2 mg/ml lysozyme (Wako). 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 two units of recombinant DNase I (Takara) 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 12 000*g* for 5 min and the supernatant was used as crude extracts. The presence of the GST-fused proteins in the crude extracts was confirmed by Western blotting using a goat anti-GST antibody (GE Healthcare) and a horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (MBL).

GST-fused proteins in the crude extracts were bound to Glutathione Sepharose 4 Fast Flow (GE Healthcare) following the manufacturer's instructions, and the resin was washed 4 times by 1× PBS. The resin was then resuspended in a solution containing purified polyhistidine-tagged AGB1 (His-AGB1), which was prepared as previously described [6], and incubated at room temperature for 60 min with gentle shaking. The resin was then washed 4 times by 1× PBS, resuspended in 20 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0, and incubated at room temperature for 15 min to elute the GST-fused proteins. The slurry of the resin was centrifuged for 3 min at 12 000*g*, and His-AGB1 in the supernatant was analyzed by Western blotting using HisProbe-HRP (Thermo Fisher Scientific). When used, HisProbe-HRP was diluted 2000 times by 1× PBS. Signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the Las 1000 image analyzer (Fuji Film).

2.4. Bimolecular fluorescence complementation (BiFC) assay

To express cYFP (the C-terminal half of yellow fluorescent protein)-fused NPH3.1, pBluescript II SK⁻ NPH3.1 (see Supplementary Method S1) was digested by *Xba*I, and the resultant *NPH3.1* ORF fragment was inserted into the *Spe*I site of pBS-35SMCS-cYFP [6], generating pBS-35S-NPH3.1-cYFP. To express nYFP (the N-terminal half of YFP)-fused AGB1, pBS-35S-nYFP-AGB1 [6] was used. A mixture of pBS-35S-nYFP-AGB1 and pBS-35S-NPH3.1-cYFP (500 ng each) was used for particle bombardment to co-express NPH3.1-cYFP and nYFP-AGB1 in onion epidermal cells. Particle bombardment was performed as previously described, and YFP fluorescence was observed by fluorescence microscopy as previously described [22]. Images were processed using Canvas X software (ACD Systems).

2.5. RT-PCR

For RT PCR, plants were grown for four weeks and sampled. Total RNA was prepared using RNeasy Plant Mini Kit (Qiagen), and cDNA was synthesized from 900 ng total RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems). The cDNA was diluted 15 times by distilled water and used as template for RT-PCR. The expression of *NPH3* was examined by semi-quantitative RT-PCR, and the expression of *AGB1* was examined by quantitative real-time RT-PCR. The real-time PCR was performed using SYBR Premix EX Taq II (Perfect Real Time) (Takara) and the StepOne Real-Time PCR Systems (Applied Biosystems). Primers used for RT-PCR are shown in Supplementary Table S2.

3. Results and discussion

3.1. AGB1 interacts with NPH3

We and another group independently performed yeast two-hybrid (Y2H) screens using AGB1 as bait, and both identified NPH3 as a potential AGB1 interactor [5,6]. In our Y2H screen, *NPH3* was detected as a 5′ region-truncated form (the position 1546-2241 from the start codon) of *NPH3.1* (see Fig. 1B for the region of NPH3 used in Y2H analysis). Yeast cells could grow on the selection medium when they were co-transformed with the constructs that contain both *AGB1* and the 5′ region-truncated version of *NPH3.1* (Fig. 1A, middle panel), but not when either *AGB1* or the truncated form of *NPH3.1* was absent in the constructs (Fig. 1A, right and left panels), confirming that AGB1 and the C-terminal region of NPH3.1 interact in yeast cells. Y2H analysis using full-length NPH3.1 and AGB1 was also attempted. However, yeast transformed with pGAD full-length NPH3.1 could not grow in the control medium (data not shown).

In an *in vitro* GST pull-down assay, polyhistidine-tagged AGB1 (His-AGB1) was detected when it was reacted with GST-fused NPH3.1, NPH3.2 or BTB+I+II, a C-terminal region-truncated form of NPH3, but not when reacted with III+IV (GST-fused form), which corresponds to the central region of NPH3 (Fig. 1C and D; see Fig. 1B for diagrammatic representations of NPH3 proteins). These