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Characterization of the *Arabidopsis* Heterotrimeric G Protein*

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We have used fluorescence resonance energy transfer and co-immunoprecipitation to analyze the interactions among the α, β, and γ1 subunits of the *Arabidopsis* heterotrimeric G protein. Using cyan and yellow fluorescent protein fusion constructs, we show that overexpressed $G\gamma_1$ localizes to protoplast membranes, but Gβ exhibits membrane localization only when the $G\gamma_1$ protein is co-overexpressed. Overexpressed Gα shows membrane localization unaccompanied by overexpression of either Gβ or $G\gamma_1$. We detect fluorescence resonance energy transfer between Gβ and $G\gamma_1$ in the absence of Gα overexpression and between Gα and $G\gamma_1$ but only when all three subunits are co-overexpressed. Both Gα and Gβ are associated with large macromolecular complexes of ~700 kDa in the plasma membrane. Gα is present in both large complexes and as free Gα in plasma membranes from wild type plants. In plants homozygous for a null allele of the Gβ gene, Gα is associated with smaller complexes in the 200–400-kDa range, indicating that its presence in the large complex depends on association with Gβγ. Activation of the Gα subunit with guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) results in partial dissociation of Gα from the complex. Hydrogen peroxide (H_2O_2) promotes extensive dissociation of the Gα complex but does not interfere with binding of GTPγS to purified recombinant Gα, suggesting that reactive oxygen species affect the stability of the large complex but not the activity of Gα itself.

The results of pharmacological and genetic studies have provided evidence that the plant heterotrimeric G protein is involved in the transmission of light (1) and hormone signals (2) as well as in the regulation of ion channels (3). *Arabidopsis gpa1* mutant plants, which lack the Gα protein, encoded by the *GPA1* gene, exhibit reduced cell division during hypocotyl and leaf formation (4), whereas overexpression of GPA1 causes ectopic cell division, including meristem proliferation (5). Homozygous *gpa1* mutant plants are less sensitive to abscisic acid inhibition of stomatal opening and guard cell inward K^+ currents than wild type plants (6). By contrast, *gpa1* mutant seeds exhibit hypersensitivity to abscisic acid in inhibition of germination and in root growth and seedling gene expression. In addition, *gpa1* mutant plants are hyposensitive to gibberellic acid and brassinolide (7, 8). *Arabidopsis agb1-2* mutant plants, which lack the Gβ protein encoded by the *AGB1* gene, show alterations in leaf, flower and fruit development, decreased hypocotyl cell division and hypersensitivity to D-glucose (9–11). There are two Gγ subunit genes in *Arabidopsis*, *AGG1* and *AGG2*, and mutant analysis indicates that each Gγ subunit participates in a subset of Gβ-related developmental processes (12).

Evidence is also accumulating that the heterotrimeric G protein mediates plant responses to bacterial and fungal pathogens and abiotic stress. Heterotrimeric G protein signaling to membrane-bound NADPH oxidase has been implicated in the development of disease resistance and in the apoptotic hypersensitive response in rice (13) and homozygous *agb1–1* mutant plants are more susceptible to necrotrophic fungal pathogens than are wild type plants (14, 15). The Gα and Gβ subunits serve both separable and synergistic functions in signaling by reactive oxygen species in the oxidative stress response (16) and the Gβ subunit of the heterotrimeric G protein mediates cell death signaling in the *Arabidopsis* unfolded protein response (UPR) (17).

By contrast to the large number of different G protein subunits in animals, *Arabidopsis* has only one Gα subunit (18), one Gβ subunit (19), and two Gγ subunits (20, 21). The *Arabidopsis* G protein subunits exhibit limited homology with their animal counterparts (22). The GPA1 protein is roughly 30% identical to the mammalian Gα subfamily proteins, the AGB1 protein shows about 42% identity to mammalian Gβ subunits, and the AGGs display ~25–35% identity with certain mammalian Gγ subunits (20, 21). At the cellular level, *Arabidopsis* Gα has been immunolocalized to the plasma membrane and endoplasmic reticulum (23), whereas *Arabidopsis* Gβ has been detected in the plasma membrane, endoplasmic reticulum and Golgi apparatus (24, 25). Interactions between *Arabidopsis* Gβ, $G\gamma_1$ and $G\gamma_2$ have been detected using a yeast two-hybrid system and by vitro binding assays (20, 21), and there is also evidence for interactions between the rice Gα and Gβ subunits (26). Structural predictions for Gα, Gβ, and $G\gamma_1$ suggest that they can form a heterotrimer similar to that formed by mammalian G protein subunits (27). Although the heterotrimerization of *Arabidopsis* G protein subunits in cowpea protoplasts was recently reported (24), there is still limited information available about the structural and biochemical characteristics of the *Arabidopsis* heterotrimeric G protein complex.

Here we provide further structural and biochemical characterization of the *Arabidopsis* heterotrimeric G protein complex using transgenic plants and transiently transformed *Arabidop-*

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sis protoplasts. First, we show interactions between Gβ and $G\gamma_1$, as well as between Gα and $G\beta\gamma_1$ in *Arabidopsis* mesophyll protoplasts using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation. We then show that the heterotrimeric G protein is part of a large complex of roughly 700 kDa using blue native (BN) gel electrophoresis. We also show that the Gα subunit is present both in the large complex and as a free monomer in the plasma membrane fraction of wild type plants and that it is also detected in smaller complexes in *agb1-2* mutant plants. We report that GTPγS[2] binds to recombinant Gα protein and promotes slight dissociation of the Gα monomer from the large complex. Treatment of the plasma membrane fraction with hydrogen peroxide (H_2O_2) promotes the extensive dissociation of Gα from this complex but does not affect binding of GTPγS to the recombinant protein. These observations suggest that reactive oxygen species activate plant G protein signaling by promoting dissociation of the G protein macromolecular complex rather than by acting directly on the Gα protein.

EXPERIMENTAL PROCEDURES

Plant Materials—We used *Arabidopsis thaliana* Col-0 plants and *agb1-2* and *gpa1-4* null mutant homozygotes in the Col-0 background (27). Plants used to isolate plasma membrane fractions were grown in MetroMix 200 (Scotts-Sierra Horticultural Products Co., Marysville, OH) in 5-cm pots (51 per flat) at 65% humidity under fluorescent light at 150 μmol of photons/m^2s with a 12-h light/12-h dark photoperiod for 5 weeks.

Vectors for Expression of Cyan and Yellow Fluorescent Proteins (YFP) Fusions—The AGB and AGG1 coding sequences were cloned by PCR from a previously described *A. thaliana* Col-0 cDNA library (28). According to sequence information in the *Arabidopsis* data base, primers were designed as follows, with the underlined sequences adding restriction sites, and the bold faced sequences adding a spacer (primers were purchased from Integrated DNA Technologies, Coralville, IA). The forward primer for AGB1 was 5′-AGATCT**GGAGGTGGAGGTAGTA**TGTCTGTCTCCGAGCTCAA-3′; the reverse primer for AGB1 was 5′-TCTAGATCAAATCACTC TCCTGTGTCC-3′. The forward primer for AGG1 was 5′-AGATCT**GGAGGTGGAGGTAGT**ATGCGAGAGGAAACTGTGGTT-3′; the reverse primer for AGG1 was (5′-TCTAGATCAAAGTATTAAGCATCTGCA-3′. To insert CFP into GPA1, the binary vector of loop CFP-GPA1 (L-CFP-GPA1) was obtained from Dr. Alan Jones (University of North Carolina, Chapel Hill, NC). The full-length sequence of L-CFP-GPA1 was cloned from the binary vector. The amplified PCR fragments were cloned into pGEM-Teasy (Promega, Madison. WI), and sequences were verified. To make the pAVA321-CFP (or YFP)-Gβ and pAVA321-YFP-$G\gamma_1$ constructs, PCR products were cleaved from pGEM-Teasy (Promega) and further cloned into pAVA321-C/YFP (a kind gift of Dr. Xuemei Chen, University of California, Riverside, CA) using the BglII and XbaI sites. To make the pAVA321-L-CFP-Gα, pVAV321-Gβ, and pAVA321-$G\gamma_1$ constructs, PCR fragments of L-CFP-GPA1, AGB1, or AGG1 were cleaved from corresponding pGEM-Teasy constructs and cloned into pAVA321 with the removal of CFP/YFP via NcoI and XbaI. Then, the entire (35S)Gβ or (35S)$G\gamma_1$ expression cassette was cut by ClaI and KpnI, and 5′ overhangs were filled by Klenow fragment to form blunt ends and then ligated into pAVA321-YFP-$G\gamma_1$ or pAVA321-YFP-Gβ using the ClaI and SmaI sites resulting in pAVA321-$G\gamma_1$-(35S)YFP-Gβ and pAVA321-Gβ-(35S)-YFP-$G\gamma_1$. Finally, the entire (35S)-L-CFP-Gα expression cassette was cloned into pAVA321-$G\gamma_1$-(35S)-YFP-Gβ or pAVA321-Gβ-(35S)-YFP-$G\gamma_1$ via SacI and SmaI sites and Klenow fragment as described above, resulting in pAVA321-L-CFP-Gα-(35S)-$G\gamma_1$-(35S)-YFP-Gβ and pAVA321-L-CFP-Gα-(35S)-Gβ-(35S)-YFP-$G\gamma_1$. The (35S)-L-CFP-Gα and (35S)-YFP-Gβ expression cassettes were further cloned into the pCAMBIA3300 *Agrobacterium* binary vector and transformed into homozygous *agb1-2* and *gpa1-4* mutant plants. Transformed plants that exhibited the wild type leaf morphology phenotype on 10 μg/ml glufosinate Murashige and Skoog basal medium plates were used for further study.

Protoplast Isolation and Transfection, Confocal Microscopy, and FRET—*Arabidopsis* mesophyll protoplasts were prepared from fully expanded leaves of 4–5-week-old plants and transfected using the polyethylene glycol method developed by Sheen and co-workers (29). An additional 21% sucrose gradient was applied to the isolated protoplasts to obtain healthy protoplasts followed by transfection with plasmids containing the FRET pair to be tested. Plasmids were isolated using a Plasmid Maxi kit (Qiagen, Chatsworth, CA). A total of ~1×10^5 cells were transfected with 50 μg of plasmid for each microscopy observation, FRET measurement, or immunoprecipitation experiment. The transfected protoplasts were incubated at 22 °C for 12–16 h before being mounted in chambers (Molecular Probes, Eugene, OR) for microscopy. The transfected protoplasts were imaged using a Zeiss LSM 510 META laser scanning microscope (Carl Zeiss, Thornwood, NY) with a 40× NA 1.2 water objective. To monitor fusion protein expression and localization, protoplasts were excited with two argon laser lines, 458 nm for CFP and 514 nm for YFP, and emission images were collected simultaneously with a 480–520-nm filter for CFP and a 530–590-nm filter for YFP.

FRET is a method widely used to identify *in vivo* interactions between two proteins tagged with a donor (CFP) and an acceptor (YFP) fluorophor pair. FRET has also been used in conjunction with fluorescence lifetime imaging microscopy to monitor protein-protein interactions in plant cells (24, 30–32). In addition to common concerns, several other factors complicating FRET analysis in plants have been identified (33, 34). These are that (*a*) CFP and YFP excitation and emission spectra have crossover problems when a conventional epifluorescence microscope is used, (*b*) detection of FRET depends not only on the microscope optics but also on the relative local concentrations of the donor CFP fusion and the acceptor YFP fusion, (*c*) interference from the high background autofluorescence of chlorophyll, cell walls, and high concentrations of phenolic

[2] The abbreviations used are: GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; PVDF, polyvinylidene difluoride; BN, blue native; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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compounds in plant cells, and (*d*) photobleaching, which is conventionally used to confirm FRET in animal cells (35, 36), often cannot be applied to plant cells because it dramatically perturbs cellular structures (33).

To obviate these difficulties, we used a Zeiss LSM510 Meta spectral confocal fluorescence microscope equipped with a chameleon multiphoton laser, which can be modulated to a two-photon laser at 820 nm without any crossover excitation of YFP (37). In addition, λ stacks of acquired images were processed using the linear unmixing function mode of the Zeiss LSM510, allowing us to remove crossover emission from our analysis. Thus, our novel approach of two photon excitation combined with spectral image analysis allowed us to avoid the major type of artifact that plagues FRET studies performed with conventional epifluorescence microscopy (concern (*a*) above). To avoid concern (*b*) above, we ensured expression consistency between donor and acceptor molecules by transcriptionally fusing their expression cassettes into one construct, which we transfected into *Arabidopsis* protoplasts (Fig. 1*B*). The linear unmixing function mode of the Zeiss LSM510 also allowed us to remove autofluorescence signals, thus avoiding concern (*c*) above. We also performed extensive attempts to utilize photobleaching to confirm the FRET results reported here. However, we observed that protoplasts deformed or burst after the photobleaching procedure, and thus, the photobleaching method could not validly be used in our studies.

In further support of the method we used, we performed FRET tests using enhanced CFP-TGA5 and enhanced YFP-TGA5 as positive controls, because these proteins were previously shown to interact (33). As negative controls, we assayed for FRET between enhanced CFP and enhanced YFP as well as between enhanced CFP-TGA5 and enhanced YFP-LexA (33). Constructs expressing the FRET fusion pairs used as positive and negative controls were kindly provided by Dr. Eric Lam. Upon CFP excitation with a two-photon laser at 820 nm, a YFP emission peak was detected in protoplasts expressing the positive FRET control pair but not the negative control pairs (data not shown).

In this report spectral color encoding was performed with the Zeiss LSM 510 software. Thus, when a FRET signal was detected, the image is displayed in a green color indicating a YFP emission spectrum at loci in protoplasts expressing positive FRET pairs, whereas when a FRET signal was not detected, the image is displayed in a cyan color, indicating a CFP emission spectrum at loci in protoplasts expressing negative FRET pairs.

Protoplasts co-transfected with CFP and YFP fusion proteins were excited with a chameleon multiphoton laser (Coherent MRU 1000), which was modulated to a 820-nm wavelength and set at 4–5% laser intensity, ideal for cross-talk-free FRET analysis. The emission spectra from selected regions of interest were recorded by a connected multi-channel spectrometer in 12 channels, each with a 10-nm bandwidth, from 464 to 584 nm, using a 650 KP dichroic mirror in the λ stack acquisition mode. Emission spectra were recorded from at least 20 individual protoplasts transfected with each G protein FRET pair tested. Spectral analysis for automatic peak detection was performed with the LSM 510 software. The fluorescence spectra of the CFP- and YFP-fused G protein subunits were corrected for background fluorescence. FRET ratios were calculated as the ratio of YFP and CFP emission, where the intensities of YFP and CFP emission at 532 and 479 nm, respectively, are recorded by the multichannel spectrometer upon excitation of the specimen with the two photon laser at 820 nm.

Immunoprecipitation and Western Blotting—The catch and release reversible immunoprecipitation system (Upstate Biotechnology, Inc., Lake Placid, NY) was used for immunoprecipitation experiments. The transfected protoplasts were lysed with 1× wash buffer provided in the kit and then centrifuged at 15,000 × *g* for 10 min. The clear extract was incubated with a rabbit polyclonal anti-Gα antibody (1:250) (gift of Dr. Alan M. Jones) and the affinity ligand (1:50) in the kit column at 4 °C for 10–12 h. The column was washed 3 times by centrifugation with 1× wash buffer. 1× denaturing elution buffer in the kit was used to elute bound proteins, which were then fractionated by electrophoresis and transferred to a membrane for Western blotting. Total extract, flow-through (concentrated using Strataclean resin from Stratagene, La Jolla, CA), and eluted proteins were loaded on a 12% polyacrylamide discontinuous gel (Bio-Rad mini electrophoresis system). After electrophoresis, proteins were transferred to Hybond-P PVDF membrane (Amersham Biosciences) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Immunoblotting was performed with mouse monoclonal anti-G/C/Y antibodies (anti-G/C/Y antibody detects GFP, CFP, and YFP proteins, 1:2000, BD Biosciences). After incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibodies, proteins were detected using ECL Plus protein gel blotting detection reagents (Amersham Biosciences) according to the manufacturer's instructions. The immunoprecipitation experiments were repeated three or more times using independent protein preparations.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)—Plasma membrane fractions for BN-PAGE were prepared using an aqueous two-phase partitioning system as described (38–41). BN-PAGE/SDS-PAGE two-dimensional gel electrophoresis was performed as described previously with some modifications (28, 42, 43). Plasma membrane proteins were incubated with solubilization buffer (20 mM Bis-Tris-HCl, pH 7.0, 250 mM ϵ-aminocaproic acid, 2 mM EDTA, 1.0% Nonidet P-40, 0.25% Coomassie Blue G 250, and 10% glycerol) for 30 min and centrifuged twice at 15,000 × *g* for 5 min. For GTPγS or H_2O_2 treatment experiments, solubilization buffer containing 100 μM GTPγS or 20 μM H_2O_2 was used. The supernatant was fractionated on a 5.5–16% blue native polyacrylamide gradient gel, and albumin bovine monomer (66 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa), and porcine thyroid (669kD) from Amersham Biosciences were loaded alongside as marker proteins. One lane of the first dimension BN-PAGE gel was transferred to Hybond-P PVDF membrane (Amersham Biosciences) and immunoblotted with anti-G/C/Y antibodies (BD Bioscience) to detect CFP-tagged Gβ complex. The remaining first dimension lanes of the BN-PAGE gel were cut and incubated in denaturing solution (1% SDS, 1% β-mercaptoethanol) for 2 h and applied to a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane for immunoblotting with anti-Gα anti-

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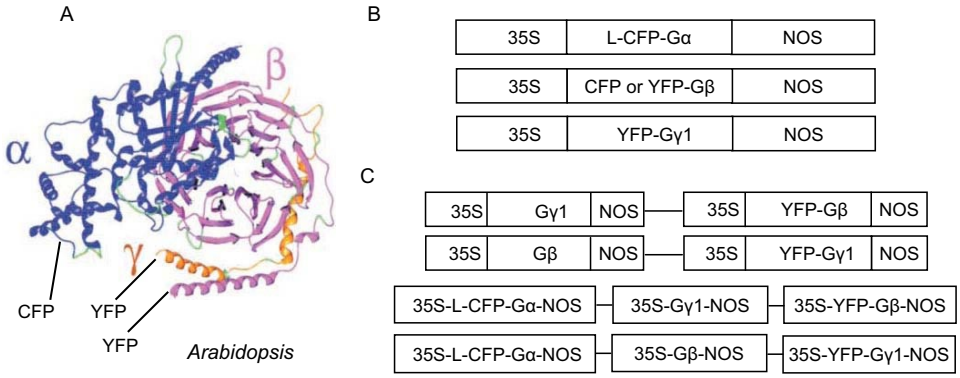

FIGURE 1. **The predicted structure of the *Arabidopsis* heterotrimeric G protein and the CFP and YFP fusion constructs used in the present study.** *A*, the predicted structure of *Arabidopsis* heterotrimeric G protein was redrawn from Ullah *et al.* (27). The sites of YFP and CFP insertion or fusion are indicated. *B* and *C*, structure of the CFP and YFP fusion constructs with *Arabidopsis* G protein subunits used in this study. *NOS*, gene terminator from agrobacterium.

body. Each experiment was repeated three times. The blots for GTPγS or H_2O_2 treatment experiments were scanned, and the intensity was quantified using Image J software. The percentage of the complex was calculated by the ratio of the value of the intensity of the complex to the value of the intensity of the total protein.

Recombinant Gα Expression, Purification, and the [^{35}S]GTPγS Binding Assay—We amplified GPA1 by PCR from an *Arabidopsis* cDNA library as described above using the forward primer 5′-GAGCTCATGGGCTTACTCTGCAGTAGAA-3′ and the reverse primer 5′-GGTACCTCATA AAAGGCCAGCCTCCAGTAA-3′. The amplified fragment was cloned into QIAexpress pQE-30 His_6 tag vector (Qiagen) at the SacI and KpnI restriction sites (underlined in the primer sequences) and expressed in BL21-CodonPlus (DE3)-RIPL strain (Stratagene). The recombinant His_6-tagged Gα was purified according to the manufacturer's instructions (The QIAexpressionist, Qiagen). Purification was confirmed by SDS-PAGE, and the purified recombinant Gα protein was dialyzed against GTPγS binding assay buffer (50 mM Tris·HCl, pH 8.0, 5 μM GDP, and 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl). The recombinant Gα was incubated with 20 nM [^{35}S]GTPγS in the above buffer for different times, and the reaction was diluted with stop solution (25 mM Tris·HCl, pH 8.0, 25 mM $MgCl_2$, 100 mM NaCl). The reaction mixture was applied to a 0.45-μm nitrocellulose membrane filter (Whatman, Florham Park, NJ), which was rinsed 3 times with stop solution to remove free [^{35}S]GTPγS. The amount of recombinant Gα-bound [^{35}S]GTPγS was then quantified by liquid scintillation spectrometry. The specific binding of [^{35}S]GTPγS with recombinant Gα was evaluated by nucleotide competition experiments with unlabeled ATP and GTP. The effect of H_2O_2 on the binding of [^{35}S]GTPγS to recombinant Gα was tested as described (44).

RESULTS

Overexpression and Subcellular Localization of Arabidopsis Heterotrimeric G Protein Subunits—Based on the predicted structure of the *Arabidopsis* G protein, we constructed genes encoding N-terminal fusions of enhanced CFPs and YFPs and Gβ and $G\gamma_1$, respectively, and expressed them from a cauliflower mosaic virus 35S promoter (Fig. 1, *A* and *B*). To minimize the possibility of interference of the CFP insertion with the structure and function of Gα, we inserted CFP into the second loop within the α helical domain of Gα (24, 35, 45), designating the construct L-CFP-Gα (Fig. 1, *A* and *B*). The effect of the C/G/YFP moiety on the function of the protein was tested for each Gα and Gβ construct by transforming it into the respective null mutant (*gpa1-4* and *agb1-2*) and affirming its ability to complement the mutant phenotype (see "Experimental Procedures").

Arabidopsis mesophyll protoplasts were transformed separately with constructs expressing L-CFP-Gα, YFP-Gβ, or YFP-$G\gamma_1$ to determine subcellular localization of each G protein subunit. Protoplasts with the lowest detectable expression levels were always chosen to rule out overexpression phenotypes. Peripheral fluorescence, consistent with the localization of the fluorescent fusion protein to the plasma membrane, was observed in both wild type and *agb1-2* mutant protoplasts transfected with L-CFP-Gα (Fig. 2*A1* and Fig. 2*B1*), indicating that the plasma membrane localization of Gα is independent of the presence of Gβ. Peripheral fluorescence was also observed in both wild type and *agb1-2* mutant protoplasts transfected with YFP-$G\gamma_1$ (Fig. 2*A3* and Fig. 2*B3*), indicating that $G\gamma_1$ localizes to the plasma membrane, and this localization does not require Gβ. Peripheral localization was not observed in protoplasts transfected only with YFP-Gβ construct; instead, we observed small fluorescent bodies in the cytoplasm of protoplasts (Fig. 2*A2* and Fig. 2*B2*). However, peripheral fluorescence was detected when protoplasts were co-transfected with YFP-Gβ-(35S)-$G\gamma_1$ (Fig. 2*A4* and Fig. 2*B4*), indicating that the $G\gamma_1$ subunit was required for plasma membrane localization of YFP-Gβ. Even when these constructs were transfected into wild type protoplasts, which contain all three subunits, the endogenous level of $G\gamma_1$ was evidently not sufficient to detect a peripheral signal from the Gβ fusion protein unless the $G\gamma_1$ protein was also overexpressed. We infer that $G\gamma_1$ is required for plasma membrane localization of Gβ, whereas Gα and $G\gamma_1$ are each able to localize to the membrane independently of Gβ.

FRET Detection of in Vivo Interaction between the Subunits of the Arabidopsis Heterotrimeric G Protein—We used FRET to ask whether the Gβ and $G\gamma_1$ subunits are in close proximity in the membrane. Modeling predictions suggested that the Gα, Gβ, and $G\gamma_1$ proteins of *Arabidopsis* can form a heterotrimer similar to those characterized in other organisms (8). Moreover, an interaction between Gβ and $G\gamma_1$ has been detected in a yeast two-hybrid system and by *in vitro* pulldown assays (20, 21). In human embryonic kidney cells and *Dictyostelium*, FRET has been used to monitor the association of subunits of the heterotrimeric G protein and the dissociation of the Gα subunit from Gβγ dimer in response to external stimuli (35, 46, 47). FRET is detected as a shift in the emission spectrum of CFP (480 ± 20 nm) to that of YFP (530 ± 15 nm) upon excitation of

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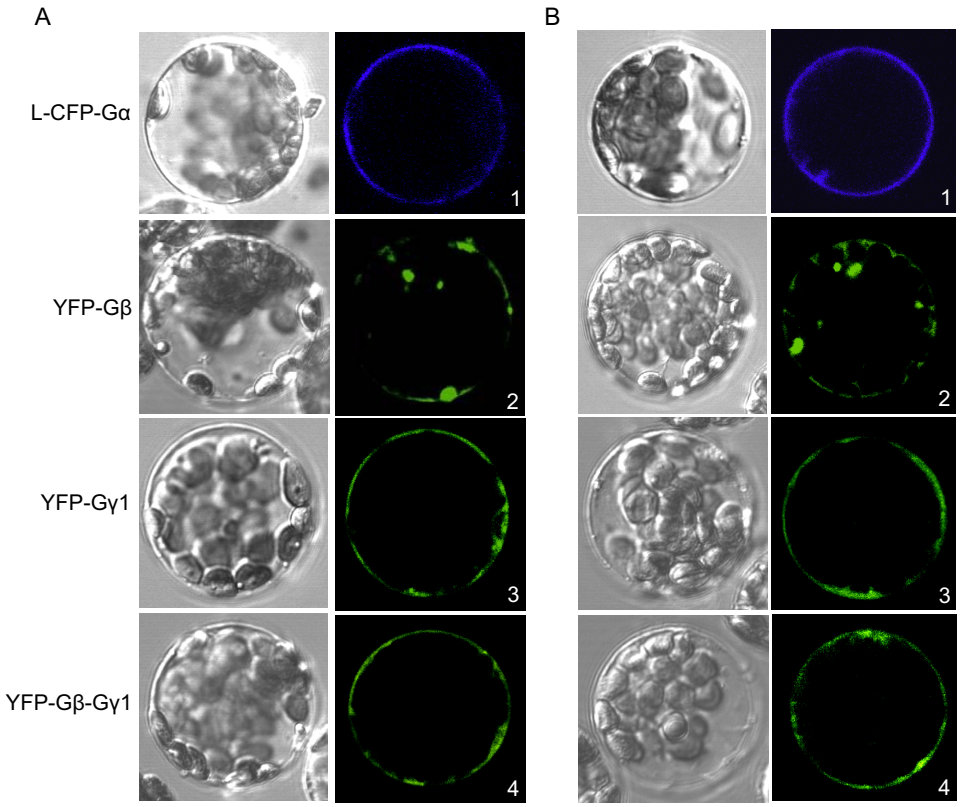

FIGURE 2. **Subcellular localization of CFP- and YFP-*Arabidopsis* G protein fusions.** *A*, images of *Arabidopsis* wild type mesophyll protoplasts transformed with the indicated constructs: *1*, L-CFP-Gα; *2*, YFP-Gβ_1; *3*, YFP-Gγ_1; *4*, (35S)-YFP-Gβ-(35S)-Gγ_1. *B*, images of *Arabidopsis agb1-2* mutant mesophyll protoplasts transformed with the indicated constructs: *1*, L-CFP-Gα; *2*, YFP-Gβ_1; *3*, YFP-Gγ_1; *4*, (35S)-YFP-Gβ-(35S)-Gγ_1.

CFP with the two-photon laser at 820 nm and occurs if the tested proteins are in close juxtaposition. The emission spectra of CFP- and YFP-G protein fusions are shown in Fig. 3*G*.

Upon co-transfection of *Arabidopsis* mesophyll protoplasts with CFP-Gβ and YFP-Gγ_1, we observed a FRET signal in peripheral areas of cells (Fig. 3*A*) and a spectral shift from 480 nm, representing the CFP spectrum, to 530 nm, representing the YFP spectrum (Fig. 3*H*). This result clearly shows that FRET occurs in the plasma membrane between CFP-Gβ and YFP-Gγ_1. We conclude that Gβ and Gγ_1 form a complex, which is required for membrane localization of the Gβ protein.

Next, we used FRET to assess *in vivo* interactions between Gα and G$\beta\gamma_1$. We co-transfected *Arabidopsis* mesophyll protoplasts with combinations of plasmids encoding the following: L-CFP-Gα and YFP-Gβ; L-CFP-Gα and YFP-Gγ_1; L-CFP-Gα, YFP-Gβ, and YFP-Gγ_1. We did not detect a FRET signal and spectral shift (Fig. 3*H*) in cells when we co-transfected the protoplasts with constructs expressing either Gβ or Gγ_1 fluorescent fusion proteins pairwise with L-CFP-Gα. (Fig. 3, *B* and *C*). However, we were able to detect a FRET signal (Fig. 3*D*) and spectral shift (Fig. 3*I*) in cells when we co-transfected the protoplasts with constructs containing the coding sequences for all three subunits. Although the FRET construct pairs were transfected into wild type protoplasts containing all three subunits, the endogenous levels of the third subunit were insufficient to allow FRET detection when only two of the three subunits were overexpressed. The presence of FRET between L-CFP-Gα, YFP-Gγ_1, and YFP-Gβ (Fig. 3, *D* and *I*) supports the inference that the subunits of *Arabidopsis* G protein form a heterotrimer *in vivo*.

To further determine whether the energy transfer is from L-CFP-Gα to YFP-Gβ or from L-CFP-Gα to YFP-Gγ_1 in the heterotrimeric G protein complex formed in *Arabidopsis* protoplasts, we created single constructs expressing all three genes with two as a FRET pair and the third one untagged as follows: L-CFP-Gα-(35S)-YFP-Gβ-(35S)-Gγ_1 and L-CFP-Gα-(35S)-Gβ-(35S)-YFP-Gγ_1 (Fig. 1*B*). We detected a FRET signal and a spectral shift in protoplasts transfected with L-CFP-Gα-(35S)-Gβ-(35S)-YFP-Gγ_1 (Fig. 3, *F* and *I*) but not in protoplasts transfected with the L-CFP-Gα-(35S)-YFP-Gβ-(35S)-Gγ_1 construct (Fig. 3, *E* and *I*). Thus, energy transfer occurs between L-CFP-Gα and YFP-Gγ_1 but not between L-CFP-Gα and YFP-Gβ, implying that the CFP of L-CFP-Gα fusion protein is in closer proximity to the YFP of the YFP-Gγ_1 fusion than to that of the YFP-Gβ in the G protein complex formed in *Arabidopsis* protoplasts. This observation implies that the Gα subunit does not interact with either Gβ or Gγ_1 alone in protoplasts but interacts with the G$\beta\gamma$ complex. Quantitative analysis of FRET ratios among the six tested FRET pairs is given in Fig. 3*J* to further demonstrate the occurrence of FRET, where a FRET signal is considered as positive when the ratio is greater than one. These results verify the successful detection of FRET between *Arabidopsis* heterotrimeric G protein subunits and indicate that they form a heterotrimeric complex *in vivo*.

We next applied an independent biochemical method to confirm that the *Arabidopsis* G protein complex contains all three subunits *in vivo*. First, we immunoprecipitated L-CFP-Gα with Gα antibody and assessed whether YFP-Gγ_1 proteins transiently expressed in protoplasts were co-immunoprecipitated. L-CFP-Gα and YFP-Gγ_1 differ in size and can be detected together in the same Western blot with anti-G/C/Y antibody to assess the efficiency of co-immunoprecipitation. We found that YFP-Gγ_1 indeed can be co-immunoprecipitated with L-CFP-Gα from protoplasts transfected with a L-CFP-Gα-(35S)-Gβ-(35S)-YFP-G$\gamma_1$construct (Fig. 4*B*) but not from protoplasts co-transfected with L-CFP-Gα and YFP-Gγ_1 (Fig. 4*A*). Thus, our biochemical results support the conclusions from FRET analysis that the subunits of the *Arabidopsis* G protein form a heterotrimeric complex *in vivo* and that the interaction of Gα and Gγ_1 requires Gβ.

The Arabidopsis Heterotrimeric G Protein Complex—We used two-dimensional PAGE to further characterize the plasma membrane-bound heterotrimeric G protein complex. We isolated the plasma membrane fraction from rosette leaves using

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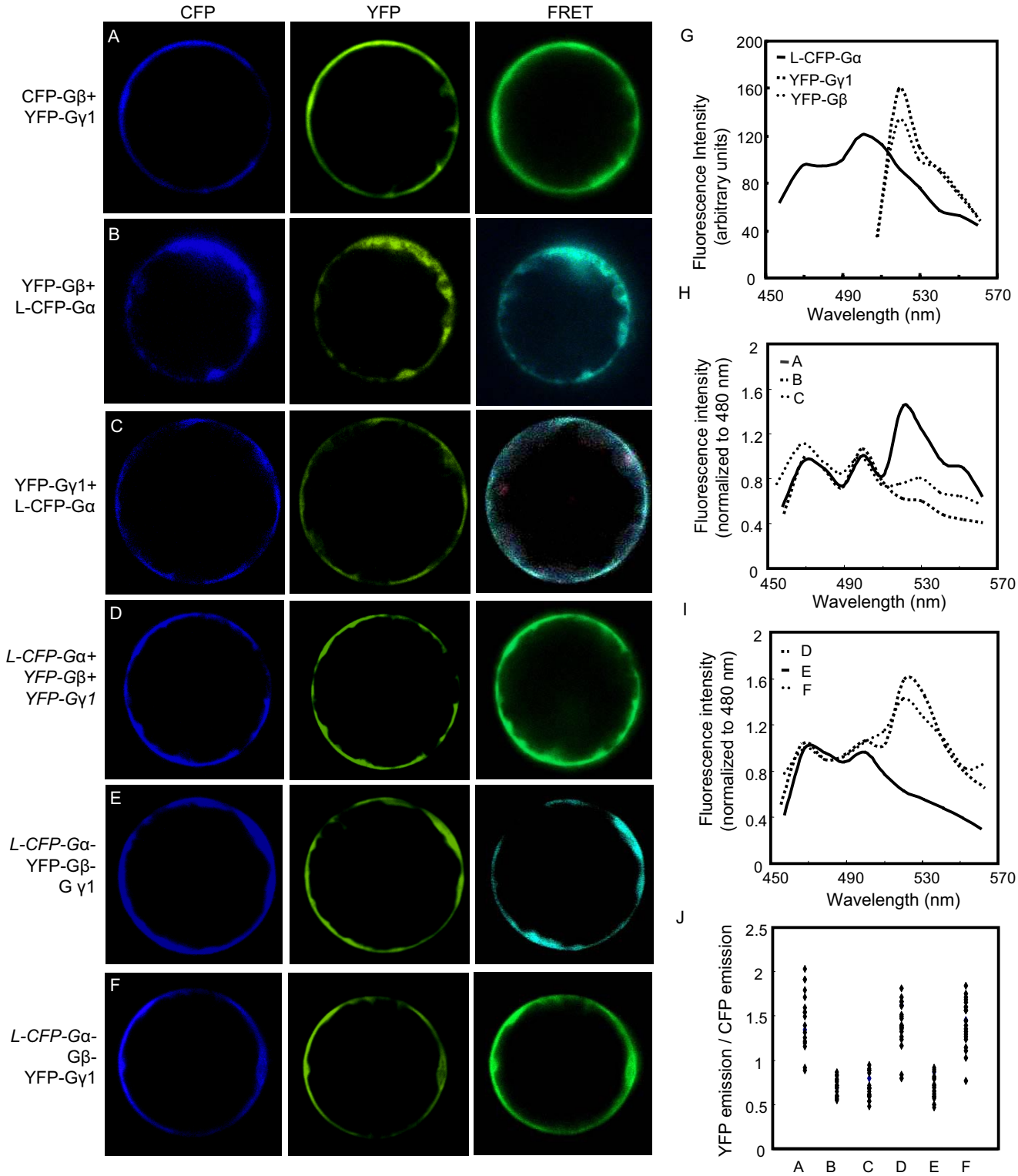

FIGURE 3. **Interaction of *Arabidopsis* G protein subunits *in vivo* detected by FRET.** *A–F*, images of *Arabidopsis* mesophyll protoplasts transformed with the indicated constructs: *A*, CFP-Gβ+YFP-Gγ_1; *B*, YFP-Gβ+L-CFP-Gα; *C*, YFP-Gγ_1+L-CFP-Gα; *D*, L-CFP-Gα+YFP-Gβ+YFP-Gγ_1; *E*, L-CFP-Gα-(35S)-YFP-Gβ-(35S)-Gγ_1; *F*, L-CFP-Gα-(35S)-Gβ-(35S)-YFP-Gγ_1. CFP, YFP, and FRET labels above the images indicate the individual optical filter conditions. FRET images were obtained by spectral encoding with the LSM510 META software of the series of images collected in the λ stack acquisition mode from 464 to 584 nm. Upon the excitation of CFP with the two photon laser at 820 nm, the image is displayed in *green* when a FRET signal was detected, whereas the image is displayed in *cyan* when a FRET signal was not detected. *G*, fluorescence emission spectra from the representative protoplasts shown in Fig. 2*A*. CFP was excited with a two-photon laser at 820 nm, and YFP was excited with an argon laser at 514 nm. *H–I*, fluorescence emission spectra from representative protoplasts in *A* to *F* upon excitation with the two photon laser at 820 nm. The spectra in *A*, *D*, and *F* show an increase in the YFP emission peak, indicative of FRET between CFP-Gβ and YFP-Gγ_1 or L-CFP-Gα and YFP-Gγ_1. Fluorescence intensity was normalized to 480 nm, which is one of the peaks of the CFP emission spectrum. *J*, quantitative analysis of the FRET ratio between tested pairs illustrated in *A* to *F*. The FRET ratio is the ratio of yellow emission (532 nm) to cyan emission (479 nm) upon excitation of CFP with the two-photon laser at 820 nm. For each of *A* through *F*, *black diamonds* show FRET ratios in 20 individual protoplasts, observed in the FRET detection mode. If more than 1 cell had the same FRET ratio, the representative diamonds cannot be distinguished so that less than 20 diamonds are apparent in *J*.

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an aqueous two-phase partitioning system (48, 49), solubilized it with Nonidet P-40, and fractionated the native complex by BN-PAGE followed by denaturing SDS-PAGE and Western blotting. In the plasma membrane fraction, Gα antibody detects the Gα protein in a complex of ~700 kDa as well as in a low molecular weight, apparently monomeric form (Fig. 5*A*). Approximately 30% of the Gα protein in wild type plants is in the high molecular weight complex in the plasma membrane. Thus, Gα protein appears to be part of a complex larger than anticipated for the heterotrimer, whose molecular mass is expected to be about 100 kDa. A complex of about the same size (~700 kDa) was detected by Western blotting with anti-CFP antibodies in total extract derived from *Arabidopsis* plants expressing a CFP-Gβ fusion protein under control of the 35S promoter (Fig. 5*B*). However, by contrast to what was observed with Gα, all of the immunoreactive Gβ protein was associated with this large complex, and none of it was detected at the expected mobility of either the heterotrimer (100 kDa) or the Gβγ heterodimer (60 kDa). Thus, both Gα and Gβ are part of large membrane-bound complexes in *Arabidopsis*, but a significant fraction of Gα is also present in the membrane as the free monomer.

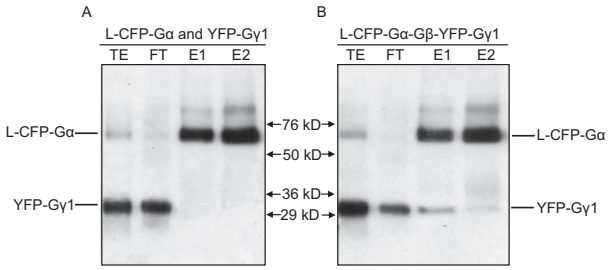

FIGURE 4. **Co-immunoprecipitation of Gα and $G\gamma_1$ is dependent on co-expression of Gβ, indicating formation of a heterotrimer.** Protein extracts of protoplasts transformed with (35S)-L-CFP-Gα and (35S)-YFP-$G\gamma_1$ (*A*) and (35S)-L-CFP-Gα-(35S)-Gβ-(35S)-YFP-$G\gamma_1$ (*B*) were immunoprecipitated with anti-Gα. *Lanes 1* of *A* and *B*, 10% of the total extract (*TE*); *lanes 2* of *A* and *B*, the concentrated flow through (20 μl) from the column (*FT*); *lanes 3* of *A* and *B*, proteins in the first eluate with denaturing buffer (*E1*); *lanes 4* of *A* and *B*, proteins in the second eluate with denaturing buffer (*E2*). Proteins were separated by SDS-PAGE gel, transferred to a PVDF membrane, and probed with anti-C/YFP antibody. Note that in *panel B*, but not *panel A*, YFP-$G\gamma_1$ is co-immunoprecipitated with Gα (compare lanes *E1* in *B versus A*).

To determine whether the Gβ subunit is required for the formation of the large complex containing the Gα subunit, we isolated Gα-containing complexes from *agb1-2* mutant plants and observed that Gα was also in the plasma membrane fraction of the *agb1-2* mutant plants. This result is consistent with our observation in imaging studies that the plasma membrane localization of Gα is independent of the presence of Gβ. However, only about 8% of the Gα protein was associated with the ~700 kDa complex in *agb1-2* mutant plants as compared with ~30% in wild type plants, as judged by the detection of Gα with anti-Gα antibodies after BN-PAGE and SDS-PAGE (Fig. 5*C*). In plants lacking the Gβ subunit, a substantial fraction of the Gα protein was associated with several complexes in the 140–400-kDa range (Fig. 5*C*); such complexes were not observed in wild type plants (Fig. 5*A*). These observations suggest that Gβ is not required for the plasma membrane localization of Gα but that Gβ promotes the association of Gα with the large complex. Alternatively, other proteins may bind to Gα in the absence of Gβ, resulting in formation of a different large complex.

Dissociation of the G Protein Complex—To examine whether G protein activation causes the dissociation of Gα from the complex, we used BN-PAGE to measure the change in the fraction of Gα in the high molecular weight and monomeric forms in the plasma membrane-bound fraction after incubation with GTPγS. GTPγS is a non-hydrolysable analog of GTP which binds Gα and locks it in its active state (50). It has been reported that the Gα subunit of the rice heterotrimeric G protein is fully dissociated from a large (~400 kDa) complex in the presence of GTPγS (26). We isolated the plasma membrane fraction from wild type *Arabidopsis* plants, incubated it with 100 μM GTPγS, and fractionated it by BN-PAGE followed by SDS-PAGE and Western blotting with Gα antibody. The fraction of Gα protein in the high molecular weight complex declined from 29 to 20% after incubation with GTPγS, a roughly 30% decrease (Fig. 6*A*). Given that GTPγS treatment is expected to result in heterotrimer dissociation, such dissociation may not result in extensive release of Gα from the large complex in *Arabidopsis*.

In view of the known involvement of reactive oxygen species in hetero-

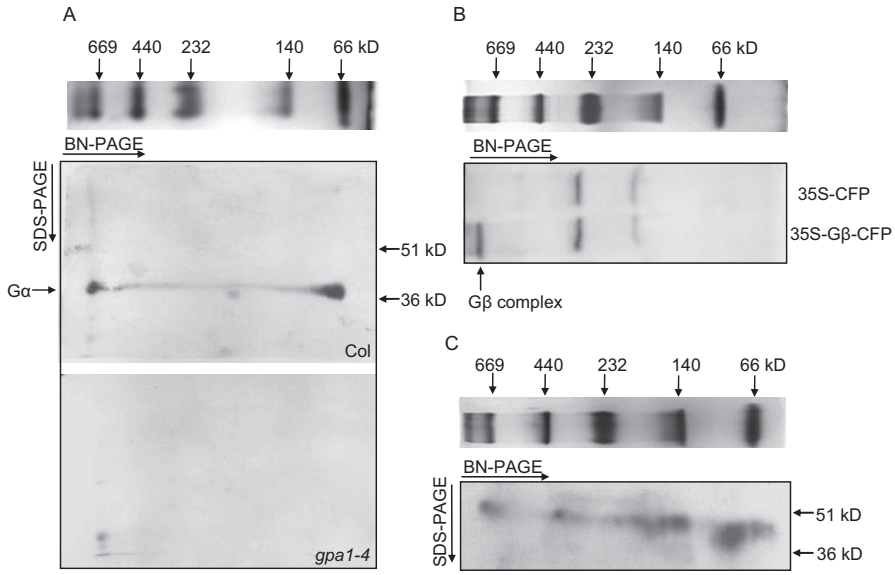

FIGURE 5. **The *Arabidopsis* Gα protein is associated with a complex of ~700 kDa.** *A*, the plasma membrane fractions from Col-0 and *gpa1-4* mutant rosette leaves were fractionated by BN-PAGE in the first dimension and SDS-PAGE in the second dimension, transferred to PVDF membranes, and probed with anti-Gα antibody. The *arrowhead* points to the location of Gα. *B*, total protein extracts of *agb1-2* plants overexpressing either CFP or Gβ-CFP were fractionated by BN-PAGE and transferred to PVDF membranes, and the blot was probed with anti-CFP antibody. The *arrowhead* points to the location of the Gβ complex. *C*, the plasma membrane fraction from *agb1-2* mutant plants was fractionated by BN-PAGE and SDS-PAGE, and the blots were probed with anti-Gα antibody. The sizes of protein mass standards are indicated.

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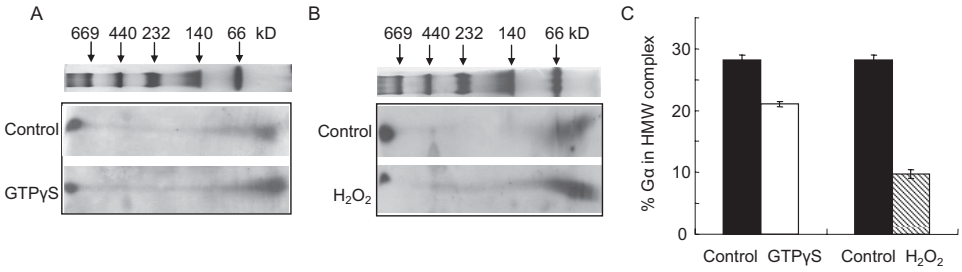

FIGURE 6. **The *Arabidopsis* Gα protein complex dissociates upon treatment with GTPγS or H_2O_2.** *A* and *B*, the isolated plasma membrane fraction from Col-0 rosette leaves was incubated with buffer containing either 100 μM GTPγS (*A*) or 20 μM H_2O_2 (*B*) and diluted with solubilization buffer containing 20 mM Bis-Tris-HCl, pH 7.0, 250 mM ϵ-aminocaproic acid, 2 mM EDTA, 1.0% Nonidet P-40, and fractionated by BN-PAGE followed by SDS-PAGE. The blots were probed with anti-Gα antibody. The sizes of protein mass standards are indicated. *C*, quantification of the fraction of Gα in the large complex in control and treated plasma membrane extracts. This experiment was repeated three times, and the *error bars* shown here are S.E.

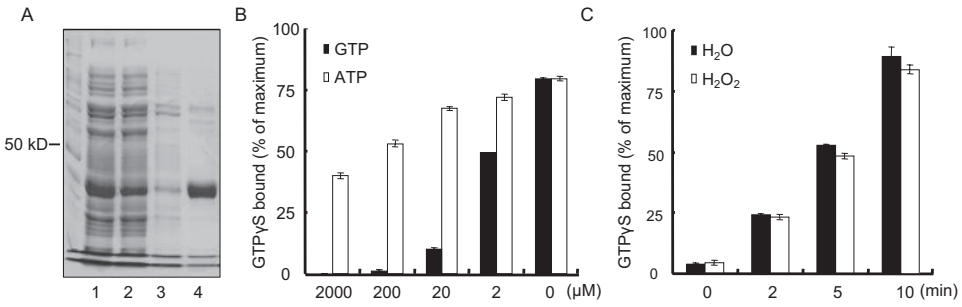

FIGURE 7. **GTPγS binds to recombinant *Arabidopsis* Gα protein.** *A*, purification of His_6-tagged Gα was assessed by 10% SDS-PAGE. *Lane 1*, total lysate of *Escherichia coli* expressing His_6-tagged Gα; *lane 2*, flow-through from nickel affinity resin; *lane 3*, wash; *lane 4*, eluate. The 50-kDa marker protein is indicated. *B*, [^{35}S]GTPγS binding to recombinant His_6-tagged Gα protein in the presence of increasing concentrations of unlabeled ATP or GTP ($n = 6$). *C*, [^{35}S]GTPγS binding to recombinant His_6-tagged Gα in the presence of either 20 μM H_2O_2 or H_2O (control) ($n = 6$). The radioactivity of the [^{35}S]GTPγS added to the reaction mixture was set as maximum (100%). This experiment was repeated three times, and the *error bars* shown here are S.E.

incubation of the recombinant protein with 20 μM H_2O_2 has little effect on its GTP binding activity, as judged by the binding of GTPγS. Thus, 20 μM H_2O_2 promotes dissociation of Gα from the membrane-bound high molecular mass complex but does not affect its GTP binding activity (Fig. 7*C*).

DISCUSSION

It was previously reported that the subunits of the *Arabidopsis* G protein interact in the plasma membrane of cowpea protoplasts (24). These experiments demonstrated the utility of using transfected constructs of fluorescent fusion proteins to assess the localization and dynamics of plant G protein subunits, a strategy employed extensively in mammalian cell lines and *Dictyostelium* (35, 47). In the present study we detected peripheral fluorescence, consistent with plasma membrane localization, in *Arabidopsis* wild type and *agb1-2* protoplasts transiently transfected with cauliflower mosaic virus 35S promoter-driven cDNAs for the Gα protein carrying a CFP in the second loop of the α-helical domain. We similarly observed peripheral localization of N-terminal-fused YFP-$G\gamma_1$ regardless of whether this construct was expressed in wild type or Gβ mutant (*agb1-2*) protoplasts. These results are consistent with imaging studies performed by Zeng *et al.* (54) but different from those of Adjobo-Hermans *et al.* (24). The latter performed heterologous expression studies of *Arabidopsis* G protein subunits in cowpea protoplasts and observed that co-expression of Gβ was required for plasma membrane localization of $G\gamma_1$.

When protoplasts expressing a similar 35S promoter-driven cDNA encoding an N-terminal-fused YFP-Gβ construct were imaged, peripheral fluorescence was not observed. In such protoplasts the detected fluorescence was cytoplasmic and particulate, and fluorescence was detected in some cells in large inclusion bodies, possibly representing Gβ aggregates. However, co-expression of a $G\gamma_1$ cDNA with the YFP-Gβ cDNA promoted peripheral localization of fluorescence, indicating that $G\gamma_1$ suffices to target Gβ to the plasma membrane. This is consistent with the presence of a prenylation sequence at the C terminus of the $G\gamma_1$ protein but not that of the Gβ protein. This observation provides an interesting contrast to the behavior of mammalian G proteins, for which localization of the Gβγ complex to the plasma membrane requires prior association of Gα and Gβγ (55).

We have reported that more than 60% of the cellular Gα protein in wild type *Arabidopsis* leaf tissue is plasma membrane-bound, whereas less than 40% of the Gβ protein is in the

trimeric G protein signaling, we also asked whether H_2O_2 (51), a known second messenger in stress signaling (52), can influence either the stability of the Gα-containing high molecular weight complex or the GTP binding capacity of recombinant *Arabidopsis* Gα protein. To determine whether H_2O_2 affects the stability of the complex, we analyzed the effect of H_2O_2 treatment on the fraction of Gα protein in the high molecular weight membrane-bound complex. We isolated the plasma membrane fraction from wild type plants and treated it with physiological levels of H_2O_2 (20 μM) (53) before BN-PAGE and SDS-PAGE fractionation. We observed that H_2O_2 treatment promotes the extensive release of Gα protein from the high molecular weight complex (Fig. 6*B*). The fraction of complexed Gα protein declined from about 30% to less than 10% after H_2O_2 treatment (Fig. 6*C*).

It has been reported that the GTP binding activity of two mammalian Gα proteins, $G\alpha_i$ and $G\alpha_o$, are directly regulated by reactive oxygen species via changes in the redox state of sulfhydryl residues in the protein (44, 51). Because the *Arabidopsis* Gα protein contains potentially regulatory sulfhydryl residues (18), we asked whether H_2O_2 affects the GTP binding activity of recombinant Gα. We purified recombinant His_6-tagged Gα (Fig. 7*A*) and first assayed its ability to bind GTP. Recombinant *Arabidopsis* Gα selectively binds GTP, as determined by the ability of GTP, but not ATP, to compete for binding of GTPγS (Fig. 7*B*). We then asked whether H_2O_2 affects the ability of the recombinant Gα protein to bind GTPγS. We observed that

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plasma membrane, and the rest is in internal membranes, including the endoplasmic reticulum (17). The difference in subcellular partitioning of Gα and Gβ is likely to be attributable to differential subcellular targeting of Gβ in dimers with $G\gamma_1$ and $G\gamma_2$. Although both $G\gamma_1$ and Gγ2 associate with plasma membranes as a consequence of prenylation, $G\gamma_1$ was also detected in internal membranes (12). This observation is consistent with our report that Gβγ has a function separable from that of the heterotrimer in the unfolded protein response (17). The *Arabidopsis* $G\gamma_2$ protein, which is *S*-acylated as well as a prenylated, localizes to the plasma membrane independently of Gβ and more extensively than $G\gamma_1$ (12, 24). These observations suggest that the two Gβγ dimers of *Arabidopsis*, Gβγ1 and $G\beta\gamma_2$, have distinct functions in different subcellular compartments. Also consistent with this concept is the observation that $G\gamma_1$ (*agg1*) and $G\gamma_2$ (*agg2*) mutants share different subsets of the several phenotypes reported for Gβ (*agb1*) mutants (12, 54).

We detected FRET between the CFP and YFP moieties of CFP-Gβ and YFP-$G\gamma_1$ fusion proteins but only when the fusions were N-terminal for both proteins. We also tested Gβ and Gγ FRET pairs in which one or both of the fusions were C-terminal (Gβ-CFP and YFP-$G\gamma_1$, CFP-Gβ and $G\gamma_1$-YFP, Gβ-CFP and $G\gamma_1$-YFP). No FRET signal was detected if even one member of the FRET pair was a C-terminal fusion, although both CFP-Gβ and Gβ-CFP fusions were functional by the criterion that they were able to complement the *agb1-2* null mutation phenotypically (data not shown). These observations are in agreement with the structure of the *Arabidopsis* heterotrimeric G protein predicted by molecular modeling, which places the N terminus of Gβ and the N terminus of $G\gamma_1$ in close proximity (27).

We detected FRET between L-CFP-Gαand YFP-$G\gamma_1$ but only when a Gβ cDNA was co-expressed with the Gα and Gγ fusion constructs in *Arabidopsis* mesophyll protoplasts. This result is different from what was observed in cowpea protoplasts, where FRET was also detected between L-CFP-Gα and YFP-$G\beta_1$ with the co-expression of $G\gamma_1$, although they did not test FRET between L-CFP-Gα and YFP-$G\gamma_1$ with the co-expression of Gβ (24). Given that different expression systems were used, it is possible that the folding of YFP-$G\beta_1$ or the conformation of the G protein complex differs in *Arabidopsis* and cowpea protoplasts, resulting in the apparent variation of proximity of G protein subunits in the complex formed. Whatever the source of this difference, the results of both our study and that of Adjobo-Hermans *et al.* (24) support the conclusion that *Arabidopsis* G proteins form a heterotrimeric complex *in vivo* that can be detected by FRET. Thus, based on our results, we conclude that both Gα and $G\gamma_1$ can be localized to the plasma membrane, and their close juxtaposition requires the presence of Gβ and, therefore, the formation of the heterotrimer. Because the protoplasts used were from wild type plants, this observation suggests that the endogenous levels of the G protein subunits are insufficient to assemble enough of the heterotrimer to generate a detectable FRET signal. We did not detect FRET in the plasma membrane between L-CFP-Gα and YFP-Gβ either with or without co-expression of $G\gamma_1$ in *Arabidopsis* protoplasts. This result differs from those reported in mammalian studies in which FRET can be detected between L-CFP-Gα and YFP-Gβ as well as between L-CFP-Gα and YFP-$G\gamma_1$ (35) and may be attributable to slight structural differences between mammalian and *Arabidopsis* heterotrimeric G proteins (27). Heterotrimer formation and Gβ-dependent interaction of Gα and $G\gamma_1$ was further supported by independent biochemical approaches showing that if Gβ was co-expressed, it was then possible to co-immunoprecipitate YFP-$G\gamma_1$ with L-CFP-Gα using Gα antibodies (Fig. 4*B*).

Roughly 30% of the native Gα protein in the plasma membranes of wild type *Arabidopsis* plants was in a large complex of ~700 kDa, and the remainder appeared to be monomeric, judging from its mobility in a native gel (Fig. 5*A*). By contrast, all of the Gβ detectable in plants stably transformed with a 35S-CFP-Gβ cDNA fusion construct was associated with a large complex of roughly the same size (~700 kDa) (Fig. 5*B*). The large Gα-containing complexes were much less abundant in *agb1-2* mutant plants lacking the Gβ subunit. Instead, the Gα subunit was present in smaller membrane-bound complexes ranging in size from 140 to 400 kDa and as free monomer. This observation suggests that the formation of the large Gα-containing complexes requires the presence of the Gβ subunit, further suggesting that the plasma membrane complexes containing Gα and Gβ are the same. A number of Gα-interacting proteins have been identified, including AtRGS (regulator of G protein signaling) (56), AtGCR1 (G protein-coupled receptor) (57), AtGCR2 (58), phospholipase Dα) (59), PRN1 (pirin) (60), THF1 (thylakoid formation) (45), and PD1 (prephenate dehydratase) (61). It remains to be determined whether any of these proteins is a component of the ~700-kDa complex.

It is well documented that Gα dissociates from the Gβγ complex upon binding of GTP to Gα in mammalian and *Dictyostelium* G heterotrimeric G proteins, with the possible exception of those containing $G\alpha_i$ (62). However, the evidence about whether plant heterotrimeric G proteins dissociate upon activation is somewhat contradictory. The fact that FRET still occurs between *Arabidopsis* Gβ and a constitutively active mutant form of the *Arabidopsis* Gα subunit has been interpreted as indicating that the plant heterotrimer does not dissociate upon activation (24). However, we observed that a significant fraction (~70%) of plasma-membrane localized *Arabidopsis* Gα is already present in the monomeric form and that GTPγS promotes partial dissociation of the remaining Gα from the large complex (Fig. 6*A*). Gel filtration experiments in rice indicate that Gα, Gβ, and the two Gγ subunits are in a 400-kDa complex, and GTPγS treatment results in almost complete dissociation of all three types of subunits from the complex (26). When a constitutively active form of rice Gα is expressed in a Gα-null background, the constitutively active subunit is predominantly found in the monomeric form (28). Because the *Arabidopsis* Gα exhibits slow GTPase activity, most if not all of the *Arabidopsis* Gα protein is proposed to be in its active GTP-bound form (54, 63). We found that H_2O_2 promotes dissociation of *Arabidopsis* Gα from the ~700 kDa complex (Fig. 6*C*), but does not affect its ability to bind GTPγS (Fig. 7*C*). In view of the evidence that Gα mediates oxidative stress signaling (18), this result suggests that reactive oxygen species affect the stability of the complex, but unlike the situation with mammalian $G\alpha_i$ and $G\alpha_o$ (51), not the activity of Gα itself.

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In conclusion, our results affirm that the *Arabidopsis* G protein complex behaves similarly to mammalian G protein complexes in that it forms a heterotrimer *in vivo*. However, our results also reveal distinct aspects of G protein biochemistry in plants, including a lack of dependence of Gβ and Gγ localization on Gα, an apparently closer interaction of Gα and Gγ than Gα and Gβ, a predominance of the monomeric form of Gα in the plasma membrane, and a lack of apparent regulation of Gα activity by reactive oxygen species. The question of how G protein signaling evolved in plants (64) to encompass these unique attributes will be an interesting topic for future studies.

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