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Heterotrimeric G protein signaling is important for cell-proliferative and glucose-sensing signal transduction pathways in the model plant organism Arabidopsis thaliana. AtRGS1 is a seven-transmembrane, RGS domain-containing protein that is a putative membrane receptor for α-glucose. Here we show, by using FRET, that α-glucose alters the interaction between the AtGPA1 and AtRGS1 in vivo. AtGPA1 is a unique heterotrimeric G protein α (Ge) subunit that is constitutively GTP-bound given its high spontaneous nucleotide exchange coupled with slow GTP hydrolysis. Analysis of a point mutation in AtRGS1 that abrogates GTPase-accelerating activity mediates sugar signal transduction during Arabidopsis development, in contrast to animals where nucleotide exchange is the limiting step in the heterotrimeric G protein nucleotide cycle.

α-glucose | G protein-coupled receptor | guanine nucleotide cycle | RGS protein | GTPase-accelerating protein

G protein-coupled receptors (GPCRs), also known as seven-transmembrane domain (7TM) receptors, compose a large superfamily of cell membrane proteins that convert extracellular signals from environmental cues to intracellular responses (1–3). Both genetic and biochemical data firmly support a role for G proteins in sugar-regulated plant cell proliferation, yet plants have a limited repertoire of heterotrimeric G protein-signaling components (4–10). Arabidopsis thaliana has one canonical G protein α (Ge) subunit (AtGPA1), one Ge β subunit, and two Ge γ subunits, but, as yet, no bona fide GPCR (although candidate GPCRs have been proposed) (10, 11). The Arabidopsis regulator of G protein signaling-1 (AtRGS1) protein contains seven transmembrane-spanning domains and a C-terminal RGS domain (5). Thus, AtRGS1 has the membrane topology and structural characteristics of a GPCR, and genetic evidence is consistent with AtRGS1 being a receptor or coreceptor for α-glucose at the plasma membrane (4, 5). The RGS domain of AtRGS1 has GTPase-accelerating activity toward AtGPA1, which is rate-limiting in the guanine nucleotide cycle.

Based on these previous findings, it has been proposed that AtRGS1 could be a guanine-nucleotide exchange factor (GEF) and/or a GTPase-accelerating protein (GAP) for AtGPA1 (3, 13). A ligand for AtRGS1 that modulates GEF or GAP activity has not been identified. However, based on the altered sugar responsiveness of Atgpa1-null mutants, it has been suggested that α-glucose is a candidate ligand (4–8). Here we examined the requirement for G protein signaling in the Arabidopsis glucose-sensing pathway. Conventional GPCRs serve as nucleotide exchange factors controlling the rate-limiting step in heterotrimeric G protein cycling: the release of GDP (14). We found that the Arabidopsis AtRGS1 protein cycling: the release of GDP (14). We found that the Arabidopsis AtRGS1 protein serves as a 7TM GAP, and it is GTP hydrolysis, not GDP release, by AtGPA1 that is rate-limiting in Arabidopsis α-glucose signal transduction.

Results and Discussion

Growth arrest during Arabidopsis development can be induced by high concentrations of α-glucose and quantified by the fraction of seedlings with green cotyledons (4, 15). At 1% α-glucose, individuals of all Arabidopsis genotypes that were tested grew normally (100% green seedlings) (data not shown), whereas at 6% glucose, approximately half of the individual wild-type seedlings arrested (Fig. 1A). As expected (4), AtRGS1-deficient plants were tolerant to 6% α-glucose, whereas AtGPA1-deficient plants were hypersensitive to glucose-induced growth arrest (Fig. 1A). Likewise, Arabidopsis overexpressing wild-type AtRGS1 were hypersensitive to glucose-induced growth arrest (Fig. 1A). Plants lacking both AtGPA1 and AtRGS1 also were hypersensitive to glucose (Fig. 1A), phenocopying the Atgpa1 mutant phenotype of glucose hypervisitility. This finding indicates that AtGPA1 and AtRGS1 work in the same glucose signal transduction pathway, and that the null Atgpa1 allele is epistatic to the null Args1 allele in this pathway. These data suggest that, in the presence of ligand (high concentrations of glucose), a signal transduction pathway inhibits plant growth and development. This pathway is modulated by heterotrimeric G protein signal transduction, and AtGPA1-GTP most likely attenuates an antiproliferative pathway (6). We hypothesize that this process is modulated by ARGS1 acting as a glucose sensor and regulating the GTPase activity of AtGPA1. Therefore, based on this hypothesis, glucose should alter the interaction between AtRGS1 and AtGPA1, thereby coupling receptor activation to the modulation of AtGPA1’s activity on seedling developmental processes.

To test this prediction in vivo, we performed FRET studies on plants coexpressing AtGPA1-CFP and AtRGS1-YFP fusion proteins (e.g., Fig. 1B–G). Within 6 min after the addition of exogenous α-glucose, FRET increased (Fig. 1G, arrows). The change in FRET was transient, peaking at 8–10 min and decaying with a half-life of ≈2 min. Interestingly, FRET signals were exclusively observed on or around plastids that were in close proximity to the plasma membrane. The observed FRET was specific to α-glucose because it was not observed upon the addition of L-glucose (Fig. 1H) or mannitol [supporting information (SI) Fig. 4]. Thus, both genetic and imaging data are consistent with the hypothesis that AtRGS1 is a membrane receptor or coreceptor for α-glucose that interacts with AtGPA1 upon glucose binding. However, the imaging data do not preclude a mechanism in which indirect modulation of FRET...
may occur by an alternative glucose-sensing pathway. The imaging data further suggest that downstream signal transduction may be spatially localized where plastids abut the plasma membrane. This finding is consistent with evidence showing that the plastid protein THF1 directly interacts with AtGPA1 at the plastid/plasma membrane interface, and that the thf1-null allele is epistatic to the Atgpa1-null allele in the sugar-sensing pathway (6).

Based on its chimeric structure (N-terminal 7TM and C-terminal RGS domains), we previously hypothesized that AtRGS1 could be a glucose-regulated GAP, GEF, or dual GAP and GEF for AtGPA1 (3). Our present analysis of the biochemical properties of AtRGS1 is consistent with the former (glucose-regulated GAP) and suggests that the AtRGS1 GTPase cycle does not require GEF activity. Recombinant AtGPA1 had a high specific activity, binding 35S-GTP with a stoichiometry of 0.91 mol of GTP/mol of protein. Equilibrium competition binding assays with a variety of purine and pyrimidine nucleotide triphosphates were able to compete with 35S-GTP binding to AtGPA1 by using intrinsic tryptophan fluorescence, and comparable kinetic data were obtained (SI Table 2). This observation demonstrates that the rate of GDP dissociation from AtGPA1 (k\text{GDP} = 12.6 min\textsuperscript{-1}) was highly concordant with the measured GTP\gamma S-binding rate.

GDP dissociation is the rate-limiting step of the heterotrimeric G protein cycle as described in animals (14). To understand the exceptionally rapid nucleotide exchange exhibited by AtGPA1 in the context of its complete nucleotide cycle, we also measured the rate of GTP hydrolysis by AtGPA1 by using a single turnover GTPase assay. The k\text{cat} value for AtGPA1 at 20°C was 0.12 min\textsuperscript{-1} (Fig. 2E), making AtGPA1 among the slowest heterotrimeric GTPases described (SI Table 2). The k\text{cat} value of Gα\text{oA} was determined in parallel (Fig. 2E) and was consistent with published values (SI Table 2). This observation demonstrates that the rate of GTP hydrolysis (k\text{cat}) by AtGPA1 is over two orders of magnitude slower than the rate of nucleotide exchange (k\text{off}). Thus, GTP hydrolysis (rather than GDP release) is the rate-limiting step in the guanine nucleotide cycle of AtGPA1. Two predictions follow from these observations: (i) The steady-state rate of GTP hydrolysis should approximate k\text{cat}, and (ii) RGS domain-mediated GAP activity should accelerate steady-state GTP hydrolysis.

We tested these two predictions by performing steady-state GTPase assays by using \[^{\gamma\text{32P}}\]GTP. The rate of GTP hydrolysis at steady state (k\text{cat}) at 20°C was 0.063 min\textsuperscript{-1} (±0.015 min\textsuperscript{-1}; n = 4) (Fig. 2F). Thus, the observed k\text{cat} approximated the rate of nucleotide hydrolysis, not nucleotide exchange, therefore satisfying the first prediction. We observed that a 5-fold molar excess of AtRGS1 gave a 35-fold increase in steady-state GTPase activity (Fig. 2F), satisfying the second prediction and validating that GTP hydrolysis is the rate-limiting step in the Arabidopsis G protein cycle in vitro. Under steady-state conditions, the fraction of G protein in the active state can be approximated by k\text{off}/(k\text{cat} + k\text{off}) (SI Table 2) (16). In the case of Gα\text{oA}, for example, the percentage of protein bound to GTP at steady state is predicted to be 10%. In stark

Fig. 1. Signal transduction by d-glucose is mediated by AtRGS1 and AtGPA1. (A) Seedlings of different genotypes were grown on 6% d-glucose, and the percentage of seedlings with green cotyledons was quantified. All genotypes had 100% green seedlings when grown on 1% d-glucose. Genotypes: Col, wild-type Columbia ecotype; AtRgs1–2, AtRgs1-null mutant; Atgpa1–4, Atgpa1-null mutant; 35S:AtRGS1 (wild type: Ox9, Ox10, Ox16), three independent wild-type AtRGS1 constitutive overexpression lines; Atgpa1–2 Atgpa1–4, double-null mutant. Statistical significance was determined by Dunnett’s test (*, P < 0.05 vs. Col). (B–G) d-glucose-induced FRET between AtRGS1-YFP and AtGPA1-CFP in Arabidopsis roots was measured in vivo. Fluorescence emission for CFP excitation/YFP emission (B and D) and CFP excitation/CFP emission (C and F) were captured 5 min (B and C) and 8 min (E and F) after the addition of d-glucose. The normalized net FRET (nF/I) at 5 min (D) and 8 min (G) after d-glucose addition is shown. PM, plasma membrane. (H) Levels of nF/I were calculated every 30 s from 5 to 30 min after addition of 6% (w/vol) d-glucose, 6% (w/vol) l-glucose, or no treatment controls. Red and blue lines show the observed FRET for the regions of interest (ROI) 1 and ROI 2, denoted in G. Black lines indicate other independent FRET efficiency measurements. Arrows indicate image capture time points of t = 5 and t = 8 min, as denoted in B–G.
AtRGS1(E320K) is deficient in its interaction with AtGPA1 as time course assays (SI Figs. 7 and 8). To test that data were obtained by using single turnover and steady-state trations. The E320K substitution reduced AtRGS1 GAP activity. AtRGS1(E320K) was ineffective over a wide range of concentrations. In contrast, the calculated value for AtGPA1 is 99% (SI Table 2). Furthermore, AtGPA1 appears to have a higher affinity for GTP than GDP (SI Table 1), further supporting the hypothesis that AtGPA1 appears to have a higher affinity for GTP in vivo. Studies from mutants in the Arabidopsis G protein pathway indicate a role for G protein signaling in regulating cell growth. Dark-grown AtGPA1-deficient lines exhibit reduced cell proliferation and consequently have a shortened hypocotyl (19), whereas AtRGS1 null seedlings have longer hypocotyls (5). In contrast, seedlings overexpressing wild-type AtRGS1 have shortened hypocotyls (5). Unlike AtRGS1(wild-type)-overexpressing plants, AtRGS1(E320K)-expressing plants had wild-type-length hypocotyls (Fig. 3F). Data obtained by using AtRGS1-GFP fusion proteins expressed in AtRGS1-deficient plants confirm that AtRGS1(E320K) cannot complement the Atrgs1-null mutation (Fig. 3G). These results indicate that GAP activity is the essential determinant in AtRGS1-mediated cell proliferative signaling.
We also measured the contribution of AtRGS1 GAP activity to glucose-mediated growth arrest. Expression of the AtRGS1(E320K) mutant was unable to complement Atrgs1-null alleles and did not elicit the glucose-hypersensitive phenotype typical of AtRGS1(wild-type)-overexpressing plants (compare Fig. 1A with Fig. 3H). This result suggests that the influence of AtRGS1 on developmental responses to environmental glucose is directly dependent on its GAP activity. This finding is consistent with the hypothesis that AtRGS1 is a glucose-regulated GAP for GTP-bound AtGPA1. Furthermore, the intrinsic biochemical properties of high spontaneous nucleotide exchange and low GTPase activity suggest that AtGPA1 is predominantly in the GTP-bound state, and this state is the likely substrate for AtRGS1 in vivo. The action of AtRGS1 is likely to be catalytic because biochemical experiments demonstrate that AtRGS1 is a potent accelerator of AtGPA1 GTPase activity at substoichiometric concentrations in vitro (Fig. 3A and SI Fig. 8). The in vitro biochemical properties of AtGPA1 imply that the Arabidopsis G protein cycle is distinct from the mammalian G protein cycle in two critical aspects: GEF activity is not required, and GDP-bound AtGPA1 may be the active signaling species in some cases. These observations are concordant with data showing that AtGPA1-GDP interacts with AtPLDα1 to regulate phosphatidic acid production and inhibition of stomatal opening (20).
In addition to AtRGS1, two other putative *Arabidopsis* GPCRs, GCR1 and GCR2, have been described (21, 22). We recently argued that GCR2 is not a transmembrane receptor, but rather a plant homolog of bacterial lanthionine synthetases that was misidentified as a GPCR (11). GCR1 is a predicted 7TM protein reported to interact with AtGPA1 (23). Physiological data suggest that GCR1 may be involved in the AtGPA1 signaling pathway (23). However, GCR1 also regulates hormonal signaling in a G protein-independent manner (24). These reports on GCR1 and GCR2 are entirely consistent with our findings presented here, in that, to date, none of these plant proteins has been shown to possess GEF activity toward the sole *Arabidopsis* 

subunit, AtGPA1. Thus, in light of the unique biochemical activities of AtGPA1 as a rapid spontaneous exchanger and poor GTpase, putative plant membrane receptors such as GCR1 also may regulate AtGPA1 activity by GEF-independent mechanisms.

*Arabidopsis* 

β subunits also are implicated in both cell proliferation and sugar-sensing pathways (6, 19). The canonical function for Gβ subunits in metazoan organisms is to participate directly in GPCR-mediated GEF activity and regulate effector pathways (1, 3). Although our data would appear to exclude the former, it does not exclude the latter. Gβγ subunits also are required for the proper membrane targeting and stability of mammalian Go subunits (25), and this finding appears to be the case in *Arabidopsis* as well (26). Moreover, FRET studies suggest that the *Arabidopsis* Goβγ heterotrimer does not dissociate upon GTP binding, but merely changes conformation (26). This result is in line with recent studies on mammalian G protein signaling by nondissociated heterotrimers (27), as well as those of the regulatory protein AGS8 shown to mammalian G protein signaling by nondissociated heterotrimers (28). Mammalian Gβγ subunits also attenuate spontaneous nucleotide exchange on Gα subunits by ≥5-fold (29, 30). It is possible that AtGPA1 constitutive activity is dampened by the *Arabidopsis* Gβγ complex in vivo. However, to have a significant physiological effect on AtGPA1 nucleotide cycling kinetics, *Arabidopsis* Gβγ would need to attenuate GDP release by an improbable three orders of magnitude (i.e., leading to a predicted GTP occupancy of ~10%), whereas a reduction in AtGPA1 GDP release rate by two orders of magnitude is predicted to maintain GTP occupancy level at >50% (data based on calculations similar to those of SI Table 2).

We described genetic, biochemical, and cellular data on AtRGS1 and AtGPA1 in support of a unique paradigm for heterotrimeric G protein action. This AtRGS1-coupled, high-glucose-sensing mechanism appears to be distinct from the most thoroughly characterized plant glucose sensor, hexokinase (4). This finding is not surprising because multiple sugar-sensing mechanisms exist in plants (9, 31). Further studies should be directed toward an unequivocal demonstration of glucose binding to AtRGS1 and glucose-mediated modulation of AtRGS1 GAP activity. This system is not unique to *Arabidopsis* because we have identified AtRGS1 orthologs in several plant species (SI Figs. 10–12). Moreover, we identified putative 7TM RGS proteins in both fungi and protozoa as well (SI Figs. 10–12). These findings suggest that many organisms may use 7TM RGS proteins for glucose sensing and the control of cell proliferation.

**Methods**

**Materials and Data Analysis.** [35S]GTPγS and [γ-32P]GTP were from PerkinElmer (Wellesley, MA). [β-32P]GDP was from MP Biomedicals (Solon, OH). BODIPYFL-GTPγS was from Molecular Probes (Eugene, OR). XTP and UTP were from Rob Nicholas (University of North Carolina), and all other nucleotides were obtained from Sigma–Aldrich (St. Louis, MO). Unless otherwise specified, all other chemicals were of the highest purity obtainable from Sigma–Aldrich or Fisher Scientific (Pittsburgh, PA). Nonlinear regression and statistical analyses were performed in Prism version 4.0 (GraphPad, San Diego, CA). All data presented are representative of three or more independent experiments. Multiple comparison tests were calculated by ANOVA by using either Dunnett’s or Bonferroni’s post-test at the 95% significance level (Prism).

**Protein Purification/Enzymology.** His6-AtGPA1-GDP and GST-AtRGS1 (amino acids 249–459) were purified as described (12). Site-directed mutagenesis was conducted by using QuikChange (Stratagene, La Jolla, CA). [35S]GTPγS binding and [γ-32P]GTP steady-state hydrolysis assays were conducted as described (32). SPR assays using an anti-GST biosensor were conducted as described (12, 18). GST, GST-AtRGS1 (wild type), and GST-AtRGS1(E320K) were immobilized to 240, 250, and 290 resonance units (RU), respectively. Bulk buffer refractive index change upon AtGPA1 injection was observed to increase response units on all sensor surfaces equally by ~200 RU. Bulk shift and nonspecific binding were accounted for by subtraction of simultaneous sensorgram curves derived from the GST-only surface.

**Intrinsic Trypsophan Fluorescence.** Tryptophan fluorescence of Gα was used as a probe for G protein activation (33). Structural and mutagenic analyses indicate that a tryptophan residue in the α2-helix (switch-II), equivalent to W207 in Go (34), shifts from a solvent-exposed area to a hydrophobic pocket upon Gα activation, resulting in an increased fluorescence quantum yield (35). AtGPA1 also has this α2-helix tryptophan (W229). Tryptophan fluorescence of AtGPA1 was measured at 20°C in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM MgCl2 by using a PerkinElmer LS55 spectrophotometer. Excitation and emission wavelengths were 282 and 340 nm, respectively, with slits widths of 5 nm.

**GDP Release.** First, 100 nM AtGPA1 was preloaded with 3.2 nM [β-32P]GDP [3,000 Ci/mmol (1 Ci = 37 GBq)] for 10 min at 20°C in 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 25 mM MgCl2, 100 mM NaCl, 1 mM DTT, and 0.1% (wt/vol) C12E10. Then, samples were placed on ice, and aliquots were taken to assess total [β-32P]GDP bound. To quantify release of bound [β-32P]GDP, 100 μM GTPγS was added, and reactions again were incubated at 20°C. Aliquots were taken at indicated times, vacuum-filtered onto HA45 nitrocellulose (Millipore, Billerica, MA), washed, and analyzed by liquid scintillation. Data were plotted as amount [β-32P]GDP bound, with the initial preloaded aliquot serving as zero time point, and fit to a single exponential decay function.

**Single Turnover GTP Hydrolysis.** The rate of GTP hydrolysis by AtGPA1 was measured by a single turnover. Mg2+ is a crucial cofactor for GTP hydrolysis (16), and thus it is typically excluded from the [γ-32P]GTP loading phase to prevent hydrolysis before initiation of the single turnover reaction (36). However, AtGPA1 was observed to be highly dependent on Mg2+ for GTP binding (data not shown). Thus, we modified our standard method (32) to account for [32P] release during preloading, as detailed in SI Fig. 7.

**Arabidopsis.** All experiments were conducted by using Columbia ecotype *A. thaliana*. Generation and characterization of the majority of *Arabidopsis* lines containing T-DNA insertions and transgenic alleles used in these studies are described (4, 5, 13). Isolation of Atrgs1–2/Atgpa1–4 double mutants also has been described (13). AtGPA1-LCFP consists of enhanced cyan fluorescent protein inserted into the loop between the predicted αA and αB helices (between Ala-97 and Gin-98) of AtGPA1 as described (6, 13).
**Plant Growth Assays.** For phenotypic analyses, wild-type and mutant seeds were sterilized; sown in Petri dishes containing 1/2 Murashige and Skoog basal medium with Gamborg’s vitamins (ICN Biomedicals, Aurora, OH), 1% (wt/vol) sucrose, and 0.5% (wt/vol) phytoagar (Research Products International, Mt. Prospect, IL); adjusted to pH 5.7; and treated at 4°C in the dark for 3 days and then moved to a growth chamber with 23°C and light intensity of 100 μmol per m²/s. For the phenotypic analysis of 2-day-old etiolated seedlings, Petri dishes were wrapped in aluminum foil and placed in the dark at 23°C. Etiolated hypocotyls were measured by ruler.

**AtRGS1 Transgenic Plants.** The AtRGS1 ORF (At3g26090) was PCR-amplified from cDNA made from seedlings grown in light for 10 days, cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA), and then subcloned into Gateway plant transformation destination binary vector pB2GW7 or pGWB42 for Agrobacterium plants of the indicated genotypes by mosaic virus. All constructs were transformed into Arabidopsis plants of the indicated genotypes by Agrobacterium-mediated transformation (37), and expression of all transgenes was verified by RT-PCR (SI Fig. 13).

**Green Seeding Assay.** The green seeding assay was performed largely according to published protocols (4). Briefly, wild-type (Col-0), mutant, and transgenic seeds were sown, chilled, light treated, and grown under identical conditions until maturation. Seeds from matched lots were sterilized with 80% (vol/vol) ethanol for 2 min, followed by 30% (vol/vol) bleach with 0.1% (vol/vol) Tween-20 for 10 min, and then washed with sterile deionized water six times under sterile conditions. Sterilized seeds were sown on plates consisting of 1/2 Murashige and Skoog basal medium with vitamins (Plantmedia, Dublin, OH) (pH adjusted to 5.7 with 1 N KOH), 0.5% (wt/vol) phytoagar (Plantmedia) and different concentrations of d-glucose (Sigma–Aldrich) and stratified at 4°C in the dark for 48 h. Then plates were moved to a 23°C growth chamber, under 16/8-h photo-period at 100 μmol/m²/s, and placed horizontally. Ten days later, the percentage of green seedling was scored as the number of green seedlings divided by the total number of seeds. Each experiment was repeated three times. Minimally, 50 seeds were scored for each treatment of each genotype.

**FRET Microscopy.** Fluorescence images of AtRGS1-YFP/AtGPA1-L-CFP seedlings were captured by using an Olympus IX81 inverted microscope (Center Valley, PA) controlled by IPLab software version 3.6 (BD Biosciences, Rockville, MA). Images of CFP, YFP, and the YFP/CFP ratio were observed through a 60× water immersion objective and simultaneously captured by a cooled charge-coupled device Photometrics Cascade Digital Camera (Roper Scientific, Tucson, AZ) equipped with CFP/YFP FRET emission filter OI-05-EM in a dual-view mounting tube. Filter sets used were YFP (excitation, 500/20 nm; emission, 535/30 nm), CFP (excitation, 436/20 nm; emission, 480/40 nm), and FRET (505dcxr; excitation, 436/20 nm; emission, 480/30 nm and 535/40 nm). Normalized net FRET was calculated in IPLab version 3.6 software using established algorithms for two-filter FRET with fluorescence microscopy (38).

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