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Two Novel GPCR-Type G Proteins Are Abscisic Acid Receptors in *Arabidopsis*

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SUMMARY

In plants, G proteins modulate signaling by the stress hormone, abscisic acid (ABA). We identify and characterize two novel *Arabidopsis* proteins that show homology to an orphan vertebrate GPCR (GPR89) and interact with the sole *Arabidopsis* G protein α subunit, GPA1, but also have intrinsic GTP-binding and GTPase activity. We have named these proteins *G*PCR-*t*ype *G* proteins (GTG1 and GTG2). *Arabidopsis* mutants lacking both *GTG1* and *GTG2* exhibit ABA hyposensitivity. GTG1 and GTG2 bind ABA specifically. The GDP-bound form of the GTGs exhibits greater ABA binding than the GTP-bound form, the GTPase activity of the GTGs is inhibited by GPA1, and *gpa1* null mutants exhibit ABA-hypersensitive phenotypes. These results predict that, unusually, it is the GDP-bound, not the GTP-bound, form of the GTGs that actively relays the signal. We propose that GTG proteins function both as a new type of G protein and as a class of membrane-localized ABA receptors.

INTRODUCTION

Signal transduction processes mediated by G protein signaling components constitute one of the most elaborate receptor-effector signaling networks (Offermanns, 2003). The central components of this network are heterotrimeric G proteins, comprised of Gα, Gβ, and Gγ subunits, and G protein-coupled receptors (GPCRs). The Gα subunit has both GTP-binding and GTPase activity and acts as a bimodal molecular switch, typically with a GDP-bound "off" mode and a GTP-bound "on" mode. GPCRs classically act as *g*uanine nucleotide *e*xchange *f*actors (GEFs), and a change in GPCR conformation upon signal perception leads to exchange of GDP for GTP at the Gα subunit. This promotes dissociation of the heterotrimer into free GTP-Gα and Gβγ dimers, both of which can interact with an array of downstream signaling elements. The intrinsic GTPase activity of Gα regenerates its GDP-bound form, permitting reassociation with the Gβγ dimer and completing the cycle (Cabrera-Vera et al., 2003). Accessory proteins also regulate the G protein cycle, most prominently the *G*TPase-*a*ccelerating *p*roteins (GAPs) exemplified by RGS (*r*egulators of *G* protein *s*ignaling) proteins (Ross, 2008) and the *g*uanine nucleotide *d*issociation *i*nhibitor (GDI) proteins that primarily inhibit dissociation of GDP from Gα. Diversity in mammalian G protein signaling is achieved by a large combinatorial repertoire of G protein signaling components and the range of effectors with which they interact. In human, there are 23 Gα, 5 Gβ, and 12 Gγ subunit proteins and >800 predicted GPCRs, generating a multitude of receptor-G protein combinations (Offermanns, 2003). Fungi and plants, however, have limited numbers of G protein components. The genome of the model plant *Arabidopsis thaliana* contains one prototypical Gα (GPA1), one Gβ (AGB1), and two identified Gγ (AGG1 and AGG2) subunits (Jones and Assmann, 2004) and one RGS protein, AtRGS1 (Chen et al., 2003). GCR1 is the best characterized GPCR-like protein in *Arabidopsis* (Pandey and Assmann, 2004), although no ligand has yet been identified for it.

Despite the paucity of components, G proteins are involved in numerous fundamental growth and developmental processes in plants (Assmann, 2004). There is also evidence suggesting that plants have evolved a scheme with added temporal and spatial aspects that allows a limited number of G protein components to act as nodes for integration and amplification of a host of abiotic, biotic, and hormonal signals (Assmann, 2004).

Based on phenotypic analyses of null mutants, G proteins modulate almost all aspects of ABA signaling in plants (Wang et al., 2001; Coursol et al., 2003; Pandey and Assmann, 2004; Pandey et al., 2006). While biochemical evidence supports the presence of both cell-surface and intracellular receptor(s) for ABA (reviewed in Assmann, 1994), a direct receptor-effector signaling module regulated by G proteins during ABA signaling has remained elusive. The intracellular ABA-binding proteins, FCA and CHLH (Razem et al., 2006; Shen et al., 2006), bear no hallmarks supporting G protein coupling, and the recent report of GCR2 as a G protein-coupled ABA receptor (Liu et al., 2007) seems to be incorrect in its conclusion that GCR2 harbors transmembrane domains (Illingworth et al., 2008; Johnston et al., 2007b). In addition, ABA-related phenotypes are mild to absent in *gcr2* mutants (Gao et al., 2007; Guo et al., 2008). Such data suggest that important ABA receptors in plants remain to be identified.

Additional GPCR-like proteins exist in plants (Gookin et al., 2008 and references therein). Our in silico analyses identified

two new GPCR-like proteins in *Arabidopsis*, At1g64990 and At4g27630, that show extensive sequence homology to a human orphan receptor, GPR89, but also have some unique features. In addition to a predicted GPCR-like topology and sequence similarity to GPR89, both proteins also have a predicted ATP-/GTP-binding domain and a degenerate GTPase-activating protein domain. We have named these proteins *G*PCR-*t*ype *G* proteins 1 and 2 (GTG1 and GTG2). We find that the GTG proteins exemplify a novel class of proteins with topology similar to GPCRs but with classic GTP-binding/GTPase activity. We provide biochemical and phenotypic evidence that GTG1 and GTG2 proteins are redundantly involved in G protein-coupled ABA signaling and are, or are parts of, ABA receptor complexes.

RESULTS

GTG1 and GTG2 Are *Arabidopsis* Homologs of Orphan GPCR, GPR89

GTG1 (At1g64990) and GTG2 (At4g27630) show 90% amino acid sequence identity with each other and 45% identity and 68% similarity with GPR89. The similarity extends throughout the length of the proteins (Figure 1A). BLAST analysis identifies close homologs of these proteins in monocot and dicot plants, vertebrates, invertebrates, fungi, and unicellular organisms. The *Arabidopsis* GTG proteins show about 80% identity and 90% similarity at the amino acid level with their plant homologs, whereas 40%–45% sequence identity and 60%–70% sequence similarity are observed with vertebrate and nonvertebrate animals. Lower homology is observed with fungi and unicellular organisms (about 20% identity and 40% similarity). To assess the evolutionary relationship between these proteins we performed a phylogenetic analysis (Figure 1B). Plants form a separate clade from other organisms and the unicellular green alga *Chlamydomonas* groups with the other lower organisms, suggesting a possible sequence divergence when unicellular and multicellular organisms diverged.

PROSITE motif analysis (http://www.expasy.ch/prosite/) identified a conserved ATP-/GTP-binding region in GTG1 and GTG2 (Figure 1A, region 382–411 for GTG1). This motif is highly conserved in all the plant proteins; however the similarity with non-plant proteins is relatively low with little conservation of the first nine amino acids at the junction of the predicted 4th intracellular loop. The polar charged glutamic acid at position 396 of GTG1 is conserved in all plant species but is replaced by a neutral, uncharged amide (glutamine) in the non-plant proteins. The large third intracellular loop of GTG1 and GTG2 also has a region showing similarity to the degenerate Ras GTPase-activating protein domain ([GSNA]-x-[LIVMF]-[FYCI]-[LIVMFY]-R-[LIVMFY](2)-[GACNS]-[PAV]-[AV]-[LIV]-[LIVM]-[SGANT]-P) with 68% and 62% similarity, respectively (amino acids 230–243 in GTG1). This region also shows high sequence conservation in all the plant proteins analyzed but is divergent elsewhere.

Both GTG1 and GTG2 have nine predicted transmembrane domains, similar to human GPR89 (Figure 1C; Table S3 available online). As anticipated, the FLAG epitope-tagged versions of both GTG1 and GTG2 proteins are detected mostly in the total microsomal fractions isolated from transgenic *Arabidopsis* plants (Figure 1D). Transient expression in *Arabidopsis* mesophyll protoplasts shows localization of GFP-tagged GTG1 and GTG2 at the cell periphery (Figure 1E).

GTG1 and GTG2 Have Specific GTP-Binding and GTPase Activity

Purified recombinant GTG1, GTG2 (Figure S1), and GPR89 proteins were analyzed for GTP-binding and GTPase activity using a real-time assay based on BODIPY-GTPγS or BODIPY-GTP, in which fluorescence increases upon binding of the fluorescently labeled nucleotide and decreases upon GTP hydrolysis (Willard et al., 2005). We first validated this method using commercially available bovine G protein (Figures 2A and 2B). As expected, the bovine protein showed specific binding of a nonhydrolyzable GTP, BODIPY-GTPγS, that could be competed with unlabeled GTP or GDP but not with unlabeled ADP (Figure 2A). The bovine G protein also showed GTPase activity against BODIPY-GTP that was competed by unlabeled GDP or GTP but not by unlabeled ADP (Figure 2B).

We then analyzed the *Arabidopsis* GTG proteins and human GPR89 for GTP binding and hydrolysis. GTG1 and GTG2 both show specific GTP binding that could be efficiently competed by nonfluorescent GDP or GTP but not by ADP (Figures 2C and 2E). Confirming binding specificity, both GTG proteins showed efficient binding with BODIPY-GDP, which could be competed with nonfluorescent GTP but not with nonfluorescent ATP. No binding with BODIPY-ATP or effect of ATP on BODIPY-GTP binding were observed (Figures S2 and S3). GTP binding was also independently confirmed by [^{35}S]GTPγS-binding assays (Figure S6C).

The GTG proteins also show GTPase activity (Figures 2D and 2F). We independently confirmed their GTPase activity by assaying the production of free Pi using the ENZchek phosphate assay kit (Figure S4) and by quantification of [^{32}P]GTP hydrolysis using thin-layer chromatography (Figure S6B). The GTPase activity of the two GTG proteins is Mg^{2+} dependent as the presence of 2 mM EDTA (free Mg^{2+} concentration 0.8 mM) abolished GTPase activity (Figures 2D, 2F, and S5), and it has a broad pH optimum (Figure S6). Recombinant human GPR89 did not exhibit GTP binding or GTPase activity under our assay conditions (Figures 2D and S4). Together, these assays firmly establish that the newly identified GTG1 and GTG2 proteins have specific GTP-binding and GTPase activity, hallmarks of signaling G proteins.

GTG1 and *GTG2* Are Widely Expressed

To evaluate the in planta functions of the *GTGs*, we first analyzed their expression patterns. Quantitative PCR analysis showed widespread expression of both *GTG* genes (Figure 3A). These results were corroborated by GUS reporter gene analysis. GUS staining was observed in all plant organs analyzed: cotyledons, leaves, stems, roots, flowers, and guard cells (Figures 3B and 3C). *GTG1* and *GTG2* transcript levels did not change after treatment with ABA or different abiotic stresses (Figure 3D; analysis of AtGenExpress microarray data [not shown]).

Isolation of T-DNA Insertional Mutants and Complementation

We pursued a reverse genetic approach to decipher GTG1 and GTG2 protein functions in planta. Single T-DNA insertional

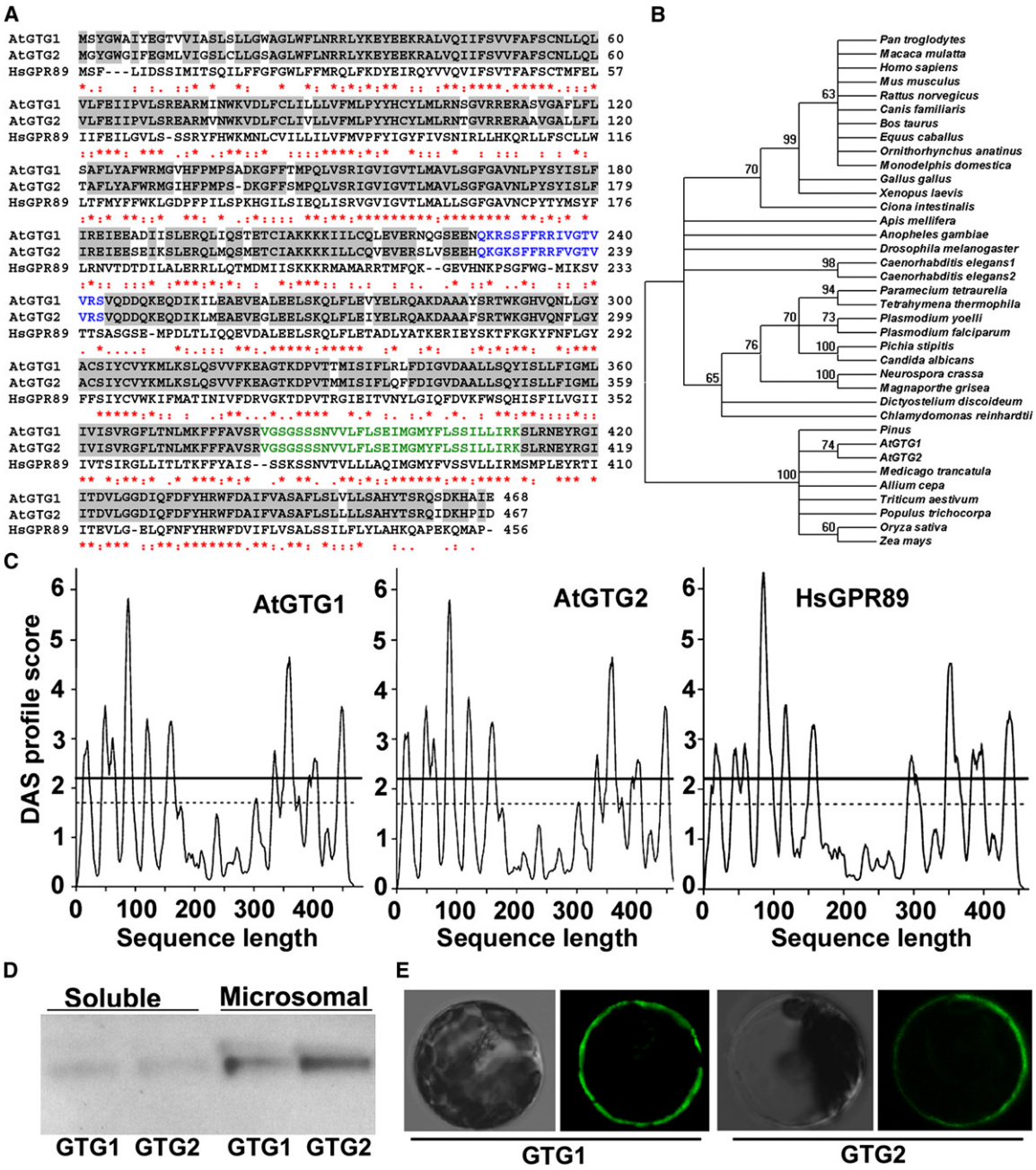

Figure 1. Identification of GTG Proteins

(A) Alignment of GTG1 and GTG2 proteins with the human GPR89A protein using Clustal W multiple alignment program. * represents amino acids conserved in all three proteins, and gray shading represents amino acid residues conserved between GTG1 and GTG2. Amino acids marked with blue represent the degenerate Ras GTPase-activating protein domain and with green represent the ATP-/GTP-binding site in GTG1 and GTG2 as predicted by PROSITE.

(B) Phylogenetic analysis of GTG1 and GTG2 was performed on full-length translated cDNA sequences from various genomes using the MEGA4.0 program (http://www.megasoftware.net/mega.html). The figure shows a nonrooted, bootstrap consensus plot, generated using the Neighbor-Joining method (Saitou and Nei, 1987), with cutoff value for condensed tree set at 60. The tree was constructed using MEGA4 (Tamura et al., 2007).

(C) Transmembrane prediction for GTG1, GTG2, and Human GPR89A (http://www.sbc.su.se/~miklos/DAS/tmdas.cgi). Solid line represents strict cutoff (2.2) and broken line represents loose cut off (1.7) values for prediction of transmembrane domains.

(D) Western blotting with proteins isolated from plants expressing GTG1-FLAG and GTG2-FLAG. GTG proteins are primarily present in the microsomal fraction.

(E) Localization of GFP-tagged GTG1 and GTG2 at the plasma membrane of mesophyll protoplasts. For each fusion protein, the left panel shows brightfield and the right panel shows confocal fluorescent image.

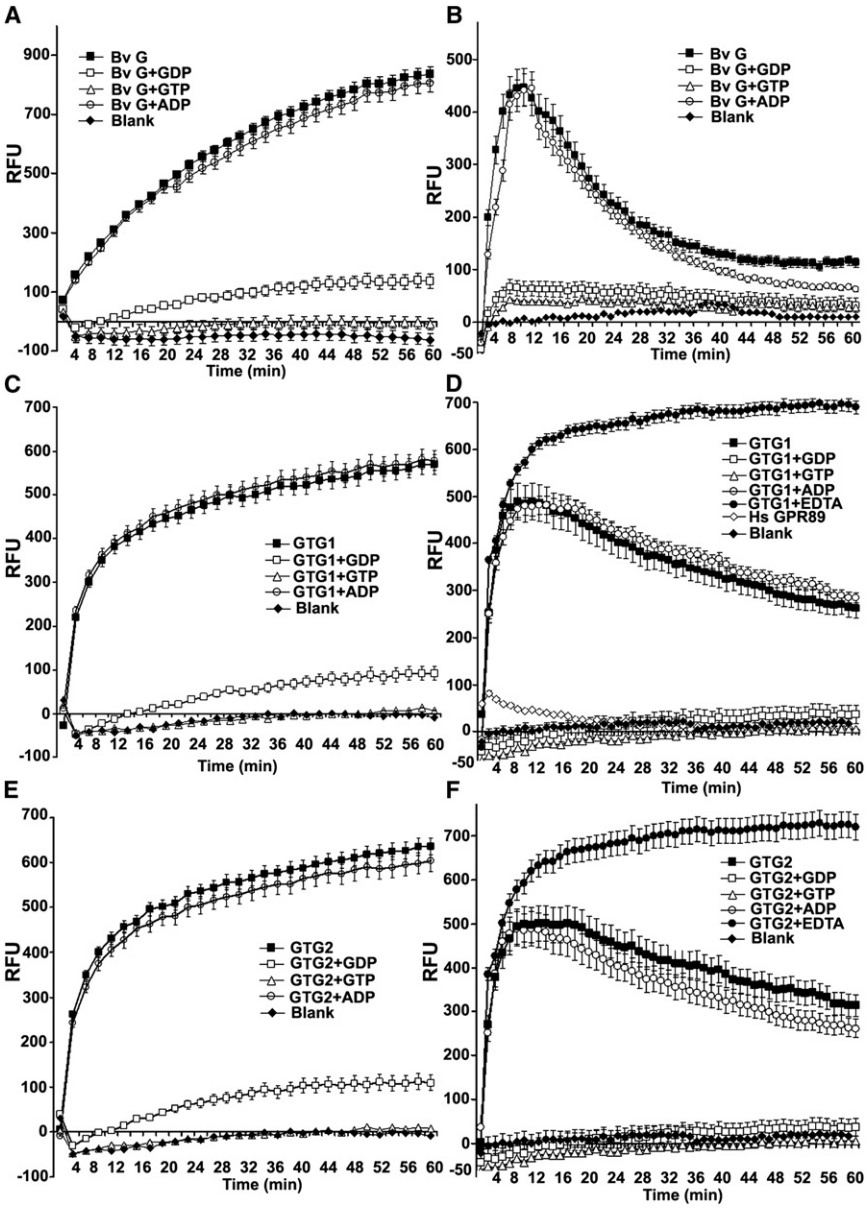

Figure 2. *Arabidopsis* GTG Proteins Specifically Bind and Hydrolyze GTP

GTP binding (assayed using nonhydrolyzable GTPγS-BODIPY FL) and GTP binding and hydrolysis (measured using GTP-BODIPY FL) in real-time fluorescence assays. RFU = relative fluorescence units. Data are one of two independent experiments, each with three replicates, means ± standard deviation (SD).

(A) Commercially available bovine G protein (Calbiochem, Cat # 371739 CA, USA) and GTPγS-BODIPY FL, used as a positive control and to validate the GTP-binding assay.

(B) GTP binding and hydrolysis validated using bovine G protein and GTP-BODIPY FL dye. Binding is represented by an increase in fluorescence over time and hydrolysis is represented by a subsequent decrease in fluorescence over time.

(C) GTG1 has specific GTP-binding activity.

(D) GTG1 has GTPase activity.

(E) GTG2 has specific GTP-binding activity.

(F) GTG2 has GTPase activity.

mutants (*gtg1-1* and *gtg2-1*) were isolated for each gene (Figure 4A). RT-PCR using the primer combinations 1P1+1P4 and 2P4+2P5, which flank the insertion sites, confirmed the absence of full-length transcripts (Figures 4B, 4C, and S7). The single mutants were used to generate the *gtg1-1 gtg2-1* double mutant (henceforth referred to as *gtg1 gtg2*). PCR with primer combinations P1+P2 and P3+P4 revealed that reduced levels of a truncated transcript could be detected for both *GTG1* and *GTG2* upstream of the insertion (Figure S7). To confirm that phenotypes of the *gtg1 gtg2* mutants are indeed due to *GTG* mutation, the double mutant was complemented with full-length *GTG1* or *GTG2* genes. For each gene, complementation was performed with two types of constructs: N- or C-terminal fusions with the FLAG epitope tag. Multiple transgenic lines were obtained for each construct and showed different levels of fusion protein expression (e.g., Figure 4D). For each gene, two independent lines of each of N- and C-terminal FLAG fusions (four lines total per gene), one showing moderate expression (e.g., #1 in upper and lower panels of Figure 4D) and the other showing higher expression (e.g., #4 in upper and lower panels of Figure 4D) were analyzed (see also Figures S8–S12).

gtg1 gtg2 Mutants Show ABA Hyposensitive Phenotypes

While the phenotypes of single *gtg* mutant plants were indistinguishable from wild-type, the double *gtg1 gtg2* mutant exhibited significant differences, related mostly to ABA signaling, both at early and adult stages. In all assays, the *gtg1 gtg2* mutant phenotype could be fully complemented with introduction of either the *GTG1* or the *GTG2* genes, implying redundancy in GTG function. No complementation was observed with the empty vector. Complemented lines with either N- or C-terminal FLAG epitope fusions showed similar results (Figures 4D, 5, and S8–S12).

The *gtg1 gtg2* mutant showed hyposensitivity to ABA in seed germination and post-germination growth assays. On 1 μM ABA, only about 50% of the wild-type Wassilewskija (Ws) seeds germinated whereas the germination percentage of *gtg1 gtg2* mutant seeds was ~80% (Figure 5A). About 40% germination was obtained with 2 μM ABA for *gtg1 gtg2* mutants, an ABA concentration at which no wild-type seeds germinated (Figures 5A and S8). The effect of ABA on post-germination growth of seedlings was analyzed by scoring percentages of germinated seeds forming green cotyledons

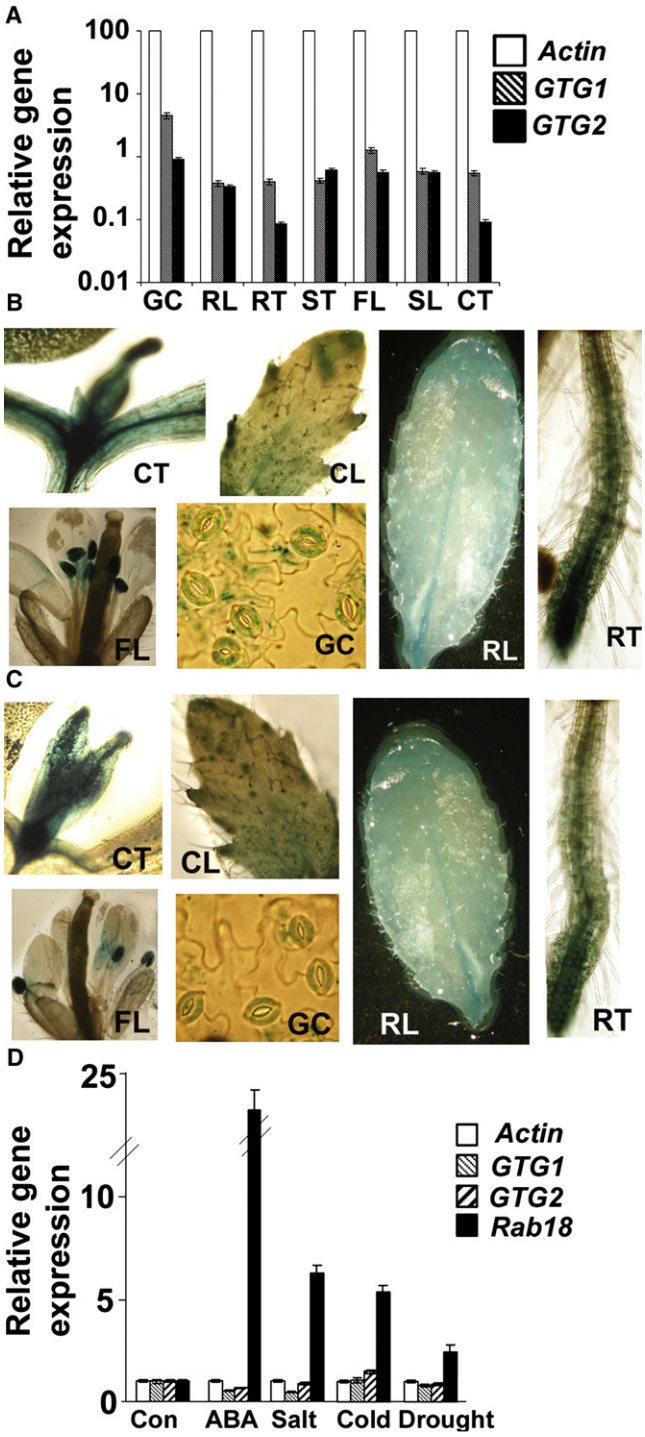

Figure 3. *GTG1* and *GTG2* Transcripts Show Widespread Expression, and Levels Are Not ABA or Stress Regulated

(A) *GTG1* and *GTG2* expression determined by real-time Q-PCR and plotted relative to expression of the *Actin* 2/8 control, set to 100% in each tissue type. GC, guard cells; RL, rosette leaves; RT, roots; ST, stem; FL, flower; SL, siliques; CT, cotyledons. Data are means ± standard error of the means (SEM), n = 3 independent experiments.

(B) Expression of *GTG1* by reporter gene (GUS) analysis.

(C) Expression of *GTG2* by reporter gene (GUS) analysis.

(D) *GTG1* and *GTG2* transcript levels in Ws seedlings treated with ABA (20 μM, 1 hr), salt (200 mM NaCl, 1 hr), cold (0°C for 3 hr), or drought (3 hr air-dried on filter paper). *Actin2/8* was used for normalization. The stress-responsive *Rab18* gene was a positive control. Data are means ± SEM, n = 3 independent experiments.

at 1 μM ABA (Chen and Jones, 2004). All *gtg1 gtg2* seedlings formed green cotyledons at this ABA concentration, whereas only about 60% of Ws or *gtg* single mutants developed green cotyledons (Figure 5B). The same trend was obtained for inhibition of primary root length by ABA. The *gtg1 gtg2* mutants showed less sensitivity to ABA, with only ~15% inhibition in root length at 20 μM ABA, a concentration sufficient to cause almost 40% inhibition of root length in Ws, single *gtg1* or *gtg2* mutants, and *gtg1 gtg2* mutant complemented with either *GTG1* or *GTG2* (Figures 5E and S9). ABA-induced gene expression was also significantly compromised in the *gtg1 gtg2* mutants, as determined by quantification of the transcript levels of a number of ABA-induced marker genes (Figures 5F and S10). For ABA induction of stomatal closure the *gtg1 gtg2* mutant plants were significantly ABA hyposensitive, exhibiting only a 17% decrease in stomatal aperture as compared to an ~40% decrease in wild-type plants (Figures 5G and S11); however, ABA inhibition of stomatal opening remained similar to wild-type (Figures 5H and S12). The adult *gtg1 gtg2* mutant plants are smaller, have a mildly stressed appearance, and flower earlier than wild-type plants (Figures 5C and 5D). The endogenous ABA level in the double mutant plants is comparable to that of wild-type (Figure S13). We also analyzed the transcript levels of other reported ABA receptors in the *gtg1 gtg2* mutant, to evaluate the possibility that the ABA hyposensitive phenotypes are due to lower expression of those receptors. No differences in transcript levels of *FCA, CHLH,* or *GCR2* could be detected in the *gtg1 gtg2* mutants as compared to wild-type (Figure S14).

GPA1 Interacts with GTG1 and GTG2 and Affects Their GTPase Activity

The GTG1 and GTG2 proteins were initially identified based on their similarity to an orphan GPCR. One of the signature properties of GPCRs is their interaction with G proteins; therefore, we evaluated the interaction of GTG proteins with the sole canonical *Arabidopsis* Gα subunit, GPA1. Both GTG1 and GTG2 interacted with GPA1 in the split ubiquitin system, designed to assess interaction of membrane proteins (Pandey and Assmann, 2004). Interaction of GPA1-CUb with GTG1- and GTG2-NUb fusions (in both orientations) was observed (Figure 6A) under very high-stringency conditions (1 mM methionine). Additional results and controls are presented in Table S1. We verified this interaction by coimmunoprecipitation. Total microsomal or soluble fractions from wild-type or *gtg1 gtg2* mutant plants expressing FLAG-tagged GTG1 or GTG2 were immunoprecipitated with FLAG antibodies and probed with GPA1 antibodies. The GPA1 protein could be detected in the total microsomal fractions but not in the soluble fractions nor in the immunoprecipitated proteins from wild-type plants (Figure 6B).

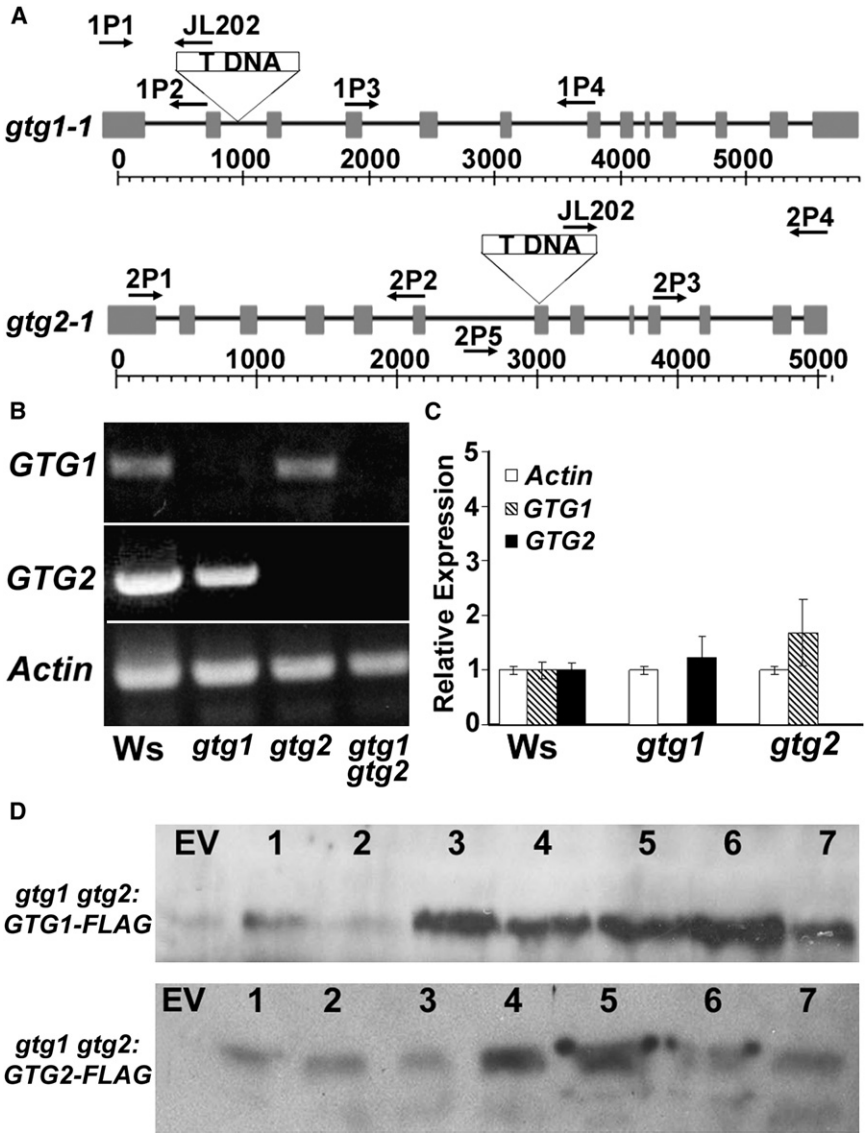

Figure 4. T-DNA Insertional Mutants of *GTG1* and *GTG2*

(A) *gtg1-1* and *gtg2-1* single mutants (depicted) were used to produce the *gtg1 gtg2* mutant. Boxes = exons, lines = introns. T-DNA insertion (not drawn to scale) position is marked. Primer pairs used in genomic PCR screening and primer pairs used in RT-PCR to assess *GTG* transcripts are indicated (see text and Figure S5 for details).

(B) Gene-specific primers flanking the insertion sites (1P1 and 1P4 for *GTG1* and 2P4 and 2P5 for *GTG2*) used in RT-PCR to confirm absence of full-length transcripts in mutants. *Actin2* = positive control.

(C) Relative expression of *GTG1* and *GTG2* genes in wild-type and single mutants with real-time Q-PCR. Expression was set to 1 in wild-type plants. *Actin2/8* gene expression was used for normalization. Data are means ± SEM, n = 3 independent experiments.

(D) Anti-FLAG western blot of different lines (#1–7) of the *gtg1 gtg2* mutant complemented with C-terminal FLAG peptide-tagged GTG1 and GTG2. Similar results were obtained with N-terminal FLAG-tagged GTG1 and GTG2 transformants. Lane EV = empty vector as a negative control.

Since GTG1 and GTG2 have G protein activity of their own, we elucidated whether interaction with GPA1 affects the GTP-binding and GTPase activity of the GTG proteins. The GTP-binding activity of the GTG1 and GTG2 proteins is accelerated by GPA1 and, remarkably, their GTPase activity is strongly inhibited (Figure 6C). To confirm that the reduced total GTPase activity is an effect of GPA1 on GTG activity and not vice versa, we used a mutant form of GPA1 (Q222L GPA1), predicted to have normal GTP binding but lack GTPase activity due to the mutation of its conserved GTPase domain (Ullah et al., 2003). Our assays with BODIPY-GTP and α^{32}P-GTP experimentally demonstrate that Q222L GPA1 indeed lacks GTPase activity, and therefore we designate it as constitutively active GPA1 (CaGPA1) (Figures 6D and S6B). Remarkably, the presence of CaGPA1 blocks the GTPase activity of the GTG proteins, confirming that the GTP-bound form of GPA1 inhibits the GTPase activity of GTG proteins.

GTG1 and GTG2 Show Specific, Saturable ABA Binding

The G protein activity and GPCR-like topology of GTG proteins along with the significantly ABA hyposensitive phenotypes of *gtg1 gtg2* mutants led to the intriguing possibility that the GTG proteins constitute all or part of an unusual class of G protein-coupled ABA perception complex(es). To test this possibility, we carried out direct ABA-binding assays with ^{3}H-ABA and purified recombinant GTG proteins. Both GTG1 and GTG2 proteins showed saturable and specific ^{3}H-ABA binding, with apparent K_D (dissociation constant) values of 35.8 nM and 41.2 nM, respectively (Figure 7A). GTG proteins did not bind ^{3}H-benzoic acid, another weak acid used as a negative control. The ABA binding of GTG1 and GTG2 could be effectively competed with unlabeled biologically active (±)-ABA, but not by the biologically inactive (−)-ABA isomer (Figure 7B). Following the implication that the GTG proteins do not bind the (−)-ABA isomer, the above K_D values would then be halved. No ^{3}H-ABA binding was observed when BSA or GPA1 was used as a control for nonspecific binding (Figures 7A and 7C). AtRGS1, which, like the GTG proteins, is a predicted multitransmembrane domain protein (Chen et al., 2003), was evaluated as a further control for nonspecific binding. In contrast to the GTG proteins, AtRGS1 showed low levels of nonspecific ABA binding (data not shown).

Both GTG1 and GTG2 showed more efficient ABA binding in the presence of GDP, as compared to binding in the presence of GTP or ATP (Figure 7C). The presence of GPA1 (in the

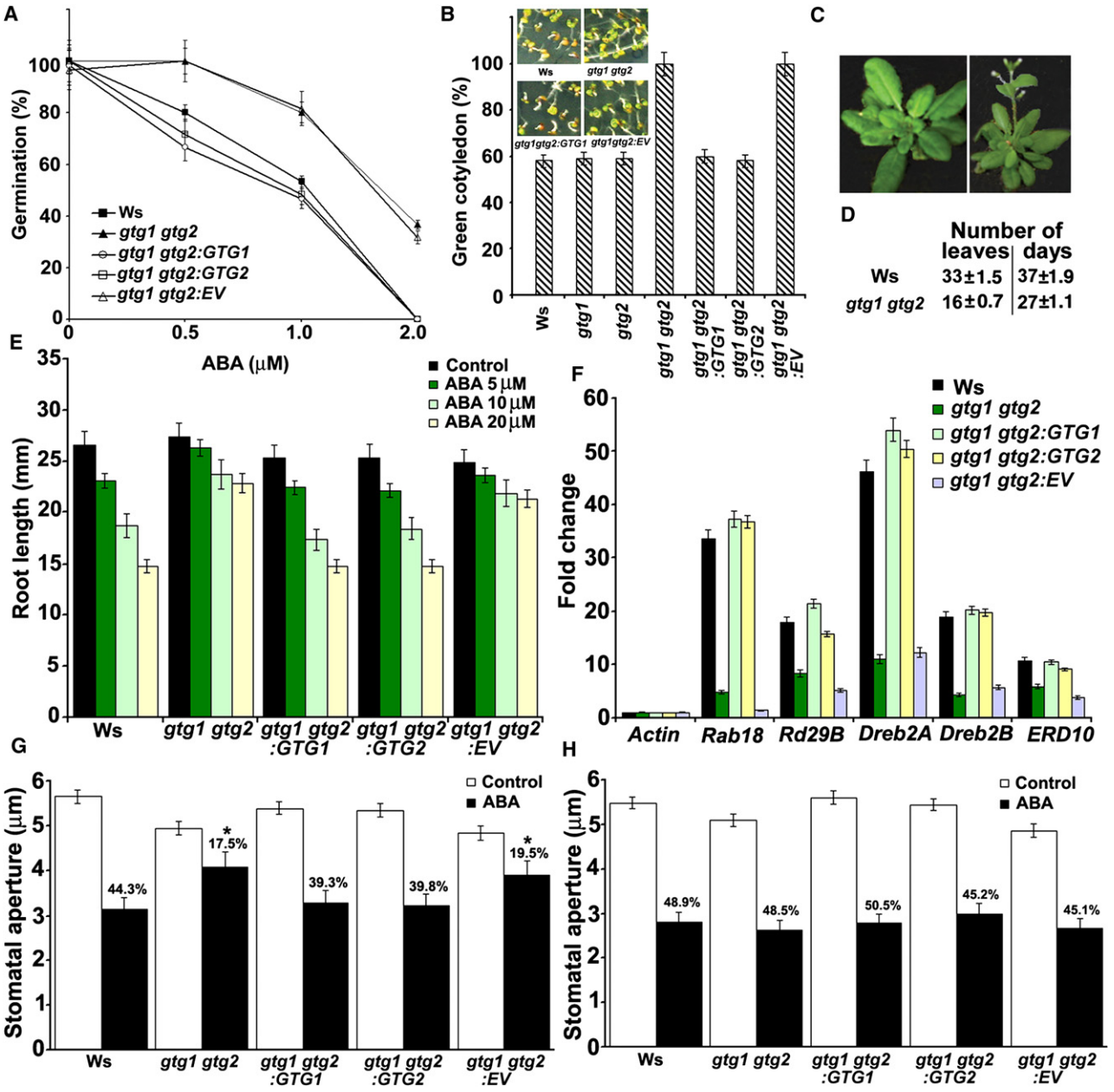

Figure 5. *gtg1 gtg2* Mutants Are Hyposensitive to ABA

For (A), (B), and (E–H), for clarity, data from only the *gtg1 gtg2* mutant and only one complemented line of each of GTG1 and GTG2 (*gtg1 gtg2*:GTG1-FLAG #4c and *gtg1 gtg2*:GTG2-FLAG #4c) and one empty vector line are presented. See Figures S8–S12 for additional data.

(A) Germination (% germination at 48 hr after transfer of seeds to light at 22°C) of wild-type, *gtg1 gtg2* mutants, and *gtg1 gtg2* mutants complemented with *GTG1* or *GTG2* or empty vector in the presence of different ABA concentrations. The experiment was repeated three times and data were averaged; values are means ± SEM, n = 100/experiment/genotype.

(B) ABA effect on post-germination growth. Data are percentage of seedlings with fully expanded green cotyledons after growth on MS media ± 1 μM ABA for 12 days. The experiment was repeated thrice. Data from one representative experiment; means ± SEM n = 60/genotype. Similar results were obtained with multiple lines (not shown). Insets illustrate the ABA-hyposensitive phenotype of the *gtg1 gtg2* mutant.

(C) The *gtg1 gtg2* mutant (right) is smaller, has reduced apical dominance, and flowers earlier than wild-type (left).

(D) Flowering time under long day. n = 20 plants per genotype: means ± SEM.

(E) ABA inhibition of primary root growth. Seedlings were germinated and grown on MS media for 3 days followed by transfer to ABA-containing media and measurement 10 days thereafter. The experiment was repeated thrice; data are means ± SEM, n = 72/genotype.

(F) Expression of ABA-responsive genes by real-time Q-PCR. *Act 2/8* expression was used for normalization. Values are means ± SEM, n = 3 independent experiments.

presence of GDP; thus presumably GDP-bound) did not affect ABA binding (Figure 7D). GPA1 alone showed no ABA binding (Figure 7D). Additionally, the presence of EDTA, which inhibits GTPase activity of the GTG proteins (Figures 2D and 2F), also abolished ABA binding (Figure 7D), consistent with the observation that GTP-GTG proteins bind ABA more weakly than GDP-GTG proteins (Figure 7C). Heat-denatured GTG proteins do not show ABA binding (Figure 7D). These results thus substantiate the specific ABA binding by GTG proteins. The observed stoichiometry of binding is low in these experiments (~0.01 mol ABA/mol protein) probably due to nonoptimal conditions of protein purification, solubilization, and renaturation. Since the expression and purification of GPCRs (and proteins with multitransmembrane domains in general) in heterologous systems remains one of the biggest challenges in the field (Lundstrom et al., 2006; Mancia and Hendrickson, 2007), further experiments are needed to find the optimal system for expression and purification of active GTG proteins. Optimized conditions, especially with respect to the lipid environment, detergent concentration plus other renaturation conditions, and posttranslational modifications will help in more accurate determination of the stoichiometry.

DISCUSSION

In contrast to the extensive G protein repertoires of vertebrates, the few G protein components in plants seem to have evolved as central nodes, integrating multiple signals and essentially ''modulating'' the response. Such a role for plant G proteins is consistent with the observation that *Arabidopsis* plants harboring mutations in Gα, Gβ, and Gγ subunits exhibit altered degrees of hormone sensitivity (hyposensitivity or hypersensitivity) but do not exhibit a complete lack of hormone sensitivity (Ullah et al., 2003; Pandey et al., 2006). The limited number of canonical G proteins in plants is also possibly compensated for by the presence of some unique proteins, e.g., the XLG proteins that have a C-terminal region homologous to GPA1 but a unique, plant-specific N-terminal half (Ding et al., 2008) and the AtRGS1 protein that has a C-terminal RGS domain and an N-terminal GPCR-like domain (Chen et al., 2003). The newly discovered GTG proteins with GPCR-like topology (Figure 1) and GTPase activity provide an additional novel dimension to G protein signaling.

GTGs Are Evolutionarily Conserved, Novel GPCR-Type G Proteins

To our knowledge, this is the first report of a GPCR-like G protein. The GTG proteins exhibit all basic characteristics of signaling G proteins: reversible binding with GTP and GDP but not with ATP or ADP (Figures 2 and S2), GTPase activity, and requirement for Mg^{2+}. A few other proteins that combine GTPase activity with additional unique functional or structural domains are known, e.g., the yeast Fzo1p is a mitochondrial membrane-localized, transmembrane GTPase involved in membrane fusion (Hermann et al., 1998) and the bacterial FeoB is a seven-transmembrane transporter that mediates GTPase-dependent iron uptake in bacteria (Marlovits et al., 2003). What sets the GTGs apart from these other GTPases is the fact that they also function in hormone perception and are coupled to and regulated by a prototypical Gα.

We speculate that the GTG proteins evolved to combine the signal perception and the initial execution steps of GPCR-G protein signaling cascades. The presence of GTG homologs across phyla implies their ancient origin (Figure 1B). Proteins like FeoB and the GTG homologs present in unicellular organisms are possibly primordial GPCRs that have proliferated as receptor-effector systems in higher organisms. Additionally, the lack of detected G protein activity in the human GPR89, at least under our conditions (Figure 2D), may suggest functional divergence among GTG homologs. It would be interesting to assess if the G protein activity of these proteins is plant specific or is conserved in other organisms, e.g., in fungi and other lower eukaryotes that also have limited G protein repertoires.

Fluorescence from GFP-tagged GTGs is observed in the region of the plasma membrane and this, together with their predominant membrane-partitioning in western analysis, suggests that the majority of GTG1 and GTG2 protein localizes at or on the plasma membrane (Figures 1D and 1E). Their N termini are predicted to be extracellular, as is also the case for conventional GPCRs, and both the GTP-binding and the GTPase-activating domains of the GTGs are predicted not to be embedded in the transmembrane segments but rather accessible to the cytosol. Since the GTG proteins interact with GPA1 (Figures 6A and 6B), we assessed the functional significance of this interaction. While the GTPase activity of GPA1 is slow (Figure 6C; Johnston et al., 2007a), GTGs have comparatively higher rates of GTP hydrolysis (Figures 6D and 6F). Additionally, GPA1 in its GTP-bound conformation (which has been predicted to predominate in planta owing to its slow rate of GTP hydrolysis; Temple and Jones, 2007; Johnston et al., 2007a) inhibits the GTPase activity of GTG proteins (Figures 6C and 6D). These results have two important implications. First, GTP-GPA1 has unusual ''anti-GAP'' activity against the GTGs, reducing their GTP hydrolysis. Second, GPA1 accelerates rather than retards GTP binding by the GTGs and may actually promote GDP release from GTGs, opposite of conventional GDI proteins (Figures 6C and 6D versus Figures 2C–2F). Thus, GTP-GPA1 maintains the GTGs in their GTP-bound form, essentially regulating their activity. It is possible that GPA1 and GTG proteins compete for the same cellular GTP pool, and GPA1 has evolved to regulate the faster GTP turnover by GTGs.

GTG Proteins Are ABA Receptors

The *GTG* genes are widely expressed (Figures 3A–3C) and *gtg1 gtg2* mutant plants are hyposensitive to ABA for classic ABA responses (Figures 5 and S8–S11). This, combined with their GPCR-like topology and specific ABA binding at physiologically

(G) *gtg1 gtg2* mutants are hyposensitive to ABA-induced promotion of stomatal closure. Values are means ± SEM, n = 3 independent experiments. For each experiment, n = 300 stomata/genotype/treatment.
(H) *gtg1 gtg2* mutants show normal ABA inhibition of stomatal opening. Sample sizes and analysis as in (G). Numbers = percent reduction in stomatal aperture by ABA compared to solvent control.

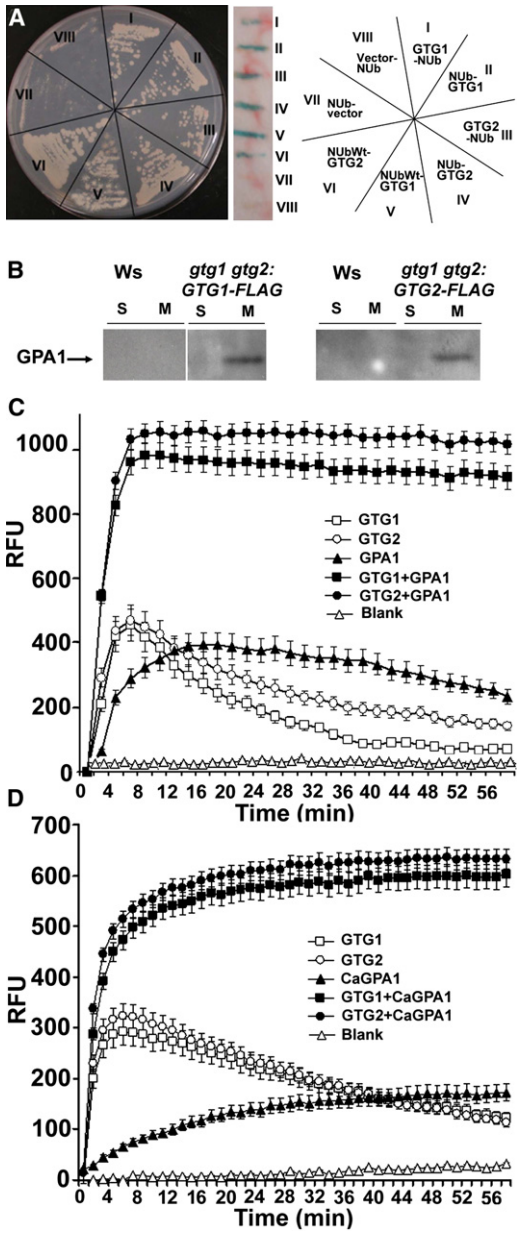

Figure 6. GTG1 and GTG2 Interact with GPA1, and GTP-GPA1 Inhibits GTG GTPase Activity

(A) Left panel: yeast growth on selective media with 1 mM methionine. Middle panel: X-Gal filter assay. Right panel: the proteins used for interaction assays; in all cases the second protein was GPA1-CUb. NUb_{Wt} fusion constructs were a positive control for interaction and NUb-vector fusions were a negative control. Yeast growth and positive X-Gal assay both indicate interaction of GTG1 (or GTG2) and GPA1.

(B) Coimmunoprecipitation of GPA1 and the GTGs. Soluble (S) and total microsomal (M) proteins were isolated from Ws or *gtg1 gtg2* mutants complemented with either GTG1:FLAG or GTG2:FLAG, precipitated with FLAG epitope antibodies, and probed with GPA1 antibodies.

(C) GTP hydrolysis rate, indicated by the rate of fluorescence decrease, is drastically slower when GPA1 is present with GTG1 or GTG2, compared to either GTG protein individually. Each experiment was repeated three times with three replicates/assay/experiment. Data are means ± SD from one representative experiment.

(D) GTG1 and GTG2 lack GTPase activity in the presence of CaGPA1, which has no GTPase activity of its own. Sample sizes and analysis as in (C).

relevant concentrations (Figures 7A and 7B), leads us to propose that the GTG proteins are ABA receptors.

Receptors for the major plant hormones have been identified recently. Some have expected receptor-like structures although with unique properties, e.g., receptors for ethylene and cytokinins are two-component histidine kinase sensors and brassinosteroid receptors are LRR-RLKs (Chow and McCourt, 2006). Receptors for auxin (Dharmasiri et al., 2005; Kepinski and Leyser, 2005) and gibberellins (Ueguchi-Tanaka et al., 2005) are unconventional, comprised of components of ubiquitin proteasome pathways. The perception system for ABA seems to be more diverse. Three ABA receptors have been proposed in plants to date: the "soluble" proteins FCA (Razem et al., 2006) and CHLH (Shen et al., 2006), and GCR2 (Liu et al., 2007). GCR2 had been proposed as a G protein-coupled, plasma membrane-localized ABA receptor (Liu et al., 2007), but characterization of GCR2 as a GPCR and ABA receptor is debatable (Gao et al., 2007; Johnston et al., 2007b; Guo et al., 2008). GCR2 lacks classic GPCR-like transmembrane topology (Illingworth et al., 2008; Table S3) and is predicted to be a homolog of bacterial LanC protein (pfam 05147, E value $3e^{-84}$, NCBI conserved domain search). Additionally, the ABA hyposensitive phenotypes of *gcr2* mutants are mild to absent (Gao et al., 2007; Guo et al., 2008).

FCA and CHLH were initially identified biochemically as ABA-binding proteins in other plant species followed by identification of their homologs in *Arabidopsis* (Razem et al., 2004; Zhang et al., 2002). Although FCA is apparently involved in ABA-regulated transition to flowering, classic ABA-mediated responses are normal in *fca* mutant plants (Razem et al., 2006), indicating that FCA is not the major plant ABA receptor (see also Jang et al., 2008). CHLH is a subunit of Mg^{2+} chelatase, has a role in chlorophyll biosynthesis (Nott et al., 2006), and is apparently a dual-function protein with distinct roles both in nuclear-chloroplast signaling and ABA perception. Plants with reduced CHLH levels show hyposensitivity in classic ABA responses whereas CHLH overexpressor plants are ABA hypersensitive (Shen et al., 2006). However, neither of these soluble proteins can account for membrane-localized ABA perception. Additionally there are no data on how ABA binding to FCA or CHLH is processed or modulated.

Our data show that the novel GTG proteins fulfill the following criteria for a classic ABA receptor involved in G protein signaling: (1) receptor-like topology and membrane localization; (2) interaction with GPA1; (3) highly specific ABA binding; (4) existence in two different conformations, GTP-bound and GDP-bound, which provides a likely structural mechanism for regulation of ABA signal propagation; (4) dependence of the efficiency of ABA binding on their conformation; (5) ABA hyposensitive phenotypes of mutants lacking GTGs; (6) no effect of ABA on expression of *GTG* transcripts, indicating that the role of GTG proteins in ABA signaling is posttranslational.

GTGs, like CHLH or the transglutaminase G proteins (Mhaouty-Kodja, 2004), could be dual-function proteins with separate functions in ABA versus G protein signaling. However,

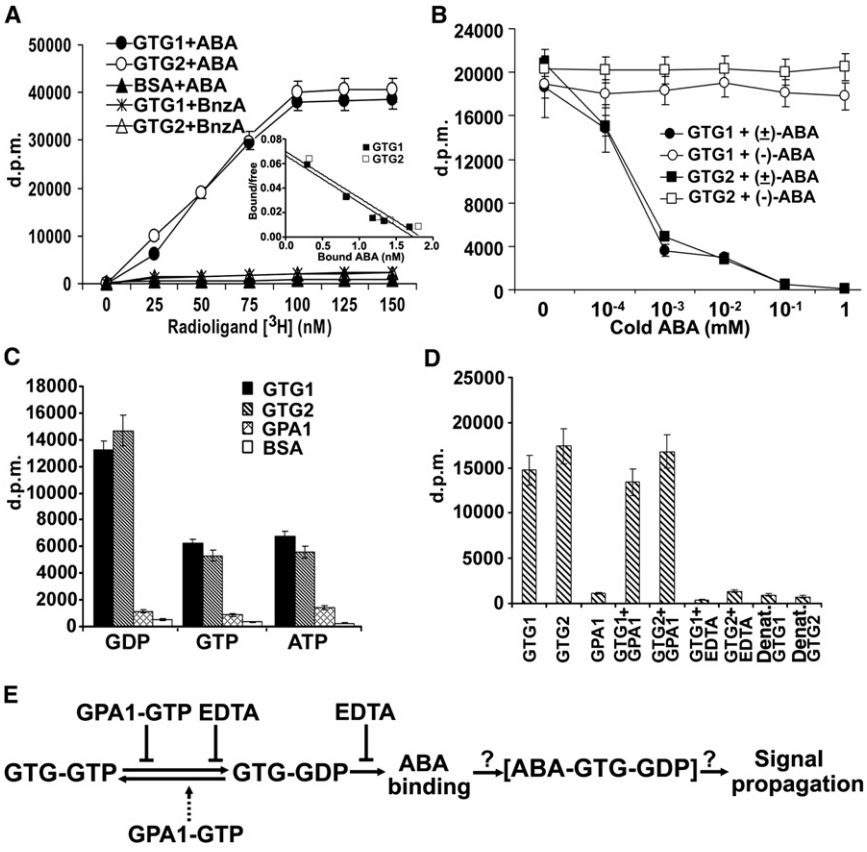

Figure 7. Specific ABA Binding by GTG1 and GTG2

Each experiment was repeated five times and data were averaged. Values are means ± SEM. Except as described in (C), the reaction mix included 5 μM GDP.

(A) Both GTG1 and GTG2 show saturable ABA binding with increasing concentrations of ^{3}H-ABA. No binding was detected when BSA instead of GTG1 or GTG2 was used or by using ^{3}H-benzoic acid (BnzA) instead of ^{3}H-ABA. Inset shows Scatchard plot analysis. Parameters derived for GTG1 are K_D = 35.8 ± 2.6 nM, with R^2 = 0.98 and for GTG2 are K_D = 41.2 ± 3.1, with R^2 = 0.97.

(B) ABA binding by GTG1 and GTG2 is competed by (±)-ABA but not by the biologically inactive stereoisomer, −(-)ABA. One hundred nanomolar ^{3}H-ABA was used, with increasing concentrations of cold ABA as indicated.

(C) GTG1 and GTG2 bind ABA more efficiently in the presence of GDP (5 μM) as compared with GTP (5 μM) or ATP (5 μM), although some ^{3}H-ABA binding is still observed in the presence of GTP, ATP, or ADP (not shown) or in the absence of any added nucleotide (not shown).

(D) GPA1 neither binds ABA alone nor affects the efficiency of binding of GTG1 or GTG2 when added together in the reaction mix in the presence of 5 μM GDP. EDTA (2 mM) essentially abolishes ABA binding by GTG1 or GTG2. Heat-denatured GTG proteins do not bind ABA.

(E) Proposed model for mechanism of action of GPA1 and GTG proteins. Question marks indicate aspects of the model inferred from, but not directly demonstrated by, experimental results to date.

we prefer a more parsimonious hypothesis. We propose that the GTGs are ABA receptors and that GPA1 acts as a ''rheostat'' on GTG, such that GTP-bound GPA1 downregulates ABA binding, and thus ABA-signal propagation, by the GTGs (Figure 7E). Conversely, GDP-bound GTGs bind ABA, and this initiates the ABA signaling cascade (note that this is opposite from conventional Gα proteins, where GDP-bound Gα ''turns off'' the system). This scenario is also consistent with results from BODIPY-GTP, [^{35}S]-GTPγS binding, and TLC assays of GTPase activity showing no effect of ABA on GTP binding or GTP hydrolysis of the GTGs (Figures S6). Thus, the *gtg1 gtg2* mutants, lacking the GTG ABA receptors, exhibit ABA hyposensitivity. GPA1-GTP (1) may slightly promote GTP binding by the GTGs (as shown by the faster rate of GTP binding by GTG+GPA1 as compared to either GTGs or GPA1 alone; Figure 6C) and (2) inhibits GTPase activity of the GTGs (Figures 6C and 6D). Both of these effects shift the GTGs toward their GTP-bound form, which exhibits weaker ABA binding and is hypothesized to be the GTG conformation that does not propagate the ABA signal. According to this model, *gpa1* mutants should exhibit ABA hypersensitivity because in the absence of GPA1, relatively more GTG will be in the GDP-bound form. In fact, *gpa1* mutants are indeed hypersensitive to ABA in seed germination, root growth, and gene regulation (Pandey et al., 2006) (stomatal regulation is discussed below). Although we did not see effects of GPA1 on the magnitude of ABA binding by GTGs (Figure 7D), these experiments were performed in the presence of GDP to facilitate efficient ABA binding, conditions under which the regulatory form of GPA1 (GTP-GPA1) would not be present and the presence of GDP would already favor the GDP-bound conformation of the GTGs. Note that because we saw no effect of GPA1 on ABA binding by GTGs under this condition, in Figure 7E we do not show any action of GDP-GPA1 on the GTG G protein cycle.

Our model (Figure 7E) is also consistent with the effects of EDTA. EDTA inhibits the GTPase activity of the GTGs (Figures 2D and 2F) and thus promotes the GTP-bound form of the GTGs, which is the conformation that binds ABA less strongly (Figure 7C). The discussion above thus consolidates all our results obtained with the GTG proteins to date. Due to nonoptimized stoichiometry of ABA binding by GTG proteins, and the fact that there is some ABA binding by the GTG proteins in the presence of GTP, we do not fully rule out the possibility of a classic G protein mechanism of signal transduction (GTP-bound form being more active, with ABA affecting GTPase activity). However, our ''rheostat'' model for GPA1 is consistent with the idea presented at the beginning of this Discussion that the plant Gα acts as a modulator of hormonal responses. We propose that the dynamics of the GTP-/GDP-bound states of GPA1 and GTGs regulate ABA signaling in wild-type plants.

All of the results discussed above for the GTGs as well as current and previously published results on GPA1 and *gpa1* mutant phenotypes are internally consistent. The one interesting

exception concerns stomatal regulation. *gpa1* stomata are hyposensitive to ABA in ABA inhibition of stomatal opening and ion channel regulation and exhibit wild-type ABA promotion of stomatal closure (Fairley-Grenot and Assmann, 1991; Wang et al., 2001; Coursol et al., 2003), while *gtg1 gtg2* mutants are the opposite: they are wild-type for ABA inhibition of stomatal opening and hyposensitive for ABA promotion of stomatal closure (Figures 5G, 5H, S11, and S12). These results are consistent with previous data showing that ABA signaling components from mesophyll cells cannot substitute for guard cell elements in ABA regulation of guard cell K^+ channels involved in stomatal opening (Sutton et al., 2000). Together these findings predict that guard cell-specific aspects of G protein-regulated ABA signaling exist and remain to be elucidated.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

All mutants are in the *Arabidopsis* Ws ecotype. Seed germination and plant growth conditions were according to Pandey et al. (2006) unless stated otherwise. The *gtg1* and *gtg2* insertion mutants were isolated by screening the Basta population of T-DNA insertion lines (Sussman et al., 2001). For complementation of double *gtg1 gtg2* mutants, full-length *GTG1* and *GTG2* cDNAs were cloned into pGW11 and pGW12 binary vectors to generate C- and N-terminal FLAG-tagged fusions, respectively. The 1.32 kb intergenic genomic sequence and a 3.12 kb genomic sequence, for *GTG1* and *GTG2* respectively, located upstream of the translational start site were identified as promoter regions in this study and were cloned into pGW3 vectors to produce GTG promoter::GUS fusion constructs. Transgenic plants were generated via Agrobacterium-mediated transformation and selected on 0.5 × MS media containing kanamycin/hygromycin. GUS staining was performed according to Tsugeki and Fedoroff (1999).

Cloning and Purification of GTG1 and GTG2

Full-length *GTG1*, *GTG2*, and *GPA1* were amplified from *Arabidopsis* seedling cDNA and cloned into the pENTR11 vector (Invitrogen, CA, USA). The clones were transferred into the pDEST17 destination vector containing the His-epitope tag (Invitrogen) by LR recombination reaction as per the manufacturer's instructions. The constructs were transformed into BL21-AI cells (Invitrogen). Protein expression was induced with 0.2% arabinose for 3 hr at 37°C. GPA1 and CaGPA1 were purified using the B-PER 6×His fusion protein purification kit (Pierce, IL, USA) following the manufacturer's instructions. GTG1, GTG2, and GPR89 proteins were purified using the same system with the following modifications. The cells were resuspended in 10 ml of B-PER reagent containing 1% Tween 20, 0.25% NP-40, and EDTA-free complete protease inhibitors (Roche, IN, USA). Cleared supernatant obtained after centrifugation was loaded on a Ni-NTA column and the column was washed with wash buffer 1 (50 mM Tris, 200 mM NaCl, 15 mM imidazol, 10% glycerol, 0.25% Tween 20, pH 7.5) five times with three column volumes each and with wash buffer 2 (50 mM Tris-HCl, 300 mM NaCl, 30 mM imidazol, 12% glycerol, 0.25% Tween 20, pH 7.0) three times with three column volumes each. The proteins were eluted with 5 ml of elution buffer (50 mM Tris-HCl, 300 mM NaCl, 300 mM imidazol, 12% glycerol, 100 ng/ml unsaturated phosphatidyl choline [UPC, Avanti Polar Lipids] 0.1% Tween 20 and 1 × EDTA-free complete protease inhibitor) in 1 ml fractions. The fractions were further loaded on the Extracti-Gel D detergent removing gel column (Pierce) and the proteins were purified according to manufacturer's instructions (see Supplemental Experimental Procedures).

GTP Binding and Hydrolysis Assays

GTP binding and hydrolysis were performed using BODIPY-GTPγS or BODIPY-GTP FL (Invitrogen). Assays were performed at 25°C in 200 μl reaction mix in BODIPY assay buffer (Tris, 20 mM, pH 8.0, and $MgCl_2$, 10 mM) according to Willard et al. (2005). Additional components (EDTA 2 mM, GTP 5 μM, GDP 5 μM, or ADP 5 μM) were added before starting the reaction by addition of BODIPY-GTPγS or BODIPY-GTP FL. Fluorescence was recorded every 30 s for up to 60 min using a fluorescence microplate reader (FLx800; Biotek Instruments, Inc., Vermont, USA) at λ_{ex}485, λ_{em}530 nm. Assays of [^{35}S]-GTPγS binding and TLC measurements of GTPase activity were performed after Seo et al. (1997). Identical buffers were used for GTG proteins and GPA1 (see Supplemental Experimental Procedures).

Expression Analysis by RT-PCR and Western Blotting

RNA isolation, cDNA synthesis, RT-PCR, and real-time quantitative PCR (Q-PCR) were as in Pandey and Assmann (2004). For gene expression analysis under abiotic stress treatments, RNA was isolated from 7-day-old stress-treated seedlings. To confirm the absence of full-length *GTG1* and *GTG2* transcripts in *gtg1-1*, *gtg2-1* single, and *gtg1 gtg2* mutants, cDNA was synthesized from RNA isolated from 2-week-old seedlings. RT-PCR or Q-PCR was performed using specific primers (cf. Figure 4A; Table S2). To confirm expression of GTG-FLAG or FLAG-GTG fusion proteins in the complemented *gtg1 gtg2* mutants, western blotting with total microsomal protein fractions was performed with anti-FLAG antibodies (Pandey and Assmann, 2004).

Split Ubiquitin Interaction Assays and In Vivo Coimmunoprecipitation

Interaction assays with GPA1, GTG1, and GTG2 were performed using the split ubiquitin assay (Pandey and Assmann, 2004). Briefly, full-length *GTG1*, *GTG2*, and *GPA1* were cloned in CUb, NUbG, and NUb_{WT} vectors by mating-based in vivo recombination cloning. Interaction was determined by growth on minimal media lacking Leu, Trp, His, and Ade but containing 1 mM methionine and also by X-gal filter assay (Pandey and Assmann, 2004). The Catch and Release system (Upstate, VA, USA) was used for in planta immunoprecipitation assays. Twelve-day-old seedlings of Ws, *gtg1 gtg2*, *gtg1 gtg2:GTG1-FLAG*, and *gtg1 gtg2:GTG2-FLAG* were used as the source of proteins. Five hundred micrograms of total microsomal proteins, 3 μg FLAG antibodies, and 10 μl of affinity ligand were added to the column and incubated at 4°C overnight. The washing and elution of the bound proteins were done according to the manufacturer's instructions. Eluted proteins were run on SDS-PAGE, immunoblotted, and probed with GPA1 antibodies (see Supplemental Experimental Procedures for details).

ABA Binding Assays

Purified GTG1 or GTG2 (0.4 μg) or GPA1 or BSA (EMD Biosciences, CA, USA) proteins (2 μg each) were resuspended in an ABA-binding buffer (Tris 50 mM, NaCl 50 mM, $MgCl_2$ 10 mM, glycerol 5%, BSA 0.025%, unsaturated phosphatidyl choline 100 ng/ml, Tween-20 0.1%, and complete protease inhibitor [Roche Applied Sciences, IN, USA] 1×) in a 50 μl reaction mix. ^{3}H-labeled ABA (Amersham, UK) was added to start the reaction. This ABA was provided from Amersham as (±)-ABA with a specific activity of 46.0 Ci/mmol or 200 μCi/ml, which corresponds to a stock concentration of ~4 μM. The final concentration used was 100 nM unless otherwise noted. Tubes were incubated on ice for 1 hr. The reaction mixture was then filtered through GF/C membranes (Whatman Inc. NJ, USA), filters were washed with 5 ml of binding buffer each, and bound radioactivity was measured using a liquid scintillation counter in Optiphase HiSafe 3 (Perkin Elmer, MA, USA). Binding constants were calculated using GraphPad Prism (GraphPad Software Inc.). For competition studies, binding assays were performed in the presence of different amounts of cold (±)-ABA (AG Scientific, CA, USA) or (−)-ABA (Sigma Chemical Co., MO, USA). EDTA, GDP, GTP, or ATP were added to the reaction mix to study the effects of these compounds on ABA binding. Binding assays with ^{3}H-labeled benzoic acid or BSA were performed under identical conditions.

Phenotypic Analyses

Seed germination (3-month-old seeds; comparable results were obtained with other seed ages [not shown]) and root growth assays were performed as in Pandey et al. (2006; see also Supplemental Experimental Procedures). The effect of ABA on post-germination growth of seedlings was recorded as in Chen and Jones (2004). For ABA-induced gene expression analysis, 7-day-old Ws and *gtg1 gtg2* mutants were treated with 50 μM ABA for 1 hr. Stomatal aperture assays were as in Pandey and Assmann (2004) except that peeled epidermes

were imaged (400× total magnification) using a digital camera (Nikon Coolpix 990). Apertures were measured (at least 30 images per sample and 10 apertures per image) using Image J (NIH). The experiments were performed blind. For flowering time estimation, wild-type and mutant plants were grown side by side in 16 hr light/8 hr darkness (light intensity $120 \cdot \mu mol \cdot m^{-2} s^{-1}$).

Localization of GTG1 and GTG2

GTG1 and *GTG2* were cloned into a modified pEarleyGate 102 vector. *GTG1*, *GTG2*, or vector DNA was isolated using an Endo free plasmid mini kit (QIAGEN, CA) and was transfected to *Arabidopsis* mesophyll cell protoplasts using a modified poly ethylene glycol method (Sheen, 2001). Transfected protoplasts were incubated at 22°C for 18–24 hr in darkness followed by localization using an Olympus FV1000 Laser Scanning Confocal Microscope (see Supplemental Experimental Procedures for details).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, fourteen figures, and four tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01632-2.

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REFERENCES

Assmann, S.M. (1994). Ins and outs of guard cell ABA receptors. Plant Cell *6*, 1187–1190.

Assmann, S.M. (2004). Plant G proteins, phytohormones, and plasticity: three questions and a speculation. Sci. STKE *264*, re20.

Cabrera-Vera, T.M., Vanhauwe, J., Thomas, T.O., Medkova, M., Preininger, A., Mazzoni, M.R., and Hamm, H.E. (2003). Insights into G protein structure, function, and regulation. Endocr. Rev. *24*, 765–781.

Chen, J.G., and Jones, A.M. (2004). AtRGS1 function in *Arabidopsis thaliana*. Methods Enzymol. *389*, 338–350.

Chen, J.G., Willard, F.S., Huang, J., Liang, J., Chasse, S.A., Jones, A.M., and Siderovski, D.P. (2003). A seven-transmembrane RGS protein that modulates plant cell proliferation. Science *301*, 1728–1731.

Chow, B., and McCourt, P. (2006). Plant hormone receptors: perception is everything. Genes Dev. *20*, 1998–2008.

Coursol, S., Fan, L., Le Stunff, H., Spiegel, S., Gilroy, S., and Assmann, S.M. (2003). Sphingolipid signalling in *Arabidopsis* guard cells involves heterotrimeric G proteins. Nature *423*, 651–654.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature *435*, 441–445.

Ding, L., Pandey, S., and Assmann, S.M. (2008). Arabidopsis extra-large G proteins (XLGs) regulate root morphogenesis. Plant J. *53*, 248–263.

Fairley-Grenot, K., and Assmann, S.M. (1991). Evidence for G-protein regulation of inward K^+ channel current in guard cells of fava bean. Plant Cell *3*, 1037–1044.

Gao, Y., Zeng, Q., Guo, J., Cheng, J., Ellis, B.E., and Chen, J.G. (2007). Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination and early seedling development in Arabidopsis. Plant J. *52*, 1001–1013.

Gookin, T.E., Kim, J., and Assmann, S.M. (2008). Whole proteome identification of plant candidate G-protein coupled receptors in Arabidopsis, rice, and poplar: computational prediction and in-vivo protein coupling. Genome Biol. *9*, R120.

Guo, J., Zeng, Q., Emami, M., Ellis, B.E., and Chen, J.G. (2008). The GCR2 gene family is not required for ABA control of seed germination and early seedling development in Arabidopsis. PLoS ONE. *3*, e2982. 10.1371/journal.pone.0002982.

Hermann, G.J., Thatcher, J.W., Mills, J.P., Hales, K.G., Fuller, M.T., Nunnari, J., and Shaw, J.M. (1998). Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J. Cell Biol. *143*, 359–373.

Illingworth, C.J., Parkes, K.E., Snell, C.R., Mullineaux, P.M., and Reynolds, C.A. (2008). Criteria for confirming sequence periodicity identified by Fourier transform analysis: Application to GCR2, a candidate plant GPCR? Biophys. Chem. *133*, 28–35.

Jang, Y.H., Lee, J.H., and Kim, J.K. (2008). Abscisic acid does not disrupt either the Arabidopsis FCA-FY interaction or its rice counterpart in vitro. Plant Cell Physiol. *49*, 1898–1901.

Johnston, C.A., Taylor, J.P., Gao, Y., Kimple, A.J., Grigston, J.C., Chen, J.G., Siderovski, D.P., Jones, A.M., and Willard, F.S. (2007a). GTPase acceleration as the rate-limiting step in Arabidopsis G protein-coupled sugar signaling. Proc. Natl. Acad. Sci. USA *104*, 17317–17322.

Johnston, C.A., Temple, B.R., Chen, J.G., Gao, Y., Moriyama, E.N., Jones, A.M., Siderovski, D.P., and Willard, F.S. (2007b). Comment on "A G protein coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid." Science *318*, 914.

Jones, A.M., and Assmann, S.M. (2004). Plants: the latest model system for G-protein research. EMBO Rep. *5*, 572–578.

Kepinski, S., and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature *435*, 446–451.

Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W.H., and Ma, L. (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science *315*, 1712–1716.

Lundstrom, K., Wagner, R., Reinhart, C., Desmyter, A., Cherouati, N., Magnin, T., Zeder-Lutz, G., Courtot, M., Prural, C., André, N., et al. (2006). Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. J. Struct. Funct. Genomics *7*, 77–91.

Mancia, F., and Hendrickson, W.A. (2007). Expression of recombinant G-protein coupled receptors for structural biology. Mol. Biosyst. *3*, 723–734.

Marlovits, T.C., Haase, W., Herrmann, C., Aller, S.G., and Unger, V.M. (2003). The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. Proc. Natl. Acad. Sci. USA *99*, 16243–16248.

Mhaouty-Kodja, S. (2004). Ghα/tissue transglutaminase 2: an emerging G protein in signal transduction. Biol. Cell *96*, 363–367.

Nott, A., Jung, H.S., Koussevitzky, S., and Chory, J. (2006). Plastid-to-nucleus retrograde signaling. Annu. Rev. Plant Biol. *57*, 739–759.

Offermanns, S. (2003). G-proteins as transducers in transmembrane signalling. Prog. Biophys. Mol. Biol. *83*, 101–130.

Pandey, S., and Assmann, S.M. (2004). The Arabidopsis putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. Plant Cell *16*, 1616–1632.

Pandey, S., Chen, J.G., Jones, A.M., and Assmann, S.M. (2006). G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. Plant Physiol. *141*, 243–256.

Razem, F.A., Luo, M., Liu, J.H., Abrams, S.R., and Hill, R.D. (2004). Purification and characterization of a barley aleurone abscisic acid-binding protein. J. Biol. Chem. *279*, 9922–9929.

Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D. (2006). The RNA-binding protein FCA is an abscisic acid receptor. Nature *439*, 290–294.

Ross, E.M. (2008). Coordinating speed and amplitude in G-protein signaling. Curr. Biol. *18*, R777–R783.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406–425.

Seo, H.S., Choi, C.H., Lee, S.Y., Cho, M.J., and Bahk, J.D. (1997). Biochemical characteristics of a rice (*Oryza sativa* L., IR36) G-protein α-subunit expressed in *Escherichia coli*. Biochem. J. *324*, 273–281.

Sheen, J. (2001). Signal transduction in maize and Arabidopsis mesophyll protoplasts. Plant Physiol. *127*, 1466–1475.

Shen, Y.Y., Wang, X.F., Wu, F.Q., Du, S.Y., Cao, Z., Shang, Y., Wang, X.L., Peng, C.C., Yu, X.C., Zhu, S.Y., et al. (2006). The Mg-chelatase H subunit is an abscisic acid receptor. Nature *443*, 823–836.

Sussman, M.R., Amasino, R.M., Young, J.C., Krysan, P.J., and Austin-Phillips, S. (2001). The *Arabidopsis* knockout facility at the University of Wisconsin-Madison. Plant Physiol. *124*, 1465–1467.

Sutton, F., Paul, S.S., Wang, X.Q., and Assmann, S.M. (2000). Distinct abscisic acid signaling pathways for modulation of guard cell versus mesophyll cell potassium channels revealed by expression studies in *Xenopus laevis* oocytes. Plant Physiol. *124*, 223–230.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. *24*, 1596–1599.

Temple, B.R., and Jones, A.M. (2007). The plant heterotrimeric G-protein complex. Annu. Rev. Plant Biol. *58*, 249–266.

Tsugeki, R., and Fedoroff, N.V. (1999). Genetic ablation of root cap cells in Arabidopsis. Proc. Natl. Acad. Sci. USA *96*, 12941–12946.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.Y., Hsing, Y.I., Kitano, H., Yamaguchi, I., and Matsuoka, M. (2005). GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. Nature *437*, 693–698.

Ullah, H., Chen, J.G., Temple, B., Boyes, D.C., Alonso, J.M., Davis, K.R., Ecker, J.R., and Jones, A.M. (2003). The β-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell *15*, 393–409.

Wang, X.Q., Ullah, H., Jones, A.M., and Assmann, S.M. (2001). G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science *292*, 2070–2072.

Willard, F.S., Kimple, A.J., Johnston, C.A., and Siderovski, D.P. (2005). A direct fluorescence-based assay for RGS domain GTPase accelerating activity. Anal. Biochem. *340*, 341–351.

Zhang, D.P., Wu, Z.Y., Li, X.Y., and Zhao, Z.X. (2002). Purification and identification of a 42-kilodalton abscisic acid-specific-binding protein from epidermis of broad bean leaves. Plant Physiol. *128*, 714–725.

Note Added in Proof

The designation of FCA as an ABA receptor has been retracted. See Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D. (2008). Retraction. The RNA-binding protein FCA is an abscisic acid receptor, Nature *456*, 824 and also see Risk, J.M., Macknight, R.C., and Day, C.L. (2008). FCA does not bind abscisic acid. Nature *456*, E5–E6.

Consistent with our lack of detection of GTP binding and GTPase activity for HsGPR89, GPR89 was recently identified to have anion channel function. See Maeda, Y., Ide, T., Koike, M., Uchiyama, Y., and Kinoshita, T. (2008). GPHR is a novel anion channel critical for acidification and functions of the Golgi apparatus. Nat. Cell Biol. *10*, 1135–1145.