Recently duplicated plant heterotrimeric Gα proteins with subtle biochemical differences influence specific outcomes of signal-response coupling

Received for publication, April 25, 2017, and in revised form, August 10, 2017 Published, Papers in Press, August 21, 2017, DOI 10.1074/jbc.M117.793380

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Heterotrimeric G-proteins, comprising Gα, Gβ, and Gγ subunits, regulate key signaling processes in eukaryotes. The Gα subunit determines the status of signaling by switching between inactive GDP-bound and active GTP-bound forms. Unlike animal systems, in which multiple Gα proteins with variable biochemical properties exist, plants have fewer, highly similar Gα subunits that have resulted from recent genome duplications. These proteins exhibit subtle differences in their GTP-binding, GDP/GTP-exchange, and GTP-hydrolysis activities, but the extent to which these differences contribute to affect plant signaling and development remains unknown. To evaluate this, we expressed native and engineered Gα proteins from soybean in an *Arabidopsis* Gα-null background and studied their effects on modulating a range of developmental and hormonal signaling phenotypes. Our results indicated that inherent biochemical differences in these highly similar Gα proteins are biologically relevant, and some proteins are more flexible than others in influencing the outcomes of specific signals. These observations suggest that alterations in the rate of the G-protein cycle itself may contribute to the specificity of response regulation in plants by affecting the duration of active signaling and/or by the formation of distinct protein-protein complexes. In species such as *Arabidopsis* having a single canonical Gα, this rate could be affected by regulatory proteins in the presence of specific signals, whereas in plants with multiple Gα proteins, an even more complex regulation may exist, which likely contributes to the specificity of signal-response coupling.

Heterotrimeric G-proteins are key regulators of signaling pathways in all eukaryotes. Composed of three dissimilar subunits, Gα, Gβ, and Gγ, the proteins act as molecular switches to link signal perception at the plasma membrane with downstream intracellular effectors. At the mechanistic level, signaling via G-proteins is controlled by the guanine nucleotide-bound state of the Gα subunit, which switches between GDP-bound heterotrimeric (GDP·Gαβγ) and GTP-bound monomeric (GTP·Gα and free Gβγ) forms, representing the inactive and active signaling states, respectively. As per the established metazoan paradigm, activation of G-protein cycle typically requires a G-protein–coupled receptor (GPCR)-mediated[2] exchange of GDP for GTP on the Gα protein of the heterotrimer, which releases the Gβγ dimer from GTP-bound Gα. Deactivation of the cycle occurs because of the inherent GTPase activity of Gα, which hydrolyzes the bound GTP to GDP. GDP·Gα reassociates with the Gβγ dimer, and the heterotrimer becomes available for the next round of activation (1–3). Proteins such as regulator of G-protein signaling (RGS) or phospholipases accelerate the deactivation of G-protein cycle by acting as GTPase activity–accelerating proteins (GAPs). The amplitude and duration of G-protein signaling is exquisitely controlled by the inherent activation/deactivation rates of Gα proteins, as well as by the activity of specific regulators (4–9).

In metazoans, the G-proteins, their regulators, and their effectors are all encoded by multiple genes. For example, the human genome codes for 23 Gα, 5 Gβ, and 12 Gγ proteins. The Gα proteins are further divided in four distinct families: $G\alpha_s$, $G\alpha_i$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$, based on significant differences in their kinetics (rates of GTP binding, hydrolysis, and exchange), as well as their interaction with specific downstream effectors (10, 11). The multiplicity of each of the subunits can result in the formation of a large number of potential heterotrimeric complexes, with varied affinity for distinct GPCRs and/or effectors and provide for the specificity of response regulation in a host of G-protein–based signaling pathways in metazoans (12–15). In contrast, classic GPCRs and well-established effector proteins such as adenyl cyclases are missing from the plant genomes, and although the heterotrimeric G-protein subunits exist in all plants, their repertoire is relatively limited. The genomes of model plants such as *Arabidopsis*, rice, *Brachypodium*, or basal plants such as *Chara* or *Selaginella* encode only one canonical Gα protein each, whereas in plants that possess more Gα proteins, such as soybean (4) or *Camelina* (3), the multiplicity is due to the recent genome duplications or polyploidy, resulting in proteins that are highly similar at the sequence level. Interestingly, despite the paucity in the number

This work was supported by National Institute of Food and Agriculture/Agriculture and Food Research Initiative Grant AFRI-2015-67013-22964 and National Science Foundation Grant IOS-1557942 (to S. P.). The authors declare that they have no conflicts of interest with the contents of this article.

[S] This article contains supplemental Figs. S1–S10.

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[2] The abbreviations used are: GPCR, G-protein–coupled receptor; GAP, GTPase activity–accelerating protein; GEF, guanine nucleotide exchange factor; RGS, regulator of G-protein signaling; ABA, abscisic acid; GA, gibberellic acid; BR, brassinosteroid; EV, empty vector; BL, brassinolide; PAC, paclobutrazol; ANOVA, analysis of variance.

of G-proteins in plant genomes, their involvement has been shown during regulation of numerous aspects of plant growth, development, and signaling. *Arabidopsis* mutants lacking the sole Gα gene exhibit altered response to multiple phytohormones such as abscisic acid (ABA), gibberellic acid (GA), and brassinosteroid (BR), as well as many abiotic and biotic stresses and other environmental changes. In addition, the mutants display variations in several developmental traits such as leaf shape, rosette size, hypocotyl lengths, and root mass, compared with the wild-type plants (16–28). How a single Gα protein regulates a wide variety of responses and how the specificity of response regulation is attained remain important questions in the plant G-protein signaling field. Some of it is certainly due to the involvement of unique components such as extra-large G-proteins (29–31), multiplicity of the extant Gγ proteins (32, 33), or tissue- or cell type-specific expression of G-proteins or due to their interactions with specific downstream effectors. However, our recent work suggests that precisely controlled biochemical regulation of the G-protein cycle itself may also play a critical role to confer specificity in modulating plant growth and development (6, 9), likely by controlling the duration of the availability of the active Gα protein and/or by the subunit-specific protein–protein interactions.

To directly test the hypothesis that variations in the inherent biochemical properties of highly similar Gα proteins can lead to distinct modes of response regulation, we investigated the soybean Gα (GmGα) proteins, because these represent four naturally occurring proteins with subtle differences in their biochemical properties. The proteins are a result of two recent genome duplication events (34, 35) and, despite being more than 90% similar at the sequence level, exhibit differences in the rate of GTP binding (*e.g.* ~4-fold difference in K_{on} for GTP binding and ~5-fold difference in K_{off} for GDP dissociation) and hydrolysis, under *in vitro* conditions (36). Complementation of the yeast Gα mutant, *gpa1*, with different GmGα proteins and their variants has confirmed that the biochemical differences observed *in vitro* are indeed biologically relevant (37). Additionally, our results with the G-protein-dependent regulation of nodule formation in soybean showed that the overexpression of *GmGα2* and *GmGα3* resulted in a significantly stronger repression of nodule development compared with the overexpression of *GmGα1* and *GmGα4* genes (38), suggesting some functional specificity between these proteins. However, the interpretation of these data remains indirect because yeast is a heterologous system, and ectopic overexpression in soybean hairy roots is not likely to determine the effects of inherently different biochemical activities of individual Gα proteins on specific signaling or developmental pathways. A direct evaluation of the effect of individual Gα protein in modulating specific growth and development phenotypes in soybean is currently extremely difficult, if not impossible. Complete knockout mutants or gene-edited lines are not available, and RNAi- or miRNA-based suppression is not subunit-specific or complete. To circumvent these challenges and to directly test the effect of differences in the biochemical activities of individual Gα proteins *in planta*, we made use of the *Arabidopsis* Gα knock-out null mutant *gpa1*. Because *gpa1* mutant exhibits a wide range of developmental and signaling phenotypes, it serves as an ideal testing ground for interrogating the possible role(s) of individual Gα proteins in defining the specificity of response regulation.

By expressing the native and engineered soybean Gα genes (and *Arabidopsis GPA1* and a variant $GPA1^{Q222L}$) with the native *GPA1* promoter in the *gpa1* mutant background, we found clear differences in their ability to complement specific phenotypes. Our data suggest that modulation of the kinetics of G-protein cycle may influence the specificity in G-protein–mediated signaling and developmental responses. In plants with a single canonical Gα, this rate could be affected by regulatory proteins in the presence of specific signals (6, 9); whereas in plants with multiple Gα proteins, an even more complex regulation of G-protein dynamics and a likely subfunctionalization of duplicated genes possibly contribute to the specificity of signal-response coupling.

Results

The developmental phenotypes of gpa1 mutants are complemented by only a subset of GmGα proteins

Based on extensive biochemical characterization, we have grouped the GmGα proteins in group I (GmGα 1 and 4) and group II (GmGα 2 and 3). Group I Gα proteins have relatively faster rates of GDP/GTP exchange and a slower rate of GTP hydrolysis, compared with the group II Gα proteins (34, 36). As expected based on their extremely high sequence similarity with *Arabidopsis* GPA1 (supplemental Fig. S1), each of the soybean Gα proteins interacted with the *Arabidopsis* Gβ protein (supplemental Fig. S2), a prerequisite to assess their *in planta* functionality in *Arabidopsis*. To determine the effect of individual GmGα proteins in the context of their varied biochemical properties, *Arabidopsis gpa1–4* mutants were transformed with native *GPA1* and *GmGα* genes, driven by the native *GPA1* promoter. Multiple T4 homozygous transgenic lines with similar levels of Gα protein expression, as confirmed by immunoblotting with GPA1 antibodies (supplemental Fig. S3), were selected for detailed phenotypic analysis. The data using two independent lines are presented in the manuscript, with the results obtained with the second line presented in the supplemental figures, unless noted otherwise.

The *gpa1* mutant displays clearly quantifiable phenotypes in its leaf shape and in rosette size when grown in short day/night cycle (14 h light/10 h dark). Compared with WT, the leaves of *gpa1* mutants are shorter, rounder, and wider, with a crinkled appearance. At the end of the vegetative growth period, the mutant also has smaller rosettes compared with the WT plants (18, 23). A comparison of leaf shape traits of different *GmGα* complemented *gpa1* plants with WT and mutant *gpa1* was performed by quantifying the length, width, and overall appearance of the 10th leaf of each genotype. Leaves from *gpa1* complemented with native *GPA1* and empty vector (EV) were used as positive and negative controls, respectively.

Visual inspection of each of the genotypes showed a clear difference in the ability of GmGα proteins to complement the mutant leaf phenotype. *gpa1* mutants complemented with native *GPA1* or *GmGα2* or *GmGα3* appeared similar to the WT plants, with elongated leaves, and no crinkled appearance. In

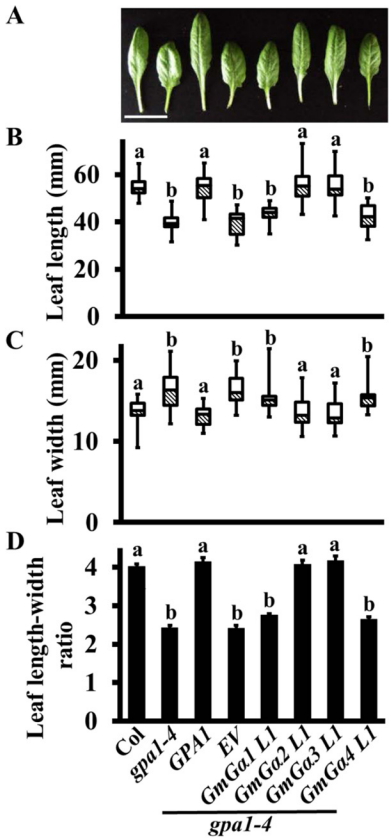

Figure 1. Leaf phenotypes of WT, *gpa1*, and *gpa1* complemented with different *Gαs*. *A*, representative images of the 10th leaf of 4-week-old WT (Col-0), *gpa1*, and *gpa1* mutant plants complemented with native *GPA1* and *GmGαs*. *Bar*, 25 mm. *B–D*, the lengths, widths and length:width ratio of the leaf blade of the 10th leaf were measured from the 4-week-old plants. The values represent the average lengths and widths of leaf blade from at least 24 leaves ± S.D. Statistical significance in *B–D* was determined using one-way ANOVA multiple comparisons. ^{a,b}, different letters indicate a significant difference (Tukey's multiple comparison test, $p < 0.05$).

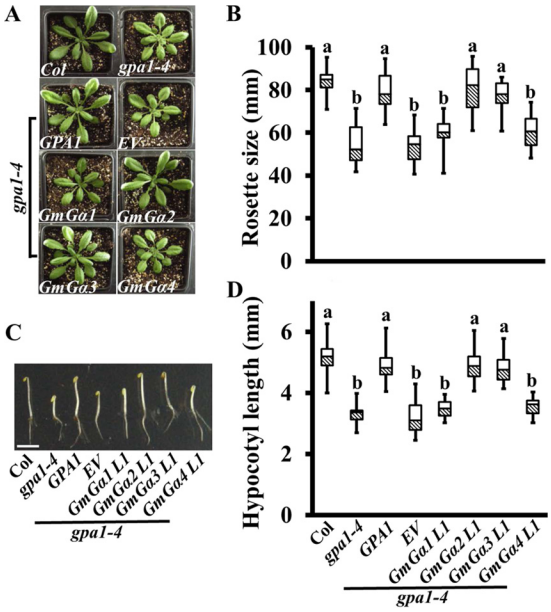

Figure 2. Rosette size and hypocotyl length phenotypes of WT, *gpa1*, and *gpa1* complemented with different *Gαs*. *A*, representative images of 4-week-old rosettes of WT (Col-0), *gpa1* mutant, and *gpa1* mutant plants complemented with native *GPA1* and *GmGαs*. *B*, the rosette diameter was measured from 4-week-old plants. The data points are average distances between the two farthest leaves from at least 24 rosettes ± S.D. *C*, representative images of 3-day-old, dark-grown hypocotyls of WT (Col-0), *gpa1* mutant, and *gpa1* mutant plants complemented with native *GPA1* and *GmGαs*. *Bar*, 2.5 mm. *D*, quantification of 3-day-old dark-grown hypocotyl lengths in *gpa1* mutant and all complemented lines compared with WT. The experiment was repeated three times, and the data were averaged ($n = 24$ plants per genotype per experiment). The *error bars* represent ± S.D. Statistical significance in *B* and *D* was determined using one-way ANOVA multiple comparisons. ^{a,b}, different letters indicate a significant difference (Tukey's multiple comparison test, $p < 0.05$).

contrast, *gpa1* complemented with *GmGα1* and *GmGα4* showed rounded, crinkled leaves, similar to the mutant complemented with an EV control construct (Fig. 1*A*), even though an equivalent level of respective protein was expressed in the transgenic plants (supplemental Fig. S3). Quantification of the leaf length, width, and the ratio of length to width of the 10th leaf confirmed these observations. For each of these traits, the phenotypes of *gpa1* mutant plants complemented with native *GPA1*, *GmGα2* or *GmGα3* were restored to the WT level (Fig. 1, *B–D*). No such recovery was observed in the mutants transformed with *GmGα1* and *GmGα4*, and these plants exhibited phenotypes similar to the EV transformed controls and to *gpa1* plants (Fig. 1, *B–D*, and supplemental Fig. S4, *A–C*).

We also quantified the rosette size of 4-week-old plants grown under short-day condition by measuring the distance between the two farthest leaves. Under these conditions, the *gpa1* rosette size is ~70% of the WT plants. Introduction of native *GPA1*, *GmGα2*, or *GmGα3* to the mutant plants led to the restoration of rosette size to the WT level (Fig. 2, *A* and *B*). Similar to the leaf shape, the introduction of group I *GmGαs* (*Gα1* and *Gα4*) to the mutant *gpa1* failed to restore the phenotype, and these plants showed smaller rosettes, comparable with the mutant or mutants transformed with an EV construct (Fig. 2, *A* and *B*, and supplemental Fig. S5*A*).

Another obvious developmental phenotype displayed by the *gpa1* mutants is the reduced length of their hypocotyls compared with the WT plants, when seedlings are grown in darkness (17, 18). Measurement of the hypocotyl lengths of 3-day-old dark grown seedlings showed a pattern similar to the leaf shape and rosette size. The *gpa1* hypocotyl length was ~60% of the length of the WT seedlings (Fig. 2, *C* and *D*). The hypocotyl lengths of the mutants were restored to the WT levels in seedlings complemented with group II Gα (*GmGα2* and *GmGα3*) but not in seedlings complemented with *GmGα1*, *GmGα4*, or EV (Fig. 2, *C* and *D*, and supplemental Fig. S5*B*). These data suggest that for vegetative growth and developmental phenotypes including light grown leaf shape, rosette size, or dark grown hypocotyl length, the group II GmGα proteins are true functional homologs of *Arabidopsis* GPA1.

An additional developmental phenotype observed in the *gpa1* mutants is its reduced stomatal density compared with the WT plants (39). Quantification of stomatal density in WT, *gpa1*, and *gpa1* transformed with native *GPA1*, different *GmGα* genes, or EV displayed a trend seen with leaf or hypocotyl phenotypes. The reduced stomatal density of *gpa1* leaves was restored to the WT level in the presence of *GPA1*, *GmGα2*, and *GmGα3* genes, but not in the presence of *GmGα*1 and *GmGα*4 or EV constructs (Fig. 3).

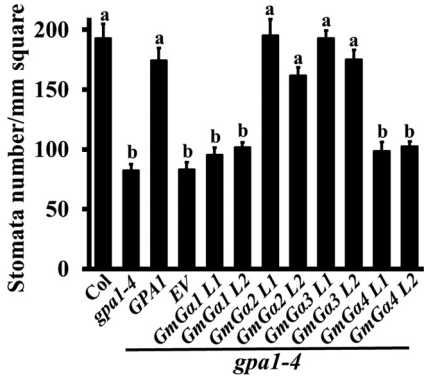

Figure 3. Quantification of stomatal density of WT, *gpa1*, and *gpa1* complemented with different *Gαs*. Stomatal density was quantified from the images of the abaxial surfaces of the fifth and sixth rosette leaves of plants at 4 weeks after germination. Measurements were made from five different regions of two leaves per genotype. *L1* and *L2* refer to the two independent lines. The *error bars* represent ± S.D. Statistical significance was measured using one-way ANOVA multiple comparisons. ^{a,b}, different letters indicate a significant difference (Tukey's multiple comparison test, $p < 0.05$).

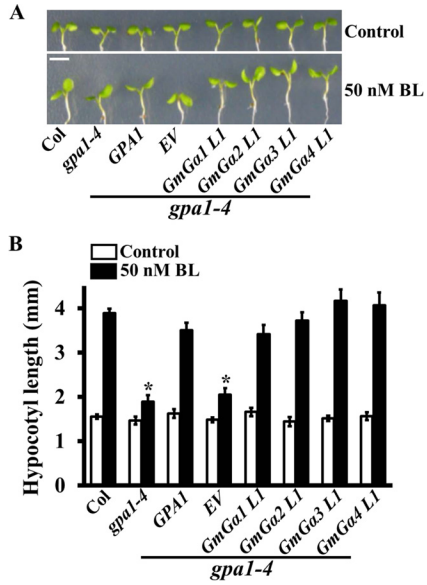

Figure 4. Effect of BL on hypocotyl length of WT, *gpa1*, and *gpa1* complemented with different *Gαs*. *A*, representative images of 5-day-old hypocotyl of WT (Col-0), *gpa1* mutant, and *gpa1* mutant plants complemented with native *GPA1* and *GmGαs*. *Upper panel*, without BL; *lower panel*, with BL. *Bar*, 4 mm. *B*, WT, *gpa1* mutant and different complemented lines were grown side by side on the same plate, and hypocotyl lengths were recorded after 5 days of growth under continuous light (22 °C; 100 μmol m^{-2} s^{-1} light) in the presence of 50 nM BL. The experiment was repeated three times, and the data were averaged ($n = 20$ plants per genotype per experiment). *Error bars* represent ± S.D. and significant difference at $p < 0.01$ (*) as determined by *t* test in comparison with WT.

Each GmGα gene can complement the altered BR and GA sensitivity of gpa1 mutants

The complementation of many developmental phenotypes of the *gpa1* mutants by only two of the four Gα proteins of soybean *i.e.* GmGα2 and GmGα3 suggested either that only these proteins are functional *in planta* or that different Gα homologs have distinct roles during regulation of specific pathways. To address these possibilities, we assessed the ability of each of the soybean Gα proteins in restoring the altered sensitivity of *gpa1* mutants to multiple phytohormones. As reported previously, *gpa1* displays reduced sensitivity to brassinolide (BL), a biologically active form of BR, in hypocotyl elongation (16). Exogenous application of BL resulted in an almost 2.5-fold increase in hypocotyl length in 5-day-old, light-grown, WT seedlings. In contrast, the *gpa1* mutants showed significantly reduced sensitivity to BL, and only a modest increase in BL-induced hypocotyl length was observed. Interestingly, *gpa1* mutants complemented with either native *GPA1* or any of the four *GmGα* genes resulted in a normal, WT-like response to exogenous BL, whereas the EV transformed plants showed phenotypes similar to the mutant as expected (Fig. 4 and supplemental Fig. S6).

GPA1 is a positive regulator of GA signaling. It has been proposed that GA-dependent seed germination in *Arabidopsis* is coupled with BR, which potentiates the response (16). To evaluate the effect of each of the GmGα proteins in mediating GA-dependent processes, we subjected WT, *gpa1*, and *gpa1* transformed with native *GPA1*, all four *GmGα* and an EV construct to a GA-dependent seed germination assay. Seeds were pretreated with paclobutrazol (PAC), a potent GA biosynthesis inhibitor to inhibit any germination, and subsequently germinated in the presence of different concentrations of exogenously applied GA_3 to evaluate its effect. No germination was observed in any of the seeds treated with PAC without GA_3 treatment (supplemental Fig. S7*A*). Application of 10^{-8} and 10^{-6} μM exogenous GA_3 resulted in up to 50 and 75% germination of the WT seeds, respectively (Fig. 5). A clear hyposensitivity was observed in *gpa1* mutant seeds, where ~20 and 40% seeds germinated at 10^{-8} and 10^{-6} μM exogenous GA_3 treatment, respectively. Similar to what was observed for BL response, each of the *GmGα* genes and native *GPA1* were able to restore the seed germination of the complemented plants to the WT level in the presence of different concentrations of GA_3, whereas the EV transformed seeds showed similar sensitivity as the mutant seeds (Fig. 5 and supplemental Fig. S7*B*). These results confirm that each of the GmGα proteins is active and functional in planta, and the differences observed in their complementation ability to a subset of developmental phenotypes is indeed due to their involvement in specific signaling pathways.

The altered ABA and glucose sensitivity of gpa1 mutants is differentially complemented by different GmGα genes

G-proteins are negative regulators of ABA- and glucose-mediated signaling pathways in *Arabidopsis* (21, 40). In contrast to GA and BR signaling, which is thought to be indirectly mediated by G-proteins, their effect on ABA (and potentially glucose) signaling is direct and relatively complex (16, 21). To further explore the role of individual GmGα proteins during regulation of ABA response, we tested the ABA-dependent inhibition of germination of different genotypes used in our experiments. Mutant *gpa1* seeds exhibit clear hypersensitivity to ABA during germination. In the presence of 1 μM ABA, ~60% WT seeds showed radical protrusion (a sign of germination) at 72 h postimbibition, whereas only ~30% *gpa1* seeds or *gpa1* seeds harboring EV constructs germinated by this time point. All *gpa1* seeds complemented with different *GmGα* constructs showed improved germination compared with the

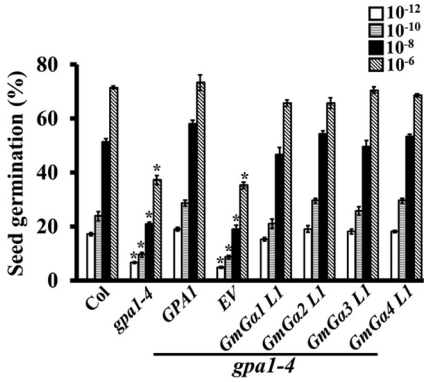

Figure 5. The effect of GA_3 on PAC-inhibited seed germination of WT, *gpa1*, and *gpa1* complemented with different *Gαs*. Seeds were pretreated with 10 μM PAC, followed by extensive washing with water and sowing on medium supplemented with different concentration of GA_3. After 48 h under continuous light (22 °C; 100 μmol m^{-2} s^{-1} light), germination was scored and expressed as a percentage of total seeds. The experiments were repeated three times, and the data were averaged (n = 100 per genotype for each experiment). The *error bars* represent ± S.D. *, $p < 0.01$ as determined by *t* test in comparison with WT at different concentration of GA_3.

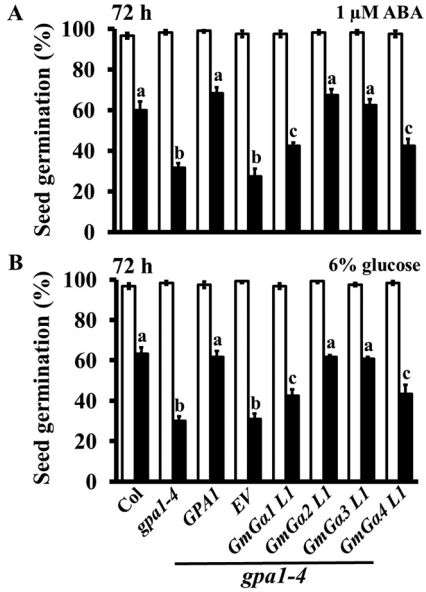

Figure 6. Altered ABA and glucose hypersensitivity of WT, *gpa1*, and *gpa1* complemented with different *Gαs*. *A*, seeds from WT, *gpa1* mutant, and different complemented lines were surface-sterilized and plated on 0.5× MS medium containing 1% sucrose in the absence or presence of 1 μM ABA. *B*, seeds were plated on 0.5× MS medium in the absence or presence of 6% glucose. In both experiments, germination was recorded at 72 h after transfer of the plates to growth chambers (22 °C; 100 μmol m^{-2} s^{-1} light) and expressed as a percentage of total seeds. The experiments were repeated three times, and the data were averaged (n = 100 per genotype for each experiment). The *error bars* represent ± S.D. All seeds of each genotype germinated on control media. Statistical significance was measured in ABA- and glucose-treated seed germination using one-way ANOVA multiple comparisons. ^{a,b,c}, different letters indicate a significant difference (Tukey's multiple comparison test, $p < 0.05$).

mutants (Fig. 6*A* and supplemental Fig. S8*A*). Although the presence of native *GPA1* and group II *GmGα* (*Gα2* and *Gα3*) restored germination of the mutant seeds to the WT level (~60% germination in the presence of ABA at 72 h), seeds complemented with *GmGα1* and *GmGα4* showed partial recovery. At 72 h postimbibition, ~40–45% seeds displayed radical protrusion, showing significant differences from both WT and *gpa1* (Fig. 6*A*). A similar trend was seen in the presence of 6% glucose, where group I and group II GmGα proteins partially or fully restored, respectively, the germination and greening phenotype of *gpa1* mutant seeds (Fig. 6*B* and supplemental Fig. 8*B*).

Engineered changes in specific Gα proteins recapitulate their effects on plant phenotypes

The data presented in previous sections establish that the inherent changes in the biochemical properties of Gα proteins potentially result in alterations of response regulation. To expand on this idea further, we generated site-directed variants of specific GmGα proteins that have been demonstrated to exhibit differences in their GTP-binding or hydrolysis activities; and evaluated their ability to influence the G-protein–mediated responses, *in planta*. Our choice of these protein variants is also informed by our previous results with their effects on complementing yeast mutant phenotypes (37).

$GmGα1^{Q223L}$ is a variant of GmGα1 where the exchange of glutamine to leucine at position 223 results in a Gα that can no longer be affected by the GAP activity of its cognate RGS protein (as has been also well-established for a corresponding mutation in mammalian Gα proteins), although the effect of additional plant-specific GAPs such as phospholipase Dα1 on its GTPase activity is currently not known (6, 8, 37). However, the rate of activation/deactivation of a G-protein cycle mediated by $GmGα1^{Q223L}$ is expected to be different from the one mediated by native GmGα1. Similarly, we have previously reported a mutation in GmGα2 ($GmGα2^{Q181E}$), which alters its GTPase activity (37) (supplemental Fig. S9). We introduced these protein variants in the *gpa1* mutant background and compared it with the native GmGα1 and GmGα2 harboring plants for their ability to complement the mutant phenotypes.

Both these protein variants showed clearly different complementation abilities when compared with their native protein versions, as was also seen with our yeast studies (37). In contrast to the native GmGα1, the $GmGα1^{Q223L}$ variant was able to rescue the leaf shape and rosette size of *gpa1* mutants to the WT levels (Fig. 7, *A* and *B*, and supplemental Fig. S10, *A* and *B*). Similarly, the dark grown hypocotyl length (Fig. 7*C*) and stomatal density (Fig. 7*D*) of $GmGα1^{Q223L}$-expressing *gpa1* plants were similar to the WT plants. Conversely, the plants complemented with $GmGα2^{Q181E}$ were not able to fully restore these developmental phenotypes of *gpa1* mutants and exhibited phenotypes distinct form the plants complemented with their native protein version (Fig. 7, *A–D*, and supplemental Fig. 10, *A* and *B*). An analogous trend was seen when comparing ABA-mediated and glucose-mediated inhibition of seed germination, where $GmGα1^{Q223L}$ and $GmGα2^{Q181E}$ complemented seeds exhibited germination levels distinct from the seeds complemented with their native protein versions (Fig. 8, *A* and *B*). $GmGα1^{Q223L}$ was able to overcome the ABA- and glucose-mediated inhibition of germination better than the native *GmGα1*, whereas the converse was true for the mutants complemented with native *GmGα2* and $GmGα2^{Q181E}$.

We extended this observation by comparing the results of complementation of the *gpa1* mutant plants with either native GPA1 or the $AtGPA1^{Q222L}$ variant (same mutation as

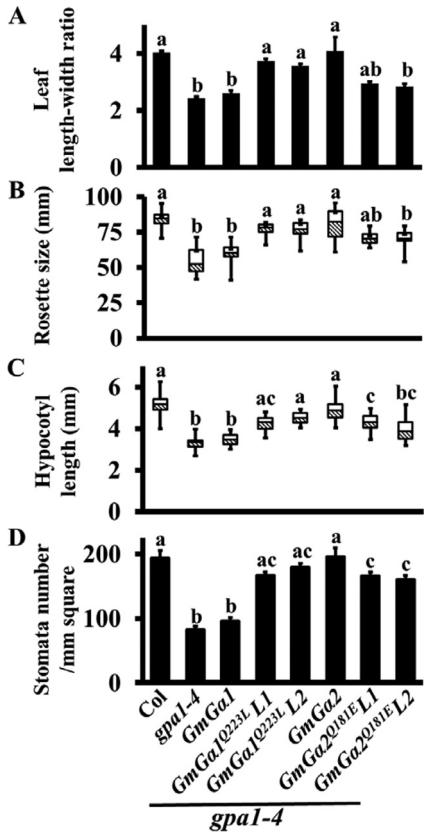

Figure 7. Vegetative growth parameters of *gpa1* plants complemented with native and engineered *Gαs*. Phenotypic characterizations of WT, *gpa1* mutant, *gpa1* mutant complemented with native *GmGα1*, variant *GmGα1* (*GmGα1*^{Q223L}), native *GmGα2*, and variant *GmGα2* (*GmGα2*^{Q181E}) were performed. *A*, the length-width ratio of the leaf blade of the 10th leaf was measured from the 4-week-old plants. The values represent the average lengths and widths of leaf blade from at least 24 leaves. *B*, the rosette diameter was measured from 4-week-old plants. The data points are average distances between the two farthest leaves from at least 24 rosettes. *C*, quantification of 3-day-old dark-grown hypocotyl lengths. The experiment was repeated three times, and the data were averaged ($n = 24$ plants per genotype per experiment). *D*, stomatal density was quantified from the images of the abaxial surfaces of the 4-week-old rosette leaves. Measurements were made from five different regions of two leaves per genotype. *L1* and *L2* refer to the two independent lines. In all experiments, the *error bars* represent ± S.D. Statistical significance was measured using one-way ANOVA multiple comparisons. ^{a,b,c}, different letters indicate a significant difference (Tukey's multiple comparison test, $p < 0.05$).

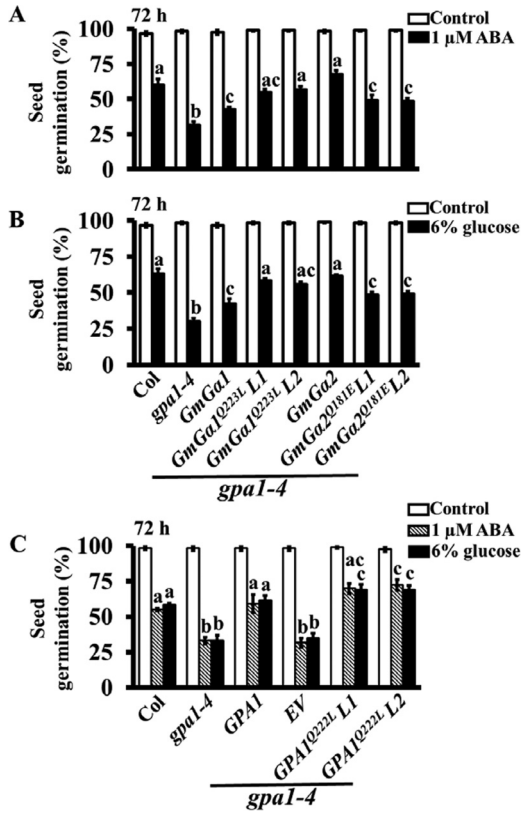

Figure 8. Effect of ABA and glucose on seed germination of *gpa1* plants complemented with native and engineered *Gαs*. *A*, seeds from identical seed lots of WT, *gpa1* mutant, *gpa1* mutant complemented with native *GmGα1*, variant *GmGα1* (*GmGα1*^{Q223L}), native *GmGα2*, and variant *GmGα2* (*GmGα2*^{Q181E}) were surface-sterilized and plated on 0.5× MS medium containing 1% sucrose in the absence or presence of 1 μM ABA. *B*, surface-sterilized seeds of indicated genotypes were plated on 0.5× MS medium in the absence or presence of 6% glucose. *C*, seeds from identical seed lots of WT, *gpa1* mutant, *gpa1* mutant complemented with native *GPA1*, and variant *GPA1* (*GPA1*^{Q222L}) were surface-sterilized and plated on 0.5× MS medium in the absence or presence of 1 μM ABA or 6% glucose. In both treatments germination was recorded at 72 h after transfer of the plates to growth chambers (22 °C; 100 μmol m^{-2} s^{-1} light) and expressed as a percentage of total seeds. The experiment was repeated three times, and the data were averaged ($n = 100$ for each experiment per genotype). *L1* and *L2* refer to the two independent lines. The *error bars* represent ± S.D. All seeds of each genotype germinated on control medium, with no difference in the timing or efficiency. Statistical significance was measured in ABA- and glucose-treated seed germination using one-way ANOVA multiple comparisons. ^{a,b,c}, different letters indicate a significant difference (Tukey's multiple comparison test, $p < 0.05$).

GmGα1^{Q223L}). Because native GPA1 could fully complement each of the phenotypes tested, to assess the effect of *AtGPA1*^{Q222L}, we chose phenotypes where the two types of GmGα proteins exhibited differences in their complementation ability, and those differences were quantitative; *e.g.* ABA or glucose-mediated inhibition of seed germination. For both these phenotypes, *gpa1* mutants complemented with the variant *AtGPA1*^{Q222L} protein exhibited improved germination rates compared with the ones complemented with the native protein or the wild-type plants (Fig. 8*C*), following the trend exhibited by the *GmGα1*^{Q223L} variant. Each of the tested proteins was able to complement the GA- and BL-dependent phenotypes of *gpa1* mutants (supplemental Fig. S11, *A* and *B*). These data confirm that alterations in the biochemical activities of Gα proteins can result in varied physiological or developmental responses. Our results suggest that the outcome of specific signaling pathways can be fine-tuned by modulating the Gα activity, which may lead to changes in its binding affinity or interactions with other proteins and in the context of the whole plant offers a glimpse of plasticity that can exist in G-protein signaling.

Discussion

Plant growth and development is incredibly plastic, and information from multiple cues, both internal and external, needs to be integrated and processed in a highly efficient manner to result in an optimum response under any given condition. Proteins such as heterotrimeric G-proteins are uniquely positioned to regulate such adaptive responses because they integrate a variety of signaling networks to modulate the overall growth and development of plants (41, 42), without being indispensable, at least in *Arabidopsis*.

As per the classical paradigm of heterotrimeric G-protein signaling, the inherent biochemical properties of Gα proteins

determine the amplitude and duration of active signaling. In metazoan systems, multiple Gα proteins with varying dynamics contribute to signal-response coupling by interaction with specific downstream effectors or regulators (15, 43–45). In contrast, the presence of a single canonical Gα in *Arabidopsis*, combined with its involvement in control of a multitude of signaling and development pathways, has always been fascinating from the point of view of the specificity of response regulation. There is evidence that additional proteins such as the extra-large Gα proteins also constitute part of canonical G-protein signaling networks; and the multiplicity of Gγ proteins or tissue-dependent and conditional expression of individual genes may provide for some degree of specificity (30, 46–48). However, the role of Gα itself and the possibility that signal-dependent changes in its dynamics can also lead to the specificity of response regulation has not been explored. This concept is relatively difficult to evaluate in plants, because canonical GPCRs with a guanine nucleotide exchange factor (GEF) activity have not been identified, unequivocally. Many of the well-established effectors of metazoan G-protein signaling do not exist in plants, and the activation mechanisms of Gα proteins or the identity of their cognate receptors remains unknown in the majority of the cases. Furthermore, except for the ion channel regulations in stomatal guard cells, most of the signaling and developmental responses affected by G-proteins in plants are slow, possibly spanning days or weeks. As a result, the fast, cell-based systems that exist for determining the activation/deactivation kinetics of metazoan G-proteins and their *in vivo* effects remain unavailable for plant G-proteins.

However, the availability of null *Arabidopsis gpa1* mutant that exhibits a range of altered phenotypes compared with the WT plants and the presence of multiple Gα proteins with slightly different kinetics in the genomes of recently duplicated plants such as soybean offer an excellent opportunity to determine their effects in an *in vivo* system. By expressing native and engineered Gα proteins with subtle differences in their biochemical properties and evaluating their roles in the regulation of multiple phenotypes, we show that the inherent properties of Gα proteins do affect the specificity of response regulation. This is achieved most likely via distinct protein–protein interactions, which depend on the activation state and/or binding affinity of individual G-proteins.

We observed three different modes of regulation by soybean Gα protein activities: (i) a stringent regulation, where only a subgroup of proteins can substitute for GPA1 function; these include the regulation of developmental phenotypes by G-proteins, namely leaf shape and size, rosette size, hypocotyl length in darkness, and stomatal density (Figs. 1–3); (ii) a relaxed regulation, where each of the soybean Gα proteins, native or engineered, are able to functionally complement for GPA1; these include GA- and BL-regulated responses (Figs. 4 and 5); and (iii) an intermediate effect, where quantitative differences are observed in the ability of different soybean Gα proteins to complement the *gpa1* mutant phenotypes. These include ABA- and glucose-mediated signaling (Fig. 6). In other words, a subset of soybean Gα proteins (Gα2 and Gα3) is more flexible and multifunctional, because they can complement all tested phenotypes of the *gpa1* mutants. The group I Gα proteins, Gα1 and Gα4, however, are relatively limited in their functionality and can complement some but not all phenotypes (Fig. 9). Because each of the Gα proteins can complement specific phenotypes, it is obvious that the differences observed are not due to some of them being non-functional, their insufficient expression, or positional effects caused by their insertion at specific sites in the chromosomes. The differences in their complementation ability are indeed due to the changes in their inherent biochemical activities. This is further corroborated by the expression of variants of specific proteins in the mutant background (Figs. 7 and 8).

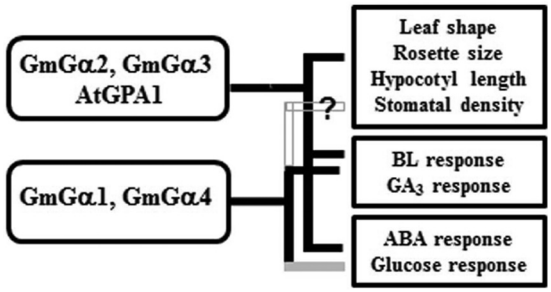

Figure 9. Summary of the response regulation by GmGα proteins. The two groups of GmGα proteins differentially modulate developmental and signaling responses. The figure includes the information for which we have presented the experimental data. *Solid black*, *solid gray*, and *open lines* represent the ability of different Gα proteins to fully, partially, or not complement the assessed phenotypes, respectively. The *question mark* shows the possibility that other proteins might be required, in addition to Gα, to complement the phenotype.

The four GmGα proteins have arisen because of two genome duplication events dating back to 59 and 13 million years ago (49). Gene duplication is an important mechanism for acquiring important developmental and regulatory genes. Many major plant agronomic traits, including those related to domestication, have arisen through deviations in gene coding sequence and their expression patterns (50–53). Our results suggest that the GmGα proteins have acquired some degree of subfunctionalization. Two of these are more adaptive and complement for each of the assayed phenotypes, whereas the other two are relatively limited in their scope, suggesting that these might have acquired unique functions *in planta*.

An interesting comparison can be drawn by judging the ability of GmGα proteins to complement mutant phenotypes of *Arabidopsis gpa1 versus* that of the yeast *gpa1*. Although the yeast *gpa1* mutant phenotypes in the pheromone response pathway were fully complemented by the soybean group I Gα and not by group II Gα (37), the opposite was seen during the complementation of developmental phenotypes of *Arabidopsis gpa1* (Figs. 1–3). Incidentally, the *Arabidopsis* GPA1 does not complement the yeast *gpa1* mutant phenotypes, similar to GmGα2 and GmGα3 (37). One key difference between yeast and plant G-protein signaling is related to the activation mechanism of G-protein cycle. Although in yeast a GPCR-dependent GDP/GTP exchange activates the cycle, classic GPCRs with GEF activity have not yet been identified in plants, and the activation mechanisms of plant Gα proteins remain unknown. One hypothesis, based on the work with *Arabidopsis* GPA1, is that the plant Gα proteins are self-activated (54, 55). It may be that the degree or rate of self-activation of plant Gα proteins

varies and influences their ability during response regulation. Alternatively, it is possible that different Gα proteins have distinct activation mechanisms, depending on direct *versus* indirect regulation of a phenotype by G-protein signaling. Under such a scenario, GmGα 1 and 4 potentially remain similar to yeast Gα protein and maintain the ability to be activated by classic mechanisms. In contrast, the group II GmGα proteins (and *Arabidopsis* GPA1) might have evolved to acquire additional, plant-specific activation or regulatory mechanisms. Such mechanisms might include the involvement of receptor-like kinases in affecting plant G-protein signaling, as has been proposed by several recent studies (38, 56–58). Additionally, there may exist yet unidentified, novel receptor-like proteins in plants that can activate the Gα protein by a classical GEF-like activity. It is conceivable that in plants with multiple Gα proteins, specific signaling pathways may employ distinct receptors to activate a particular signaling pathway, whereas in plants with a single Gα, multiple mechanisms of activation via distinct receptors exist. The net outcome of such plastic regulation of the Gα activity could result in signal-dependent changes in the dynamics of the G-protein cycle, even though the involved G-protein components remain the same. The biochemical diversity arising from such regulations could be a potential mechanism to compensate for the paucity in the number of the G-protein components in plants. Another equally compelling possibility is that the Gα proteins have different interaction specificity with various downstream components in response to particular signal or environmental or developmental cues. Plant G-proteins have been proposed to exist as large macromolecular complexes (59), and the composition of such complexes may be signal- or tissue type–dependent.

How might the changes in the dynamics of a signaling complex be able to determine the specificity of response regulation or linked to altered protein–protein interaction specificity, especially in plants with a single canonical Gα? We can speculate that a Gα protein that is available in its GTP-bound, active form for a longer duration or with higher frequently, is able to interact with a wide variety of effector proteins compared with a Gα protein, which is relatively short-lived in its active form; or the type of effector proteins may vary depending on the duration of the GTP-bound form of Gα proteins. The dynamics of the active *versus* inactive state of Gα also determines the duration of the availability of Gβγ to interact with downstream effectors, which can add another dimension to the G-protein–dependent regulatory processes. In addition, because G-proteins are expected to be a part of larger signaling complexes, the composition or stability of such complexes could also be affected by the G-protein activity. A similar situation has been reported in auxin-induced degradation of Aux/IAA proteins, where specifically controlled protein turnover dynamics have been shown to determine the occurrence of lateral root formation (60).

Overall our data show that relatively subtle changes in the inherent biochemical properties of Gα proteins can affect the type or strength of signal input and result in precisely controlled, specific outputs, likely by modulating the protein–protein interaction networks. In sessile organisms as plants, such plastic regulatory mechanisms might be essential for their optimal growth, development and productivity.

Experimental procedures

Plant materials and growth conditions

All *Arabidopsis* plants used in this study were of the Columbia-0 ecotype. The *gpa1–4* (Salk_001846) mutants used in this study have been previously described and were confirmed by genotyping. Surface-sterilized seeds from WT, mutant, and complemented transgenic lines were sown on ½ MS agar (1%) medium with 1% sucrose and cultured for 10 days in a growth chamber (22 °C; 14/10 h day/night; 100 μmol m^{-2} s^{-1} light). Robust seedlings were transferred to Soilrite and grown at 22 °C; 14/10 h day/night; 200 μmol m^{-2} s^{-1} light until maturity. All genotypes were grown together under identical conditions, and seeds were collected from mature dry siliques. Seed stocks were maintained in the dark at 4 °C.

Genetic complementation

Arabidopsis GPA1 promoter (~1.5 kb) was amplified from Columbia-0 ecotype by genomic PCR and cloned into pCR8 GW® vector (Invitrogen). The promoter, together with a Myc-epitope tag containing Gateway cassette from the pEarlyGate vector 203 (61), was subcloned into pFGC5941 binary vector. The *Arabidopsis GPA1* or the four *GmGα* genes (*Gα1–4*) were cloned into modified pFGC5941 vector by LR Clonase (Invitrogen) reaction. All constructs including an EV control were transformed into *Arabidopsis gpa1–4* mutants via *Agrobacterium tumefaciens* strain GV3101-mediated floral dip transformation (62). Transgenic plants were identified by selection on medium containing 25 μg/ml Basta. A minimum of six independent transgenic plants were selected for each transformation. Seeds collected from two independent homozygous T3 transgenic lines (T4 seeds) were used for phenotypic characterization. For the sake of clarity, the results obtained with the second line are presented in the supplemental materials, unless noted otherwise.

Physiological analysis

For hypocotyl length measurement, seeds of WT, *gpa1* mutant, and complemented plants were plated onto ½ MS medium with 1% sucrose and grown horizontally in darkness for 72 h in growth chamber. To measure the rosette and leaf phenotypes, plants were grown as previously described (6). Seedlings, leaves, or mature plants were photographed, and hypocotyl lengths and rosette/leaf size were measured from individual pictures using ImageJ software. Twenty-four plants with three biological replicates were measured for each genotype.

To detect the stomatal density, abaxial epidermis was peeled from the fully expanded leaves of 4-week-old plants. Two leaves were sampled per plant, and ~24 images were taken with the Nikon Eclipse E800 microscope. Stomatal density was determined for each image using ImageJ software. The scale was determined by photographing a slide micrometer. Five replicates were measured for each genotype.

To study the effect of ABA on seed germination, sterilized seeds were plated on treatment (1 μM ABA; Caisson Labs) or

control (equimolar amount of EtOH) media directly and grown in a growth chamber under continuous light (22 °C; 100 μmol m^{-2} s^{-1} light). For the effect of sugar on seed germination, sterilized seeds were plated on filter sterilized 6% glucose (Sigma) media. In both cases, the radicle emergence was counted as germination, and germination rates were expressed compared with control plates.

To study the effect of BL (Brassinolide, $C_{24}H_{48}O_6$; Pubchem) on hypocotyl length WT, mutant and different transgenic lines were grown side by side on the same plate, and hypocotyl lengths were recorded after 5 days of growth in a growth chamber under continuous light (22 °C; 100 μmol m^{-2} s^{-1} light). To study the effect of GA_3 on seed germination, seeds were first treated with 10 μM of PAC (Chem Service) and kept in darkness at 4 °C for 48 h. The seeds were washed six times with sterile water to remove PAC before plating them on ½ MS agar medium containing different concentrations of GA_3 (Caisson Labs) as previously described (63). After 48 h at 22 °C, germination was scored and expressed as a percentage of total seed. All hormonal experiments were repeated at least three times, and the data were averaged.

Immunoblotting

WT, *gpa1–4* mutant and transgenic lines and control (containing empty vector) *Arabidopsis* seedlings were grown on ½ MS agar plates at 22 °C under continuous light for 10 days. Total proteins (25 μg) were extracted from the whole seedlings and transferred on to nitrocellulose membrane for Western blotting with GPA1 antibodies (Plant Antibody Facility, Ohio State University; catalog no. AB00099) as described previously (21).

Protein–protein interaction assays

Split ubiquitin-based protein–protein interaction assay was performed to study the interaction of soybean Gα proteins with *Arabidopsis* Gβ protein (AGB1). At least two independent transformations were performed for the split ubiquitin-based assay as previously described (36). To quantify the interaction between GmGα and *Arabidopsis* AGB1 proteins, GATEWAY-based yeast two-hybrid assay was performed (ProQuest Two Hybrid System; Invitrogen). The GmGα1–4 genes and *Arabidopsis* AGB1 were cloned into pDEST32 bait vectors (containing DNA-binding domain) and pDEST22 prey vectors (containing DNA-activating domain). Assays were performed as per the manufacturer's instruction. The quantitative strength of interaction was determined by β-galactosidase expression assay using *o*-nitrophenyl-β-D-galactopyranoside as a substrate (35). Strong, weak, and −ve controls are provided with the ProQuest two-hybrid system (Invitrogen).

Phosphate release assay

ENZchek phosphate assay kit (Invitrogen) was used to determine the amount of phosphate release from wild-type and variant GmGα proteins in presence of AtRGS1. Equal amounts of GmGα proteins (2.5 μM) were preloaded with GTP (1 mM) and incubated with different concentration of RGS proteins. Phosphate (P_i) production was detected by Tecan Infinite® 200 PRO microplate readers, as described previously (36).

Statistical analysis

Statistical analysis of the results from experiments was performed using a one-way ANOVA (Graph Pad Prism V5). The plant phenotypic differences including leaf shape, rosette size, and stomata number were considered to be statistically significant when $p < 0.05$. In the case of plate-based assays, hypocotyl length and seed germination were considered to be statistically significant when $p < 0.01$.

Author contributions—S. P. conceived and directed this study. S. R. C. conducted all of the experimental work. Overall supervision of the present study was undertaken by S. P. Both authors contributed to designing of experiments, interpretation of results, and writing of the manuscript.

Acknowledgments—We thank Henry Hellmuth and Caitlin Brenner for help with plant care, seed collection, and selection of transgenic plants.

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