G-Protein Complex Mutants Are Hypersensitive to Abscisic Acid Regulation of Germination and Postgermination Development[1[W]]

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Abscisic acid (ABA) plays regulatory roles in a host of physiological processes throughout plant growth and development. Seed germination, early seedling development, stomatal guard cell functions, and acclimation to adverse environmental conditions are key processes regulated by ABA. Recent evidence suggests that signaling processes in both seeds and guard cells involve heterotrimeric G proteins. To assess new roles for the Arabidopsis (*Arabidopsis thaliana*) Gα subunit (GPA1), the Gβ subunit (AGB1), and the candidate G-protein-coupled receptor (GCR1) in ABA signaling during germination and early seedling development, we utilized knockout mutants lacking one or more of these components. Our data show that GPA1, AGB1, and GCR1 each negatively regulates ABA signaling in seed germination and early seedling development. Plants lacking AGB1 have greater ABA hypersensitivity than plants lacking GPA1, suggesting that AGB1 is the predominant regulator of ABA signaling and that GPA1 affects the efficacy of AGB1 execution. GCR1 acts upstream of GPA1 and AGB1 for ABA signaling pathways during germination and early seedling development: *gcr1 gpa1* double mutants exhibit a *gpa1* phenotype and *agb1 gcr1* and *agb1 gcr1 gpa1* mutants exhibit an *agb1* phenotype. Contrary to the scenario in guard cells, where GCR1 and GPA1 have opposite effects on ABA signaling during stomatal opening, GCR1 acts in concert with GPA1 and AGB1 in ABA signaling during germination and early seedling development. Thus, cell- and tissue-specific functional interaction in response to a given signal such as ABA may determine the distinct pathways regulated by the individual members of the G-protein complex.

Abscisic acid (ABA) is an important phytohormone that regulates numerous aspects of plant growth, development, acclimation to environmental stress conditions (for review, see Leung and Giraudat, 1998), and flowering (Razem et al., 2006). ABA-regulated processes in plants can be divided into two broad and overlapping categories: (1) signaling in seeds, including maintenance of dormancy and inhibition of germination and early seedling development (Lopez-Molina et al., 2001; Nambara and Marion-Poll, 2003); and (2) abiotic stress responses of developmentally advanced plants, including guard cell functioning and ion channel regulation (Himmelbach et al., 1998; Fan et al., 2004). In both of these categories, ABA signaling is not a linear signal-response event, but a complex network involving a number of different signals and effectors. For example, during seed germination and early seedling development, there is integration of signaling inputs from ABA, gibberellins (GAs), brassinosteroids (BRs), sugars, ethylene, and auxins. A number of ABA response mutants have been uncovered in screens designed for mutants of sugar signaling pathways (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Gibson et al., 2001) and ethylene signaling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000), confirming cross talk between these signals.

ABA signaling is regulated at a number of different levels, including rapid signaling events independent of transcription and translation, transcriptional regulation, and posttranscriptional control of gene expression (Fedoroff, 2002). ABA signaling processes in both seeds and guard cells involve components of the heterotrimeric G-protein complex (Wang et al., 2001; Assmann, 2002; Ullah et al., 2002; Coursol et al., 2003; Lapik and Kaufman, 2003; Chen et al., 2004; Pandey and Assmann, 2004; Perfus-Barbeoch et al., 2004). In the classical signaling paradigm, the G-protein complex consists of three different subunits, Gα, Gβ, and Gγ, which form a heterotrimeric complex. Upon activation by agonist binding to an associated G-protein-coupled receptor (GPCR), the inactive G-heterotrimeric complex converts to an active conformation causing Gα to

[1] This work was supported by the National Science Foundation (grant no. MCB–0209694 to S.M.A. and grant no. MCB–0209711 to A.M.J.) and the National Institute of General Medical Sciences (grant no. GM65989–01 to A.M.J.).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Sarah M. Assmann (sma3@psu.edu).

[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.079038.

exchange GDP for GTP. The GPCR thus acts as a guanine nucleotide exchange factor. As a result of GTP binding, Gα-GTP separates from the G$\beta\gamma$ dimer and either Gα or G$\beta\gamma$ or both participate in signaling to downstream effectors. Intrinsic GTPase activity of Gα hydrolyzes GTP to GDP, thereby allowing Gα to reassociate with the G$\beta\gamma$ dimer. The Arabidopsis (*Arabidopsis thaliana*) genome has a limited number of gene(s) for each member of the G-protein complex; one canonical G-protein α subunit (*GPA1*), one G-protein β subunit (*AGB1*), two G-protein γ subunits (*AGG1* and *AGG2*), at least one candidate GPCR (*GCR1*), and one regulator of G-protein signaling (*RGS1*) protein (for review, see Jones and Assmann, 2004). Recent database searches have identified additional proteins such as a family of heptahelical receptors similar to human adiponectin and progestin receptors (Hsieh and Goodman, 2005); however, their role(s) in Arabidopsis G-protein-coupled signaling pathways remains to be ascertained.

While there are no published data on ABA-regulated processes in *agb1* mutants, null mutants of *GPA1* are hypersensitive to ABA in seed germination (Ullah et al., 2002; Lapik and Kaufman, 2003). A cupin domain-containing protein AtPirin1 was identified as a GPA1-interacting protein in a yeast two-hybrid screen. Mutation of this gene also leads to ABA hypersensitivity of germination, implicating AtPirin1, a possible transcriptional cofactor, in G-protein-mediated ABA signaling (Lapik and Kaufman, 2003). *gpa1* seeds are hyposensitive to GA and less sensitive to BR rescue of germination arrest induced by the GA biosynthesis inhibitor, paclobutrazol (Ullah et al., 2002). Seeds that overexpress GPA1 are many-fold more sensitive to GA than wild-type seeds, yet still require GA for germination (Ullah et al., 2002). Consistent with the fact that BR regulates GA sensitivity, BR biosynthesis and response mutants have reduced sensitivity to GA in seed germination, similar to *gpa1* mutants, and it is proposed that GPA1 may couple BR potentiation of GA signaling (Ullah et al., 2002).

Compared to germination, lack of GPA1 confers ABA hyposensitivity in stomatal guard cells. *gpa1* mutants exhibit reduced sensitivity to ABA in inhibition of stomatal opening and inward K^+ channel regulation and show altered ABA regulation of slow anion channels, although they have wild-type sensitivity to ABA in promotion of stomatal closure (Wang et al., 2001). *gpa1* mutants also have altered stomatal responses to a sphingolipid metabolite sphingosine-1-P (S1P; Coursol et al., 2003, 2005), a signaling molecule that appears to function upstream of ABA and, in mammalian cells, is a ligand for GPCRs (see Spiegel and Milstien, 2003).

GCR1 is a candidate GPCR in Arabidopsis. GCR1 has a predicted seven transmembrane domain structure and shows similarity to Dictyostelium and Drosophila GPCRs in the transmembrane region (Chen et al., 2004; Pandey and Assmann, 2004). Overexpression of GCR1 causes loss of seed dormancy and expression of a germination marker gene *PP2A* (Colucci et al., 2002). Our recent work has shown that GCR1 interacts with GPA1 both in vitro and in planta (Pandey and Assmann, 2004), providing experimental evidence that GCR1 forms a complex with GPA1. T-DNA insertional mutants of GCR1 show hyposensitivity to GA- and BR-mediated germination processes. However, some of these responses may not be directly coupled to G-protein signaling: The double mutant combinations *gcr1 gpa1* and *agb1 gcr1* and the triple mutant *agb1 gcr1 gpa1* have mostly synergistic or additive responses to GA and BR, implying that at least in these two hormonal responses, GCR1 can act independently of the heterotrimeric G protein (Chen et al., 2004).

Our data on the function of GCR1 in guard cells suggest that this protein may have a more central role in ABA signaling. *gcr1* mutants are hypersensitive to both ABA (and S1P) inhibition of stomatal opening and ABA (and S1P) promotion of stomatal closure (Pandey and Assmann, 2004) and we propose that GCR1 acts as a negative regulator of GPA1 in guard cell signaling. At the whole seedling level, *gcr1* plants show enhanced expression of ABA-regulated genes, as revealed by quantitative reverse transcription-PCR and hypersensitivity to ABA inhibition of root elongation. Mature *gcr1* plants also show improved recovery from drought (Pandey and Assmann, 2004).

Based on the above information, we wanted to study the roles of AGB1 and GCR1 in ABA control of the first category of ABA-regulated processes: germination and early postgermination growth and development. We made use of single, double, and triple G-protein complex mutants available in Arabidopsis to address the following questions vis-à-vis ABA signaling in germination and the early stages of sporophyte development: (1) Are there differential dependencies on GPA1 and AGB1? (2) Do null mutations of *GPA1* and *GCR1* result in opposite ABA sensitivities, as in guard cells? (3) does GCR1 act in the same pathway as GPA1 and AGB1 or independently? Our genetic analyses indicate that for these ABA-regulated responses, GCR1 acts in the same pathway and in concert with GPA1 or AGB1. Moreover, plants harboring an *agb1* mutation show the strongest phenotype in many cases, indicating a predominant role for AGB1 in seed and seedling ABA responses.

RESULTS

Expression of G-Protein Complex Genes during Germination

Knockout mutations of GPA1 and GCR1 show altered sensitivity to signals that regulate seed germination in Arabidopsis (Ullah et al., 2001; Chen et al., 2004), however, little is known about the expression patterns of the G-protein complex genes during this process (Weiss et al., 1993; Ullah et al., 2003), and

whether ABA affects expression of these genes. We used real-time quantitative PCR with gene-specific primers to determine the expression levels of *GCR1*, *GPA1*, and *AGB1* genes during germination and postgermination growth in Arabidopsis. Wild-type (ecotype Columbia of Arabidopsis [Col]) seeds were stratified at 4°C for the first 48 h during imbibition and then transferred to growth chambers (16-h light/8-h darkness regime) at 22°C. As shown in Figure 1A, *GCR1* and *AGB1* were expressed at the earliest time point tested, 6 h postimbibition, and both genes showed a gradual increase in expression level during germination and postgermination growth. *GPA1* transcript, however, could not be detected until 48 h postimbibition and showed very low levels at 1 d after transfer of seeds to 22°C. By this time, approximately 70% of seeds had germinated. *GPA1* level increased dramatically 2 d after transfer of seeds to 22°C, showing more than a 10-fold increase in the transcript level. We extended this observation by analyzing *GPA1* transcript in nonstratified seeds. As shown in Figure 1A inset, *GPA1* transcript was detectable in the nonstratified seeds at the earliest time point tested and showed a more than 10-fold increase in level after 96 h postimbibition, similar to the increase seen in the stratified seeds. In Figure 1A, values are represented as relative change in expression level of the gene with respect to the time point its expression was first detected. We further confirmed this observation by analysis of GPA1 protein. Western blotting of microsomal proteins with anti-GPA1 antibodies showed the presence of a faint GPA1-specific signal beginning 1 d after transfer of stratified seeds to 22°C, followed by a marked increase in protein level at later time points (2 and 3 d; Fig. 1B). In nonstratified seeds, however, GPA1 protein was detectable even at the earliest time point tested (Fig. 1A, inset).

We also wanted to determine if ABA affected the expression level of G-protein complex genes in wild-type seeds during germination. Quantitative real-time PCR with RNA isolated from control versus ABA-treated seeds during germination and postgermination growth did not show any significant effect of ABA on the transcript level of these genes (Fig. 1C). GPA1 protein level was also not affected by ABA treatment until the germination stage (1 d after transfer to 22°C, data not shown) although, as shown in the inset (Fig. 1C), ABA slightly increased the level of GPA1 protein at the postgermination stage (2 d after transfer to 22°C). Thus, it appears that the inhibitory action of ABA toward seed germination cannot be due to ABA-induced decreases in GPA1 transcript or protein level.

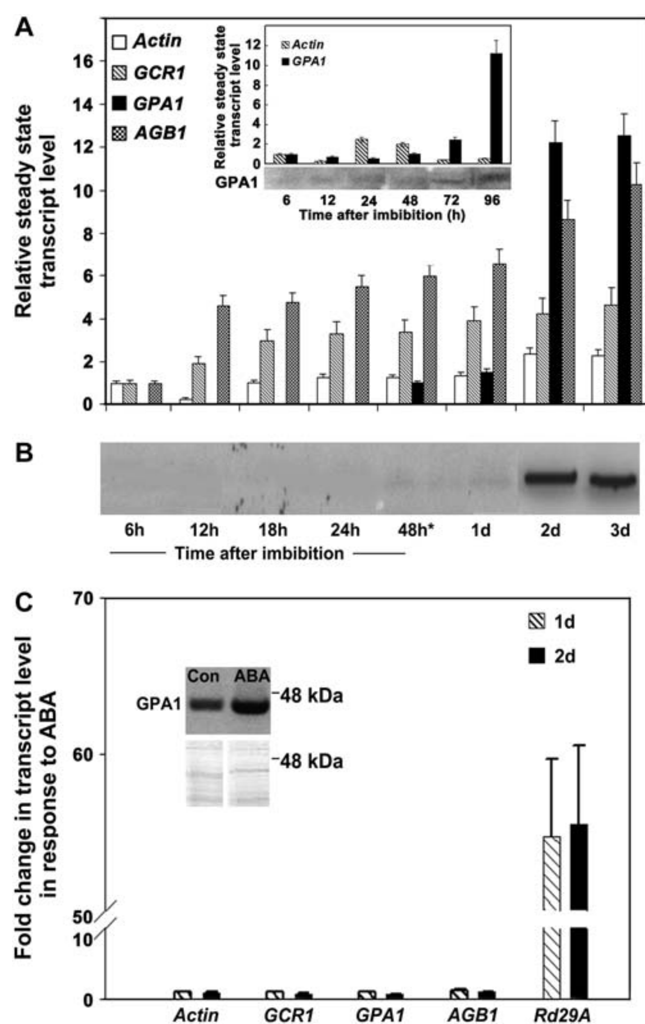

Figure 1. Expression of G-protein complex component genes during germination. A, Expression levels of *GCR1*, *GPA1*, and *AGB1* genes. Expression was determined by real-time quantitative PCR using SYBR green dye. Expression of the *Actin* gene under identical conditions served as an internal control. Expression was determined starting 6 h postimbibition until 3 d after transfer of seeds to growth chambers (16-h light/8-h darkness regime) at 22°C. Asterisk indicates the time point when seeds were transferred to 22°C. Inset shows relative expression of *Actin* and *GPA1* genes and expression level of GPA1 protein in nonstratified seeds. The experiment was repeated three times and data were averaged. The error bars represent SD. B, Western blotting with GPA1 antibodies. Steady-state level of GPA1 protein was determined in the microsomal fraction prepared from seeds and seedlings during germination and postgermination growth. Faint GPA1-specific signal appeared 1 d after transfer of seeds to 22°C and showed a large increase at day 2 (2 d), consistent with the expression profile obtained by real-time PCR. C, Effect of ABA on expression of *GCR1*, *GPA1*, and *AGB1*. Expression of *GCR1*, *GPA1*, *AGB1*, and *Rd29A* (a known ABA-responsive gene, used as a positive control) genes was determined by real-time quantitative PCR with control and ABA-treated seeds at 1 and 2 d after transfer to 22°C. Values represent fold change in expression level upon ABA treatment compared to control (ethanol) treatment. The experiment was repeated three times and data were averaged. The error bars represent SD. Inset shows the level of GPA1 protein at day two (2 d) after transfer of seeds to 22°C, before and after ABA treatment. Top, Western blot with GPA1 antibody; bottom, Coomassie Blue-stained gel.

Null Mutants of the G-Protein Complex Subunits Show Hypersensitivity to ABA and Glc during Germination

For stimulatory-germination signals such as GA and BR, the single knockout mutants of *GCR1*, *GPA1*, and *AGB1* show hyposensitivity, whereas they are hypersensitive to the GA biosynthetic inhibitor, paclobutrazol

(Chen et al., 2004). We examined the effects of the germination-repressive signals, ABA and Glc, on the G-protein complex knockout mutants. One micromolar ABA was found to be optimal to study the effect of ABA, based on the dose-response curve as shown in Supplemental Figure 1. Figure 2 shows germination of Col and mutant genotypes at different time points after transfer to 22°C, on control or ABA media with seeds pretreated with water (Fig. 2, A–D) or the ABA biosynthetic inhibitor fluridone (Fig. 2, E–H). For the sake of clarity, data on only one allele of the single mutants *gcr1* (*gcr1-2*), *gpa1* (*gpa1-4*), and *agb1* (*agb1-2*), are presented. These same alleles were used to generate the double and triple mutant combinations (*agb1-2 gpa1-4*, *gcr1-2 gpa1-4*, *agb1-2 gcr1-2*, and *agb1-2 gcr1-2 gpa1-4*). Data on the second alleles of the single mutants are presented in Supplemental Figure 2. For the water pretreated seeds on control media plates, the wild-type Col seeds, as well as all the mutant genotypes (except *agb1-1*; see Supplemental Fig. 2) showed 70% to 80% germination with no significant difference by 24 h after transfer to 22°C (Fig. 2A). However, in the presence of 1 μM ABA the G-protein complex mutants showed differential germination rates. Compared to the Col seeds that showed 60% germination at 24 h, the germination percentage of *gcr1-2* seeds was about 45% to 50%, showing a small but consistent ABA hypersensitivity (Fig. 2B). The *gpa1-4* mutant allele was also moderately hypersensitive, showing about 30% germination under these conditions (Fig. 2B). The strongest hypersensitivity was observed for the *agb1-2* allele. By 24 h after transfer to 22°C, approximately 15% of seeds germinated compared to 60% germination in Col (Fig. 2B). Analysis of the double and triple mutants shows that the Gβ subunit plays a predominant role in ABA signaling during germination. All the mutant combinations showed normal germination rates on control media, showing 70% to 80% germination at 24 h after transfer to 22°C (Fig. 2C). However, all the genotypes lacking the Gβ subunit were severely hypersensitive to ABA, showing less that 20% germination. The ABA hypersensitivity of all mutant genotypes containing the *agb1-2* allele was also confirmed in dose-response assays (Supplemental Fig. 1). The *gcr1 gpa1* double mutants showed moderate hypersensitivity to ABA (30% germination at 24 h time point), similar to the *gpa1* single mutants (Fig. 2D). The *agb1-1* allele, unlike *agb1-2*, is a point mutation. The mutation alters the splice donor site at the first intron of *AGB1*, leading to production of an altered transcript with a premature stop codon (Lease et al., 2001). This allele behaved differently from *agb1-2* for the time course of germination, possibly suggesting that an aberrant transcript or polypeptide is produced that affects germination, or that additional mutations are present. The ABA hypersensitivity of *agb1-1* was similar to *agb1-2*, but germination was delayed by approximately 28 h in the *agb1-1* mutant under both control and ABA-treated conditions (Supplemental Fig. 2).

We extended these observations with seeds pretreated with the ABA biosynthetic inhibitor fluridone (Fig. 2, E–H). Under our experimental conditions, seeds pretreated with fluridone showed delayed germination at early time points compared to the nontreated seeds, possibly due to an indirect effect of fluridone on GA/BR biosynthesis (Bartels and Watson, 1978; Devlin et al., 1980; Chae et al., 2004). By 48 h, this effect had disappeared and all the seeds were fully germinated. On control plates, 80% to 90% germination was observed at the 36 h time point for the wild-type Col as well as the G-protein complex mutant genotypes (Fig. 2E). In the presence of 1 μM ABA, approximately 60% of Col seeds germinated at 36 h. A slight hypersensitivity was observed for the *gcr1-2* mutants showing 45% to 50% germination (Fig. 2F). The *gpa1-4* mutants showed greater hypersensitivity to ABA after fluridone treatment compared to the non-fluridone treated *gpa1* seeds and about 40% germination was achieved at 36 h (Fig. 2F). The germination percentage for fluridone pretreated seeds of all the double and triple mutants was comparable to wild-type Col on control media (Fig. 2G). However on ABA-containing media, single *agb1* mutants (Fig. 2F), as well as double *agb1 gpa1* and *agb1 gcr1* mutants and triple *agb1 gcr1 gpa1* mutants showed strong hypersensitivity, showing less than 20% germination at 36 h (Fig. 2H). The double *gcr1 gpa1* mutant seeds showed sensitivity similar to the single *gpa1* mutants with about 40% germination at 36 h (Fig. 2H). Note that these phenotypes are not synergistic or additive, unlike BR and GA signaling in seed germination (Chen et al., 2004).

As these results were obtained with nondormant, imbibed seeds we further analyzed these results with nonstratified seeds of G-protein complex mutants. As shown in Figure 3, A to D, the relative ranking of G-protein complex mutants with respect to ABA hypersensitivity of germination remains identical to that of stratified seeds even though small differences were also observed without ABA treatment (Fig. 3, A and C). In the presence of 1 μM ABA, *gcr1* seeds show slight hypersensitivity (approximately 80% germination compared to approximately 90% germination in Col at 72 h), *gpa1* shows moderate hypersensitivity (approximately 60% versus approximately 90% for Col), and the *gcr1 gpa1* double mutant shows similar hypersensitivity to *gpa1*, suggesting that GPA1 acts in the same pathway as GCR1 for this signaling pathway. All the genotypes harboring an *agb1* mutation remained severely hypersensitive, showing about 40% germination at 72 h (Fig. 3, B and D). These observations thus confirm and extend the results obtained with stratified seeds. The relative ABA hypersensitivity of different mutant alleles was also confirmed in a dose-response curve (Supplemental Fig. 3).

Similar to ABA, high concentrations of exogenous Glc also inhibit germination of Arabidopsis seeds and early seedling development in which the G-protein complex is involved (Ullah et al., 2002; Chen et al.,

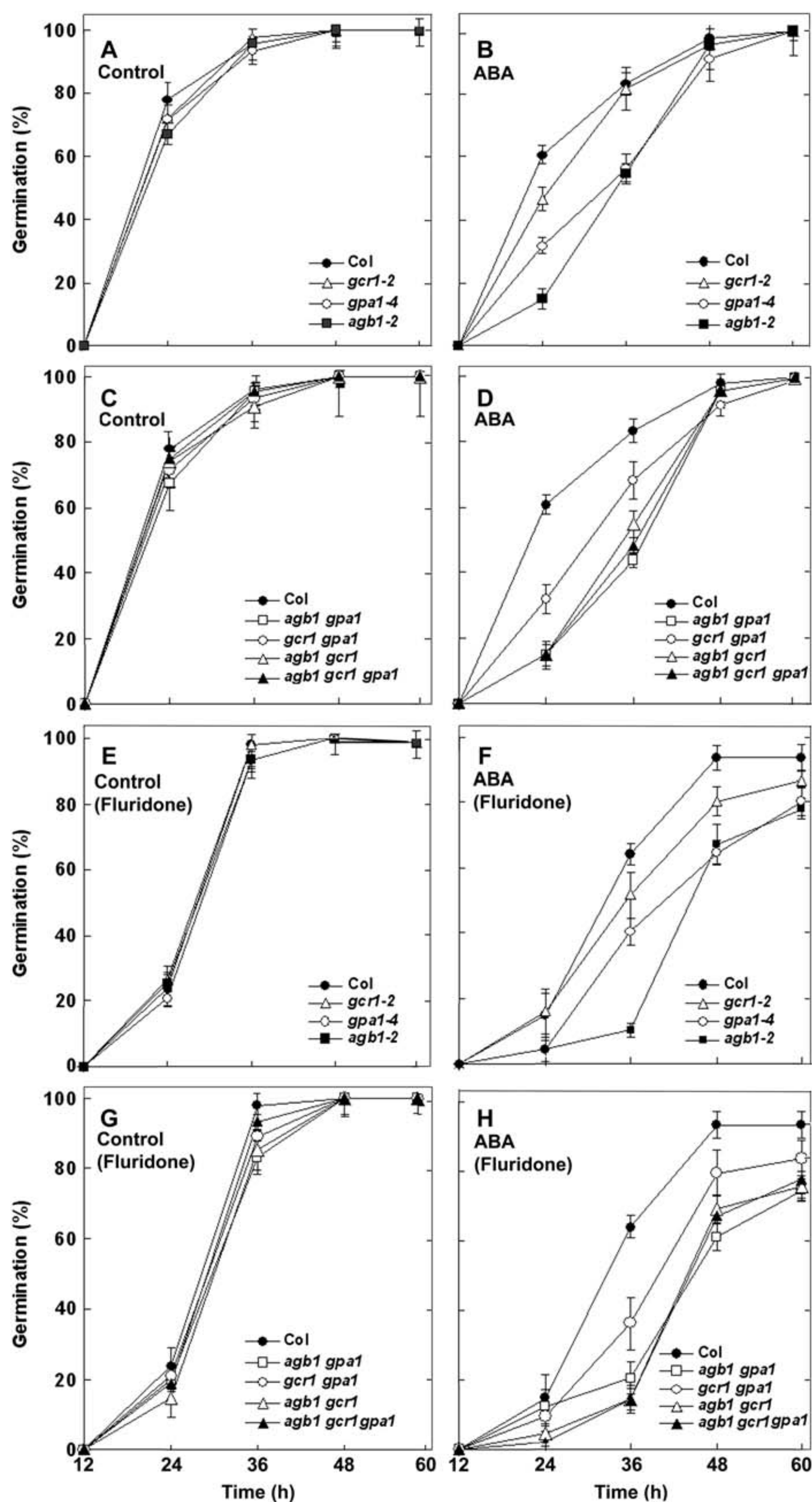

Figure 2. Stratified seeds of null mutants of G-protein complex show increased sensitivity to ABA-induced inhibition of seed germination. Seeds from matched seed lots were surface sterilized and pretreated with water (A–D) or 100 μmol fluridone (E–H) at 4°C in darkness for 48 h. Seeds were washed extensively with water and plated on 0.5 × MS media plates containing 1% Suc in the absence or presence of 1 μM ABA. Plates were kept at 4°C in darkness for 48 h and then transferred to growth chambers (16-h light/8-h darkness regime) at 22°C. Germination was recorded starting 12 h after transfer of the plates to growth chambers until 60 h and expressed as a percentage of total seeds. The figure shows germination of the single mutant alleles *gcr1-2*, *gpa1-4*, and *agb1-2* compared to Col seeds in the absence (A) or presence (B) of ABA. Germination of the double and triple mutant seeds *agb1 gpa1*, *gcr1 gpa1*, *agb1 gcr1*, and *agb1 gcr1 gpa1* in the absence and presence of ABA is shown in C and D, respectively. Germination percentage of fluridone pretreated seeds of the single mutants *gcr1-2*, *gpa1-4*, and *agb1-2* is shown in absence of or in the presence of ABA compared to Col seeds in E and F, respectively, and of the double and triple mutant seeds *agb1 gpa1*, *gcr1 gpa1*, *agb1 gcr1*, and *agb1 gcr1 gpa1* in the absence and presence of ABA is shown in G and H, respectively. The experiment was repeated three times and data were averaged, $n = 60$ for each experiment. The error bars represent SD. $P < 0.05$ for *gcr1-2*, <0.001 for *gpa1-4* and *gcr1 gpa1*, and <0.0001 for all the genotypes with *agb1* mutation as determined by *t* test in comparison to Col control at 24 h time point for water pretreated seeds and at 36 h time point for fluridone pretreated seeds.

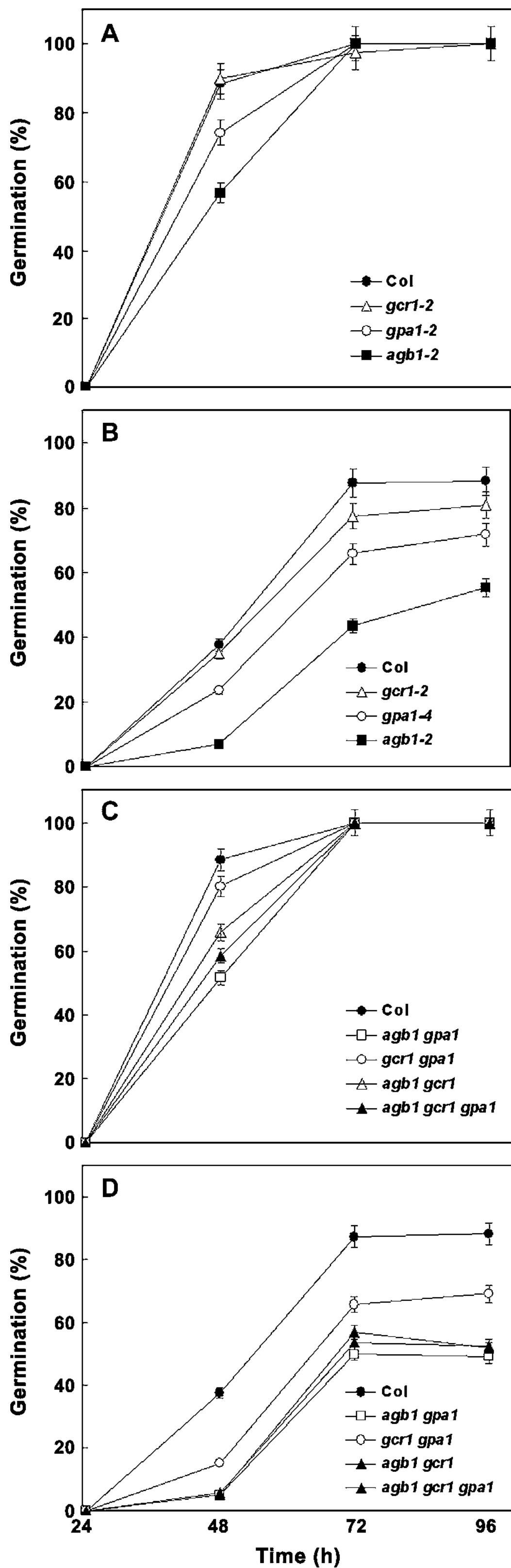

Figure 3. Nonstratified seeds of null mutants of G-protein complex show increased sensitivity to ABA-induced inhibition of seed germination. Seeds from matched seed lots were surface sterilized and plated on 0.5 × MS media plates containing 1% Suc in the absence or presence of 1 μM ABA. Plates were transferred to growth chambers (16-h light/8-h darkness regime) at 22°C. Germination was recorded starting 12 h after transfer of the plates to growth chambers until 92 h and expressed as a percentage of total seeds. The figure shows germination of the single mutant alleles *gcr1-2*, *gpa1-4*, and *agb1-2* compared to Col seeds in the absence (A) or presence (B) of ABA. Germination of the double and triple mutant seeds *agb1 gpa1*, *gcr1 gpa1*, *agb1 gcr1*, and *agb1 gcr1 gpa1* in the absence and presence of ABA is shown in C and D, respectively. The experiment was repeated three times and data were averaged, $n = 60$ for each experiment. The error bars represent SD. For B and D, $P < 0.05$ for *gcr1-2*, <0.001 for *gpa1-4* and *gcr1 gpa1*, and <0.0001 for all the genotypes harboring an *agb1* mutation as determined by *t* test in comparison to Col (ABA) at 72 h time point.

2003; Price et al., 2003; Chen and Jones, 2004; Dekkers et al., 2004). To study the effect of Glc on germination of G-protein complex mutants, seeds were plated on 0.5 × Murashige and Skoog (MS) media containing 1% Suc and 6% Glc. This Glc concentration delays germination, and thus protrusion of the radicle becomes obvious at a much later time point. We recorded germination in the presence of Glc at 60 h after transfer of plates to 22°C, as at least 50% of wild-type Col seeds germinated by this time point. The G-protein complex mutants showed hypersensitivity to Glc-induced inhibition of germination, similar to the results with ABA. At 60 h, about 50% of wild-type Col seeds germinated compared to approximately 40% germination of single *gcr1-1*, *gcr1-2*, *gpa1-3*, *gpa1-4*, and *gcr1 gpa1* double mutants and about 30% germination in the *agb1-2* single mutant, *agb1 gpa1* and *agb1 gcr1* double mutants, and *agb1 gcr1 gpa1* triple mutant. Similar to delayed germination in response to ABA, *agb1-1* mutants also showed delayed germination in the presence of Glc. At 60 h less than 10% of *agb1-1* seeds had germinated (Fig. 4).

As delay of germination by Glc is proposed to require endogenous ABA (Ullah et al., 2002; Price et al., 2003; Dekkers et al., 2004), seeds were also pretreated with the ABA biosynthetic inhibitor fluridone and plated on media plates containing 6% Glc. The overall germination of seeds in the presence of Glc increased after fluridone treatment compared to nonfluridone treated seeds; however, the sugar hypersensitive phenotypes were retained for G-protein complex mutants. As shown in Figure 4, more than 80% of wild-type Col seeds germinated at 60 h, whereas the germination percentage of single *gcr1-1*, *gcr1-2*, *gpa1-3*, *gpa1-4*, and double *gcr1 gpa1* mutants was 60% to 65% and of single *agb1-2* mutants, double *agb1 gcr1* and *agb1 gpa1* mutants, and the triple *agb1 gcr1 gpa1* mutant was about 50%. The delayed germination phenotype observed with *agb1-1* mutants was not affected by the presence of fluridone, suggesting that it is not related to ABA and at 60 h only about 10% of these seeds had germinated.

To analyze that the results obtained were not due to osmotic stress, seeds were also germinated in the presence of an equimolar concentration of sorbitol.

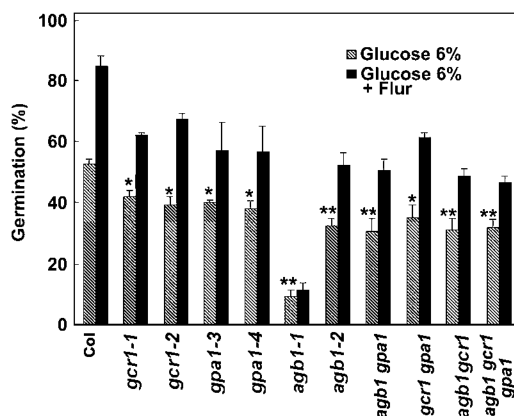

Figure 4. Null mutants of G-protein complex show increased sensitivity to Glc-induced inhibition of seed germination. Seeds from matched seed lots were surface sterilized and pretreated with water or 100 μM fluridone at 4°C in darkness for 48 h. Seeds were washed extensively with water and plated on 0.5× MS media plates containing 6% Glc. Germination was recorded at 60 h after transfer to growth chambers at 22°C. The experiment was repeated three times, $n = 60$ for each experiment. The error bars represent SD. $P < 0.005$ for water pretreated seeds of genotypes lacking *GCR1*, *GPA1*, or both, and <0.001 for all genotypes lacking *AGB1* compared to Col as determined by *t* test. For fluridone pretreated seeds, $P < 0.001$ for all the genotypes (except *agb1-1*) compared to Col, as determined by *t* test. For *agb1-1* genotype $P < 0.0001$ relative to Col. Single asterisks (*) represent significant ($P < 0.005$) and double asterisks (**) represent highly significant ($P < 0.001$) values.

Germination efficiency of all the mutant seeds was identical to wild-type Col under these conditions (data not shown).

Effect of ABA on Postgermination Growth of G-Protein Complex Mutants

Lack of G-protein complex proteins also led to ABA hypersensitivity of postgermination responses (Fig. 5). Similar to the ABA inhibition of germination, the *agb1-1* and *agb1-2* single mutants, *agb1 gcr1* and *agb1 gpa1* double mutants, and *agb1 gcr1 gpa1* triple mutants were more hypersensitive to ABA-induced growth retardation than the single *gcr1-1*, *gcr1-2*, *gpa1-3*, and *gpa1-4* and double *gcr1 gpa1* mutants (Fig. 5A). For the sake of clarity, data on only one allele of the single mutants, *gcr1* (*gcr1-2*), *gpa1* (*gpa1-4*), and *agb1* (*agb1-2*), are presented. Similar results were obtained with the second alleles of *gcr1* (*gcr1-1*), *gpa1* (*gpa1-3*), and *agb1* (*agb1-1*). The effect of ABA was visible on *agb1*, *agb1 gcr1*, *agb1 gpa1*, and *agb1 gcr1 gpa1* mutants at as low as 0.5 μM ABA and at 5 μM, the growth of germinated seedlings was completely arrested (data not shown).

In contrast to germination, the later stages of the postgermination growth of G-protein complex mutants was also affected by 0.4 M sorbitol (especially for the *agb1-2* mutant and its combinations), possibly showing their differential responsiveness to osmotic stress. After 12 d of growth, all the mutants lacking the Gβ subunit were very dark green and accumulated large amounts of anthocyanin compared to wild-type Col plants (data not shown).

Inhibition of primary root growth is another classic response mediated by ABA. Presence of ABA affects both cell extensibility and cell division during primary root growth (Finkelstein et al., 2002). Under normal growth and development conditions with no exogenous ABA, the primary roots of all the G-protein complex mutants were not significantly different from the wild-type Col plants from the initial period of postgermination growth until the primary roots were about 3 cm long (Fig. 5B; J.G. Chen and A.M. Jones, unpublished data). However, exogenous ABA treatment affected the primary root growth of the various mutants differentially (Fig. 5B). Interestingly, the effect of ABA on primary root growth of G-protein mutants was dependent on the stage the germinated seeds were transferred to the ABA-containing media plates. Under conditions when seeds were germinated on control MS media for 24 h followed by transfer to 2 μM

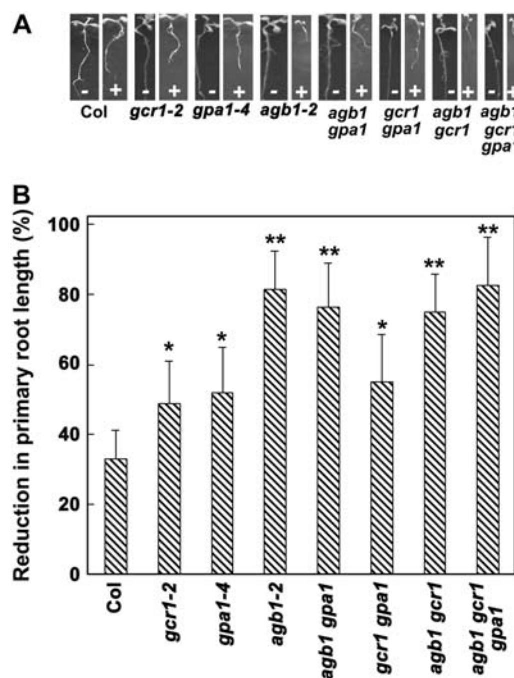

Figure 5. Effect of ABA on postgermination growth of G-protein complex mutants. Seeds germinated on 0.5× MS media were transferred after 24 h to control plates (no ABA) or 1 μM ABA-containing media plates under similar growth conditions and growth was recorded after 5 d (A). Representative seedlings from control and ABA-containing plates are shown. B, Percentage reduction in length of primary roots by ABA in different mutant genotypes. Each value represents average length of 20 seedlings. The experiment was repeated twice independently. Data from one experiment are presented. Error bars represent SD. For the difference in root length without and with ABA, $P < 0.001$ for *gcr1*, *gpa1*, and *gcr1 gpa1* and <0.0005 for all the genotypes with *agb1* mutation relative to Col, as determined by *t* test. Single asterisks (*) represent significant (<0.005) and double asterisks (**) represent highly significant (<0.001) values.

ABA-containing media, the primary roots of single *gcr1*, *gpa1*, and *agb1* mutants as well as all the double and triple mutant combinations showed hypersensitivity to ABA inhibition of primary root elongation compared to wild-type Col plants. After 8 d of growth on media containing 2 μM ABA, Col plants showed about 35% reduction in root length compared to plants growing on control media. The inhibition was 50% to 55% for the genotypes lacking either *GCR1* and *GPA1* alone or in combination, whereas plants lacking the Gβ subunit (single *agb1*, double *agb1 gpa1*, *agb1 gcr1*, and triple *agb1 gcr1 gpa1* mutants) showed the greatest (80%–85%) reduction in root length (Fig. 5B) compared to the plants growing on control media. Root growth was severely arrested at 5 μM ABA. However, if seeds were allowed to grow on the control media for 60 h after transfer to 22°C, and then transferred to the ABA-containing plates, the primary roots were not as responsive to exogenous ABA. Comparable inhibition of root growth could only be observed at much higher ABA concentrations. Wild-type Col plants showed about 50% reduction in root growth at 20 μM ABA, compared to growth on control media at 8 d (Supplemental Fig. 4). Inhibition of primary root length of the G-protein mutants was different under these conditions compared to their growth when transferred to ABA after 24 h postgermination (Fig. 5B). Seedlings lacking *GCR1* and *GPA1* or both continued showing a slight hypersensitive response to ABA compared to the wild-type Col plants, however, plants lacking the Gβ subunit (single, double, or triple mutants) were no longer hypersensitive to ABA. In fact, under some conditions, plants lacking AGB1 showed slight insensitivity to ABA compared to Col roots (Supplemental Fig. 4). This altered sensitivity to ABA under different conditions could be a developmental stage-dependent phenomenon. Although the primary root lengths of all the G-protein mutants are not significantly different during early development until the roots are about 3 cm long (Supplemental Fig. 4, control), during later stages, plants lacking a functional *AGB1* gene have a higher rate of root growth compared to wild-type Col plants (J.G. Chen and A.M. Jones, unpublished data), leading to longer primary roots in these mutants. Thus it appears that once the seeds pass the postgermination stage, an increased rate of root growth, independent of ABA, compensates for the ABA hypersensitivity observed with younger seedlings of mutants lacking *AGB1*. Alternatively, the specific functional role of AGB1 in root ABA signaling may change with seedling age.

Development of lateral roots in Arabidopsis is dependent on the integration of signals from auxin, ABA, and nutrient availability (Casimiro et al., 2003). However, whether ABA inhibition of lateral root formation is altered or affected in G-protein complex mutants has not been evaluated. As seen in Figure 6, on control media, all the mutant plants lacking the Gβ subunit had almost 200% more and longer lateral roots compared to wild-type plants (an average of 10.8 lateral roots in Col compared to an average of 21.8 lateral roots in mutant plants lacking the Gβ subunit). Plants lacking the Gβ subunit also had more adventitious roots compared to the wild type. The lateral root number of single *gcr1*, *gpa1*, and the double *gcr1 gpa1* mutants were not significantly different from the wild-type Col plants. The *gpa1* phenotype is different from what has been reported previously (Ullah et al., 2003); this could be due to ecotypical differences (ecotype Wassilewskija versus Col) or due to different growth conditions used.

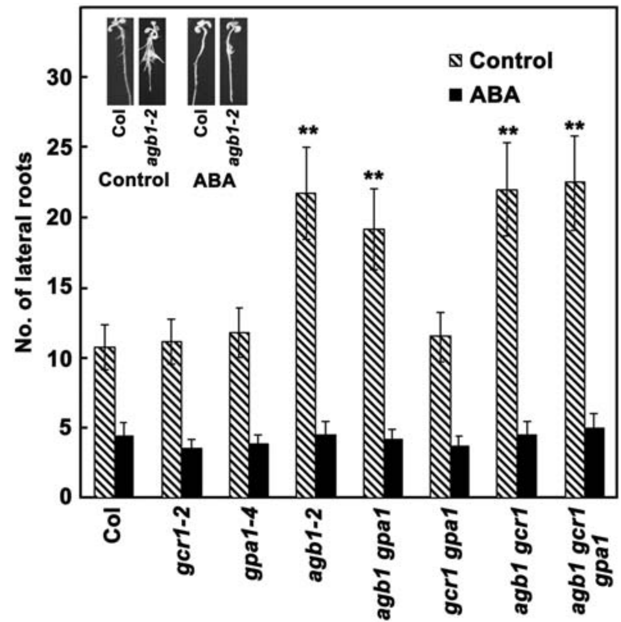

Figure 6. Inhibition of lateral root growth in G-protein complex null mutants by ABA. Seeds germinated on 0.5 × MS plates were transferred after 60 h to control plates (no ABA) or plates containing 2 μM ABA and seedlings were allowed to grow vertically for 12 d under 16-h light/8-h dark condition. Number of lateral roots was counted. Figure shows number of lateral roots in different genotypes in presence or absence of ABA. Inset shows representative Col and *agb1-2* mutant seedlings without or with 2 μM ABA treatment. Each experiment represents the average value from 40 seedlings. The experiment was repeated twice independently and data were averaged. Error bars represent SD. For difference in number of lateral roots without and with ABA, $P > 0.5$ for *gcr1*, *gpa1*, and *gcr1 gpa1* and <0.0005 for all the genotypes with *agb1* mutation relative to Col, as determined by *t* test.

The presence of 2 μM ABA drastically affected the number of lateral roots and all the plants showed very few (approximately 2 lateral roots/cm of primary root) lateral roots irrespective of the genotype (Fig. 6). Thus, due to the initial difference in the number of lateral roots, the changes were more significant in the genotypes lacking the Gβ subunit (Fig. 6). The reduction in number of lateral roots was about 50% for the Col and *gcr1*, *gpa1*, and *gcr1 gpa1* mutant plants compared to more than 80% inhibition for the mutant plants lacking the Gβ subunit. Phenotypes of typical Col and *agb1-2* plants in the absence and presence of ABA are shown in Figure 6 (inset).

We extended the work of Ullah et al. (2003) by showing that ABA inhibition of lateral root formation was independent of exogenous auxin. In the presence of 100 nM exogenous napthalene-1-acetic acid (NAA), the wild-type Col as well as mutant plants developed approximately 4 times more lateral roots (Fig. 7A). However, 1 μM ABA inhibited lateral root formation even in the presence of NAA (De Smet et al., 2003). It should be noted that the plants remained responsive to NAA in the presence of ABA, as shown by a reduction in length of primary roots in 100 nM NAA compared to no NAA treatments (Fig. 7B). NAA sensitized roots lacking *AGB1* to ABA. When AGB1 was absent, root growth was inhibited approximately 3-fold by exogenous ABA.

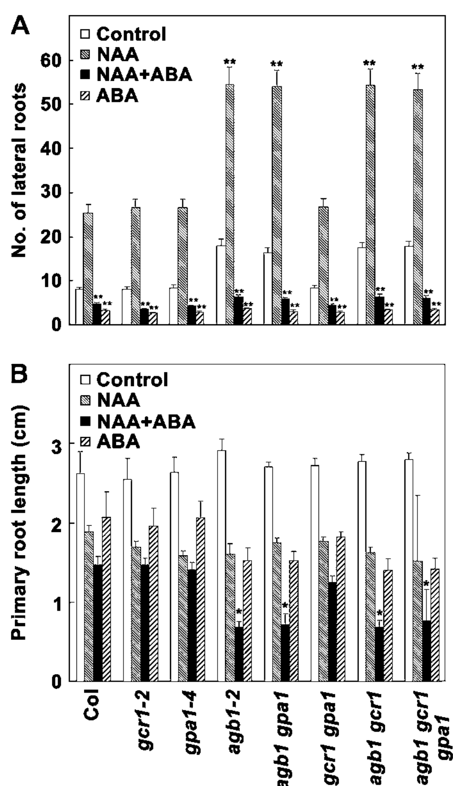

Figure 7. Effect of ABA in the presence of auxin on lateral root formation in G-protein complex mutants. Seeds germinated on 0.5 × MS plates were transferred after 60 h to control plates (no ABA) or 0.5 × MS plates containing 2 μM ABA, 100 nM NAA, or both. Seedlings were allowed to grow vertically for 12 d in 16-h light/8-h dark condition and number of lateral roots (A) as well as length of primary root (B) was measured. Each experiment represents the average value from 40 seedlings. The experiment was repeated twice independently and data were averaged. Error bars represent SD. For number of lateral roots in the presence of NAA, $P > 0.5$ for *gcr1*, *gpa1*, and *gcr1 gpa1* and <0.0001 for all the genotypes with *agb1* mutation, relative to Col. For number of lateral roots in the presence of ABA alone or both NAA + ABA, $P < 0.0001$ for all the genotypes compared to in the presence of NAA alone and $P > 0.5$ for all genotypes relative to Col as determined by *t* test. For inhibition of primary root length in the presence of NAA or ABA, relative to control media $P < 0.001$ for all the genotypes. In the presence of both NAA and ABA, $P > 0.5$ for *gcr1*, *gpa1*, and *gcr1 gpa1* and $P < 0.001$ for all the genotypes lacking *agb1* subunit, compared to Col as determined by *t* test. Single asterisks (*) represent significant (<0.005) and double asterisks (**) represent highly significant (<0.001) values.

G-Protein Complex Mutants Are Hypersensitive to ABA Induction of Gene Expression

A number of genes serve as molecular markers for ABA and stress-responsive pathways in plants. Genes such as *Kin1* and *Rd29A* are responsive to a number of different stresses, whereas genes like *Rab18* are more exclusively regulated by ABA (Ghelis et al., 2000). Since in this study the G-protein complex mutants showed hypersensitivity to a number of ABA-regulated responses, we investigated the expression profiles of key stress- and ABA-regulated genes (Seki et al., 2002). To accurately compare responses of all genotypes under current experimental conditions (including *gcr1*, which was previously analyzed under slightly different conditions; Pandey and Assmann, 2004), all genotypes were grown under identical conditions to the postgermination stage. For the *Rab18* gene (Fig. 8), the mutants lacking AGB1 showed significantly more hypersensitivity to ABA induction of gene expression (approximately 250-fold) compared to the *gcr1*, *gpa1*, or *gcr1 gpa1* double mutant plants (approximately 75- to 100-fold) or wild-type Col plants (approximately 50-fold), consistent with our observations on germination and postgermination growth. However for other genes, while in general the increase in transcript level of a given gene was greater in the G-protein complex mutant plants compared to Col plants, each gene had its own distinctive expression profile. For example, for the *Rd29A* gene (Fig. 8), *gcr1* mutants showed only 2-fold more expression compared to Col plants (approximately 10-fold induction by ABA in both *gcr1* mutants compared to approximately 5-fold induction in Col), whereas all the other mutant genotypes showed approximately 20- to 25-fold induction in the presence of ABA. Data obtained with other genes tested are summarized in Table I.

DISCUSSION

Interaction of G-Protein Complex Components during Seed Germination

Seed germination involves a complex interplay of positive and negative regulatory signals. Of particular interest is the antagonism between the phytohormones GA and ABA (Koornneef et al., 2002). Genetic screens performed in past years have shown that in many cases the same loci are involved in responsiveness to multiple signals, e.g. *aba* mutants have been obtained in screens for suppressors of nongerminating GA-deficient lines and screens designed to obtain sugar-insensitive mutants have identified *abi* mutant alleles (Finkelstein et al., 2002 and refs. therein).

Mutation of the Gα genes of Arabidopsis and rice (*Oryza sativa*) has shown a role for Gα in GA signaling

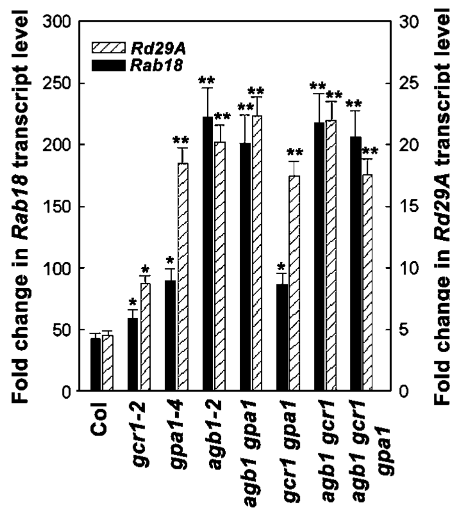

Figure 8. Expression of stress-induced genes in G-protein complex null mutants by ABA. Five-day-old seedlings were treated with ethanol (control) or 100 μM ABA for 1 h and cDNA was synthesized. cDNA was used for real-time PCR in the presence of SYBR-green dye. Data are expressed as fold change in response to ABA based on $2^{-\Delta\Delta\ Ct}$ values ($\Delta\Delta$ Ct = Δ Ct specific gene expression $-$ Δ Ct *Actin* gene expression). The experiment was repeated three times and data were averaged. Error bars represent SD. Single asterisks (*) represent significant (<0.005) and double asterisks (**) represent highly significant (<0.001) values for change in ABA-induced gene expression compared to Col as determined by *t* test.

(Ueguchi-Tanaka et al., 2000; Ullah et al, 2002; Chen et al., 2004) and Arabidopsis Gα mutants also show moderate ABA hypersensitivity in seed germination (Ullah et al., 2002; Lapik and Kaufman, 2003). The data presented in this report augment our knowledge about the roles of the G-protein complex proteins in responses to two negative germination signals: ABA and Glc. Loss of either GPA1 or AGB1 confers ABA and Glc hypersensitivity (Figs. 2–4), suggesting that in wild-type plants both subunits participate in inhibiting relay of these negative germination signals. Since embryonic ABA induces dormancy, whereas externally applied ABA affects seed development and germination, in the nonstratified seeds both these factors play a role together leading to higher hypersensitivity for individual genotypes, compared to the stratified seeds (Leubner-Metzger, 2003). Lack of the AGB1 subunit makes the seeds somewhat more hypersensitive to ABA and Glc compared to lack of the GPA1 subunit alone (Figs. 2 and 3), and the phenotype of the *agb1 gpa1* double mutant is more similar to that of *agb1* than to that of *gpa1*. One interpretation of the epistasis analyses is that *AGB1* acts downstream of *GPA1* in this signaling pathway, rather than at the same level, as in the classic paradigm of G-protein signaling. Our data, however, show that the *GPA1* transcript or protein could not be detected prior to germination (Fig. 1) in the stratified seeds, although low levels of *GPA1* transcript and protein were present in nonstratified seeds. Available public microarray data show that *GPA1* transcript is undetectable in stage 10 seeds (the green cotyledon stage of embryogenesis), which is the final stage of seed development preceding desiccation (Bowman, 1994). In ecotype Landsberg *erecta* of Arabidopsis (L*er*) seeds, *GPA1* transcript is present in dry seeds and during imbibition, and is moderately down-regulated by cold treatment (L*er* seeds, 4°C versus 22°C; www.genevestigator.ethz.ch). Currently there are no public data available that compare the exact conditions under which we tested *GPA1* expression levels; however, our data indicate that GPA1 levels in Col seeds are down-regulated by the stratification treatment, which would be consistent with the down-regulation by cold observed in L*er* seeds.

Absence of GPA1 during germination of stratified seeds raises the interesting possibility that the role of GPA1 during germination could result from an inherited epigenetic effect of the presence or absence of GPA1 on the expression of other genes whose products subsequently regulate germination (Baroux et al., 2002). It is interesting to note that at least one of the mammalian Gα genes, *Gnasx1*, has been shown itself to be subject to imprinting, and that loss of appropriate imprinting results in the human disease pseudohypoparathyroidism (Weinstein et al., 2002; Liu et al., 2005). Alternatively, the effects of the absence of GPA1 on seed germination could result from a parental effect such as altered seed provisioning or altered seed coat properties (Finkelstein, 1994; Zhang, 1998; Munir et al., 2001; Delphi and Mutikainen, 2003) in the *gpa1* lines.

Free G$\beta\gamma$ subunits may have a more direct role, as *AGB1* transcripts could be detected throughout the germination process, and plants harboring lesions in *AGB1* showed the strongest phenotype. ABA treatment did not affect the transcript levels of *GCR1*, *GPA1*, or *AGB1* during germination (Fig. 1C), suggesting that the differential response of G-protein mutants versus wild type in ABA regulation of seed germination does not result from ABA control of subunit abundance.

Table I. *Expression of some stress-induced genes in G-protein complex null mutants by ABA*

Values shown here represent ratio of change in expression of a gene in G-protein complex mutant relative to change in expression of the gene in wild-type Col plants (always defined as +). The experiment was repeated three times and data were averaged. Fold change values between 1 and 2 are represented by ++, between 2 and 3 are represented by +++, between 3 and 4 are represented by ++++, and values corresponding to 4 and above are represented by +++++.

Genotype	*ERD10*	*COR47*	*Dreb2A*	*ABI3*	*ABI5*
Col	+	+	+	+	+
gcr1-2	++	++	++	++	+++
gpa1-4	++	+++	++++	++	++
agb1-2	++++	++++	+++++	+++++	++++
agb1 gpa1	+++	+++	++++	++	++
gcr1 gpa1	++	+++	++	++	+++
agb1 gcr1	+++	+++++	++++	++	++++
agb1 gcr1 gpa1	+++	+++	+++	++++	++

Lack of GCR1 also leads to ABA and Glc hypersensitivity of seed germination (Figs. 2–4), whereas overexpression of GCR1 causes reduced seed dormancy (Colucci et al., 2002). According to the classical scenario for G-protein cycling, whereby GPA1 and AGB1 function downstream of the GPCR, one would predict by epistasis analysis that addition of the *gcr1* mutation would not alter the phenotypes of the *gpa1* and the *agb1* mutants. This prediction was supported: the *gcr1 gpa1* double mutant exhibited the *gpa1* phenotype while the *agb1 gcr1* and *agb1 gcr1 gpa1* mutants exhibited the *agb1* phenotype (Figs. 2–4). Thus for ABA and Glc repression of germination, GCR1, GPA1, and AGB1 all act in the same pathway. By contrast, for GA and BR regulation of seed germination, GCR1 and the heterotrimer seemingly act in separable, parallel pathways (Chen et al., 2004). Therefore, our results demonstrate that even in a single developmental process (germination) and in the same cell/tissue type, the components of the G-protein signaling complex can show different genetic interactions depending on the nature of the initial hormonal stimulus. Additionally, our results indicate a positive coupling between GCR1 and the G-protein subunits in ABA regulation of seed germination. This result is opposite to the negative coupling found in guard cells, where *gpa1* mutation confers reduced ABA sensitivity and *gcr1* mutation confers enhanced ABA sensitivity (Pandey and Assmann, 2004), indicating that for the same upstream signal (ABA) the regulatory role of GCR1 is cell and tissue dependent.

Interaction of G-Protein Complex Components during Postgermination Growth and Early Seedling Development

Genetic interaction between G-protein complex components was also observed for postgermination growth responses mediated by ABA, such as reduction in primary root length (Fig. 5). The predominance of the *agb1* mutation in conferring a strong phenotype is similar to the result obtained for seed germination, at least under the conditions when germinated seeds are exposed to exogenous ABA. However, if the seeds are allowed to grow on control media until the postgermination growth is complete, and then exposed to exogenous ABA, seedlings lacking either GCR1, GPA1, or both (*gcr1 gpa1* double mutant) continue to show ABA hypersensitivity but not the seedlings lacking AGB1. Moreover, the predominance of AGB1 in regulating the root length is also not observed in seedlings that have completed postgermination growth on ABA-free media (Supplemental Fig. 4). Interestingly, in the young seedlings, unlike in pregermination and newly germinated seeds, GPA1 protein is expressed (compare with Figs.1B, 2D, and 3D). These results thus imply that during germination and early postgermination growth, Gβ (or more likely the G$\beta\gamma$ dimer) is primarily responsible for the negative regulation of ABA/Glc response (i.e. the inhibition of an inhibitor). However, the scenario for the young seedling differs, possibly indicating that Gα acts as the primary transducer of the ABA signal at this stage, with Gβ or G$\beta\gamma$ acting via interaction with Gα. Thus, the primary transducer of ABA signal may differ in different developmental stages of the same tissue/organ, a conclusion also supported by comparison of the data in Figure 5 and Supplemental Figure 4.

How might loss of Gα affect signaling via G$\beta\gamma$ in germinating seeds, especially given that, once the G protein is activated, Gα is not thought to physically interact with G$\beta\gamma$? One possible model is that the signal is indeed primarily transduced by G$\beta\gamma$, but the transduction occurs when G$\beta\gamma$ is in the intact heterotrimer, a geometry in which the absence of Gα could interfere with G$\beta\gamma$ achieving its appropriate signaling conformation. Such a scenario is thought to be rare for metazoan G-protein signaling, but has been described for yeast, with a nondissociable heterotrimer (Klein et al., 2000; Levitzki and Klein, 2002). An alternative model is that loss of Gα results in mislocalization of Gβ and thus reduced efficiency of G$\beta\gamma$ signaling. Further research is required to distinguish between these possibilities.

During the formation of lateral roots, both ABA and auxin integrate the signals for cell division and cell expansion (Friml et al., 2002; Casimiro et al., 2003; Ullah et al., 2003). In the absence of ABA, all the plants lacking AGB1 (*agb1*, *agb1 gpa1*, *agb1 gcr1*, and *agb1 gcr1 gpa1*) showed increased lateral root formation, compared to wild type, regardless of whether or not auxin was applied. ABA strongly inhibited the formation of lateral roots in the presence of auxin although plants retain their ability to respond to auxin (Fig. 7B) and this is true for both wild type and mutants. Because the mutants lacking *agb1* have the greatest number of lateral roots, the percent reduction of lateral roots by ABA is greatest in these genotypes, even though the absolute number of lateral roots under plus ABA conditions is similar across all nine genotypes. This implies that either the role of G proteins during ABA inhibition of lateral root formation is upstream of their role during auxin promotion of lateral root formation or that ABA inhibition of lateral root development is parallel and independent of auxin effects on this process. De Smet et al. (2003) have also provided evidence for a checkpoint in lateral root development that is auxin independent and ABA sensitive.

Regulation of ABA-Induced Gene Expression in G-Protein Complex Mutants during Postgermination Growth and Development

In general, the plants lacking one or more subunits of the G-protein complex showed hypersensitivity to ABA in transcript abundance compared to the Col plants (Table I), confirming the previously suggested role of GCR1 as a G-protein-coupled negative regulator of ABA induction of gene expression during postgermination growth (Assmann, 2004), and indicating

that GPA1 and AGB1 are also negative regulators. However, unlike the above-described phenotypic analyses, the overall picture for transcript levels does not show uniformly greater ABA response in mutants containing the *agb1* mutation; rather the effect of the G-protein complex mutation on transcript level seems to be gene specific (Fig. 8; Table I). This lack of direct correlation between the gene expression data versus other phenotypic data could be due to the use of whole seedlings for gene expression analyses, thus precluding observation of any tissue-specific effects, or due to involvement of posttranscriptional regulation during ABA signaling. Interestingly, the ABI3 and ABI5 transcription factors show higher expression in the G-protein mutants than in the wild-type plants (Table I). ABI3/ABI5 factors are an interaction point between these ABA and auxin signaling pathways (Suzuki et al., 2001; Brady et al., 2003). These proteins, however, act at the level of transcription and the integration of these two signals may be an earlier event. We propose that G proteins might be the nodal points for such signal convergence (Assmann, 2004).

Signaling Mechanisms of G-Protein Complex Components in Arabidopsis

Despite the reduced set of G-protein components in plants compared with mammalian systems (Jones and Assmann, 2004), G-protein involvement has now been shown in a multitude of processes including cell division (Ullah et al., 2001; Chen et al., 2003), ion channel regulation (Wang et al., 2001; Coursol et al., 2003), seed germination (Ullah et al., 2002), biotic and abiotic stress responses (Joo et al., 2005; Llorente et al., 2005; Trusov et al., 2006), and blue light-mediated response (Warpeha et al., 2006). Combining previous work using null mutants of the Arabidopsis G-protein complex components and the new observations in this manuscript leads to the emergence of several scenarios. (1) For some phenotypes, e.g. leaf shape, loss of either *gpa1* or *agb1* results in a similar phenotype. This could occur in at least three ways. First, the response may be transduced by the Gα subunit (GPA1). In this case, loss of the Gβ subunit (AGB1) can have a phenotypically similar effect to loss of Gα when loss of Gβ results in improper Gα localization and/or protein-protein interaction; this is commonly observed in animal systems (Evanko et al., 2000; Schwindinger and Robishaw, 2001; Cabrera-Vera et al., 2003). Second, the response may be requisitely mediated by both GPA1 and AGB1 interactions with downstream effectors, in a manner such that loss of just one of these subunits is sufficient to confer the mutant phenotype. Third, the response may be, unconventionally, transduced by the intact heterotrimer (Levitzki and Klein, 2002). (2) Some phenomena, such as root mass of adult plants (Ullah et al., 2003), show opposite responses to loss of GPA1 versus AGB1. This result is interpreted to signify that loss of GPA1 increases the abundance of free G$\beta\gamma$ subunits available to interact with downstream effectors (Offermanns and Simon, 1998; Mirshahi et al., 2002) while loss of AGB1 de facto prevents such interactions. (3) Some phenomena support functional coupling of GCR1 with GPA1, while others do not. Our previous work has shown that phenotypes of single *gpa1* and *gcr1* mutants could be opposite, supporting negative regulation of GPA1 by GCR1 (Pandey and Assmann, 2004), possibly independent (e.g. GA- and BR-related seed germination phenotypes; Chen et al., 2004), or similar, supporting positive coupling between GCR1 and GPA1 (e.g. ABA- and sugar-related germination phenotypes; this article). These studies emphasize that, given the limited number of heterotrimeric G-protein components in plants, the interaction between these components within the cell-specific environment and developmental stage appears to be paramount in determining the explicit nature of signal-response coupling.

MATERIALS AND METHODS

Plant Material

All the mutants used in this study were in the Col background and have been described previously (Lease et al., 2001; Jones et al., 2003; Ullah et al., 2003; Chen et al., 2004). The single mutant alleles used for this study were *gcr1-1*, *gcr1-2*, *gpa1-3*, *gpa1-4*, *agb1-1*, and *agb1-2*. The *gcr1-2*, *gpa1-4*, and *agb1-2* alleles were used for generating double mutants between *gcr1 gpa1*, *agb1 gcr1*, *agb1 gpa1*, and a triple mutant *agb1 gcr1 gpa1*. The locus identifier for *GCR1* is At1g48270, for *GPA1* is At2g26300, and for *AGB1* is At4g34460.

Seed Germination Assays

The germination capacity of seeds is greatly influenced by temperature, humidity, light, chilling (stratification), and storage conditions (Bewley, 1997). Thus, all these factors must be controlled in comparative studies on germination. For this study we used seed lots that were produced, harvested, and stored under identical conditions for 6 months before the experiments. Sixty seeds from each genotype, wild-type Col, *gcr1-1*, *gcr1-2*, *gpa1-3*, *gpa1-4*, *agb1-1*, *agb1-2* single mutants, and *agb1 gpa1*, *gcr1 gpa1*, *agb1 gcr1*, and *agb1 gcr1 gpa1* plants were plated on the same plate (150 mm). In all cases, seeds were surface sterilized and sown on 0.8% agar (Sigma) containing 0.5× MS salts (Sigma), and 1% (w/v) Suc, chilled at 4°C in the dark for 48 h (stratified), and germinated at 22°C, in 16-h light/8-h dark conditions (light intensity 120 μmol·m^{-2}·s^{-1}), or directly moved to a 22°C, 16-h light/8-h dark regime (nonstratified). For ABA (AG Scientific) treatments, indicated quantities of ABA were also included in the MS media. To study the effect of sugars, seeds were germinated on the same media combination, also containing 6% (w/v) Glc (equivalent to 0.33 M Glc) or 0.4 M sorbitol. To study the effect of fluridone (Flur, Chem Service), an inhibitor of ABA biosynthesis, seeds were surface sterilized and pretreated with 100 μM Flur and 0.01% (v/v) Tween 20 (Sigma) for 48 h in darkness. As a control, seeds were pretreated with water under identical conditions. Seeds were then washed extensively with water and sown on plates with desired media combinations. Germination is defined here as an emergence of the radicle through the seed coat. Postgermination growth was recorded after 5 d of growth on the indicated media combinations. The experiments were repeated at least twice. The data shown are averages of all the experiments ± SD, unless stated otherwise.

Root Growth Assays

Seeds were germinated on MS media plates as described above and transferred to plates without (control) or with ABA and/or NAA (Sigma), 24 or 60 h after transfer to 22°C. For inhibition of primary root growth by ABA or NAA, seedlings were grown vertically in 16-h light/8-h dark conditions and root lengths were recorded after 8 d. For inhibition of lateral root growth, the

number of lateral roots and developing primordia was counted after 12 d of growth on control, ABA, or NAA plates under a dissecting microscope.

Expression Studies by Relative, Quantitative Reverse Transcription-PCR

To study the expression of *GCR1*, *GPA1*, and *AGB1* during germination, seeds were frozen at different time points. Total RNA was isolated and reverse transcriptase, real-time quantitative PCR was performed essentially according to Pandey and Assmann (2004). To study the effects of ABA on gene expression, 5-d-old Arabidopsis (*Arabidopsis thaliana*) seedlings (postgermination stage) were treated with 100 μM ABA (stock solution in ethanol) or sterile distilled water containing equimolar concentration of ethanol, and seedlings were harvested 1 h later. The sequences of primers used for amplification were as follows: *GCR1* forward (5′-atgggcattcggcattatta-3′) and *GCR1* reverse (5′-tggaagccatcgatatagcc-3′); *GPA1* forward (5′-atgagaata-cacacgctgct-3′) and *GPA1* reverse (5′-tctgatagacattggcatga-3′); *AGB1* forward (5′-tctggtaccggaatggctgtc-3′) and *AGB1* reverse (5′-ttcgttggatcctcttcaaat-3′); *RD29A* forward (5′-atcacttggctccactgttgttc-3′) and *RD29A* reverse (5′-acaaaa-cacacataaacatccaaagt-3′); *RAB18* forward (5′-cagcagcagtatgacgagta-3′) and *RAB18* reverse (5′-cagttccaaagccttcagtc-3′); *DREB2A* forward (5′-aaggtaaag-gaggaccagag-3′) and *DREB2A* reverse (5′-acacaaccaggagtctcaac-3′); *ERD10* forward (5′-tctctgaaccagagtcgttt-3′) and *ERD10* reverse (5′-cttcttctcaccgtctt-cac-3′); *ABI3* forward (5′-aattaccgccagtgatggag-3′) and *ABI3* reverse (5′-aaa-acgatccttccgaggtt-3′); *ABI5* forward (5′-acctaatccaaacccgaacc-3′) and *ABI5* reverse (5′-agcaaacacctgcctgaact-3′); *MPK3* forward (5′-tcacaatgaggatgcga-aaa-3′) and *MPK3* reverse (5′-attcgggtcgtgcaatttag-3′); and *MPK5* forward (5′-gcgaaggaaattgaatcagc-3′) and *MPK5* reverse (5′-tcgcaatctcttcgtgtgtc-3′). Amplification of *ACTIN2/8* (forward primer 5′-ggtaacattgtgctcagtggtgg-3′ and reverse primer 5′-aacgaccttaatcttcatgctgc-3′) genes was used as an internal control (Charrier et al., 2002).

Protein Extraction and Western Blotting

Protein extraction and western blotting with anti-GPA1 antibodies was performed essentially according to Pandey and Assmann (2004).

ACKNOWLEDGMENTS

We thank Dr. Lei Ding for important discussions regarding the root growth experiments and Liza Wilson for excellent technical assistance.

Received February 10, 2006; revised March 17, 2006; accepted March 19, 2006; published March 31, 2006.

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