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Role of a Heterotrimeric G Protein in Regulation of Arabidopsis Seed Germination[1]

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Seed germination is regulated by many signals. We investigated the possible involvement of a heterotrimeric G protein complex in this signal regulation. Seeds that carry a protein null mutation in the gene encoding the alpha subunit of the G protein in Arabidopsis (*GPA1*) are 100-fold less responsive to gibberellic acid (GA), have increased sensitivity to high levels of Glc, and have a near-wild-type germination response to abscisic acid and ethylene, indicating that GPA1 does not directly couple these signals in germination control. Seeds ectopically expressing *GPA1* are at least a million-fold more responsive to GA, yet still require GA for germination. We conclude that the GPA1 indirectly operates on the GA pathway to control germination by potentiation. We propose that this potentiation is directly mediated by brassinosteroids (BR) because the BR response and synthesis mutants, *bri1-5* and *det2-1*, respectively, share the same GA sensitivity as *gpa1* seeds. Furthermore, *gpa1* seeds are completely insensitive to brassinolide rescue of germination when the level of GA in seeds is reduced. A lack of BR responsiveness is also apparent in *gpa1* roots and hypocotyls suggesting that BR signal transduction is likely coupled by a heterotrimeric G protein at various points in plant development.

Seeds integrate many intrinsic signals to control germination (Koornneef et al., 2002). For example, since the original observation by Chrispeels and Varner (1966), it has been repeatedly shown that GA induces germination and that abscisic acid (ABA) antagonizes the GA effect (Koornneef and Van der Veen, 1980; Karssen et al., 1989; Gilroy and Jones, 1994; Ritchie and Gilroy, 1998; Lovegrove and Hooley, 2000). Seed germination of GA synthesis mutants can be rescued by the application of GA and ABA synthesis and insensitive mutants lack the ABA inhibition of GA-induced germination as well as being viviparous (Koornneef et al., 1982, 1984; Finkelstein and Somerville, 1990; Koornneef and Karssen, 1994; Leon-Kloosterziel et al., 1996). Brassinosteroid (BR) probably acts downstream of GA because BR is able to rescue germination of GA-deficient (Steber and McCourt, 2001) and GA response (Steber et al., 1998) mutant seeds. These authors argue that the BR input is likely to reside upstream of ABA's attenuating effect on GA-induced germination because BR synthesis and response mutants have slightly altered ABA sensitivity.

The inhibitory effect of high concentrations of sugars on germination may occur via ABA. The evidence supporting this comes from measurements of ABA in Glc-treated seedlings (Arenas-Huertero et al., 2000) and from the observation that ABA synthesis and response mutants are insensitive to Glc (Laby et al., 2000; Rook et al., 2001). Ethylene controls the Glc inhibition of germination (Zhou et al., 1998). Evidence supporting this includes the observations that high concentrations of ethylene antagonize the Glc repression of germination (Ghassemian et al., 2000), the *ctr1* (constitutive ethylene response mutant; Gibson et al., 2001), and *eto1* (ethylene-overproducing mutant) are high-Glc insensitive, and the *etr1* (ethylene-insensitive mutant) is hypersensitive to high Glc levels (Zhou et al., 1998). Epistasis analysis between various sugar and ethylene-insensitive mutants predict that ABA functions downstream of ethylene consistent with the conclusion that ethylene blocks Glc-induced ABA synthesis (Beaudoin et al., 2000; Ghassemian et al., 2000).

The mechanism by which these many signals are integrated to control germination is only now being addressed. One possible mechanism involves coupling of and cross talk between signals by heterotrimeric G proteins. A heterotrimeric G protein may mediate many of the signals that seeds use to control germination (Assmann, 2002). For example, the Arabidopsis *gpa1* protein-null mutants completely lack ABA-induced inhibition of stomatal pore opening and K^+ inward channels (Wang et al., 2001a). The *D1* (*Dwarf1*) gene in rice (*Oryza sativa*) encodes an alpha subunit of a heterotrimeric G protein (Ashikari et al., 1999) and *d1* mutants require a 10-fold higher amount of GA to induce α-amylase activity to wild-type levels (Ueguchi-Tanaka et al., 2000). Additional evidence to indicate that a heterotrimeric G protein

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mediates GA signaling is that Mas7, an activator of Gα, is as effective as GA in stimulating α-amylase in aleurone cells (Jones et al., 1998). Preliminary evidence also suggested that *gpa1* mutants have altered sensitivities to ABA, ethylene, brassinosteroid, and Glc (Ullah et al., 2001). Because seeds integrate many of these same signals to control germination, we chose germination as our biological context in which to test a role for heterotrimeric G proteins in signal cross talk. The questions we address are: Which of these hormones are coupled by Gα, and which are not? In addition, we attempt to distinguish if GPA1 is directly coupling a hormone to a downstream response that leads to germination control or whether GPA1 indirectly controls the sensitivity toward that signal, in essence behaving as a potentiator, rather than a coupler. Because seed germination is the outcome of an integration of many signals, determination of the hormone sensitivities requires isolation of one hormone pathway from another, either pharmacologically or genetically. This was accomplished in some cases by blocking the synthesis of a hormone and measuring the response to that hormone and to others.

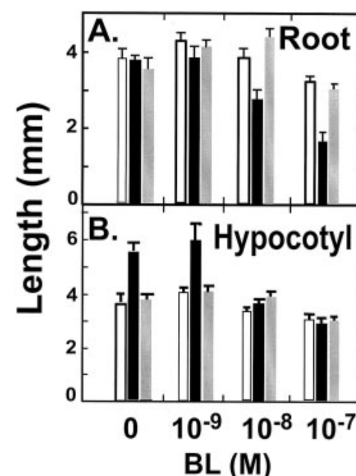

Figure 1. Altered brassinolide responsiveness in *gpa1* roots and hypocotyls. Roots length (A) and hypocotyl length (B) of 2-d-old etiolated Wassilewskija (Ws; black bars), *gpa1-1* (white bars), and *gpa1-2* (gray bars) seedlings grown on plates supplemented with the indicated concentrations of brassinolide (BR) were measured. The highest concentration used for the root assay was 10^{-7} M BR. The values represent the mean lengths from at least 10 seedlings. The error bar indicated is the SE of the mean. Student's *t* tests were performed pair wise on 0 and 0.1 μM BL treatments. The probability supporting the null hypothesis that values for wild-type root and hypocotyl lengths are same in both control and BL treatments is less than 0.001; for the null hypothesis being correct for the *gpa1* mutants is 0.2.

RESULTS

gpa1 Seedlings Have Altered Brassinolide and Sugar Sensitivities

During the early characterization of the *gpa1* seedlings (Ullah et al., 2001), it was found that the mutants may have qualitative differences in the responsiveness to brassinolide (BL), sugars, and aminocyclopropane-1-carboxylic acid (ACC); therefore, relative responsiveness to these compounds was quantitated here. Figure 1 shows that BL-inhibited growth of etiolated hypocotyls and roots is severely reduced in the *gpa1* mutants. In the presence of 0.1 μM BL, growth of wild-type roots and hypocotyls are reduced 40% and 50%, respectively, whereas this concentration of BL does not significantly inhibit growth of either hypocotyls or roots in the *gpa1* mutants. The reduced hypocotyl's length of the mutants in the absence of BL is sugar dependent. This is discussed further below.

Ethylene sensitivity in seedlings was determined by measuring the triple response of seedlings grown on plates supplemented with the ethylene precursor, ACC, and assayed 2 d after moving plates to 23°C. As shown in Figure 2A, the hypocotyls of *gpa1* mutants, like the wild-type control, became shorter and thicker. ACC-induced changes in hook angle for both wild-type and *gpa1* hooks were approximately 22% of the starting angle. Thus, based on the triple response, *gpa1* mutants have the same relative responsiveness to ethylene. Note, however, that the baseline and ACC-induced hook angles differ from wild type, indicating that although hooks are responsive to ACC, they are fundamentally different to wild type.

When *gpa1* mutants are grown on plates supplemented with the typical 1% (w/v) Suc, they are shorter than wild-type Ws seedlings grown in the dark for 2 d as previously reported (Ullah et al., 2001). The short hypocotyl in the *gpa1* mutants is due to a reduction by one-half in hypocotyl cell number, whereas the maximum cell lengths and length distribution along the hypocotyl are the same as for wild-type hypocotyls. If Suc is omitted, *gpa1* hypocotyl lengths are the wild-type values (Fig. 2B); thus, the short hypocotyl phenotype observed in the dark is Suc dependent. However, the increased length of *gpa1* hypocotyls on Suc dropout plates is due to compensation by cell elongation. Etiolated *gpa1* hypocotyls still have approximately one-half the number of cells as wild type, regardless of whether they are grown in the absence or presence of sugars (data not shown). Note also that the wild-type hook angle is reduced and similar to the mutant in the absence of sugars.

gpa1 Seeds Are Hypersensitive to Glc and Suc But Not Mannitol

It has been shown previously that mature *gpa1* leaves completely lack ABA inhibition of stomatal pore opening (Wang et al., 2001a). This observation, taken together with the current observations (Figs. 1 and 2), indicate that *gpa1* mutants are impaired in multiple signal pathways or that the defect is in a single pathway that impinges on other pathways.

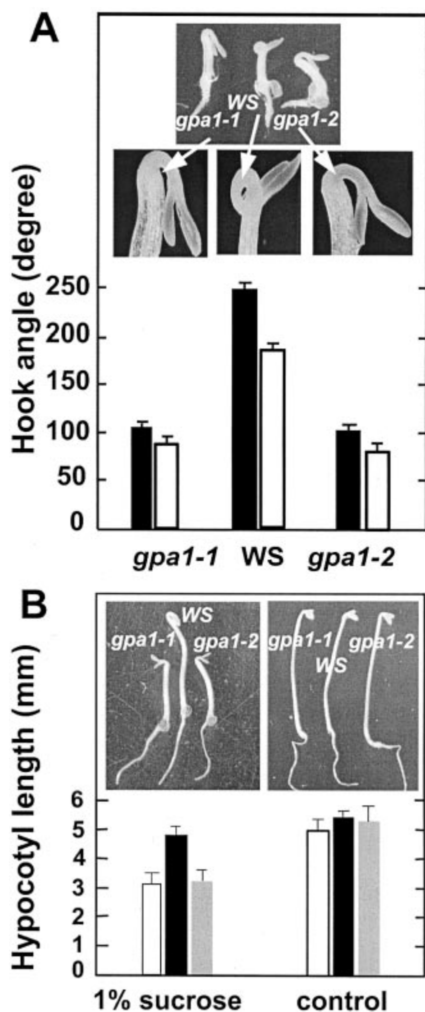

Figure 2. Altered sugar and ethylene sensitivity in *gpa1* mutants. A, Mean hook angle of seedlings grown in the dark for 2 d on 10 μM ACC. Insets show the whole seedlings representing the phenotype corresponding to the indicated genotype. Note that in the presence of ACC, hypocotyls of the *gpa1* mutants are shorter and thicker as are the wild type (WS). The percent change in hook angle for both wild-type and *gpa1* mutants in the presence of ACC is approximately the same. Solid bars, minus ACC; white bars, plus ACC. A minimum of 10 measurements were taken to generate each of the means. B, Mean hypocotyl lengths of 2-d-old dark-grown seedlings grown in the presence or absence (control) of 1% (w/v) Suc. Inset, Representative seedlings of the indicated genotype. Solid bars represent wild type (Ws ecotype), white bars represent *gpa1-1* and gray bars represent *gpa1-2* mutants. Error bars equal the SE. Each experiment was repeated at least five times with the same results.

Thus, to test this, we chose seed germination as the biological context because it involves a signaling network encompassing those signals that appear to have altered sensitivities in *gpa1*. Under normal conditions, fully after-ripened, stratified *gpa1* seeds germinate with 100% efficiency. The phenotype of *gpa1* seeds is only apparent after treatment of seeds with sugars, and as discussed below, under certain conditions.

The Suc dependency for the dark-grown short hypocotyls of the *gpa1* mutants suggested that these mutants have either altered sugar sensitivities or osmotic stress responses. To distinguish between these two possibilities and to more precisely quantify the phenotype in an all-or-nothing response, seed germination was assayed at various concentrations of Glc, Suc, and the non-metabolizable sugars mannitol and sorbitol. As shown in Figure 3, A and B, *gpa1* seeds are hypersensitive to Glc and Suc but behave as wild type on mannitol and sorbitol (Fig. 3, C and D), suggesting that the *gpa1* mutants are impaired in sugar signaling rather than osmotic stress sensing or signal transduction. Moreover, germination of *gpa1* seeds is inhibited similar to wild type by low concentrations of the toxic Glc analog, Man (Fig. 3E).

gpa1 Seeds Retain Wild-Type Sensitivity to ACC

Ethylene blocks the Glc inhibition of germination (Zhou et al., 1998). Evidence supporting this includes the observations that high concentrations of ethylene antagonize the Glc repression of germination (Ghassemian et al., 2000); the *ctr1* mutant (Gibson et al., 2001) and the *eto1* mutant are high-Glc insensitive; and the *etr1* mutant is hypersensitive to high-Glc levels (Zhou et al., 1998). To determine ethylene sensitivity in wild-type and *gpa1* seeds, germination was inhibited by 6% (w/v) Glc and the rescue of this inhibition by ethylene was scored at increasing con-

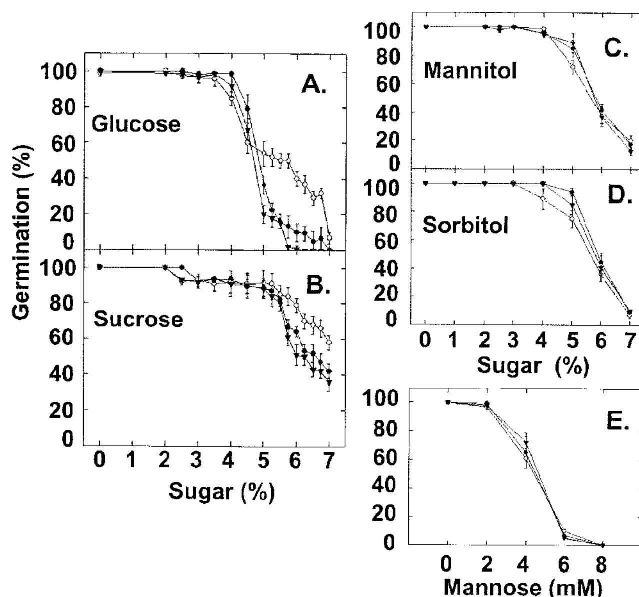

Figure 3. Sugar-specific hypersensitivity of *gpa1* seeds. Ws (white circles), *gpa1-1* (black circles), and *gpa1-2* (black inverted triangles) from matched seed lots were germinated on plates supplemented with the indicated concentration of Glc (A), Suc (B), mannitol (C), sorbitol (D), and Man (E) and scored for germination at times later. The percent germination at d 5 is shown. Each value is the mean percent germination based on at least 50 seeds and the error indicated is the SE.

centrations of ACC. Seeds were pretreated with ACC for 96 h at 4°C, then moved to 23°C under low-fluence, continuous white light. The results shown in Figure 4 represent germination scored at 7 d and confirms the sugar hypersensitivity of *gpa1* seeds and indicates that ACC is able to block hypersensitivity to wild-type levels, indicating that GPA1 does not couple ethylene regulation of sugar inhibition of germination.

gpa1 Seeds Mediate Suc-Induced ABA Inhibition Similarly to Wild Type

Arenas-Huertero et al. (2000) proposed that sugar inhibition of germination operates by increasing the ABA level. Although ABA levels in seeds were not specifically examined in that study, they showed that young seedlings grown on 7% (w/v) Glc have 3- to 6-fold higher levels of ABA. Their hypothesis fits the genetic data showing that ABA-biosynthetic and some response mutant seeds are insensitive to Glc (Beaudoin et al., 2000; Ghassemian et al., 2000). To determine if the hypersensitivity to Glc observed with *gpa1* seeds is operating via this ABA pathway, seeds were pretreated with the ABA synthesis inhibitor, fluridone, for 48 h at 4°C in the dark, and sown on plates supplemented with GA and 6% (w/v) Glc. Figure 5 shows that when endogenous ABA levels are reduced, the hypersensitivity to Glc in the *gpa1* seeds is eliminated. This is consistent with the hypothesis that the Suc effect is operating via ABA and that this pathway is normal in the *gpa1* seeds. However, we measured ABA levels in Glc-treated seeds and did not find a statistically significant difference in ABA levels 24 h after 6% (w/v) Glc treatment in either wild-type or *gpa1-2* seeds (Table I). Note also that the ABA pool size between untreated wild-type and *gpa1-2* seeds is the same.

The results of Figure 5 also indicate that GA can rescue the inhibitory effect of high levels of sugars. This suggests that either GA sensitivity or levels in the mutants are reduced. Alternatively, it may indicate that the antagonism of ABA on the GA pathway is increased in the mutants. This point is addressed in the following section.

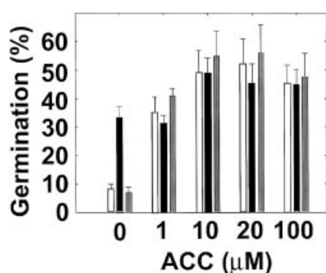

Figure 4. The effect of ACC on Suc-inhibited seed germination. Ws (black bars), *gpa1-1* (white bars), and *gpa1-2* (gray bars) were germinated in liquid cultures supplemented with the indicated concentrations of ACC and scored for germination times later. Media contained 6% (w/v) Glc to reduce germination. After 7 d at 23°C under low fluence, continuous white light, germination was scored and expressed as a percent of total seed. Error bars represent the SE.

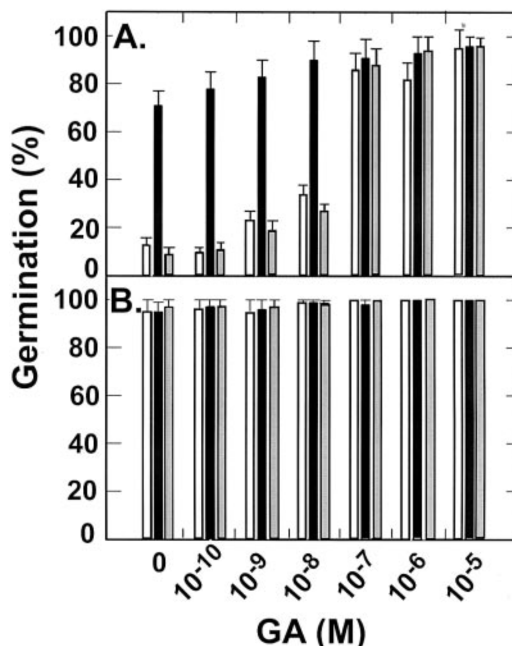

Figure 5. Role of ABA in Glc-inhibited germination. Ws (black bars), *gpa1-1* (white bars), and *gpa1-2* (gray bars) seeds were pretreated with water (A) or 100 μM fluridone (B) for 48 h at 4°C in the dark, washed, and sown on plates containing 6% (w/v) Glc and the indicated amounts of GA. After 48 h at 23°C in the dark, germination was scored and expressed as a percent of total seed. Error bars represent the SE.

gpa1 Seeds Have Near-Wild-Type Sensitivity to ABA

The sensitivity to ABA was measured in two ways: First, untreated seeds were germinated on plates supplemented with ABA between 0.5 and 10 μM. A second method was to reduce endogenous ABA levels and examine the effect of exogenous ABA over a broader concentration range. As shown in Figure 6A, when untreated seeds are germinated on plates supplemented with ABA, 50% germination occurs at approximately 3 and 4.5 μM ABA, for the *gpa1* mutants and Ws control, respectively, indicating that

Table I. *ABA levels in wild-type and gpa1 seeds*

Sterilized seeds were kept in darkness at 4°C for 2 d, moved to 3.0 mL of 1× Murashige and Skoog medium plus or minus 6% (w/v) Glc. After being cultured in light at 23°C for 24 h, the seeds and medium were lyophilized in the same tube used for incubation and the ABA contents were determined.

Treatment	ABA	
	Ws	*gpa1-2*
	nmol g dry wt^{-1}	
Control	3.26 ± 0.49	3.11 ± 0.88
6% (w/v) Glc	2.90 ± 0.35	3.16 ± 0.35

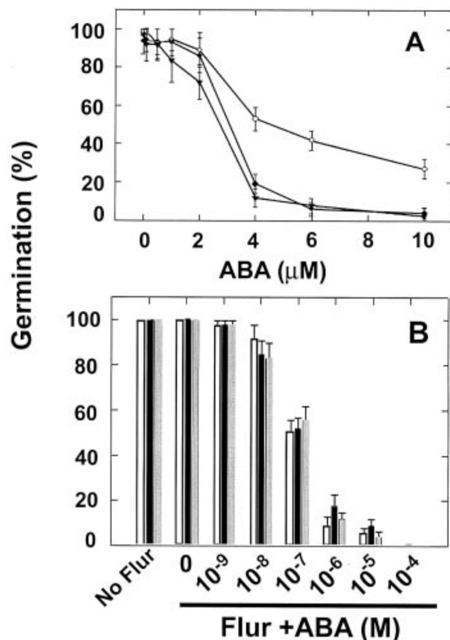

Figure 6. ABA sensitivity of *gpa1* seeds. A, Ws (white circles), *gpa1-1* (black circles), and *gpa1-2* (black inverted triangles) from matched seed lots were germinated on plates supplemented with the indicated concentration of ABA. After 48 h at 23°C under continuous white light, germination was scored and expressed as a percent of total seed. B, Ws (dark bars), *gpa1-1* (white bars), and *gpa1-2* (gray bars) seeds were pretreated with 100 μM fluridone or water ("No Flur") for 48 h at 4°C in the dark, washed, then sown on plates containing 6% (w/v) Glc and the indicated the amounts of ABA and scored as for A. Error bars in A and B represent the SE.

either *gpa1* seeds have more ABA or are hypersensitive to ABA. To more precisely determine ABA sensitivity and to analyze the effects of lower concentrations of exogenous ABA, the endogenous ABA levels were reduced using fluridone. Seeds were pretreated with 100 μM fluridone for 48 h at 4°C in the dark and then sown on plates supplemented with ABA from 1 nM to 100 μM and scored for germination as in Figure 6A. This concentration of fluridone has been shown to be effective at reducing ABA pool sizes (Lang and Palva, 1992; Grappin et al., 2000; Moreno-Fonesca and Covarrubias, 2001). After 48 h at 23°C in the dark, germination was quantified. Figure 6B shows that *gpa1* and Ws wild-type seeds have statistically similar ABA sensitivities. Therefore, the hypersensitivity of *gpa1* seeds to ABA in the absence of fluridone shown in Figure 6A must be due to different resting ABA levels or to an alteration in the GA pathway promoting germination. Direct measurements of ABA in the untreated wild-type and mutant seeds rules out the former possibility (Table I). Moreover, exogenous GA, as low as 10 pM, completely restores germination in the presence of 6% (w/v) Glc when GA pools are reduced with paclobutrazol (PAC; data not shown) or not (Fig. 5), consistent with the conclusion that the hypersensitivity to ABA in *gpa1* seeds is due to reduced GA responsiveness.

gpa1 Seeds Are Less Sensitive to GA

To directly test if *gpa1* seeds are altered in the GA response, seeds were germinated in the presence of the GA synthesis inhibitor, PAC, at 6 μM for 48 h at 4°C in the dark and then sown on plates supplemented with GA from 0.1 pM to 1 μM. After 48 h at 23°C in the dark, germination was scored. Figure 7A shows that *gpa1* seeds are at least 100-fold less re-

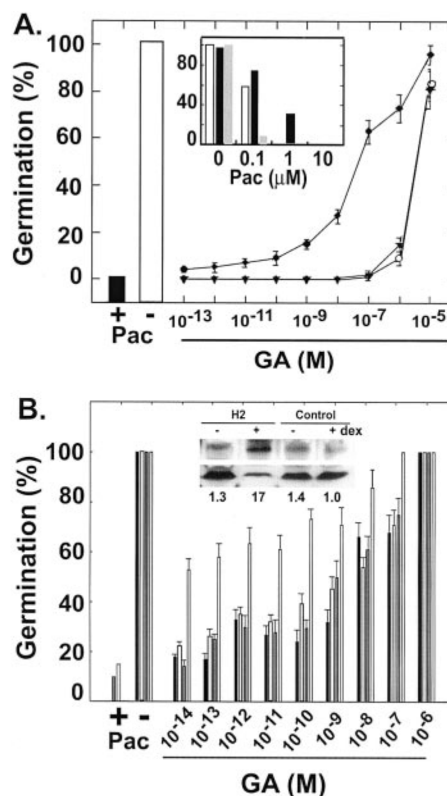

Figure 7. Role of GPA1 in GA signaling in seed germination. A, Ws (black circles), *gpa1-1* (white circles), and *gpa1-2* (black inverted triangles) from matched seed lots were pretreated with 10 μM PAC (Pac), washed, then sown on plates supplemented with the indicated concentration of GA. Seeds sown on plates without GA were either pretreated with PAC (dark bar) or water (white bar). After 48 h at 23°C, germination was scored and expressed as a percent of total seed. The inset sows the percent germination of Ws (black bars), *gpa1-1* (white bars), and *gpa1-2* (gray bars) seeds on plates supplemented with the indicated amount of PAC. B, Control seed transformed with vector only (black and white bars) and seed transformed with a dexamethasone (Dex)-inducible GPA1 construct (dark- and light-gray bars) were sown on plates containing 1 μM Dex (white and light gray bars) plus the indicated concentrations of GA. Seeds sown on plates without GA, but with the indicated (by bar color) presence or absence of Dex, were either pretreated with PAC (+) or water (−). Error bars in A and B represent the SEM. The inset illustrates the Dex-inducible expression of GPA1 as described in "Materials and Methods." Upper, Immunoblot probed with antiserum directed against recombinant GPA1 (Weiss et al., 1997); lower, nonspecific band recognized by the anti-GPA serum. Samples were either treated (+) or not (−) with Dex. The numbers below the lower panel represent the normalized ratio of GPA1 band intensity to the loading control band in the lower panel.

sponsive to exogenous GA. Consistent with this is the observation that *gpa1* seeds are hypersensitive to PAC (Fig. 7A, inset).

Inducible, ectopic expression of *GPA1* also indicated that GPA1 plays a role in GA signaling. A Dex-inducible *GPA1* line designated H2 (Ullah et al., 2001) was utilized to control GPA1 levels. Dex (1 μM) induces GPA1 levels 17-fold over vector-only control seedlings (Fig. 7B, inset). Control and H2 seeds were pretreated with PAC and sown on plates supplemented with GA as before and scored for germination. As shown in Figure 7B, over expression of *GPA1* increases GA sensitivity to germination at least 1 million-fold. This increase in GA responsiveness is dependent on *GPA1* expression because seeds not treated with Dex had the control sensitivity.

The effect of both loss and gain of *GPA1* function on seed germination indicates that GPA1 either directly couples GA perception to a downstream effector on a pathway parallel to a second less sensitive pathway or that GPA1 does not couple the GA response, but rather potentiates GA action in a single pathway. The latter possibility could occur if GPA1 is involved in regulating GA sensitivity or levels. The former possibility has been proposed by Ueguchi-Tanaka et al. (2000) to explain the reduced sensitivity of *d1* rice mutant internodes to GA.

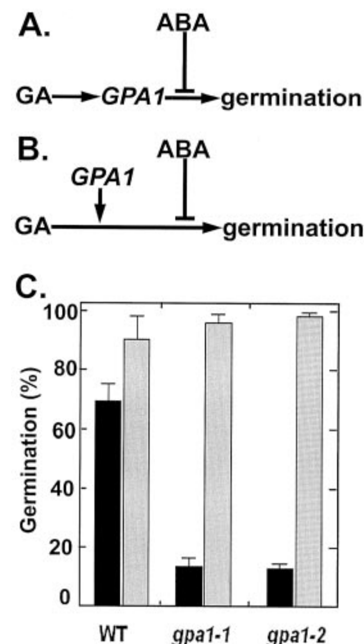

Figure 8. Direct versus indirect roles for GPA1 in the GA response. A, Model shows GPA1 couples GA response in seed germination. B, Model shows GPA1 potentiates GA response in seed germination. C, The indicated genotypes of seed from matched seed lots were pretreated with 100 μM fluridone (gray bars) or water (black bars) for 48 h at 4°C in the dark, washed, and sown on plates containing 6% (w/v) Glc. After 48 h at 23°C, germination was scored and expressed as a percent of total seed. Error bars represent the SE.

GPA1 May Potentiate GA Sensitivity

In an attempt to distinguish between the two possibilities described above, the relative GA responsiveness in Glc-treated seeds that have been pretreated with or without fluridone to reduce ABA pools was determined. The rationale for this experimental design is as follows: Because *gpa1* mutant seeds germinate with 100% efficiency, they contain sufficient GA to overcome the endogenous ABA levels that act on the pathway (Fig. 8A); specifically, the main GA effect in *gpa1* seeds must be operating via this ABA-inhibited pathway. Therefore, if GPA1 directly couples GA perception to downstream elements that are attenuated by ABA, then both GPA1 and ABA inhibition are on the same pathway. If this is true, then both *gpa1* and wild-type seed are expected to have the same sensitivity to fluridone. On the other hand, if GPA1 is not directly coupling GA perception to ABA-attenuated control of germination, but rather potentiating the GA pathway, then dramatically different sensitivities to fluridone are expected (see Fig. 8B).

The following experiment was performed to distinguish GPA1 coupling from potentiation of the GA pathway. Glc (6% [w/v]) was added to reduce wild-type seed germination to approximately 70% in the absence of fluridone to assess the ABA effect by comparing ± fluridone pretreatments (Fig. 8C). Reducing ABA by fluridone pretreatment restored wild-type seed germination to near 100%, a difference of 30%. In contrast, a 90% difference in germination rate between plus and minus fluridone pretreatment was observed for *gpa1* seeds in the presence of 6% (w/v) Glc. This suggests that GPA1 does not directly couple GA to downstream effectors leading to germination, but rather potentiates GA action.

GPA May Couple BR Potentiation of GA Signaling

GPA1 appears to couple some signal that potentiates GA signaling; therefore, we tested if GPA1 coupled BR signaling. This was done in two ways. First, we asked if a BR synthesis (*det2-1*) and a response (*bri1-5*) mutant shared the reduced responsiveness to GA as observed for *gpa1* mutants (Fig. 7A). *det2-1* (Col background), *bri1-5*, Ws, and Columbia wild-type seeds were pretreated with PAC to reduce the GA pool, plated on GA, and scored for germination after 4 d. Both *det2* and *bri1* share the low-GA responsiveness as shown for the *gpa1* mutants, consistent with the hypothesis that GPA1 and BR operate on the same pathway (Fig. 9A). A second and more direct approach was to determine BR responsiveness in the *gpa1* mutants. To do this, the GA pools of Col, Ws, *bri1-5*, and *gpa1* seeds were reduced by pretreatment with PAC and plated on BL. BL at low levels effectively rescues germination in wild-type seeds pre-

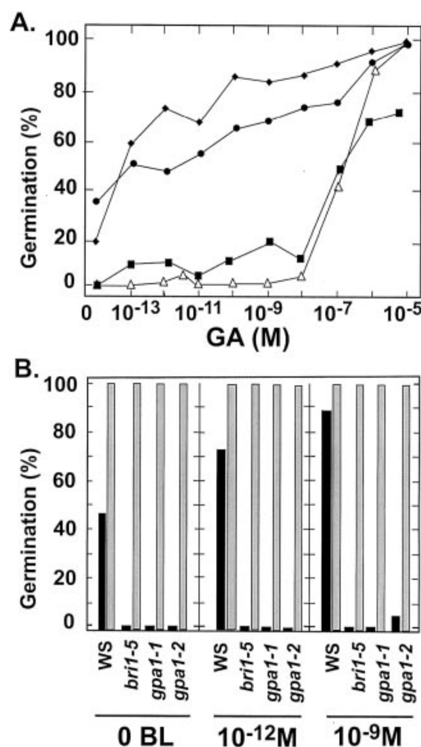

Figure 9. Role of GPA1 in BR signaling. A, Columbia ecotype (black diamonds), *det2-1* in Col background (dark squares), Ws ecotype (black circles), and *bri1-5* (white triangles) in the Ws ecotype were pretreated with PAC and sown on plates supplemented with the indicated concentrations of GA as described in Figure 7. B, Seeds were pretreated with PAC (dark bars) or water (white bars) and sown on plates supplemented with the indicated concentration of brassinolide (BL). Germination in A and B was scored and expressed as a percent of total seed after 4 d.

treated with PAC, but is unable to rescue the *bri1-5* or *gpa1* mutant seed. On the other hand, BL is able to rescue germination of *det2* seeds (data not shown). The complete lack of BL sensitivity in the *gpa1* seeds suggests that BR signaling is coupled by GPA1. Note that the Ws seed germination scored at 4 d (Fig. 9A) is greater than at 2 d (Fig. 7A), but that the GA responsiveness of wild-type seeds relative to the mutants is unchanged.

DISCUSSION

Arabidopsis lacking the alpha subunit of a heterotrimeric G protein are insensitive to BL with regard to BL's inhibitory effect on vegetative growth as well as its promotive effect on seed germination (Figs. 1 and 9). The latter is observed when endogenous GA pools are reduced. BL is probably perceived by a receptor kinase (He et al., 2000; Wang et al., 2001b); thus, the role of a G protein in a receptor kinase pathway in plants needs to be examined further and possibly reconciled. Recent evidence strongly indicates cross talk between receptor kinase and G protein-coupled receptor (GPCR) pathways in animals (Selbie and Hill, 1998; Liebmann and Bohmer, 2000; Marinissen and Gutkind, 2001). There is also evidence that receptor kinases are directly coupled by G proteins. For example, both Gα and G$\beta\gamma$ interact with the insulin growth factor-like 1 receptor (IGF-1R) in a specific manner because association could not be demonstrated to similar receptors for epidermal growth factor or insulin (Dalle et al., 2001). The addition of IGF-1 increased Gα and decreases G$\beta\gamma$ association to IGF-1R. The IGF-1R appears to utilize a Gα from the Gi subfamily, whereas the insulin receptor appears to utilize a Gq/11 subfamily member because insulin addition stimulated Gq phosphorylation (Imamura et al., 1999). Moreover, the expression of a constitutively active Gq conferred a constitutively active IGF-1 response and injected anti-Gq antibody blocked the insulin response.

Although G proteins clearly operate in plants, there is no direct evidence for a GPCR. Most classical GPCRs in animals are heptahelical transmembrane proteins. The only confirmation of a heptahelical structure for a plasma membrane protein in plants is a barley (*Hordeum vulgare*) protein designated MLO (Devoto et al., 1999). The MLO family in Arabidopsis comprises approximately 15 members and neither a ligand nor a function for these putative GPCRs has been assigned. In contrast to animals where classical GPCR genes comprise the largest gene family, plants have few candidates, if any. With only equivocal evidence supporting heptahelical receptors in plants, the intriguing possibility remains that some of the large number of receptor-like kinases in plants are GPCRs. The physiological evidence shown here suggesting that GPA1 and BRI1 operate on the same pathway raises the possibility that signaling via BRI1 is coupled by GPA1.

Plants lacking GPA1 appear to be impaired in a variety of signal transduction pathways operating throughout plant development despite near wild-type phenotypes when grown under laboratory conditions. Even within a specific context such as seed germination, *gpa1* mutants appear to be altered in sugar, ABA, GA, and BR signaling. However, because each of these signal pathways impinge on the other, the possibility exists that GPA1 couples only one signal within this signal network; i.e. the cause of alteration in multiple signaling in *gpa1* seeds may be due to the singular BR defect. Here, we conclude that BR is coupled by a heterotrimeric G protein to potentiate GA-induced seed germination. Because ABA attenuates GA-induced seed germination, the defect in *gpa1* seeds is manifested as hypersensitivity to ABA and sugars (Fig. 10).

Arenas-Huertero et al. (2000) concluded that the inhibitory effect of high Glc on germination and young seedling development was mediated by increased ABA synthesis. This result is consistent with the observation that ABA synthesis mutants are insensitive to high Glc (Arenas-Huertero et al., 2000;

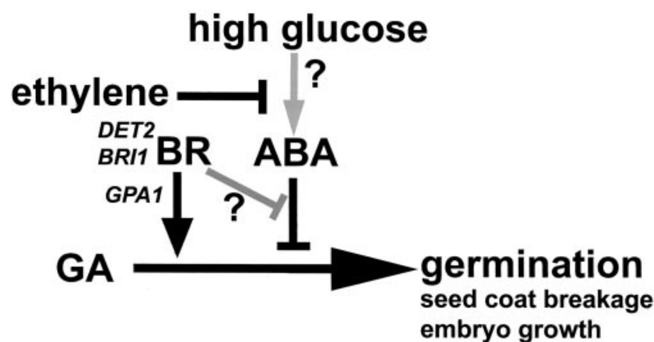

Figure 10. GPA1 couples BR potentiation of GA-stimulated germination. Black arrows indicate pathways tested for GPA1 coupling in signal control of seed germination. The results here suggest that only BR signaling is coupled by GPA1 because the BR response and biosynthetic mutants, *bri1* and *det2*, respectively, lack BR rescue of GA-dependent germination (see Fig. 9A) and because *gpa1* mutants are completely insensitive to BR with regard to rescue of GA-dependent germination (Fig. 9B). Gray arrows indicate potential pathways not yet ruled out or in. BR may also control ABA sensitivity or levels. The mechanism of sugar inhibition is unclear. On the one hand, results here show that the inhibition by high sugar on germination is dependent on ABA synthesis (see Fig. 5), but a sugar-induced increase in ABA was not detected in seeds (Table I).

Laby et al., 2000; Rook et al., 2001) as well as by our work here showing that pretreatment with fluridone blocks the Glc inhibition (Fig. 6). A 3- to 6-fold increase in ABA levels in young seedlings was also found (Arenas-Huertero et al., 2000), consistent with this conclusion. However, ABA levels in seeds were not analyzed; thus, there had not been a direct test of this hypothesis until now. We rationalized that the proposed increase in ABA levels must precede germination, first evident at 24 to 48 h. Therefore, we directly tested this hypothesis by measuring ABA levels in control and 6% (w/v) Glc-treated seeds. Whole seeds do not show a significant increase in the ABA pool size at this early time after high Glc pretreatment. In conclusion, either this component of the model is refuted or the hypothesized high-Glc-induced increase in ABA levels occurs in specific cells controlling germination and are too few for ABA changes to be detected in whole seed measurements.

era mutants have enhanced sensitivity to ABA (Cutler et al., 1996). *ERA1* encodes the beta subunit of a protein farnesyl transferase and it has been enigmatic as to how a general modifying enzyme could play a specific role in signal transduction. Cutler et al. (1996) speculated that the substrate for ERA1 could be a heterotrimeric G protein. Prenylation of Gγ is important to stabilize the complex at the membrane and for effector activation (Higgins and Casey, 1996; Fogg et al., 2001). We show here that Arabidopsis seeds lacking a complete heterotrimeric G protein complex have enhanced responsiveness to ABA when tested in the manner in which the *era1* mutants were originally screened and found. We show that ABA sensitivity is not altered in the *gpa1* mutants and this apparent enhancement in ABA responsiveness is due to reduced GA signaling. A similar characterization of *era1* seeds may reveal many characteristics shared with the *gpa1* seeds, including reduced GA signaling and BR insensitivity.

The use of hormone biosynthesis mutants and inhibitors of hormone biosynthesis have been extremely powerful tools in isolating signal transduction chains and elucidating mechanism of action. However, neither mutants nor inhibitors are without problems and these limit any study, as they did here. The concern with an inhibitor is its specificity, which can never really be known. One might expect that a compound that inhibits or stimulates a particular enzyme activity may have effects, large or small, on enzymes in other pathways. Combinations of inhibitors with large structural differences typically are utilized to assuage some concern about inhibitor specificity. Although a null mutation in a specific enzyme is thought to be a cleaner inhibition of an activity, this approach also has problems, including specificity. For example, loss of function of an enzyme early in a biosynthetic pathway could affect pool sizes of other compounds, including signaling molecules, that are formed from branch pathways downstream of the lesion. Altered pool sizes in precursors resulting from loss of function at a particular point in a pathway may also cause indirect, "nonspecific" effects. Finally, many hormone biosynthesis mutants have abnormal development and one must exercise caution in extrapolating normal function in abnormal cells.

An important distinction between GPA1 directly coupling and potentiating GA signaling is made here. In the former, GPA1 is operating in the classical manner; GA binding to its receptor leads to activation of GPA1 and subsequent downstream effector activation via either GPA1 or the released Gβγ subunits, or both. In the latter, GPA1 operates to increase the sensitivity of GA signaling or level of GA. The current data favor GA potentiation over coupling: First, *gpa1* null mutants remain GA responsive, although with at least 100-fold less sensitivity, suggesting either that the *gpa1* mutation uncovers an alternative GA pathway or that it acts to increase GA sensitivity or levels (potentiation). Second, endogenous ABA has a slightly greater effect on *gpa1* germination than wild type, suggesting either that ABA levels in *gpa1* mutants are higher or that GA sensitivity in the *gpa1* seeds is reduced. Direct measurements of ABA preclude the former. Third, overexpression of *GPA1* increases the sensitivity of GA a million-fold but still retains an absolute requirement for GA for full germination. Recently, Okamota et al. (2001) have shown that overexpression of the wild-type *GPA1* in Arabidopsis can activate light signaling equally well as a constitutively active mutant form of GPA1. Therefore, if GPA1 directly couples GA signaling, 100% germination in the absence of GA is

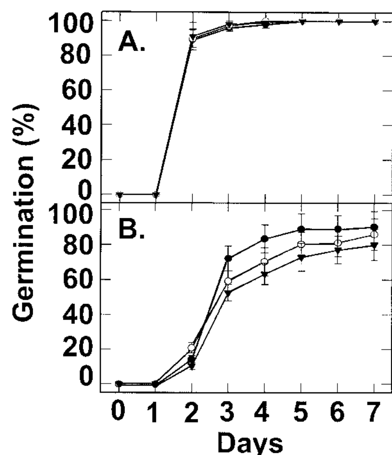

Figure 11. Quality control of matched seed germinations. Ws (white circle), *gpa1-1* (black circles), and *gpa1-2* (black inverted triangles) seeds were either plated with (A) or without (B) pretreatment at 4°C for 48 h at 4°C in the dark and sown on standard plates without supplements. After the indicated times at 23°C, germination was scored and expressed as a percent of total seed. Error bars represent the SE.

expected when GPA1 is ectopically expressed. Because this was not observed, we interpret the data to mean that GPA1 potentiates rather than couples GA signaling in seed germination (Fig. 10).

The mechanism of this potentiation is unknown; however, based on the results using 2-week-old seedlings, Bouquin et al. (2001) suggest that BR potentiates GA signaling by positively affecting GA5, a key GA biosynthesis enzyme whose activity regulates active GA levels and this may be extrapolated to germination. Alternatively, it is also possible that GA sensitivity is regulated by BR in seeds and this is consistent with data from Steber and McCourt (2001) showing that BR rescues severe GA biosynthesis mutants.

The current data, indicating that *gpa1* seeds are completely insensitive to BL potentiation of GA-induced seed germination, raise the exciting possibility that GPA1 couples BR signaling in seeds. Because young seedlings also are insensitive to BL inhibition of root and shoot growth, the role of GPA1 in other developmental stages is possible. However, because *gpa1* and *bri1* mutant phenotypes do not completely overlap, some aspects of BR signaling cannot involve GPA1 coupling.

MATERIALS AND METHODS

Plant Material

The genotypes of the *gpa1* alleles are described by Ullah et al. (2001), and the *bri1-5* allele is described by Noguchi et al. (1999). The construction of the Dex::GPA1 vector and generation of the H2 line is described by Ullah et al. (2001).

Seed Germination Quality Control

Germination is a complex trait. Full germination capacity is not realized until after several weeks of an after-ripening process for which temperature and humidity are important influences. Light and chilling (stratification) are typically required for germination, and as such, must also be controlled in comparative studies on germination. Finally, germination potential is lost over time and is influenced by storage conditions. Thus, germination assays requires exceptional quality control checks. In this study, we compared germination between seeds lots that were produced and harvested identically. For germination assays, seeds of comparative lots were stored, sown, light treated for 12 h, and chilled (4°C, 48h) under identical conditions. In all cases except where indicated in the figure legend, seeds were sterilized and sown on plates containing Murashige and Skoog salts with Gamborg vitamins (Sigma, St. Louis), and 1% (w/v) Suc, chilled in the dark for 48 h, pretreated with light (75 μmol m^2 s^{-1}) for 12 h, wrapped in aluminum foil, and germinated at 23°C. As shown in Figure 11, Ws, *gpa1-1*, and *gpa1-2* seeds were tested for germination potential with and without the chilling pretreatment and scored for germination over 7 d. No significant differences in germination potential either in amount or timing were found between the *gpa1* mutants and their isogenic background. Germination is defined here as an obvious protrusion of the radicle through the seed coat. Each experiment was repeated at least once. A minimum of 50 seeds was scored for each treatment of each genotype.

Growth Assays

Sugar plates were made with 1× Murashige and Skoog by adding filter-sterilized sugars after autoclaved medium cooled down to approximately 55°C. Ethanolic stock solution of BL and ACC were made at 1,000× strength and added to sterile media. Stock solutions (100 μM) of fluridone (Chem Service, West Chester, PA) were freshly prepared for each experiment. The stock was prepared by dissolving 16.5 mg of fluridone in 1 mL of ethanol and then adding 0.5 mL of Tween 20. The volume of dissolved fluridone was brought to 500 mL with deionized (DI) water. After sterilizing, seeds were incubated with 100 μM fluridone solution in the dark at 4°C for 48 h. Seeds were washed five times with an ample amount of DI water to remove fluridone before plating them on the indicated plates. PAC (Chem Service) was added to sterilized seeds from a 1,000× ethanolic stock solution to a final concentration of 6 μM for seeds in the Ws background or 10 μM for seeds in the Col background. PAC treatment occurred in dark at 4°C for 48 h. Seeds were washed five times with ample amount of DI water to remove PAC before plating them on the indicated supplemented plates. Ethanolic stocks of GA_3 (Sigma) were prepared as 1,000× stocks and added to sterile Murashige and Skoog media. Hook angles were measured according to Raz and Ecker (1999).

ABA Analysis

Sterilized seeds were kept in darkness at 4°C for 2 d and moved to 3.0 mL of 1× Murashige and Skoog medium, plus or minus 6% (w/v) Glc. After being cultured in light at 23°C for 24 h, the seeds and medium were lyophilized in the same tube used for incubation and shipped to Nanjing Agricultural University where the following procedures were performed. Seed samples were extracted in 2 mL of cold 80% (v/v) aqueous methanol overnight at 4°C with butylated hydroxytoluene (10 mg L^{-1}). The supernatant was collected after centrifugation at 10,000*g* (4°C) for 15 min. The crude extract was passed through a C_{18} Sep-Pak cartridge (Waters, Milford, MA). The efflux was collected and 300 μL was removed and dried in N_2 gas. The residue was dissolved in 200 μL of methanol for methylation with freshly synthesized ethereal diazomethane. The solution was dried under N_2 gas and redissolved in 300 μL of phosphate-buffered saline (PBS) for ABA ELISA.

The procedure of direct competitive ELISA measurements is based on a monoclonal antibody of high specificity for ABA methyl ester that has been described by Zhou et al. (1996). The main steps are as follows: Microtitration plates (Nubc, Roskilde, Denmark) were precoated overnight at 4°C with rabbit anti-mouse immunoglobulin. Then, the wells were coated with anti-ABA methyl ester monoclonal antibody in PBS (0.01 M, pH 7.4) at 37°C for 70 min. Authentic ABA methyl ester or sample was added 30 min before the addition of horseradish peroxidase-labeled ABA. After a 1-h incubation at 37°C, the wells were washed with PBS containing 0.05% (v/v) Tween 20. The buffered enzyme substrates, hydrogen peroxide and *ortho*-phenylenediamino, were added, and the enzyme reaction was carried out in darkness at 37°C for 15 min. The reaction was terminated with 3 M H_2SO_4 and the absorbance was recorded at 490 nm. ABA was determined three times on the same extract, and samples were assayed in duplicate or triplicate.

Protein Isolation and Immunoblot Analysis

GPA1 over expressing (H2 line) and control (containing empty vector) Arabidopsis seedlings were grown on one-half-strength Murashige and Skoog plates (plus or minus 1.0 μM Dex) at 23°C under continuous light for 8 d. Membrane proteins (20 μg) were extracted from the whole seedlings and subjected to immunoblot analysis with a polyclonal antiserum against recombinant GPA1 (Weiss et al., 1997). The blots were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL). A nonspecific band (bottom blot) was also recognized by the GPA1 antiserum, which served as an internal loading control. Quantitation of band volume (i.e. intensity times area) was determined on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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