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Heterotrimeric G Protein γ Subunits Provide Functional Selectivity in Gβγ Dimer Signaling in Arabidopsis [OA]

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The *Arabidopsis thaliana* heterotrimeric G protein complex is encoded by single canonical Gα and Gβ subunit genes and two Gγ subunit genes (*AGG1* and *AGG2*), raising the possibility that the two potential G protein complexes mediate different cellular processes. Mutants with reduced expression of one or both Gγ genes revealed specialized roles for each Gγ subunit. AGG1-deficient mutants, but not AGG2-deficient mutants, showed impaired resistance against necrotrophic pathogens, reduced induction of the plant defensin gene *PDF1.2*, and decreased sensitivity to methyl jasmonate. By contrast, both AGG1- and AGG2-deficient mutants were hypersensitive to auxin-mediated induction of lateral roots, suggesting that Gβγ1 and Gβγ2 synergistically inhibit auxin-dependent lateral root initiation. However, the involvement of each Gγ subunit in this root response differs, with Gβγ1 acting within the central cylinder, attenuating acropetally transported auxin signaling, while Gβγ2 affects the action of basipetal auxin and graviresponsiveness within the epidermis and/or cortex. This selectivity also operates in the hypocotyl. Selectivity in Gβγ signaling was also found in other known AGB1-mediated pathways. *agg1* mutants were hypersensitive to glucose and the osmotic agent mannitol during seed germination, while *agg2* mutants were only affected by glucose. We show that both Gγ subunits form functional Gβγ dimers and that each provides functional selectivity to the plant heterotrimeric G proteins, revealing a mechanism underlying the complexity of G protein–mediated signaling in plants.

INTRODUCTION

Heterotrimeric G proteins are an important element of transmembrane signal transduction, coupling stimuli as diverse as light, neurotransmitters, odorants, tastants, and hormones. They are found in a variety of eukaryotic organisms, including plants, fungi, and animals. The classical heterotrimer consist of three different subunits, α, β, and γ, which are organized in a highly conserved structure and typically bound to specific G protein–coupled receptors. Activation of the receptor by ligand binding induces a conformational change in Gα, catalyzing the exchange of GDP to GTP. GTP loading causes a protein conformational change that promotes dissociation of the heterotrimer into two functional signaling elements: the Gα subunit and the Gβγ dimer. These two elements (functional subunits) interact with specific effector molecules controlling downstream signaling. The inherent GTPase activity of the Gα subunit hydrolyzes its bound GTP, leading to the reassociation of Gα and the Gβγ dimer, returning the heterotrimer to its inactive GDP-bound state. While interaction between Gα and the Gβγ dimer is dependent on the conformational status of the Gα subunit, interaction between Gβ and Gγ is essentially nondissociable; therefore, the Gβγ dimer acts as a single functional unit in the cell (Gautam et al., 1998).

It was initially thought that signaling in animals only occurred via the activated Gα subunit, with the role of Gβγ being to inhibit the action of Gα by reforming the inactive heterotrimer and guiding Gα back to the receptor for reactivation. However, it is now established that the Gβγ dimer is an active signaling factor in at least as many processes as the Gα subunit (Clapham and Neer, 1997). Among others, the Gβγ dimer is able to interact with adenylyl cyclases, potassium channels, and phospholipases (Clapham and Neer, 1993; Scott et al., 2001). Aside from the activation of specific downstream effectors, the Gβγ dimer is involved in receptor recognition (Lim et al., 2001), membrane targeting, and activation of the Gα subunit (Evanko et al., 2000, 2001). Binding between Gα and Gβγ occurs at a molecular interface largely contained within the β-propeller structure of Gβ. With the exception of Gβ5, there is little binding preference between Gα and Gβ pairs. Therefore, it is assumed that Gγ provides a major share of the structural requisite for the selective coupling of the heterotrimer to the receptor and the Gβγ dimer to its effectors (Gautam et al., 1990; Simon et al., 1991; Hou et al., 2000; Myung and Garrison, 2000; Azpiazu and Gautam, 2002; Chen et al., 2005; Myung et al., 2006). Recent evidence indicates that some animal Gβγ dimers can move from the plasma

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membrane to the Golgi upon receptor activation, providing an extra element of spatial segregation to the Gβγ dimer in G protein–mediated signaling. The Gγ subunit type and the Gα subunit nucleotide exchange properties strongly influence the rate of translocation (Akgoz et al., 2004, 2006; Azpiazu et al., 2006).

A characteristic of mammalian systems is the existence of gene families for each of the G protein subunits. At least 23 Gα subunits, 6 Gβ subunits (including an alternatively spliced variant), and 12 Gγ subunits (Gautam et al., 1998; Balcueva et al., 2000) have been reported in humans, but not all possible combinations are present in the cell, with combinatorial multiplicity of Gβγ dimers being restricted by the specific expression patterns of the genes and selective interactions between different Gβ and Gγ subunits. Nevertheless, a wide range of Gβγ dimers, serving as distinct signal transduction elements involved in different processes, have been described (Camps et al., 1992; Katz et al., 1992; Chen et al., 1997; Clapham and Neer, 1997; Gautam et al., 1998; Bommakanti et al., 2000; Mirshahi et al., 2002; Krystofova and Borkovich, 2005).

In contrast with mammalian systems, only one canonical Gα subunit gene (*GPA1*) (Ma et al., 1990), one canonical Gβ subunit gene (*AGB1*) (Weiss et al., 1994), and two Gγ subunit genes (*AGG1* and *AGG2*) (Mason and Botella, 2000, 2001) have been found in the *Arabidopsis thaliana* genome. The same number of G protein subunits were reported in the monocot species rice (*Oryza sativa*) (Ishikawa et al., 1995, 1996; Iwasaki et al., 1997; Kato et al., 2004); however, two Gα subunits were described for legume species (Kim et al., 1995; Gotor et al., 1996; Marsh and Kaufman, 1999). G proteins are implicated in a large variety of processes in plants (Jones and Assmann, 2004; Perfus-Barbeoch et al., 2004; Assmann, 2005; McCudden et al., 2005; Temple and Jones, 2007); nevertheless, specific signaling roles for the Gα subunit or Gβγ dimers remained elusive until recently. Analysis of T-DNA and ethyl methanesulfonate mutants lacking functional Gα or Gβ subunits showed that both Gα and Gβγ could be involved in specific and independent pathways (Ullah et al., 2003; Joo et al., 2005; Chen et al., 2006a; Pandey et al., 2006; Trusov et al., 2006) as well as in the same processes (Ullah et al., 2003; Pandey et al., 2006). Studies using *Arabidopsis* demonstrated that the Gβ-deficient *agb1-1* and *agb1-2* mutants have flowers with elongated peduncles, shortened flat-top siliques, rounded rosette leaves with crinkled surfaces, and increased root mass (Lease et al., 2001; Ullah et al., 2003). Detailed studies revealed that Gβ modulates lateral root formation by interfering with auxin-dependent cell division (Ullah et al., 2003). It was shown that Gβ-mediated signaling, but not Gα, plays a distinct part in plant resistance against necrotrophic pathogens (Llorente et al., 2005; Trusov et al., 2006). Specific changes in seed germination were also ascribed to Gβ activity (Pandey et al., 2006; Trusov et al., 2006). Finally, analysis of transgenic tobacco (*Nicotiana tabacum*) plants with reduced Gβ subunit levels due to antisense expression of the Gβ subunit mRNA suggested that the Gβ subunit is involved in regulation of the reproductive phase of the tobacco life cycle, particularly in stamen development and pollen maturation (Peskan-Berghofer et al., 2005).

Strong interaction between plant Gβ and each Gγ subunit was demonstrated in vitro (Mason and Botella, 2000, 2001) as well as in vivo (Kato et al., 2004; Adjobo-Hermans et al., 2006, Chakravorty and Botella, 2007). However, despite sequence similarity (48% amino acid identity), the interaction between each of the two *Arabidopsis* Gγ subunits and Gβ seems to be centered in different domains of the protein (Mason and Botella, 2000, 2001; Temple and Jones, 2007).

Nothing is known about the cellular and physiological roles of either of the two known Gγ subunits, their possible functional redundancy, and whether the two potential dimers, Gβγ1 and Gβγ2, are involved in the same or different signaling pathways. We took advantage of the extensive phenotypic characterization of loss-of-function *agb1* mutants, and using this inventory of phenotypes, we asked which of the Gγ subunits acts with Gβ to regulate a specific function. Fungal resistance, root development, and glucose sensing were the three well-characterized AGB1-signaling pathways examined in this study. By a genetic approach, we dissected the roles of the Gγ subunits in G protein signaling in these pathways. Our results show that the different Gγ subunits form independent signal-transducing Gβγ dimers and impart functional selectivity to the heterotrimeric G protein signaling network.

RESULTS

The Expression Profiles of *AGG1* and *AGG2* Are Distinct but Together Overlap *AGB1* Expression

Expression patterns for Gα and Gβ subunit genes were previously reported in various plant species (Weiss et al., 1993; Huang et al., 1994; Kaydamov et al., 2000; Perroud et al., 2000; Chen et al., 2006c). In order to study the tissue-specific and developmental regulation of the *AGB1*, *AGG1*, and *AGG2* genes, transgenic *Arabidopsis* (Col-0) plants were produced containing the promoter regions of each gene fused to the β-glucuronidase (GUS) reporter gene. At least three independent lines were characterized for each of the promoter constructs. Transgenic plants did not show any obvious morphological alterations, suggesting that inserts did not disrupt functional genes. GUS histochemical assays revealed that all three genes are active during early seedling development, with GUS activity detected throughout the plant but highest at the hypocotyl–root junction in 2-d-old *AGB1:GUS* seedlings (Figure 1A). *AGG1:GUS* staining was observed in the hypocotyl, while *AGG2:GUS* staining occurred in the upper part of the root, including root hairs, and gradually declined along the root (Figure 1A).

During later development, all three genes always showed cell/tissue-specific expression patterns, although the overall intensity of the stain was always higher in soil-grown versus plate-grown plants. In rosette leaves of *AGB1:GUS* plants, intense GUS staining was detected in veins and guard cells (Figures 1B and 1C). *AGG1* expression was restricted to veins, while *AGG2* expression was observed primarily in guard cells (Figure 1C). Interestingly, all three genes were found to be expressed in hydathods, specialized leaf organs responsible for the excretion of excessive water and/or salts, but while *AGG2* always showed strong staining, *AGB1* and *AGG1* only occasionally did so (Figure 1B; see also Figure 3A below).

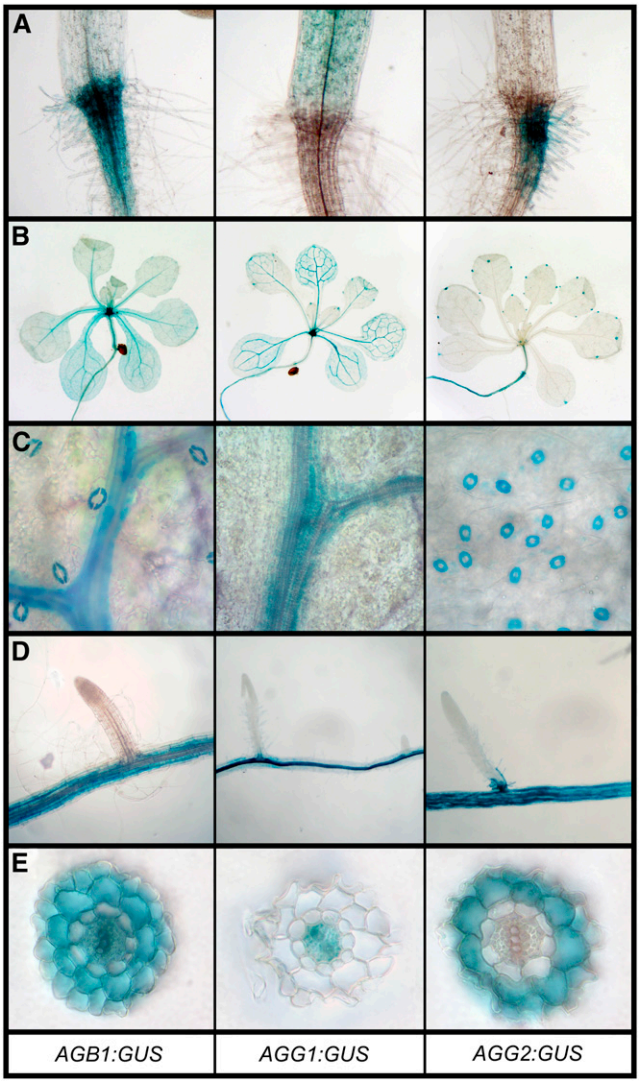

Figure 1. In Situ *AGB1*, *AGG1*, and *AGG2* Expression Patterns.

Histochemical analysis of GUS expression in transgenic *Arabidopsis* plants carrying *AGB1*, *AGG1*, or *AGG2* promoter:*GUS* fusions as indicated.

(A) Shoot–root junction of 2-d-old, dark-grown seedlings.

(B) Two-week-old light-grown seedlings.

(C) Higher magnification of 2-week-old true leaves.

(D) Four-week-old roots.

(E) Cross section through 4-week-old roots.

In roots, *AGG1* expression was restricted to the stele (Figures 1D and 1E). By contrast, *AGG2* expression was, with one exception, excluded from the stele yet found in the cortex and epidermis (Figure 1E). Neither *AGG1* nor *AGG2* expression was homogeneous in its respective tissues along the root length. The exception to the exclusion of *AGG2* expression in the stele was found in young plants (5 to 7 d old) grown on Murashige and Skoog (MS) medium, in which weak *AGG2* expression was observed in the central cylinder and not in outer tissues. *AGB1* is expressed in all root cell types (Figures 1D and 1E) (Chen et al., 2006c). Three distinct expression patterns were observed in *AGB1:GUS* plants: only in the stele, the cortex, or the entire section, with the least intensity or no staining in endodermis/pericycle cells (Figure 1E). It is interesting that throughout the plant, *AGG1* and *AGG2* expression patterns rarely overlapped and together matched the expression of *AGB1* in most tissues (with the exception of flowers and siliques).

Loss-of-Function Mutants for the Gγ Subunits

In order to study the function of both Gγ subunits in *Arabidopsis*, mutants carrying T-DNA insertions in *AGG1* (*agg1-1w*, on the Wassilewskija [Ws] ecotype) and *AGG2* (*agg2-1*, on the Columbia-0 [Col-0] background) genes were identified. An AGG1-deficient mutant in the Col-0 background was generated by genetic introgression over eight successive generations, resulting in a line designated *agg1-1c* (backcross to Col-0). In *agg1-1w*, the T-DNA insertion is positioned within the second intron, splitting the protein in approximately two equal halves, while in *agg2-1*, two tandem and opposing T-DNA insertions are located in the third intron, disrupting the C-terminal region of the hypothetical protein (Figure 2A). RT-PCR analysis showed that neither allele (*agg1-1c* or *agg2-1*) produces a detectable functional transcript for its respective gene (Figure 2C). In addition, the absence of *AGG1* expression in the *agg1-1c* mutants did not result in any observable changes in *AGG2* expression, due to possible compensatory effects (Figure 2C; data not shown). The reverse applies to *agg2-1* mutants. A double knockout of the *AGG1* and *AGG2* genes was obtained by hybridization of the *agg1-1c* and *agg2-1* mutants (*agg1 agg2*). As expected, this line lacked detectable expression of each of the two Gγ subunit genes (Figure 2C).

In addition to the T-DNA mutants, transgenic lines containing RNA interference (RNAi) constructs designed to individually silence either *AGG1* or *AGG2* (*agg1RNAi* and *agg2RNAi*, respectively) were produced in Col-0 (Figure 2B). After screening a large number of individual transgenic lines for each targeted gene, a single-insertion, homozygous line with no detectable expression was selected for further analysis (Figure 2C). For the sake of clarity, *agg1-1c* and *agg1RNAi* lines will be collectively referred to as *agg1* mutants in the text, while *agg2-1* and *agg2RNAi* lines will be collectively named *agg2* mutants.

Gβγ-Mediated Defense against Necrotrophic Fungi Is Selectively Mediated by AGG1 but Not AGG2

It was shown previously that Gβγ-mediated signaling, but not Gα-mediated signaling, is involved in resistance against necrotrophic fungi (Llorente et al., 2005; Trusov et al., 2006). Therefore, we sought to determine whether there is a specific Gγ subunit engaged with Gβ in this process or whether both subunits play redundant or synergistic roles. In preliminary experiments, we analyzed the behavior of all three genes (*AGB1*, *AGG1*, and *AGG2*) in response to attack by necrotrophic pathogens using transgenic plants carrying the promoter:*GUS* fusion constructs. *Alternaria brassicicola* is an air-borne avirulent pathogenic fungus of *Arabidopsis* ecotype Columbia (Penninckx et al., 1996; Schenk et al., 2000, 2003; Thomma et al., 2000; van

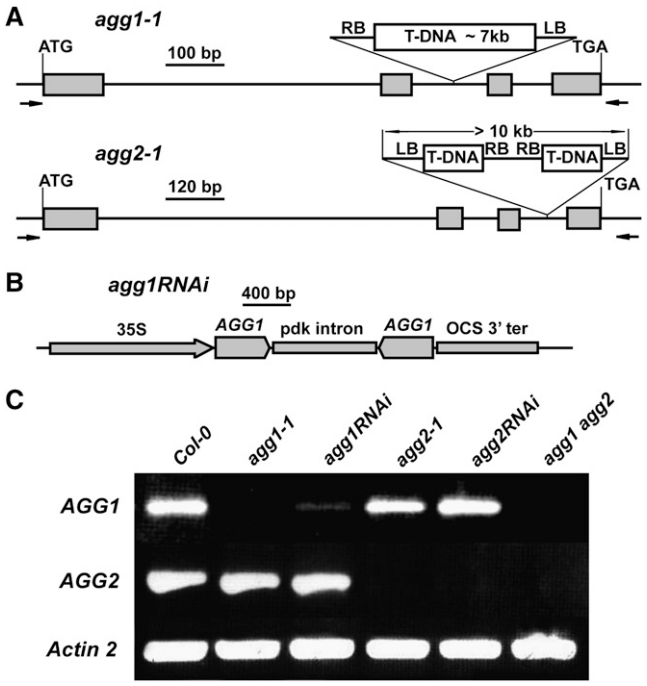

Figure 2. Molecular Characterization of *agg1-1*, *agg2-1*, and RNAi Mutants.

(A) T-DNA insertion sites in the *agg1-1* and *agg2-1* mutants. Gray boxes represent exons. Arrows show the positions of forward and reverse primers used for PCR and RT-PCR. The T-DNA insert is not in scale. ATG and TGA, start and stop codons, respectively; LB, T-DNA left border; RB, T-DNA right border.

(B) RNAi construct used in the production of the *agg1RNAi* lines. A similar construct was generated with the *AGG2* cDNA for the *agg2RNAi* lines.

(C) RT-PCR analysis of the *AGG1* and *AGG2* transcripts. Total RNA extracted from 1-week-old seedlings was used for cDNA synthesis as a template for PCR. Forward and reverse primers depicted in **(A)** were used to perform PCR. *Arabidopsis ACTIN2* was used as a control.

Wees et al., 2003), even though some isolates can reproduce at a very low rate under favorable conditions (van Wees et al., 2003). When plants were inoculated with a suspension of *A. brassicicola* spores, elevated GUS activity was detected 24 h after infection in *AGB1:GUS* and *AGG1:GUS* but not in *AGG2:GUS* transgenic plants (Figure 3A). GUS staining was restricted to the inoculation site and did not spread throughout the entire leaf.

Fusarium oxysporum (f. sp *conglutinans*) is a soil-borne necrotrophic fungus that uses the root tip, secondary root formation foci, and wounds as entry points. It subsequently colonizes the plant by traveling through the vascular system (Mauchmani and Slusarenko, 1994; Agrios, 2005). In contrast with *A. brassicicola*, *F. oxysporum* is a virulent pathogen of *Arabidopsis* (Berrocal-Lobo and Molina, 2004). Surprisingly, inoculation of roots with *F. oxysporum* did not induce GUS activity in root tissue above background levels in any of the three reporter lines; however, significant induction was detected in leaves of *AGB1:GUS* and *AGG1:GUS* plants (Figure 3B). No induction was observed in *AGG2:GUS* plants; rather, a slight decrease in gene expression was observed in leaves and roots. Taken together, our findings indicate that leaf expression of *AGB1* and *AGG1* is systemically activated by *F. oxysporum* and locally by *A. brassicicola*.

To understand the roles of Gγ1 and Gγ2 in resistance against necrotrophic pathogens, we assayed the response of the T-DNA mutants *agb1-2*, *agg1-1c*, *agg2-1*, *agg1 agg2*, and the RNAi lines (*agg1RNAi* and *agg2RNAi*) to *A. brassicicola* and *F. oxysporum* inoculation. Roots of 2-week-old mutant and wild-type plants were infected with bud cell suspensions of *F. oxysporum*, and disease progression was monitored over time from the development of the first symptoms until plants died. Figure 3C illustrates the appearance of typical disease symptoms at an early stage of infection. The advanced chlorosis observed in veins and leaves of *agb1-2*, *agg1-1c*, *agg1RNAi*, and *agg1 agg2* mutants gives a qualitative indication that there is increased susceptibility to *F. oxysporum* in these lines compared with the wild type as well as *agg2-1* and *agg2RNAi* mutants. To quantify the levels of resistance, the number of decayed plants in all mutant lines and wild-type controls was determined (Figure 4A). Plants lacking green leaves were considered decayed. *agg1-1c*, *agg1RNAi*, and *agg1 agg2* lines showed similar dynamics to *agb1-2*, all of them exhibiting a faster rate of disease progression than wild-type plants, while the behavior of *agg2-1* and *agg2RNAi* mutants resembled that of the wild type. To test whether the loss of AGG1 had a similar effect in the Ws background, we compared the *agg1-1w* mutant (in Ws) with wild-type Ws and the Gα subunit null mutant *gpa1-1* (also in the Ws ecotype) (Ullah et al., 2001). Unfortunately, no *agb1* mutants are yet available in the Ws background. We previously showed that the Gα subunit null mutants *gpa1-3* and *gpa1-4* (Col-0 ecotype) have slightly enhanced resistance to *F. oxysporum* (Trusov et al., 2006). After performing inoculation and disease evaluation as for Col-0 lines, it was evident that disease progressed faster in *agg1-1w* plants than in the Ws wild type, while *gpa1-1,* as expected, displayed slightly enhanced resistance (Figure 4C). The differences in disease progression observed between *agg1*, *agb1*, and *agg1 agg2* mutants compared with wild-type Col-0 and *agg2* mutants were statistically significant ($P < 0.05$). Similarly, the differences observed between *agg1-1w* and the wild type and between *gpa1-1* and the wild type in the Ws ecotype were statistically significant ($P < 0.01$). All experiments were repeated at least twice with similar results.

Vegetative growth was also impaired, albeit to different degrees, in wild-type and mutant plants infected with *F. oxysporum*. Figure 4B shows the inhibition of rosette growth expressed as relative size (rosette diameter) of *Fusarium*-inoculated versus mock-inoculated plants of the same genotype. The growth of both *agg1* mutants, the *agg1 agg2* double mutant, and *agb1-2* was significantly affected by the pathogen at 5 d after inoculation ($P < 0.05$), while *agg2* mutants and wild-type plants were almost indistinguishable from their respective mock-inoculated controls. By day 15, the rosette diameter of *Fusarium*-infected wild-type and *agg2* mutants was almost half that of their mock-inoculated controls, while the *agg1* mutants, the *agg1 agg2* double mutant, and *agb1-2* were more severely affected. Absolute values (day 15) for the mean rosette diameter of mock-inoculated wild-type (Col-0), *agb1-2*, *agg1-1c*, *agg1RNAi*,

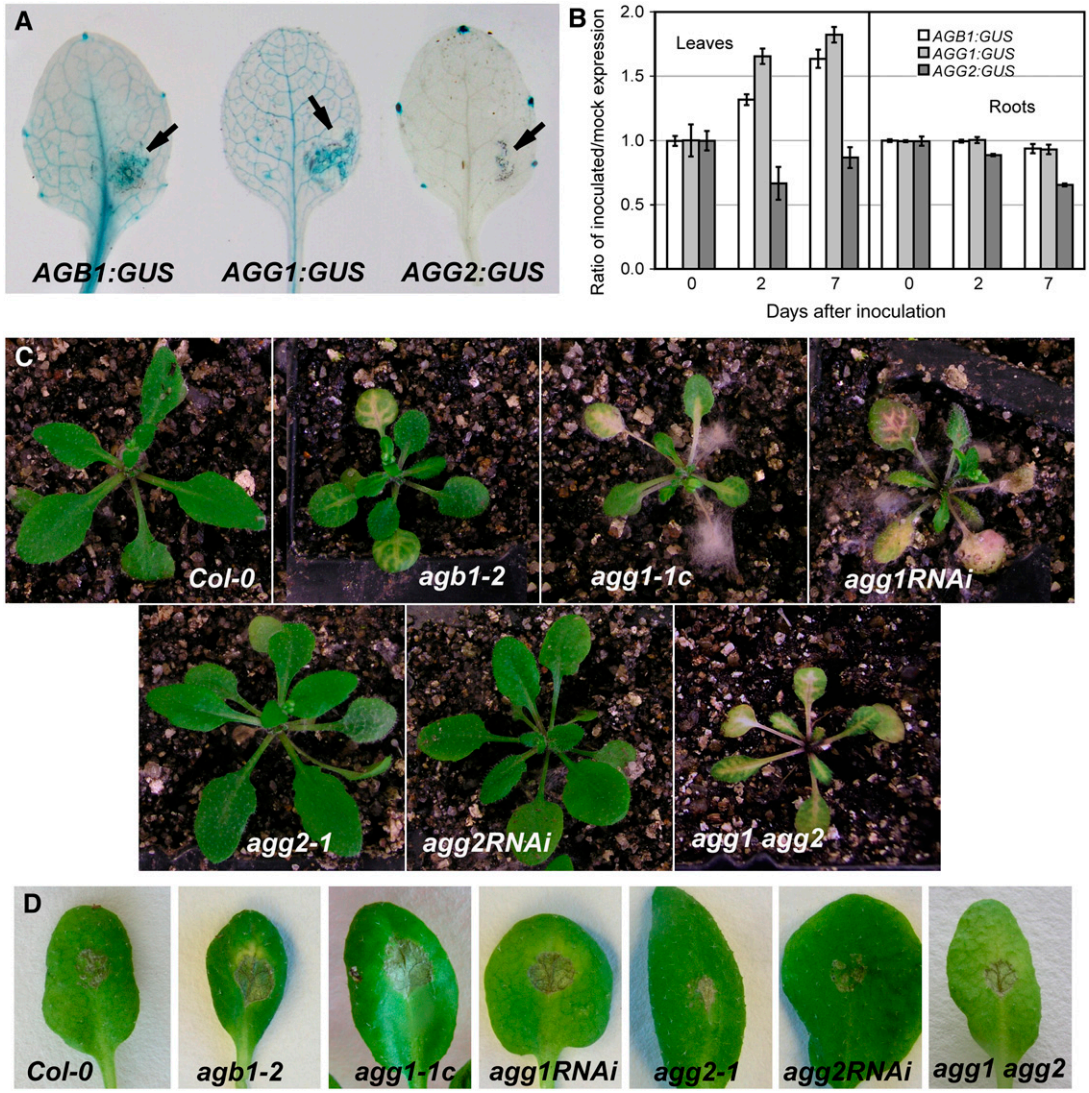

Figure 3. The Gγ Subunit Is Involved in Defense against Necrotrophic Fungi.

(A) Induction of GUS activity by *A. brassicicola* in leaves of transgenic plants expressing the designated promoter:*GUS* fusion constructs. Arrows indicate the region of infection.

(B) Fluorometric assessment of GUS activity in leaves and roots of transgenic plants expressing the designated promoter:*GUS* fusion constructs after inoculation of roots with *F. oxysporum*. The bars represent expression ratios of pathogen-inoculated versus mock-inoculated plants. Error bars represent SE of three replicates.

(C) Characteristic disease symptoms caused by *F. oxysporum* at 8 d after inoculation.

(D) Lesion development at 3 d after inoculation with *A. brassicicola*.

agg2-1, *agg2RNAi*, and *agg1 agg2* plants were 55.2 ± 6.7, 41.1 ± 6.1, 53.9 ± 8.1, 54.3 ± 6.0, 57.5 ± 9.2, 58.1 ± 11.5, and 49.9 ± 9.3 mm, respectively (shown as averages ± SE), while leaves inoculated with *F. oxysporum* displayed measurements of 34.8 ± 5.1, 11.3 ± 3.5, 18.7 ± 4.4, 17.3 ± 5.6, 30.6 ± 8.4, 33.2 ± 8.1, and 15.0 ± 4.6 mm, respectively.

We previously showed that Gβ is also involved in resistance to *A. brassicicola* (Trusov et al., 2006). Application of spores (10^6 spores/mL) on the leaf surface of *Arabidopsis* plants causes necrotic lesions that are clearly different in the wild type and Gβ-deficient mutants. *agb1-2*, *agg1*, *agg2*, and *agg1 agg2* mutants along with wild-type Col-0 plants were inoculated with *A. brassicicola* (Figure 3D), and disease progression was quantified by measuring the necrotic lesion area (given as a percentage of the droplet-inoculated area) (Figure 4E). Statistical analysis showed two very distinct groups that are significantly different from each other ($P < 0.05$). Lesions on *agb1-2*, *agg1*, and *agg1 agg2* mutant leaves occupied ~50 to 60% of the inoculated area, in contrast with wild-type plants and *agg2* mutants, in which an average of 30% of the inoculated area became

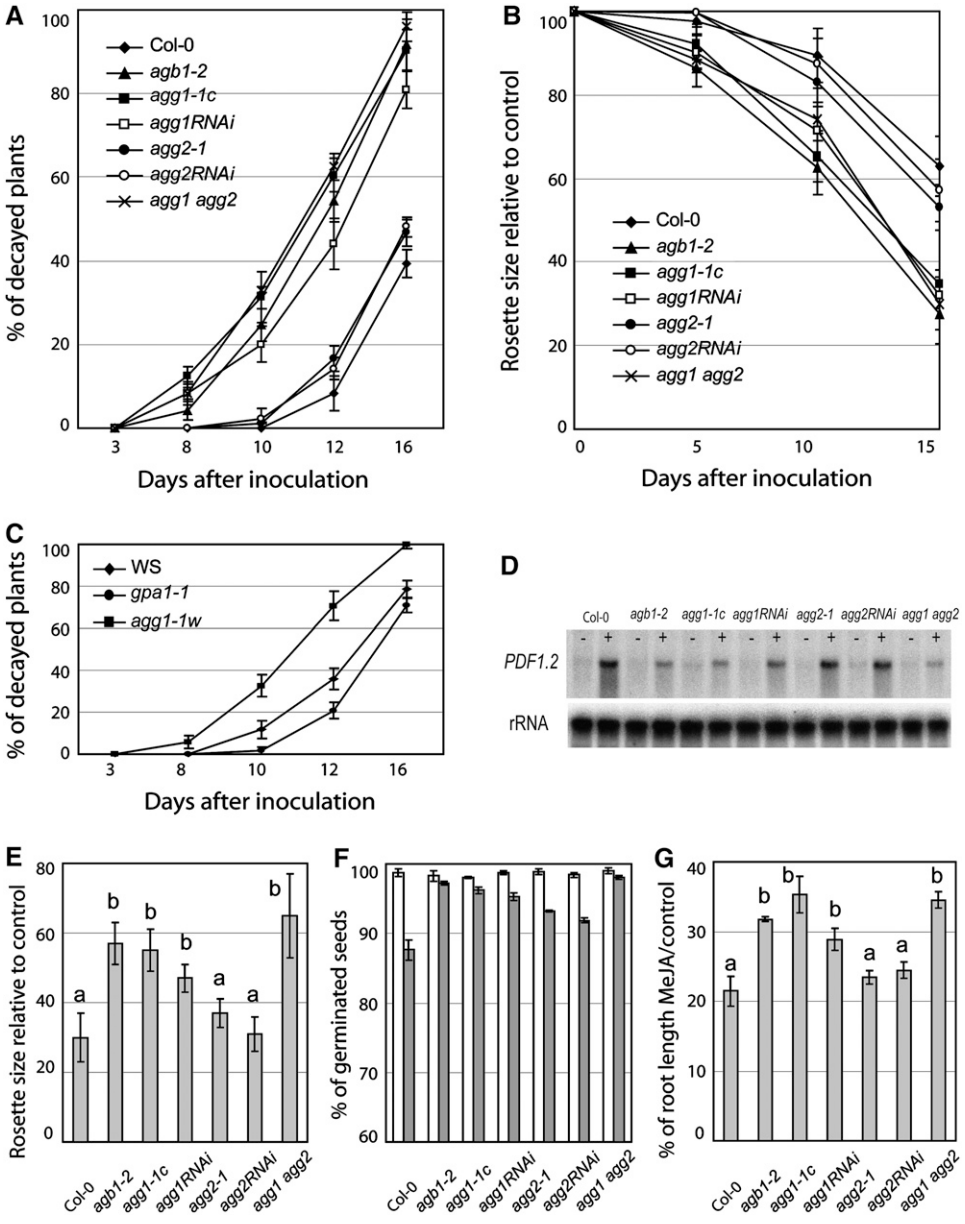

Figure 4. Differential Responses of Gγ-Deficient Mutants to Pathogen Attack and MeJA Treatment.

(A) Susceptibility of wild-type plants (Col-0) and Gβ- and Gγ-deficient mutants to *F. oxysporum*. For each genotype, 48 plants were inoculated and the average percentage of decayed plants per line was scored in three independent experiments. Error bars represent SE.

(B) Inhibition of rosette growth after *F. oxysporum* inoculation expressed relative to the mean growth of the same genotype after mock inoculation. Mean values and corresponding SD were calculated from 48 inoculated and 24 mock-inoculated plants for each genotype.

(C) Same as **(A)** for wild-type Ws ecotype and *gpa1-1* and *agg1-1w* mutants.

(D) Expression of the defense-related gene *PDF1.2* in response to *A. brassicicola* infection. Two-week-old wild-type and mutant plants were sprayed with an *A. brassicicola* spore solution (10^6 spores/mL). Total RNA was extracted from infected leaf tissue at 20 h after inoculation. The blot was hybridized with a *PDF1.2* probe, stripped, and reprobed with a ribosomal probe as a control.

(E) Quantitative estimation of lesion development after *A. brassicicola* infection (10^6 spores/mL). The area covered by necrotic tissue was expressed as a percentage of the inoculated area. Data points represent averages with SD of at least 30 lesions for each genotype. Letters indicate statistically significant differences between genotypes (Student's *t* test, $P < 0.05$, $n = 20$).

(F) Germination percentages of at least 100 seeds pretreated with 10 μM paclobutrazol and sown on 0.5× MS, 1% sucrose, and 0.8% agar plates with or without 50 μM MeJA. Germination was assessed at 2 d after transferring plates to 23°C in continuous light. Bars represent averages with SE of three independent experiments.

(G) Root growth inhibition in response to MeJA treatment. Seedlings were grown for 14 d on 1× MS and 2% sucrose plates supplemented with or without 50 μM MeJA. At least 30 seedlings were measured for each genotype. Data are presented as percentages of the length of treated roots compared with their respective nontreated controls. Bars represent averages with SD. Letters indicate statistically significant differences between genotypes (Student's *t* test, $P < 0.05$, $n = 30$).

necrotic. In agreement with these observations, RNA gel blot hybridization revealed that 20 h after infection with *A. brassicicola*, steady state levels of the plant defensin *PDF1.2* transcript were reduced in *agb1-2*, *agg1*, and *agg1 agg2* mutants compared with the wild type and *agg2* mutants (Figure 4D).

It was previously established that the increased susceptibility to fungal necrotrophic pathogens that was observed in Gβ-deficient mutants correlates with a decreased sensitivity to methyl jasmonate (MeJA). Therefore, we assayed MeJA sensitivity using a germination assay. All mutants showed reduced sensitivity to MeJA compared with wild-type plants (Figure 4F), although to different degrees: *agb1-2* = *agg1 agg2* < *agg1* < *agg2* < wild type. MeJA sensitivity was also assayed using root length inhibition assays (Figure 4G). Two statistically different groups ($P < 0.05$) were observed, the first one showing decreased sensitivity to MeJA in *agb1-2*, *agg1 agg2*, and *agg1* mutants and the second one containing the wild type and *agg2* mutants.

AGG1 and AGG2 Act Additively in Gβγ-Mediated Lateral Root Development

It has been established that Gβ, but not Gα, attenuates auxin-induced cell division leading to lateral root proliferation, although it does not directly couple auxin signaling (Ullah et al., 2003; Chen et al., 2006a). Figure 5A shows the number of lateral roots in 2-week-old wild-type plants and mutants deficient in Gβ, Gγ1, Gγ2, or both Gγ subunits grown on vertical plates (0.5× MS, 1% sucrose, and 0.8% agar, 16:8 day:night cycle, 23°C). All mutants produced more lateral roots than wild-type plants, but three statistically distinct groups ($P < 0.05$) were observed within the mutants: *agb1-2* and double *agg1 agg2* mutants had the highest number of lateral roots, *agg2-1* and *agg2RNAi* mutants produced fewer lateral roots, while *agg1-1c* and *agg1RNAi* had even fewer roots (Figure 5A). Alteration of the growth conditions, such as an increase in MS salt concentration (from 0.5× to 1×) and reduced temperature (from 23 to 21°C) substantially (more than three times) decreased the total number of lateral roots (Figure 5C, white bars) as well as the differences among the various mutants and between mutants and the wild type.

To assay responsiveness to exogenous auxin, seedlings were grown on medium supplemented with the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) and then transferred to growth medium (1× MS) in the presence or absence of 1-naphthaleneacetic acid (NAA) for 5 d before scoring the number of lateral roots (Figure 5B) (Himanen et al., 2002; Ullah et al., 2003). All of the tested G protein mutants showed increased sensitivity to NAA compared with wild-type plants. The ratio of lateral roots developed on NAA-containing medium versus control medium gives an additional indication of the relative sensitivity to NAA: Col-0, 2.2; *agb1-2*, 3.9; *agg1-1c*, 4.1; *agg1RNAi*, 3.6; *agg2-1*, 3.4; *agg2RNAi*, 3.6; and *agg1 agg2*, 4.0.

Exposure of *Arabidopsis* plants to high temperature (29°C) results in an increase in endogenous auxin levels (Gray et al., 1998). Although that original work focused on the effect of endogenous auxin induction on hypocotyl elongation, an increased number of lateral roots was also observed (Gray et al., 1998). In addition, it has been established that shoot-derived auxin is required for the emergence of lateral root primordia (Reed et al., 1998). *agb1-2*, *agg1*, *agg2*, and *agg1 agg2* mutants along with wild-type Col-0 plants were grown at either 21 or 29°C (1× MS), and the number of lateral roots was determined in 2-week-old plants. All genotypes showed a marked increase in the number of lateral roots when grown at high temperature, with the smallest effect (~2.5-fold increase) observed in wild-type plants (Figure 5C). *agg1-1c* and *agg1RNAi* mutants displayed 5.5- and 4.6-fold increases, respectively, while *agg2-1* and *agg2RNAi* showed 3.2- and 3.5-fold increases, respectively. Both *agb1-2* and double *agg1-1 agg2-1* mutants produced approximately seven times more lateral roots when grown at 29°C (Figure 5C). In addition, adventitious roots were frequently observed (80 to 90% of seedlings) on hypocotyls of *agb1-2*, *agg1 agg2*, and *agg1* mutants but never in wild-type plants or *agg2* mutants (data not shown).

AGG1 and AGG2 Are Involved in the Modulation of Acropetally and Basipetally Transported Auxin Activity, Respectively

AGG1 and *AGG2* expression in roots is cell-specific (Figure 1D), correlating with acropetal and basipetal auxin streams, respectively (Mitchell and Davies, 1975; Jones, 1998). Therefore, we hypothesized that Gβγ1 represses lateral root development from the central cylinder by attenuating the activity of acropetally transported auxin, while Gβγ2 represses lateral root formation or growth through the cortex/epidermis by affecting basipetal auxin. It was established that shoot-derived auxin is the predominant source of auxin in young (5- to 7-d-old) *Arabidopsis* roots, controlling lateral root emergence during early development, while later in development, the root system gradually reduces the dependence on shoot-derived auxin by synthesizing a sufficient amount within the root tip at 10 d after germination (although shoot-derived auxin is still important for primordial outgrowth) (Bhalerao et al., 2002; Ljung et al., 2005). Therefore, seedlings were grown for 7 d (1× MS) to allow maximal root elongation before the root tip started to produce auxin, and then acropetal auxin transport was inhibited by the method described by Reed et al. (1998). Seedlings with the auxin transport inhibitor NPA block placed at the root tip had only acropetal auxin transport in the area of the root above the block, while seedlings with the NPA block placed at the shoot–root junction should develop lateral roots mainly under the control of basipetal transport, with the exception of the fraction of roots initiated by early acropetal auxin. The dynamics of lateral root emergence was recorded during the 2-week period after the application of the NPA block (Figures 6A and 6B). As expected, the rate of lateral root production after both treatments was highest in the *agb1-2* and *agg1 agg2* mutants and lowest in wild-type plants. *agg1-1c* seedlings produced abundant lateral roots (statistically indistinguishable from *agb1-2* and *agg1 agg2*), despite the arrest of basipetal transport (Figure 6A). Inhibition of acropetal transport resulted in an initially high number of lateral roots in *agg1-1c* seedlings (day 13 in Figure 6B), probably as a result of early acropetal auxin flux before the block was applied. After the initial peak, the rate of lateral root formation was similar to that in wild-type plants (Figure 6B). By contrast, suppression of basipetal

transport reduced lateral root numbers in *agg2-1* to wild-type levels (Figure 6A), while arrest of acropetal transport resulted in elevated levels of lateral roots, statistically indistinguishable from those of *agb1-2* and *agg1 agg2* mutants (Figure 6B). Similar behavior was exhibited by the RNAi lines (data not shown).

To provide further evidence for the selective roles of the Gγ1 and Gγ2 subunits in roots, we analyzed two specific processes dependent upon the two different auxin streams, adventitious root formation in hypocotyls and root gravitropism. Adventitious root formation predominantly relies on auxin transported within the hypocotyl stele (Liu and Reid, 1992; Nicolas et al., 2004). Aseptically excised wild-type and mutant hypocotyls were incubated with the synthetic auxin NAA. *agb1-2*, *agg1-1c*, and *agg1 agg2* mutants formed adventitious roots throughout the entire hypocotyl, while in wild-type plants and the *agg2-1* mutant adventitious roots were not formed or were present only near the ends of the hypocotyl segments (Figure 6C).

Rashotte and coworkers (2000) showed that inhibition of basipetal auxin transport in roots completely blocked its gravity response, while inhibition of acropetal transport only partially reduced it. Therefore, we assayed the gravitropic response of wild-type and G protein mutant roots by measuring the root angle (measured from the horizontal position) at 24 h after gravistimulation. Figure 6D shows that *agb1-2*, *agg2-1*, and *agg1 agg2* mutants were less responsive to gravistimulation than wild-type plants and *agg1-1c* ($P < 0.001$). Interestingly, *agg1-1c* was slightly less responsive than the wild type ($P < 0.05$), probably due to a limited participation of the acropetal auxin in the gravity response (Rashotte et al., 2000).

AGG1 and AGG2 Are Involved in Different Responses during Germination

Two recent reports established that Gβ signaling plays a role in germination (Pandey et al., 2006; Trusov et al., 2006). To determine the specific roles of each of the partner Gγ subunits in this process, mutants lacking Gβ, Gγ1, Gγ2, or both Gγ subunits were subjected to germination tests. Since germination efficiency is extremely sensitive to the growth conditions experienced by the parental plant and postharvest storage, all seed lots were collected at the same time from plants grown simultaneously under the same conditions and were stored for 2 months at 4°C in the dark. Approximately 100 sterilized seeds of all tested lines were planted on the same Petri dish for a single treatment.

Germination and early development are regulated by many Gβγ-mediated signals, and glucose is arguably the best characterized of those signals to date (Ullah et al., 2002; Pandey et al., 2006; Wang et al., 2006). As shown in Figure 7A, there was a clear difference between wild-type and mutant plants when germinated in the presence of 6% glucose, while 4% glucose did not discriminate among the different genotypes and 2% glucose resulted in nearly 100% germination. Because light intensity also has an effect on germination, we used two different intensities of continuous light irradiation (63 and 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The higher light intensity resulted in faster germination rates, reaching 90% by day 6 on glucose and by day 3 on mannitol (Figures 7C and 7E, respectively), obscuring any differences between genotypes. By contrast, the slower germination rates observed using a lower light intensity accentuated the differences among genotypes. When sown on glucose under low light intensity, *agb1-2*, *agg1*, and *agg1 agg2* mutant seeds showed drastically reduced germination rates compared with wild-type seeds, with <50% germination after 2 weeks (Figure 7B). By contrast, at higher light intensities, the differences between wild-type and *agb1* and *agg1* mutant seeds were only observed at day 2 (Figure 7C). Interestingly, *agg2* mutants also displayed significant inhibition of germination on glucose, albeit at notably lower levels than *agg1* mutants. Again, the difference was statistically significant in lower light (Figure 7B), while at higher light this difference was insignificant (Figure 7C).

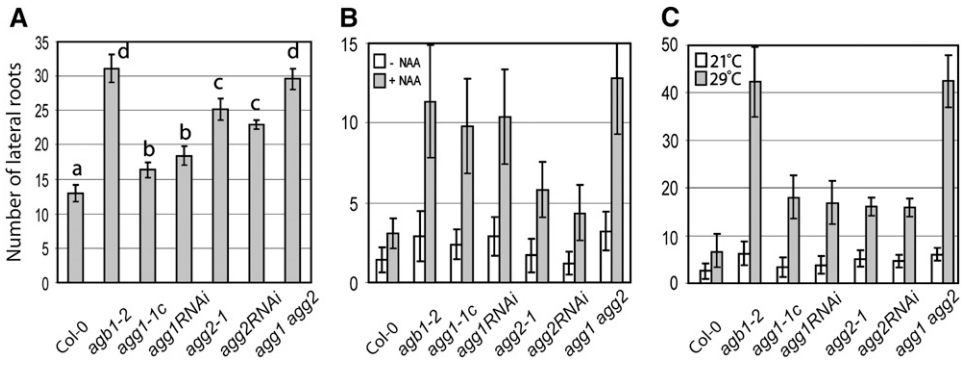

Figure 5. Effect of the Loss of Gγ Subunits on Lateral Root Formation.

(A) Average number of lateral roots in 2-week-old seedlings grown on vertical plates (0.5× MS, 1% sucrose, and 0.8% agar, 23°C, 16:8 light:dark cycle). Error bars represent SE. Letters indicate statistically significant differences between genotypes (Student's *t* test, $P < 0.05$, $n = 15$).

(B) Auxin-induced lateral root development. Seedlings were grown for 9 d on 5 μM NPA and transferred to plates with or without 0.1 μM NAA for an additional 5 d under continuous light on vertical plates. The SD is based on at least 15 seedlings.

(C) High temperature–induced lateral root development. Seedlings were grown at 21 and 29°C for 10 d, and the number of lateral roots was scored. The SD is based on at least 15 seedlings.

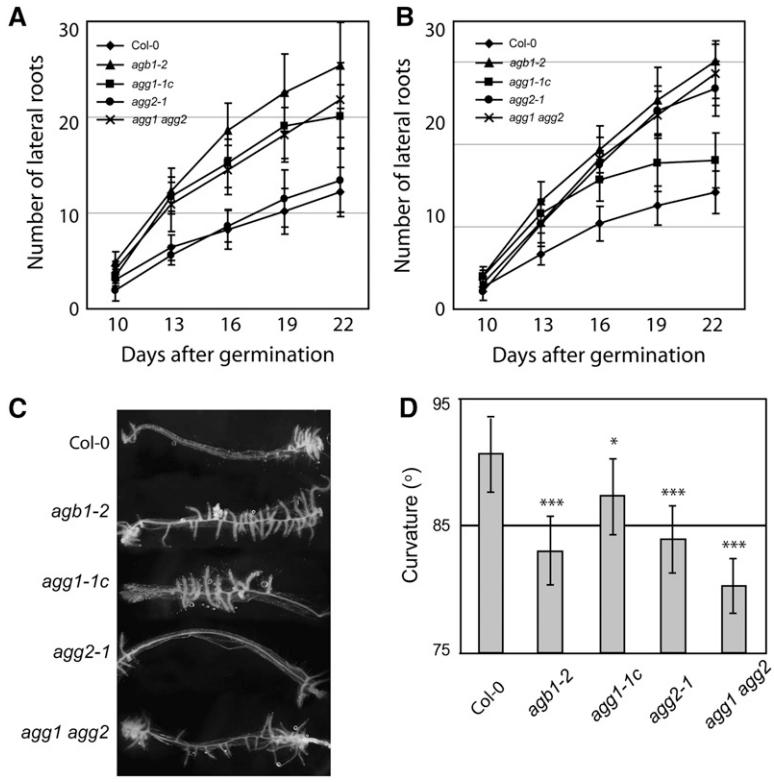

Figure 6. Specific Roles of *AGG1* and *AGG2* in the Regulation of Auxin Response.

(A) and **(B)** Dynamics of lateral root formation after the arrest of basipetal **(A)** and acropetal **(B)** auxin transport. Conditions are described in the text. At least 15 plants for each genotype were used in the assay. Error bars represent SD.

(C) Adventitious root development on excised hypocotyl explants. Seedlings were grown for 4 d in the dark and then for 1 d under light. Hypocotyls were excised aseptically and transferred to plates containing 1 nM NAA. Excised hypocotyls were grown for an additional 10 d under continuous light and photographed.

(D) Response to gravistimulation. Fifty to 60 seedlings of each genotype were grown on 1× MS plates for 5 d under continuous light and then moved into darkness for an additional 24 h, and the plates were rotated 90°. Bars represent average deviation of the angle (curvature) from the horizontal line. Asterisks indicate statistically significant differences relative to the wild type (***$P < 0.001$, *$P < 0.05$). Error bars indicate SD.

To discriminate between the signaling effect and the osmotic stress component observed when plants are exposed to high levels of sugar, we determined the effect of the osmotic agent mannitol on germination at two light intensities. Surprisingly, mannitol severely decreased germination rates in *agb1-2*, *agg1*, and *agg1 agg2* mutants at all time points under the lower light intensity (Figure 7D) and at day 2 under higher light (Figure 7E). By contrast, *agg2* mutants initially showed low germination rates but quickly reached wild-type levels by day 6 under low light (Figure 7D) and were indistinguishable from the wild type under higher light intensity at all time points (Figure 7E).

DISCUSSION

Previously, the functional selectivity of Gγ subunits was largely unrecognized, with the general view that Gγ function is limited to anchoring the Gβγ dimer to the membrane. However, Gγ recently emerged as an important element that provides effector specificity as well as receptor selectivity for the heterotrimer (Gautam et al., 1990; Hou et al., 2000; Akgoz et al., 2002; Azpiazu and Gautam, 2002; Myung et al., 2006).

The initial discovery of single Gα and Gβ subunits in *Arabidopsis* challenged the concept that plants use combinatorial subunit composition to define G protein receptor/effector specificity (Arabidopsis Genome Initiative, 2000), as proven in mammalian systems (Robishaw and Berlot, 2004). With the recent discovery of two Gγ subunits in *Arabidopsis* (Mason and Botella, 2000, 2001), we must now address this possibility. Since both plant Gγ subunits share a number of similarities with animal Gγ subunits, such as the strong interaction with Gβ and the presence of isoprenylation domains, it is reasonable to expect that there are two operational Gβγ subunits in *Arabidopsis*. A number of logical questions follow, such as whether the two subunits mediate the same processes or whether they specialize in different developmental, biotic, or abiotic responses. In this respect, it is interesting that *AGG1* and *AGG2* in situ expression profiles show a high degree of

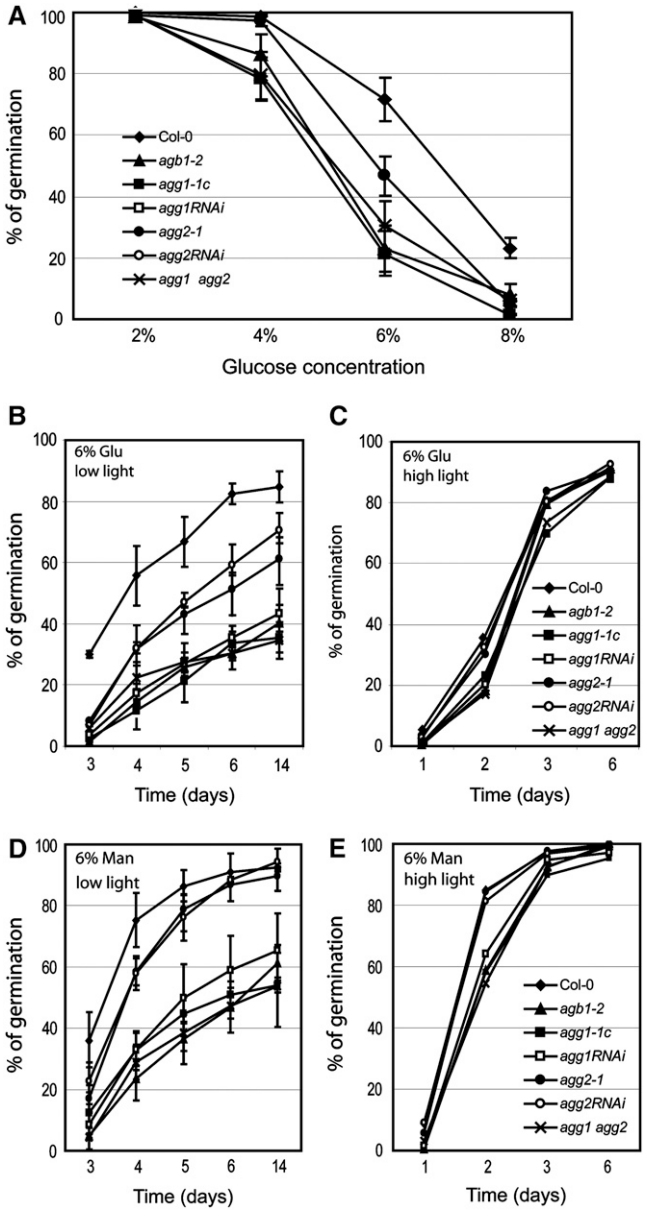

Figure 7. Germination Assays in Gγ-Deficient Mutants.

(A) Germination rates of wild-type plants and the indicated mutants at 5 d after transfer to 23°C in the presence of different concentrations of glucose.

(B) and **(C)** Germination dynamics of wild-type plants and mutants on medium (0.5× MS and 0.8% agar) containing 6% glucose under two different light intensities, 63 μmol·m^{-2}·s^{-1} **(B)** and 150 μmol·m^{-2}·s^{-1} **(C)**.

(D) and **(E)** Germination dynamics of wild-type plants and mutants on medium (0.5× MS and 0.8% agar) containing 6% mannitol under two different light intensities, 63 μmol·m^{-2}·s^{-1} **(D)** and 150 μmol·m^{-2}·s^{-1} **(E)**.

Error bars indicate SD.

tissue specificity and that, even though the sum of their individual expression patterns mimics the overall Gβ expression, the two Gγ gene expression patterns rarely overlap. This raises the possibility that Gγ subunits impose selective functionality restricted by expression patterns.

The functions of the two Gγ subunits are intrinsically linked to Gβ, since, based on mammalian studies, the Gβγ dimer operates as a single signaling unit. The Gβ subunit has been associated with a number of processes using loss-of-function mutants (Lease et al., 2001; Ullah et al., 2003; Llorente et al., 2005; Pandey et al., 2006; Trusov et al., 2006). However, according to the classical mechanism of heterotrimeric G protein action, the lack of a functional Gβ subunit affects not only processes directly mediated by Gβ but also those mediated by Gα; therefore, some of the processes affected in Gβ mutants are actually regulated by Gα (Ullah et al., 2003). In general, those phenotypes shared by Gα- and Gβ-deficient mutants are most likely due to disruption in processes mediated by Gα, while disruption of processes mediated by Gβ results in different or even opposite phenotypes (Ullah et al., 2003). Therefore, to avoid complications in interpretation, we chose processes with predominant Gβ signaling, namely, resistance against necrotrophic pathogens (Llorente, et al., 2005; Trusov, et al., 2006), auxin-regulated lateral root development (Ullah et al., 2003), and D-glucose inhibition of germination (Ullah et al., 2002; Chen et al., 2006b; Pandey et al., 2006; Wang et al., 2006).

Involvement of Gβγ1 in Resistance against Fungal Pathogens

Quantitative and in situ gene expression studies in transgenic *Arabidopsis* reporter lines using two different pathogens gave the first indication of the involvement of Gγ1 along with Gβ in the defense mechanisms against necrotrophic fungi. These observations were confirmed by the fact that the Gβ-deficient mutant *agb1-2* and all of the mutants lacking AGG1 (*agg1-1c*, *agg1RNAi*, and *agg1 agg2*) showed increased susceptibility to *F. oxysporum*, with no statistically significant differences observed between them. The increased susceptibility of Gγ1-deficient mutants to *F. oxysporum* was shown for Col-0 and Ws. The slight increase in resistance observed for Gα-deficient mutants suggests that, in defense-related processes, Gα acts by sequestering the Gβγ1 dimer to the inactive heterotrimeric complex, thus effectively lowering the free available Gβγ1 pool (Llorente et al., 2005; Trusov et al., 2006). This is consistent with the finding that the expression of *GPA1* is not altered by pathogen exposure (Y. Trusov and J.R. Botella, unpublished data). Even though *A. brassicicola* and *F. oxysporum* are both necrotrophic fungi, their infection mechanisms are different. As for *F. oxysporum*, the responses of all AGG1-deficient mutants and *agb1-2* to *A. brassicicola* were statistically indistinguishable, being more severely affected than in the wild type. This finding suggests that the complete Gβγ1 dimer is required for defense. By contrast, mutants deficient in AGG2 but not AGG1 (*agg2-1* and *agg2RNAi*) showed a wild-type phenotype in their behavior against both pathogens, thus precluding any significant role of the Gβγ2 dimer in pathogen resistance.

The susceptibility data are consistent with the molecular observations showing reduced induction of the plant defensin *PDF1.2* by *A. brassicicola* in *agb1-2* and all mutants lacking AGG1 (*agg1-1c*, *agg1RNAi*, and *agg1 agg2*) but wild-type induction in *agg2* mutants. In addition, all AGG1-deficient mutants showed reduced responses to MeJA (statistically indistinguishable from Gβ-deficient mutants), supporting the hypothesis that MeJA signaling could be the link between G proteins and the defense response (Trusov et al., 2006).

Regulation of Lateral Root Development by Gβγ1- and Gβγ2-Mediated Signaling

In the young *Arabidopsis* primary root, auxin transport occurs acropetally through the stele tissue from the first true leaves, where it is primarily synthesized (Bhalerao et al., 2002). This auxin stream initiates early lateral root primordia (Reed et al., 1998; Bhalerao et al., 2002) and augments root-mediated auxin synthesis (Ljung et al., 2005). At a later stage, the root meristem synthesizes auxin, which moves up from the root tip through the epidermis (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978; Jones, 1990, 1998; Rashotte et al., 2001), influencing lateral root initiation (Bhalerao et al., 2002; Ljung et al., 2005). Thus, auxin in both streams initiates lateral root formation, but different signaling mechanisms had not been distinguished previously.

We showed that *AGB1*, *AGG1*, and *AGG2* are each expressed in roots, with *AGB1* expression being observed in the stele, cortex, and epidermis, whereas *AGG1* expression is restricted to the stele and *AGG2* is predominantly active in the cortex and epidermis. Interestingly, none of the genes was expressed in lateral root primordia or in pericycle cells, which become the initials to lateral root meristems. Gβ attenuates auxin signaling during lateral root formation (Ullah et al., 2003), and we extended this finding by showing the Gγ subunits provide specificity in this response. While both AGG1 and AGG2 are involved in the inhibition of auxin-dependent lateral root initiation and both possible dimers, Gβγ1 and Gβγ2, exert a synergistic effect in auxin signaling attenuation, neither Gβγ dimer type is able to compensate for loss of the other. A likely explanation is that each dimer acts on different branches of the auxin/lateral root pathway. This duality does not occur in hypocotyls, as Gβγ1, and not Gβγ2, attenuates auxin-induced adventitious roots in the hypocotyl.

Considering that *AGG1* is expressed in the root stele, where acropetal auxin transport occurs, while *AGG2* is expressed in the cortex and epidermis, which are known to accommodate basipetal auxin transport, we hypothesized that Gβγ1 and Gβγ2 could be specifically involved in signaling for each of the two auxin streams. Consistent with this, we found that inhibition of acropetal auxin transport at the shoot–root junction affected *agg1* mutants, while *agg2* mutants were more responsive to the inhibition of basipetal auxin transport arising from the root tip. Furthermore, support for our hypothesis was provided by studying the gravitropic response, a process that is dependent on basipetal auxin transport. The reduced responsiveness of *agb1-2* and the *agg2* mutants is consistent with a signaling role for basipetally moving auxin in the root. Taking into account the localization of the proteins, we speculate that Gβγ1 could mediate internal signals while Gβγ2 could be involved in external/environmental signaling. Brassinosteroids and ethylene are logical candidates to be such internal signals, since both brassinosteroids and ethylene signal transduction pathways are influenced by heterotrimeric G proteins at various stages of plant development (Ullah et al., 2002) and there is evidence that brassinosteroids and ethylene promote lateral root development by increasing acropetal auxin transport (Bao et al., 2004) and by increasing auxin content locally at pericycle founder cells (Aloni et al., 2006). On the other hand, it is well known that a wide range of soil characteristics, such as availability of water or nutrients, can dramatically affect lateral root development (Vanneste et al., 2005). Signaling from one or more of these factors could be coupled by Gβγ2.

Germination and G Protein Signaling

The role of G proteins in seed germination is intriguing and complicated, since these proteins affect gibberellic acid, abscisic acid, brassinosteroids, MeJA, ethylene, and auxin signaling (Ashikari et al., 1999; Ueguchi-Tanaka et al., 2000; Wang et al., 2001; Ullah et al., 2002; Lapik and Kaufman, 2003; Chen et al., 2004; Pandey et al., 2006) as well as D-glucose sensitivity (Ullah et al., 2002; Chen et al., 2006b; Pandey et al., 2006; Wang et al., 2006). The *gpa1* and *agb1* null mutants show a number of alterations in seed germination, suggesting that GPA1 and AGB1 are involved in this process, although their specific roles are not known (Ullah et al., 2002; Chen et al., 2006b; Pandey et al., 2006). Here, we focused on traits dependent on Gβ-mediated signaling to establish the specificity of the Gγ subunits. The D-glucose hypersensitive phenotype of the Gβ null mutants is more severe than that for the Gα null mutants, implying that the predominant signaling element in D-glucose–regulated germination is the Gβγ dimer (Pandey et al., 2006; Wang et al., 2006). Our results indicate that both Gβγ1 and Gβγ2 dimers mediate this response, although their involvements are different. Gβγ1 is mostly involved in the osmotic component of the glucose response, although involvement in glucose signaling cannot be discounted, while Gβγ2 plays a role in glucose signaling but not in osmotic stress. The apparent contradiction of our results with the previously reported wild-type sensitivity of *agb1-2* to a different osmotic agent, sorbitol (Pandey et al., 2006), can be explained by the masking effect that light intensity (used in that study) has on osmotic response (cf. Figure 6E with 6D). These data further

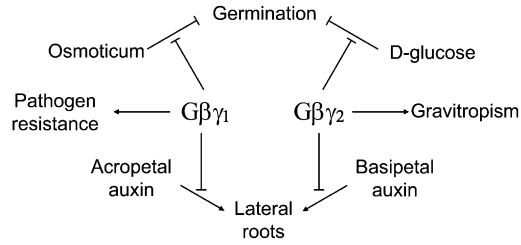

Figure 8. Two *Arabidopsis* Gγ Subunits Provide Functional Selectivity to the Gβγ Dimer.

Summary of the involvement of each Gβγ dimer in pathogen resistance, germination, lateral root development, and gravitropism.

illustrate the complexity of the germination process, implicating at least two independent signaling pathways involving Gβγ1 and Gβγ2 dimers and the additional effect of light intensity.

The fact that AGB1- and AGG1-deficient mutants are hypersensitive to osmotica raises the attractive possibility of the involvement of Gβγ1 signaling in osmoregulation (Zhu, 2002). The high expression levels observed for *AGB1* and *AGG1* in hydathods, highly specialized osmoregulatory organs, also suggests such a speculation.

γ Subunits Provide Functional Selectivity to the Gβγ Dimer

There are substantial similarities, but also important differences, between animal and plant heterotrimeric G proteins. They are structurally similar, suggesting a conserved mechanism of action (i.e., once a G protein–coupled receptor is activated, the associated G protein will dissociate and transduce the signal to downstream effectors through two functionally distinct subunits, Gα and Gβγ). However, plant G proteins lack the multiplicity of genes encoding each of the subunits, as in animals. It is this multiplicity that provides numerous combinatorial possibilities to the whole heterotrimer in order to mediate the action of hundreds of receptors in animal systems. Having single Gα and Gβ subunits begs the question of how plant G proteins are involved in a large variety of plant processes (Jones, 2002; Assmann, 2004; Jones and Assmann, 2004). The existence of two different Gγ subunits provides functional diversity to the entire heterotrimer for effector activation and receptor specificity. The similarities of the phenotypes displayed by Gβ- and Gγ-deficient mutants provide a functional association between the Gβ subunit and each of the Gγ subunits in plants, showing that both Gγ subunits form functional Gβγ dimers. We also showed that the two Gγ subunits serve independent, redundant, or complementary roles in planta, depending on the process and the tissue being studied. In some processes, such as defense against necrotrophic fungi, only one Gγ subunit is involved (AGG1). In other processes, such as auxin signaling and the development of lateral roots, both subunits are involved but are mechanistically different in their operation. In other processes, such as germination, both Gγ subunits are involved but with independent roles, with AGG2 implicated in glucose signaling and AGG1 mediating the response to osmotica (Figure 8).

In summary, the differential behavior of the Gγ mutants in known Gβ-mediated response pathways demonstrates that Gγ subunits provide functional selectivity to the plant heterotrimeric G proteins, providing a mechanism underlying the complexity in G protein–mediated signaling in plants.

METHODS

Plant Materials

The *agg1-1* mutant allele of *AGG1* in the Ws ecotype of *Arabidopsis thaliana* was generated and provided by the Institut National de la Recherche Agronomique (Versailles) (FLAG flanking sequence tag number 197F06) (Brunaud et al., 2002; Samson et al., 2002). The *AGG2* allele *agg2-1* in the Col-0 ecotype was obtained from the Salk *Arabidopsis* T-DNA mutant collection (Alonso et al., 2003) (SALK_010956). For each line, homozygous plants were selected using a three-primer PCR approach. PCR products across the insertion points were sequenced to confirm the exact position of the T-DNA.

The *agg1-1* allele was introgressed into the Col-0 background by crossing *agg1-1w* with wild-type Col-0 plants and the hybrids backcrossed to wild-type Col-0 for eight successive generations. Isolation of the hybrids and backcrosses carrying the *agg1-1* allele was performed by selecting for BASTA resistance conferred by the *BAR* gene present on the T-DNA (Samson et al., 2002). The final mutant line was designated *agg1-1c*. The double *agg1 agg2* mutant was obtained by crossing *agg1-1c* with *agg2-1*. Plants carrying both homozygous alleles were identified from the segregating F2 population using BASTA selection and PCR analysis.

AGG1 and *AGG2* RNAi constructs were generated as follows. An ~400-bp cDNA fragment for each of the genes was amplified by PCR using elongase (Invitrogen) and the following primers: for *AGG1*, 5′-CTCGAGGAATTCCTCTCTCTGACGTTGTCAGATC-3′ and 5′-ATCGATTGGTACCCATGTAAAATGATATCCTAGC-3′; for *AGG2*, 5′-CTCGAGATCTAGAGATGGAAGCGGGTAGCTCAA-3′ and 5′-AAGCTTGGATCCCCAATTACATCAAATTCACTG-3′. Restriction sites (underlined) were added at the ends of each primer for cloning into the pKANNIBAL vector (Wesley et al., 2001). Subsequently, the hairpin cassette was cloned into the binary vector pUQC477 obtained from Bernard J. Carroll (University of Queensland, Australia). *Arabidopsis* plants (Col-0 ecotype) were transformed by floral dipping (Clough and Bent, 1998). Primary transformants were selected with BASTA. Fifteen and 12 independent transgenic lines were obtained for *agg1RNAi* and *agg2RNAi*, respectively, and analyzed by RNA gel blot hybridization for downregulation of the corresponding genes. Lines with no detectable levels of mRNA were subjected to RT-PCR to confirm the lack of detectable message.

The promoter regions of *AGB1*, *AGG1*, and *AGG2* were amplified from wild-type *Arabidopsis* (Col-0 ecotype) genomic DNA using the following primers: for *AGG1*, 5′-CACCGCCGAGGAATCGATCTGGCAT-3′ and 5′-TTGCAGAAAAATGCCAAAACGCCCAA-3′; for *AGG2*, 5′-CACCCTTGGCTCGTACTTCGAT-3′ and 5′-CAAAATTTCTCGAATTCAACCCTCA-3′; for *AGB1*, 5′-AACTCGAGTTACAAGCGAGCTTG-3′ and 5′-TTGGATCCATTCCGGGATCAGACTTAGGCTTC-3′. Restriction sites (underlined) were added at the ends of each primer for cloning purposes. Primers were generally designed to amplify the 5′ upstream region of each gene starting immediately upstream of the start codon. *AGG1:GUS* and *AGG2:GUS* lines were generated as described by Chen et al. (2006c). The *AGB1* promoter fragment was cloned into pGEM-T Easy vector (Promega) and then transferred using *Xho*I and *Bam*HI into the pAOV-intron-GUS vector (Mylne and Botella, 1998). The constructs were transformed into *Arabidopsis* (Col-0 ecotype) by *Agrobacterium tumefaciens*–mediated transformation (Bechtold et al., 1993). GUS staining was performed as described by Petsch et al. (2005).

Pathogen Preparation and Inoculations

Fusarium oxysporum (f. sp *conglutinans*) (BRIP 5176; Department of Primary Industries, Queensland, Australia) and *Alternaria brassicicola* (isolate UQ4273) were grown and plants were inoculated as described previously (Trusov et al., 2006).

Plate Assays

All plates contained 0.5× or 1× MS basal salts (PhytoTechnology Laboratories), 0.8% agar, and 1% sucrose unless stated otherwise. Stock solutions of MeJA and abscisic acid were added to autoclaved medium cooled to ~55°C at the designated concentrations. Seeds were sterilized in a 50% ethanol:1.5% peroxide solution and washed with sterile water or by incubation in a chamber filled with chlorine gas. After sowing, all seeds were stratified for 72 h at 4°C in darkness. Germination was determined as an obvious protrusion of the radicle. For root assays, seedlings were grown on vertical plates for 14 or 21 d, and the number of

lateral roots was counted using a microscope. For gravitropic response assays, sterilized seeds were germinated and seedlings were grown vertically for 5 d under continuous light on square plates and then moved into darkness for another 24 h. Then, the plates were rotated 90° and left in darkness for 24 h. Seedlings were photographed and angle was measured from the digital images using NIH ImageJ software.

Isolation of RNA and Transcription Analysis

Total RNA for RNA gel blot analysis and RT-PCR was extracted as described previously (Purnell and Botella, 2007). Probes for RNA gel blots were labeled using the Rediprime II ^{32}P radiolabeling kit (Amersham). Membranes were hybridized overnight in Church buffer (Church and Gilbert, 1984) at 65°C, washed twice in 0.1% SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS solution, and exposed to PhosphorImager plates for analysis (Molecular Dynamics). For RT-PCR, reverse transcription and PCR amplification were performed as described by Cazzonelli et al. (2005). PCR amplifications were performed using 35 cycles with the following parameters: 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. The primers used for the *AGG1* and *AGG2* genes were as follows: agg1f, 5′-TGCGAGAGGAAACTGTGGTTTACG-3′; agg1r, 5′-CATCTGCAGCCTTCTCCTCCATTT-3′; agg2f, 5′-TGTATCCAACC-AGTAACAAATGG-3′; agg2r, 5′-CGGCAGTGAATTTGATGTAATTG-3′. The *ACTIN2* gene was used as a control for the RT-PCR experiments.

Accession Numbers

The Arabidopsis Genome Initiative identifiers for the genes described in this article are as follows: *GPA1* (At2g26300), *AGB1* (At4g34460), *AGG1* (At3g63420), *AGG2* (At3g22942), *PDF1.2* (At5g44420), and *ACT2* (At3g18780).

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