Arabidopsis Heterotrimeric G-protein Regulates Cell Wall Defense and Resistance to Necrotrophic Fungi


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ABSTRACT The Arabidopsis heterotrimeric G-protein controls defense responses to necrotrophic and vascular fungi. The agbl mutant impaired in the Gβ subunit displays enhanced susceptibility to these pathogens. Gβ/AGB1 forms an obligate dimer with either one of the Arabidopsis Gγ subunits (γ1/AGG1 and γ2/AGG2). Accordingly, we now demonstrate that the agg1 agg2 double mutant is as susceptible as agbl plants to the necrotrophic fungus Plectosphaerella cucumerina. To elucidate the molecular basis of heterotrimeric G-protein-mediated resistance, we performed a comparative transcriptomic analysis of agb1-1 mutant and wild-type plants upon inoculation with P. cucumerina. This analysis, together with metabolomic studies, demonstrated that G-protein-mediated resistance was independent of defensive pathways required for resistance to necrotrophic fungi, such as the salicylic acid, jasmonic acid, ethylene, abscisic acid, and tryptophan-derived metabolites signaling, as these pathways were not impaired in agb1 and agg1 agg2 mutants. Notably, many mis-regulated genes in agb1 plants were related with cell wall functions, which was also the case in agg1 agg2 mutant. Biochemical analyses and Fourier Transform InfraRed (FTIR) spectroscopy of cell walls from G-protein mutants revealed that the xylose content was lower in agb1 and agg1 agg2 mutants than in wild-type plants, and that mutant walls had similar FTIR spectra types, which differed from that of wild-type plants. The data presented here suggest a canonical functionality of the Gβ and Gγ subunits in the control of Arabidopsis immune responses and the regulation of cell wall composition.

Key words: Plant immunity; cell wall; indole glucosinolates; xylose; necrotropic fungi; G-protein.

INTRODUCTION

Heterotrimeric G-proteins are members of the superfamily of GTP hydrolyzing proteins (G-proteins) that function as signal mediators in the transduction of developmental cues and stress-induced stimuli in mammals, yeast, and plants (Digby et al., 2006; Temple and Jones, 2007). The G-protein heterotrimer is composed of α, β, and γ subunits (Gα, Gβγ, and Gγ), organized in a highly conserved structure, which is in complex with transmembrane G-protein-coupled receptors (GPCR). Recognition of specific ligands by GPCR activates the exchange of GTP for GDP at the binding site of Gα, which leads to the dissociation of the heterotrimer into two functional elements: the GTP-bound Gα subunit and the Gβγ dimer. Each of these
subunits can independently interact with their corresponding downstream effectors to transduce the signal (Digby et al., 2006; Temple and Jones, 2007). The endogenous GTPIase activity of Gα eventually returns it to its inactive form and leads to the re-association of the heterotrimer (Johnston et al., 2008).

The complexity of heterotrimeric G-protein subunits and GPCRs in plants is low compared to metazoans (Temple and Jones, 2007). In Arabidopsis, there is only one gene each for the Gα (GPA1) and Gβ (AGB1) subunits and three genes encoding Gγ1, Gγ2, and Gγ3 subunits (AGG1, AGG2, and AGG3; Temple and Jones, 2007; Chakravorty et al., 2011). Tight physical interactions between Gβ and the Gγ1 and Gγ2 subunits have been demonstrated in vitro and in vivo, and these interactions were found to be essential for the localization of Gβ to the plasma membrane (Mason and Botella, 2001; Kato et al., 2004; Adjobo-Hermans et al., 2006). These results and other genetic data led to suggest a canonical functionality of the Arabidopsis Gβi and Gγ1γ2 subunits (Marrari et al., 2007). However, some non-redundant and specific functions of the Gγ1γ2 dimers have been also described (Trusov et al., 2006, 2008b). The atypical Gγ3 subunit (AGG3), which is only expressed in stomata, flowers, and reproductive tissues, has been shown to interact weakly with the Gγ1 subunit (Chakravorty et al., 2011). To date, there are no known plant GPCRs that activate Gα subunits in a manner as in animals, but a single regulator of G signaling (RG51) has been identified in Arabidopsis that may exert this activity (Chen et al., 2003).

Heterotrimeric G-proteins are involved in different cellular processes in eukaryotes (Zhang et al., 2006). In plants, they control many developmental processes, such as abscisic acid (ABA) sensitivity during seed germination (Ullah et al., 2002; Pandey et al., 2006), stomata aperture (Wang et al., 2001), D-glucose signaling, and sugar sensing during cell division (Chen et al., 2003; Huang et al., 2006; Wang et al., 2006; Grigston et al., 2008), rosette leaf, flower and silique development (Lease et al., 2001; Ullah et al., 2003), and auxin signaling in roots (Trusov et al., 2007). Also, plant heterotrimeric G-proteins regulate stress responses, including reactive oxygen species (ROS) production and cell death progression upon ozone exposure (Joo et al., 2005), and resistance to bacteria and fungi (Suharsono et al., 2002; Llorente et al., 2005; Trusov et al., 2006; Zhu et al., 2009). For the latter, the Arabidopsis agb1 mutant was found to be more susceptible than wild-type plants to the necrotrophic fungi Botrytis cinerea, Plectosphaerellacucumerina, and Alternariabrassicicola, and to the vascular fungus Fusarium oxysporum, whereas the resistance of agb1 plants to virulent and avirulent isolates of the biotrophic oomycete Hyaloperonospora arabidopsisis and the bacterium Pseudomonas syringae did not differ from that of wild-type plants (Llorente et al., 2005; Trusov et al., 2006). In contrast, the resistance of the gpa1 mutant to the fungus mentioned above was slightly higher than that of wild-type plants (Llorente et al., 2005; Trusov et al., 2006). The double gpa1 agb1 mutant was as susceptible as agb1 to F. oxysporum, indicating that the effect of GPA1 on resistance may lie in its ability to sequester Gβ into the inactive heterotrimer (Trusov et al., 2006). The aggl agg2 and aggl plants, but not the agg2 mutant, were initially described to display enhanced susceptibility to F. oxysporum and A. brassicicola, suggesting that Gβγ1 is the specific dimer involved in the control of the immune response against necrotrophic and vascular fungi, and that AGG1 and AGG2 do not have redundant functions in regulating this defensive response (Trusov et al., 2006). However, an in-depth analysis of the resistance to F. oxysporum of Gγ1 and Gγ1γ2 double mutants indicated that the susceptibility of the aggl and agg2 mutants was higher than that of wild-type plants, but lower than that of the aggl agg2 plants (Trusov et al., 2010). These data are consistent with the hypothesis that AGG1 and AGG2 form an obligate dimer with AGB1, and have redundant functions in the control of resistance to vascular fungi. This is in contrast to the specific function of these subunits in other developmental-regulated processes: thus, the agb1 plants, but not the aggl, agg2, and aggl agg2 mutants, are specifically impaired in some responses observed in wild-type plants such as the hypersensitivity to ABA inhibition of seed germination and the hyposensitivity to ABA inhibition of stomata opening and guard cell inward K+ currents (Trusov et al., 2006, 2008b).

Necrotrophic fungi constitute a large group of plant pathogens attacking a broad range of hosts (van Kan, 2006). Arabidopsis resistance to these fungi is genetically complex (Denby et al., 2004; Llorente et al., 2005) and depends on the precise regulation of a large subset of signaling pathways, including those mediated by the hormones ethylene (ET), jasmonic acid (JA), salicylic acid (SA), ABA, and auxins (Thomma et al., 1998; Berrocal-Lobo et al., 2002; Adie et al., 2007; Hernández-Blanco et al., 2007; Llorente et al., 2008). Also, secondary metabolites, such as phytoalexins (e.g. camalexin) or other tryptophan(trp)-derived metabolites (e.g. indol glucosinolates, IGs), contribute to Arabidopsis resistance to these fungi as mutants blocked in the biosynthesis of camalexin (e.g. pad3) or both IGs and camalexin (e.g. cyp79B2 cyp79B3 double mutant) are more susceptible to necrotrophs than wild-type plants (Bohman et al., 2004; Kliebenstein et al., 2005; Glawischnig, 2007; Bednarek et al., 2009; Sánchez-Vallet et al., 2010). The immune response to F. oxysporum mediated by Arabidopsis G-protein includes defensive mechanisms independent of the SA-, JA-, ET-, and ABA-signaling pathways, but, at late stages of infection, a weak contribution to disease progression of some components of JA-signaling (e.g. COI1 and JIN1/MYC2) was found, indicating that Gβ may act upstream of these regulators in the JA pathway (Trusov et al., 2008a). Expression analyses of defensive marker genes in the agb1-P. cucumerina interaction are in line with these results, and support the participation of additional, uncharacterized defensive mechanisms in Gβ-mediated resistance (Llorente et al., 2005).

Resistance to necrotrophic and vascular fungi is also genetically determined by plant cell wall composition (Hernández-Blanco et al., 2007; Cantu et al., 2008; Sánchez-Rodriguez et al., 2009; Ramírez et al., 2011). Thus, Arabidopsis mutants
impaired in cellulose synthase (CESA) subunits required for secondary (e.g. irx irregular xylem) and primary (e.g. prc1/ixf1/iev1, proteus1/isoxaben resistant 1 constitutive expression of VSP1) cell wall formation showed enhanced resistance to different necrotrophic and vascular pathogens (Ellis and Turner, 2001; Hernández-Blanco et al., 2007). Also, the ERECTA (ER) Receptor-Like Kinase (RLK) has been implicated in the regulation of cell wall-mediated resistance to P. cucumerina (Sánchez-Rodríguez et al., 2009). Interestingly, a potential connection between heterotrimeric G-protein and ER exists as the agb1-1/elk4 mutant allele exhibits an erecta-like (elk) developmental phenotype similar to that of er plants (Lease et al., 2001; Lorente et al., 2005).

To gain insight into the molecular mechanism controlling G-protein-mediated resistance, a comparative transcriptomic analysis of the agb1-1 mutant allele and wild-type plants was performed upon inoculation with the fungus P. cucumerina. Here, we show that a significant subset of genes encoding proteins with cell wall-related functions were differentially regulated in both agb1 and agg1 agg2 mutants compared to wild-type plants. Analysis of the cell wall structure/composition of these genotypes demonstrated that the walls of agb1 and agg1 agg2 mutants showed similar alterations in their composition, which differed from that of wild-type plants. These results suggest that the modification of the cell wall architecture of agb1 and agg1 agg2 plants might contribute to the enhanced colonization of these mutants by necrotrophic fungi.

RESULTS

Resistance to P. cucumerina Is Similarly Impaired in the agg1 agg2 and agg1 Mutants

AGG1 rather than AGG2 seems to control Arabidopsis resistance to the necrotrophic fungus A. brassicicola, as the susceptibility of agg1 and agg1 agg2 plants to this pathogen is almost identical (Trusov et al., 2006). To further characterize the role of the Gβγ1/Gγ2 dimers in the regulation of Arabidopsis resistance to necrotrophic fungi, we inoculated 3-week-old Col-0 wild-type plants, the agg1-1, agg2-1, and gpa1-4 single mutants, two agg1 alleles (agg1-1/elk4 and agg1-2), and the agg1-1 agg2-1 double mutant, with a spore suspension (4 × 10^6 spores ml^-1) of P. cucumerina BMM (PcBMM), a necrotrophic fungal pathogen that causes disease in different Arabidopsis ecotypes (Sánchez-Vallet et al., 2010). The progression of the infection was examined at different hours/days post inoculation (hpi/dpi) by trypan blue staining (TB) of the inoculated leaves, determination of fungal biomass by quantitative real-time PCR (qRT-PCR) of the P. cucumerina β-tubulin gene, and by macroscopic evaluation of disease rating (DR) of the inoculated plants (Sánchez-Vallet et al., 2010). TB staining at 20 hpi revealed a higher spore germination rate on leaf surface of the two agg1 alleles and the agg1-1 agg2-1 double mutant as compared to Col-0, gpa1-4, agg1-1, and agg2-1 plants (Figure 1C). The enhanced spore germination in the leaves of agg1 alleles and agg1-1 agg2-1 double mutant was accompanied by an increase in plant cell death (Figure 1C). At 7 dpi, fungal biomass in the agg1 alleles and the agg1-1 agg2-1 double mutant was similar but higher than that determined in Col-0 plants (Figure 1A). As previously reported (Lorente et al., 2005), the gpa1-4 mutant showed a slight reduction in fungal growth in comparison with Col-0 plants (Figure 1A). Fungal biomass in the agg1-1 and agg2-1 single mutants was lower than that determined in the agg1-1 agg2-1 double mutant. However, the agg1-1 plants supported more fungal growth than the Col-0 plants whereas fungal biomass in agg2-1 was similar to that of Col-0 plants (Figure 1A).

A positive correlation was found between fungal biomass and the macroscopic disease symptoms determined at different dpi (Figure 1A and 1B). In the agg1 and agg1-1 agg2-1 mutants, the DR caused by the fungus increased over time, leading to leaf tissue collapse and plant decay, whereas no such disease symptoms were observed in the agg1-1 and agg2-1 plants (Figure 1B and 1D). At early stages of infection (0–9 dpi), we observed a faster progression of the disease in the agg1-1 agg2-1 plants than in agg1 mutants; however, at latter stages (10–12 dpi), no discernable differences between the disease symptoms of these genotypes were observed (Figure 1B and data not shown). Taken together, these data suggest that the AGG1 and AGG2 proteins have redundant functions in the control of Arabidopsis immune response to the necrotrophic fungus PcBMM, and that they might form an obligate dimer with AGB1. These results are in contrast to the suggested specific and independent roles of AGG1 and AGG2 in the regulation of resistance to the necrotrophic fungus A. brassicicola (Trusov et al., 2006), but are consistent with the proposed canonical functionality of Gβ and Gγ1/Gγ2 subunits in the control of the Arabidopsis immune response to the vascular fungus F. oxysporum (Trusov et al., 2010).

Comparative Transcriptomic Analyses Revealed that Immune Responses Required for Resistance to Necrotrophic Fungi Are Not Impaired in the agg1 and agg1 agg2 Mutants

To investigate the mechanisms controlling heterotrimeric G-protein-mediated resistance to necrotrophic fungi, we performed comparative gene expression analyses of 3-week-old wild-type plants (Col-0) and the agg1-1 mutant allele. First, we studied the transcriptome of these genotypes before fungal inoculation (0 dpi) to determine whether the enhanced susceptibility to PcBMM of agg1-1 mutant may be the result of the mis-regulation of constitutively expressed defensive genes. Also, we performed comparative transcriptomic analyses of Col-0 and agg1-1 plants 1 d after water spraying (mock-treatment) or inoculation with PcBMM, to identify potential differences in the regulation of induced defense responses in agg1 compared to Col-0 plants. Out of the 22000 genes tested in these transcriptomic analyses, 9762 showed statistically significant values (ANOVA 0.01%) and were selected for further studies. From these genes, 15 were found to be...
differentially regulated in the non-inoculated (0 dpi) agb1-1 mutant in comparison to Col-0 plants (Table 1). Among the constitutively down-regulated genes in the agb1-1 allele, we found a class B acidic phosphatase (At4g29260), the Pathogen-Circadian-Controlled 1 (PC1, At3g22237), and a protein with unknown function (At3g22240), which have been associated with defense responses, and the endogenous AGB1 gene (At4g34460; Table 1) that is known to be aberrantly transcribed in the agb1-1 plants (Lease et al., 2001). Among the set of constitutively up-regulated genes in agb1-1, we identified the defensin PDF1.2a (At5g44420), the AtPP2-A13 (At3g61060), and SEN1 (At4g35770), which have been involved in response to wounding, and a QQS protein that regulates the starch metabolism (Table 1). The differential expression of these genes in the agb1-1 allele was validated by qRT-PCR (Supplemental Figure 1A).

Similarly, we found 126 genes that were differentially expressed (110 down-regulated and 16 up-regulated) in the mock-treated agb1-1 mutant in comparison to Col-0 genotype (plants grown for 24 h at 24°C and high relative humidity (80–85%); Supplemental Table 1). We performed a functional classification of these genes by using tools of the Bio-Array Resources (BAR) for Plant Biology (http://bar.utoronto.ca) and the ARANET Gene Ontology (http://aranet.mpimp-golm.mpg.de; Mutwil et al., 2010). Notably, among the most statistically relevant functional categories we found ‘response to abiotic and biotic stimulus’ (p-value < 10^{-5}) and ‘cell wall’ (p-value < 0.047). The latter comprised 20 genes encoding well-known cell wall-related proteins such as IRX9-L, FEI1, FLA2, several expansins and hydrolases, a pectin methylasease, and a pectin lyase (Supplemental Table 1).

In the P. cucumerina-inoculated agb1-1 and Col-0 plants, 2 511 and 1 480 genes were, respectively, differentially regulated in comparison to the mock-treated plants (Supplemental Tables 2 and 3). Of these genes, only 346 showed a significant differential expression in agb1-1 mutant compared to Col-0 plants (120 down-regulated and 226 up-regulated; Supplemental Table 4). The expression of a representative set of these genes was validated by qRT-PCR of mRNA samples from agb1-1 and Col-0 plants at 1 d after mock treatment or inoculation with PcBMM (Supplemental Figure 1B). The majority (2165) of these 2 511 genes differentially regulated

![Figure 1](image1.png)

**Figure 1.** The Susceptibility of agg1 agg2 Double Mutant to *P. cucumerina* Is Similar to that of agb1.

Plants of the showed genotypes were spray-inoculated with spores (4 × 10^6 sp ml^{-1}) of *P. cucumerina* BMM. (A) Relative quantification of fungal DNA (*P. cucumerina* /tubulin) by qRT-PCR at 7 dpi post inoculation (dpi). Values are represented as the average (± SD) of the n-fold-increased expression compared with wild-type plants (Col-0). Letters indicate values statistically different among genotypes (ANOVA p < 0.05, Bonferroni Test).

(B) Average disease rating (DR ± SD) of the indicated genotypes at different dpi. DR varies between 0 (no symptoms) and 5 (dead plant).

(C) Lactophenol Trypan Blue staining of inoculated leaves at 20 hpi. Bar represents 50 μm.

(D) Disease symptoms of the indicated genotypes at 7 dpi. Data (A–D) are from one out of three independent experiments, which gave similar results.
in the PcBMM-inoculated agb1-1 plants were found to show similar expression patterns (up- or down-regulated) in PcBMM-infected Col-0 plants. However, the n-fold induction or repression of a significant subset of these genes in the inoculated agb1-1 mutant was stronger than that observed in Col-0 plants.

Among the 2,511 genes that were differentially expressed in agb1-1 plants upon PcBMM inoculation, we found a remarkable number of defensive genes whose expression is regulated by either the SA-, JA-, ET-, or ABA-signaling pathways (Figure 2A and Supplemental Tables 2 and 3). Similarly, the expression of genes encoding proteins from the trp-derived metabolites pathway (e.g. CYP79B2, CYP79B3, PAD3, and CYP81F2), which is required for Arabidopsis resistance to both adapted and non-adapted *P. cucumerina* isolates (Bednarek et al., 2009; Sánchez-Vallet et al., 2010), was also up-regulated in the agb1-1 mutant (Supplemental Tables 2 and 3). To corroborate these results, we performed qRT-PCR expression analyses of some representative marker genes from these immune response pathways in Col-0, agb1-1, and agb1-2 alleles and in the aggl-1 agg2-1 plants at different time points after PcBMM inoculation. We analyzed the expression of *PR1, LOX2, PR4, MYC2, and PDF1.2A*, marker genes of the SA-, JA-, ET-, ABA-, and ET/IA-signaling pathways, respectively, and of *CYP79B2, CYP79B3, CYP81F2, and PAD3* genes, which encode proteins from the trp-derived metabolites pathway. All the genes tested were found to be up-regulated in agb1-1 and agb1-2 alleles, and in aggl-1 agg2-1 double mutant upon PcBMM inoculation (Figures 2B and 3A). The induction of the majority of these genes was stronger in the PcBMM-inoculated mutants than in the Col-0 plants (Figures 2B and 3A). This observation might be explained by the enhanced growth of PcBMM in the G-protein mutants in comparison to the progression of fungal colonization in wild-type plants (Figures 2B and 3A). Together, these data indicate that the SA, ET, JA, ABA, and the trp-derived metabolite pathways were not impaired in the agb1 alleles and in the aggl-1 agg2-1 double mutant.

To further demonstrate that the accumulation of trp-derived metabolites, such as camalexin or IGs, was not blocked in agb1 plants during PcBMM infection, we performed a comparative metabolite profiling of leaf extracts from wild-type plants and one of the agb1 alleles (agb1-1) at 1 and 3 dpi. As shown in Figure 3B, we found that both agb1-1 and Col-0 plants had significant increase in the accumulation of two representative metabolites of these pathways, namely 4-methoxy-indol-3-ylmethylglucosinolate (4MI3G) and cama­lexin (the products derived from CYP81F2 and PAD3 activities, respectively; Schuhegger et al., 2006; Bednarek et al., 2009, 2011). The accumulation of camalexin was enhanced in the agb1-1 mutant compared to Col-0 plants at 3 dpi (Figure 3B). This observation can probably be explained by the enhanced growth of the fungus in the mutant in comparison to Col-0 plants.

Together, these results suggest that the resistance mechanism mediated by AGG1/AGG2 and AGB1 genes may be similar, which is consistent with the functional canonical model proposed for the *Arabidopsis* heterotrimeric G-protein in the regulation of some biological processes (Mason and Botella, 2001; Kato et al., 2004; Adjibio-Hermans et al., 2006). These data also demonstrate that G-protein-mediated resistance to necrotrophic fungi is not dependent on the

### Table 1. Genes Differentially Regulated in Non-Inoculated 3-Week-Old agb1-1 Plants.

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Locus</th>
<th>n-fold&lt;sup&gt;1&lt;/sup&gt; expression</th>
<th>References&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
<td>Unknown protein</td>
<td>Atg922440</td>
<td>0.20</td>
<td>Kreps et al. (2002)</td>
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<td>HAD superfamily, subfamily IIIB acid phosphatase</td>
<td>Atg292650</td>
<td>0.12</td>
<td>Liu et al. (2005)</td>
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<td>GABA; GTP binding protein beta 1</td>
<td>Atg34460</td>
<td>0.16</td>
<td>Weiss et al. (1994)</td>
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<tr>
<td>GDA1/CD39; nucleoside phosphatase family protein</td>
<td>Atg11430</td>
<td>0.56</td>
<td>Ascencio-Ibáñez et al. (2008)</td>
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<td>ATTPCK1; phosphoenolpyruvate carboxylase kinase 1</td>
<td>Atg06850</td>
<td>0.57</td>
<td>Hartwell et al. (1999)</td>
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<tr>
<td>PCC1; pathogen and circadian controlled 1</td>
<td>Atg322231</td>
<td>0.61</td>
<td>Segarra et al. (2010)</td>
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<td>Copper amine oxidase family protein</td>
<td>Atg31690</td>
<td>0.62</td>
<td>Fukao et al. (2003)</td>
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<tr>
<td>ATGCN1; transporter family protein</td>
<td>Atg60790</td>
<td>0.62</td>
<td>Sánchez-Fernández et al. (2001)</td>
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<td>ACT7; actin 7</td>
<td>Atg90810</td>
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<td>McDowell et al. (1996)</td>
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<td>Copper transport protein family</td>
<td>Atg52760</td>
<td>1.86</td>
<td>Taki et al. (2005)</td>
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<td>AtPP2-A13; phloem protein 2-A13</td>
<td>Atg61060</td>
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<td>Dinant et al. (2003)</td>
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<td>PDF1.2A; plant defensin 1.2</td>
<td>Atg44440</td>
<td>2.18</td>
<td>Penninckx et al. (1996)</td>
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<td>ATSE1; rhodanese/cell cycle control phosphatase superfamily</td>
<td>Atg35770</td>
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<td>Oh et al. (1996)</td>
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<td>QQS; qua-quine starch</td>
<td>Atg30720</td>
<td>11.87</td>
<td>Li et al. (2009)</td>
</tr>
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</table>

1 Genes (ANOVA p < 0.01) with n-fold expression in agb1-1 higher than 1.755 (induced) or lower than 0.625 (repressed) compared to wild-type plants (Col-0).

2 See supplementary information.
SA-, JA-, ET-, and ABA-pathways and on the trp-derived metabolites pathway, and point to some additional, uncharacterized mechanism determining heterotrimeric G-protein function in *Arabidopsis* immunity responses.

Transcriptomic Analyses Revealed that Cell Wall-Related Genes Are Differentially Regulated in *agbl* Mutant

To try to elucidate the molecular bases that determine heterotrimeric G-protein-mediated resistance to necrotrophic fungi, we performed functional classification of the 346 genes differentially regulated in *agbl*-1 plants upon *PcBMM* inoculation by using BAR and the ARANET Gene Ontology tools. The most statistically relevant functional categories found with the BAR tool included ‘response to stress’, ‘response to abiotic and biotic stimulus’, and ‘receptor binding activity’, which further corroborated that the *agbl*-1 and wild-type plants displayed differential responses to biotic and abiotic stresses (Figure 4A). Interestingly, the ‘cell wall’ cellular component category was also over-represented (p-value < 0.001) with 43 genes (Figure 4A and Supplemental Table 4). These cell wall-related genes were found to cluster with cellulose and pectin biogenesis, hydrolase activities, and xyloglucan biosynthesis/modification, vesicle transport of cell wall precursors, and lipid metabolism and cell wall formation (Supplemental Table 4; Brown et al., 2005; Persson et al., 2005; Mutwil et al., 2010). These data were consistent with the significant over-representation (p-value < 0.047) of the ‘cell wall’ functional category found among the genes differentially regulated in mock-treated *agbl*-1 mutant compared to Col-0 plants (Supplemental Table 1). Together, these results suggest that impairment of AGB1 function might cause mis-regulation of genes associated with biogenesis/regulation of cell wall structure/composition.

Notably, among the genes that were similarly regulated (up or down) in *PcBMM*-inoculated Col-0 and *agbl*-1 plants (Supplemental Tables 2 and 3), we also found a significant over-representation of the ‘cell wall’ functional category (82 genes from 1477 in Col-0 and 115 genes from 2165 in *agbl*-1 clustered in this category with p-values of $1.77 \times 10^{-25}$ and $2.43 \times 10^{-18}$, respectively). These data indicate that, upon necrotrophic fungal infection, a substantial reprogramming of the cell wall architecture occurs in both wild-type and *agbl*-1 plants.
Figure 3. The Trp-Derived Biosynthetic Pathway Is Not Altered in the Heterotrimeric G-Protein Mutants.
(A) Wild-type plants (Col-0) and agb1-1, agb1-2, and aggl aggl mutants were spray-inoculated with PcBMM as described in Figure 1. Expression of the CYP79B2, CYP79B3, PAD3, and CYP81F2 genes was quantified by qRT-PCR at 3 dpi in mock-treated (Mk) and PcBMM-inoculated (Pc) samples. Values were normalized to Arabidopsis UBIQUITIN21 expression levels and represented as relative expression values. Bars represent the average (± SD) of two technical replicates. Data are from one out of three independent experiments, which gave similar results.
(B) Average relative content (nmol g⁻¹ Fresh Weight (FW) ± SD) of camalexin and 4-methoxy-indol-3-ylmethyl glucosinolate (4MI3G) in mock or PcBMM-treated wild-type plants and the agb1-1 mutant at 1 and 3 dpi. Asterisks indicate differences statistically significant with Col-0 plants (ANOVA p < 0.05, Bonferroni Test). The analyses were repeated three times and similar results were obtained.
The differential expression of some of the cell wall-related genes in PcBMM-inoculated plants (n-fold induction/repression compared to Mock samples) was validated by comparative qRT-PCR analysis in Col-0 plants, in the agb1-1 and agb1-2 alleles, and in the aggl-1 agg2-1 double mutant (Figure 4B). Remarkably, the expression patterns of these genes in the aggl-1 agg2-1 double mutant were similar to those observed in the agb1 alleles (Figure 4B). Among the protein encoded by the validated gene were the NAC36 transcription factor (AT2G17040) that regulates several processes including sugar (e.g. xylose) transport (Kato et al., 2010), the xyloglucan endotransglucosylase/hydrolase 4 (XTH4) involved in xyloglucan remodeling (Nishitani and Tominaga, 1992), two pectin-methyl esterase (PME) inhibitors (PME1: AT5G62360 and AT2G45220) that are involved in the inhibition of the de-esterification of cell wall pectins by PMEs in muro (Lionetti et al., 2007; Raiola et al., 2010), the polygalacturonase-inhibiting protein 1 (PGIP1, AT5G06860; Ferrari et al., 2006) that blocks the activity of fungal PGs, and a β-1,3-glucanase that has been implicated in the deposition and hydrolysis of callose (Levy et al., 2007). These data suggested that the cell wall structure/composition might be altered in agb1 and aggl-1 agg2-1 mutants in comparison to wild-type plants, and that PcBMM infection leads to a substantial transcriptional reprogramming of cell wall-related genes in the Gβ and Gγ1/2 mutants.

The agb1 and aggl agg2 Mutants Hold Altered Cell Wall Architecture

The cell wall has been proposed to play a key role in the regulation of Arabidopsis immunity based on the identification of cell wall mutants with altered disease resistance responses (Ellis and Turner, 2001; Vogel et al., 2002, 2004; Hernández-Blanco et al., 2007; Ramirez et al., 2011). Moreover, the characterization of the SGB1 gene (suppressor of agb1 hypersensitivity to glucose), which encodes a hexose transporter located in the Golgi, led to the suggestion that heterotrimeric G-protein may be involved in the regulation of hexoses (e.g. glucose) transport into the Golgi, which consequently affects cell wall biosynthesis (Wang et al., 2006). Based on these observations, and the over-representation of cell wall-related genes among those genes differentially regulated in the agb1 and aggl-1 agg2-1 mutants (Figure 4 and Supplemental Tables 1 and 4), we decided to explore the cell wall composition of the agb1 and aggl-1 agg2-1 mutants.

Cell walls from non-inoculated leaves of 3-week-old plants from the agb1-2 null allele, the aggl-1, agg2-1, agg1-1 agg2-1, gpa1-4, and Col-0 genotypes were subjected to Fourier Transform InfraRed (FTIR) spectroscopy to obtain qualitative a better comparison with transcriptomic data were represented as n-fold compared to the mock-treated plants. Bars represent the average (± SD) of two technical replicates. Asterisks indicate differences statistically significant with Col-0 plants (ANOVA p < 0.05, Bonferroni Test). Data are from one out of three independent experiments, which gave similar results.

Figure 4. Cell Wall-Related Genes Are Mis-Regulated in agb1 and aggl agg2 Mutants.

(A) The 346 genes differentially regulated in the PcBMM-inoculated agb1-1 compared with Col-0 plants were classified using tools of the Bio-Array Resources (BAR) for Plant Biology (http://bar.utoronto.ca/) and the ARANET Gene Ontology (http://aranet.mpimp-golm.mpg.de; Mutwil et al., 2010). The normalized score value for each functional class is represented. Only over-represented classes in comparison with the whole genome (normalized score value > 1) are shown.

(B) Gene validation of differentially expressed genes in agb1-1, agb1-2, and aggl agg2 mutants. Expression of up- or down-regulated gene upon PcBMM infection genes was quantified by qRT-PCR in mock-treated and PcBMM-inoculated wild-type plants (Col-0) and agb1-1, agb1-2, and aggl agg2 mutants. Values were normalized to Arabidopsis UBIQUITIN21 expression levels and for
Figure 5. FTIR Analysis of the Cell Walls from Wild-Type Plant and Heterotrimeric G-Protein Mutants.

(A) FTIR average spectra (n = 15) from 3-week-old rosettes of Col-0 wild-type plants (WT), agb1-2, aggl-1, agg2-1, agg1-1 agg2-1, and gp1a-4 mutants. Wavenumbers 1034, 1072, and 1155 cm\(^{-1}\) may correspond to galactan side-chains (with a \((\beta-1 \rightarrow 6)\)-D-Galp backbone) in Rhammnogalacturonan I (RGI). The 1078 and 1106 cm\(^{-1}\) bands are common to several other cell wall polysaccharides (Kauráková et al., 2000). Wavenumbers 1541 cm\(^{-1}\) (Amide II; N-H deformation and C-N amide stretching) and 1637-1664 cm\(^{-1}\) (Amide I; C=O stretching and C-N) might be associated with proteins. Bands at 1425 and 1600 cm\(^{-1}\), which may correspond to unesterified pectins (COO- symmetric and antisymmetric stretching, respectively (Kauráková et al., 2000; Wilson et al., 2000)).

(B) Biplot analysis showing a clear separation of agb1-2 and a partial segregation of agg1-1 agg2-1 from WT, aggl-1, agg2-2, and gp1a-4.

(C) Mid-infrared spectra were analyzed by the covariance-matrix approach for principal component analysis 1 (PC1).

spectratypes (i.e. cell wall phenotypes). The comparison of averaged FTIR spectra (n = 15) revealed significant differences between agb1-2 and the rest of the genotypes (Figure 5A). The double mutant agg1-1 agg2-1 had an intermediate spectratype between both groups (Figure 5A). Much of the total sample variation (86%) was explained by principal component 1 (PC1; Figure 5B). Wavenumbers at 1034, 1072, and 1155 cm\(^{-1}\), which may correspond to galactan side-chains (with a \((\beta-1 \rightarrow 6)\)-D-Galp backbone) in Rhammnogalacturonan I (RGI), and bands at 1 078 and 1 106 cm\(^{-1}\), which are common to several other cell wall polysaccharides were relatively more abundant in agb1-2 and, to a lower extent, in agg1-1 agg2-1 than in wild-type cell walls (Figure 5B). In addition, some bands (1 541 and 1 600 cm\(^{-1}\)), which may correspond to proteins, were also relatively more abundant in agb1-2 than in the rest of the genotypes (Figure 5C). On the contrary, bands at 1 425 and 1 600 cm\(^{-1}\), which may correspond to unesterified pectins, are more intense in Col-0, agg1-1, agg2-1, and gp1a-4 than in agb1-2 and agg1-1 agg2-1 (Figure 5C).

To obtain a more quantitative analysis of the cell wall changes in agb1 and agg1-1 agg2-1 walls, we compared the non-cellulosic neutral monosaccharide composition of leaves from non-inoculated 3-week-old Col-0 plants, the agg1-1 agg2-1 double mutant, and the agb1-2 and agg1-1 alleles (Blakeney et al., 1983; Reiter et al., 1993). Notably, the amount (% mol) of xylose was found to be lower in agb1-2, agg1-1, and agg1-1 agg2-1 samples than in the Col-0 wild-type plants (Figure 6A and Supplemental Figure 2). A slight increase in mannose content was also detected in agg1-1 agg2-1, but not in the agg1 alleles, compared to Col-0 plants (Figure 6A and data not shown). Not significant differences were observed for the rest of the neutral sugars among the genotypes tested (Figure 6). We also determined the cellulose and uronic acid content of the walls from agb1-2, agg1-1 agg2-1, and Col-0 leaves, and we found that these levels were similar in the mutants and wild-type plants (Figure 6B).

Together, these analyses revealed that mutations in the Gp (AGB1) or Gy1y2 (AGG1+AGG2) subunits lead to similar alterations in the cell wall structure/composition of the agb1 and agg1-1 agg2-1 mutants. These data pointed to a putative function of Gp (AGB1) and Gy (AGG1/AGG2) subunits in the regulation of cell wall structure/composition, and in particular in the control of the wall xylose, a sugar that is present in glucuronoxylans and xyloglucans wall polymers (Somerville et al., 2004). To further determine the relevance of the wall xylose content on Arabidopsis resistance to necrotrophic fungi, we tested the resistance to PcbMM of additional Arabidopsis wall mutants that have enhanced level of xylose (e.g. det3 and irx-1; Brown et al., 2005; Rogers et al., 2005) or alterations in the structure of...
The agbl-1 Mutation Interferes with the Disease Resistance Phenotypes of Arabidopsis Cell Wall Mutants

The AGB1 and the ER RLK might take part of the same developmental signaling pathway (Lease et al., 2001). Notably, ER has been suggested to regulate both cell wall structure/composition and the immune response to several pathogens including PcBMM (Sánchez-Rodríguez et al., 2009). The wall of the er mutant contains less xylose, like agbl-1, but more uronic acid than wild-type walls (Sánchez-Rodríguez et al., 2009). With these data in mind, we tested the genetic interaction between er and one of the agbl-1 alleles (agbl-1) to study whether the enhanced susceptibility to PcBMM of these two mutants was determined by similar alterations in their wall compositions. We therefore inoculated 3-week-old Col-0 wild-type plants, the er-105 and agbl-1 single mutants, and the agbl-1 er-105 double mutant with PcBMM, and fungal growth was measured by qRT-PCR at early stage of infection (1 dpi) to avoid plant decay that would impede fungal biomass quantification. As shown in Figure 7, the susceptibility of the agbl-1 er-105 double mutant was higher than that of the single agbl-1 and er-105 mutants. This indicates that ER and AGB1 are not components of the same signaling pathway controlling Arabidopsis resistance to the necrotrophic fungus PcBMM. These results are in line with the described genetic interaction of AGB1 and ER in the regulation of developmental processes; for example, the agbl-1 er-105 plants showed stronger ‘er-associated’ features than the respective single mutants, and hence this interaction was additive rather than epistatic (Lease et al., 2001).

We also checked the genetic interaction between agbl-1 and the secondary cell wall mutant irx1-6, which shows a broad enhanced resistance to necrotrophic fungi, including PcBMM (Hernández-Blanco et al., 2007). The double agbl-1 irx1-6 mutant was generated and its resistance to PcBMM was determined at different dpi. As shown in Supplemental Figure 4, the introduction of the irx1-6 mutation in the agbl-1 background led to the partial suppression of the agbl-1 susceptibility phenotype, as the agbl-1 irx1-6 double mutant supported more fungal growth than Col-0 plants, but less than agbl-1. These data indicate that inactivation of IRX1/CESA8 function in agbl-1 partially restored to wild-type levels the susceptibility phenotype of the Gβ mutant.

**DISCUSSION**

In plants, as in animals, a canonical model for heterotrimeric G-protein signal transduction has been proposed that...
hypothesizes that the Gy subunits act as a functional monomer (Marrari et al., 2007; Trusov et al., 2007). This model is supported by in vivo biochemical interactions between AGB1 and AGG1/AGG2/AGG3 subunits (Kato et al., 2004; Adjobo-Hermans et al., 2006; Chakravorty et al., 2011) and by the demonstration that Arabidopsis plants lacking both Gy1 and Gy2 subunits display phenotypes that resemble those observed in the agb1 mutant (Trusov et al., 2007, 2008b, 2010). However, specific functions of Arabidopsis Gy1 and Gy2 subunits have also been reported for example in the resistance to the necrotrophic fungus A. brassicicola or in response to auxins (Trusov et al., 2006, 2007, 2008b). In addition, some discrepancies were found between some developmental-associated phenotypes of Gβ and Gγ1/Gγ2-deficient mutants, which led to the suggestion that additional Gy subunits or some functional autonomy of G-protein subunits might exist in Arabidopsis (Trusov et al., 2008b). Some of these discrepancies can be explained by the functional contribution to these processes of the recently described atypical Gy3 subunit (Chakravorty et al., 2011).

Here, we demonstrate that the Gβ/Gγ subunits act as a functional monomer and that the Gy1 and Gy2 subunits play redundant functions in the regulation of Arabidopsis defense response to the necrotrophic fungus PcBMM, as the susceptibility of aggl-1 agg2-1 double mutant to this pathogen is similar to that of the agb1 plants, but higher than that of the aggl-1 and agg2-1 single mutants (Figure 1). In line with this model, we show that the gpa1-4 mutant impaired in the Gα subunit has a slight enhanced resistance to PcBMM, which contrasts with the hyper-susceptibility of the Gβ/Gγ-deficient mutants (Figure 1; Llorente et al., 2005). The role of the Gβ/γ functional monomer in the regulation of Arabidopsis immune response to PcBMM is also supported by the finding that the majority of the genes differentially regulated in agb1 mutant alleles upon PcBMM infection show a similar expression pattern in the aggl-1 agg2-1 double mutant (Figures 3 and 4), which further indicates that inactivation of either Gβ or Gγ1/Gγ2 subunits results in a similar transcriptional reprogramming of the immune response upon necrotrophic fungal infection. These results contrast with the previously reported specific function of AGG1 in the control of Arabidopsis resistance to the necrotrophic fungus A. brassicicola (Trusov et al., 2006). However, our results are in agreement with the described canonical functionality of Gβ/γ in the control of Arabidopsis resistance to the vascular fungus F. oxysporum (Trusov et al., 2010). Additional analysis will be required to determine whether the Gβ/γ functional monomer also controls Arabidopsis defense response to other fungal pathogens, such as the necrotrophic fungus B. cinerea, which is more virulent on the agb1 mutant than on wild-type plants (Llorente et al., 2005).

The molecular basis of the defense response controlled by plant heterotrimeric G-proteins has not been characterized in detail. In rice, the Gα subunit controls the resistance to blast fungus (Magnaporthe grisea) by regulating the production of hydrogen peroxide and the induction of pathogenesis-related genes (PR1 and PB21) upon fungal infection (Suharsono et al., 2002). In Arabidopsis, where the defense response is controlled by the Gβγ dimer (Figure 1), the accumulation of callose, an early immune response, was found to be impaired in agb1 mutant upon infection with PcBMM (Llorente et al., 2005). However, callose accumulation might not be essential for mounting an effective resistance against this fungal pathogen (Stein et al., 2006). A genetic analysis of the interaction between the Gβ subunit and the JA, SA, ET, and ABA signal transduction pathways, which play different roles in regulating resistance to F. oxysporum and A. brassicicola, revealed that AGB1-mediated resistance was independent of these pathways and that Gβ can act upstream of MYC2 and COII, two regulators of JA-signaling (Trusov et al., 2008a). Here, by using a transcriptomic comparative analysis of agb1 and wild-type plants upon PcBMM infection, we demonstrate that the SA-, ABA-, JA-, and ET-signaling and the trp-derived metabolites pathways, which are required for resistance to necrotrophic fungi (Thomma et al., 1998; Berrocal-Lobo et al., 2002; Sánchez-Vallet et al., 2010), are functional in both agb1 and aggl-1 agg2-1 mutants (Figure 2A). Also, we show that, upon PcBMM infection, the expression of defensive marker genes from these pathways is up-regulated in the mutants as in wild-type plants (Figure 2B). Interestingly, we find that the accumulation of camalexin and 4MI3G, two essential trp-derived metabolites for Arabidopsis basal and non-host resistance, is not impaired in agb1 mutant (Figure 3). This suggests that the enhanced susceptibility of agb1 mutant alleles to virulent fungal pathogens (e.g. PcBMM; Figure 1) and the described impairment of non-host resistance to non-adapted fungal isolates of M. oryzae and P. cucumerina in agb1 plants (Maeda et al., 2009; Sánchez-Vallet et al., 2010) might not be associated with a defect in the biosynthesis of these trp-derived metabolites.

The cell wall is a dynamic and responsive structure that regulates plant responses to external stimuli or stresses, including pathogen attack (Humphrey et al., 2007; Cantu et al., 2008). Specific and genetically induced changes in Arabidopsis cell wall composition can therefore result in altered immune responses to different types of pathogens, including the fungal pathogens P. cucumerina, F. oxysporum, and G. cichoracearum (Ellis and Turner, 2001; Vogel et al., 2002, 2004; Hernández-Blanco et al., 2007). Comparative transcriptomic analyses of mock-treated and PcBMM-inoculated agb1 and wild-type plants revealed that a significant subset of genes encoding proteins with putative or well-characterized cell wall-related functions were differentially regulated in agb1 compared to wild-type plants, further suggesting a putative function of G-protein in controlling cell wall composition (Figure 4). Notably, the majority of these genes show a similar pattern of expression in agb1 and aggl-1 agg2-1 mutants, which is consistent with the proposed G-protein canonical model (Figures 4). Among these wall-related genes, we find some that encoded putative regulators (e.g. NAC36
transcription factor), proteins with a direct function in cell wall biosynthesis or remodeling (e.g. two PMEIs and the PGIP1 and XTH4 proteins), or proteins that form part of the cell wall proteome (Bayer et al., 2006). Also, we find genes whose expression is down-regulated upon Arabidopsis treatment with cellulose synthase inhibitors (Bischoff et al., 2009). Interestingly, some of these cell wall-related genes have been associated with plant resistance to necrotrophic fungi, such as those encoding PGIP1, PMEIs, and XTHs (Ferrari et al., 2006; Lionetti et al., 2007; Miedes and Lorences, 2007), or with callose turnover upon pathogen infection (Levy et al., 2007).

The transcriptomic data suggested that agb1 and agg1-1 agg2-1 plants might have some alterations in their cell wall architecture that could explain their enhanced susceptibility to PcBMM. Consistent with this hypothesis, we demonstrate that the cell wall structure/composition of 3-week-old agb1 and agg1 agg2 plants is similar, but differ from that of wild-type plants (Figures 5 and 6). The complexity of the PC1 FTIR spectrum indicates that the cell wall differences between wild-type, agg1-1, and agg2-1 plants and the agb1-2 and agg1-1 agg2-2 genotypes cannot be assigned to a defect in just a single cell wall polymer. While FTIR analysis of agb1 and agg1-1 agg2-2 mutants suggested a higher content of galactan side-chains and a lower content of unesterified pectins (Figure 5), neutral monosaccharide composition analyses of the non-cellulosic material showed that, in these mutants, only the content of xylose was reduced compared to wild-type plants (Sanchez-Rodriguez et al., 2009). Together, these data suggest that the alteration in the content of xylose, which is present in glucoronoxylans and xyloglucans (Somerville et al., 2004), might explain, at least in part, the enhanced susceptibility to PcBMM observed in the GjGy mutants. In line with this hypothesis, we found that Arabidopsis mutants with increased xylose content, such as det3 and irx6-1 (Brown et al., 2005; Rogers et al., 2005), or with alterations in the xyloglucan structure (e.g. xyl1-2; Sampedro et al., 2010), showed an enhanced resistance to the necrotrophic fungus PcBMM (Supplemental Figure 3). These data suggest that wall xylose content might be one of the determinants of Arabidopsis susceptibility to necrotrophic fungi.

In addition to xylose content, other molecular modifications of the cell wall might contribute to explain the susceptibility phenotype of agb1 and agg1-1 agg2-1, as the FTIR and neutral monosaccharide composition analyses suggest the presence of additional and specific alterations in the walls of these mutants (Figures 5 and 6). In line with this hypothesis, we found that the susceptibility to PcBMM of agb1 er-105 double mutant was stronger than that of the single mutants (Figure 7). By contrast, we find that the susceptibility phenotype of agb1 can be partially suppressed by irx1-6 secondary cell wall mutant (Supplemental Figure 4), whose xylose content is similar to that of wild-type plants (Brown et al., 2005). The data presented here do not exclude that additional, uncharacterized molecular mechanisms might also contribute to explaining the enhanced susceptibility to necrotrophic fungi of G-protein.

Our results point to a potential function of heterotrimeric G-protein in the regulation of cell wall architecture. This is consistent with the previously suggested function of heterotrimeric G-protein in the regulation of cell wall biogenesis, which was mainly supported by the characterization of SGB1 gene (Wang et al., 2006). SGB1 encodes a member of a family of Golgi-localized hexose transporters that have been implicated in the translocation of sugars, including xylose, and in de novo wall synthesis, most notably pectins and hemicelluloses (Wang et al., 2006). The constitutive activation of the SGB1 gene in agb1 restores to wild-type levels the hypersensitivity of agb1 to D-glucose (Wang et al., 2006), but does not restore the enhanced susceptibility of agb1 to PcBMM (Supplemental Figure 5), indicating that SGB1 does not play a function in immunity. Heterotrimeric G-protein could also function as a cell wall integrity regulator responding to environmental stress, including pathogen attack, as has been described in yeast for the WSC system (for cell wall integrity and stress response), which is formed by integral membrane proteins acting as surface sensors (Philip and Levin, 2001). In plants, a similar sensing system to that of yeasts has not been found, but several RLKs members have been proposed to play this function (Hématy and Höfft, 2008). The characterization of GjGy-mediated downstream signaling as well as the identification of AGB1 or AGG1/AGG2 targets will be needed to corroborate the function of heterotrimeric G-protein in the regulation of cell wall architecture/composition, and to link plant wall features with specific disease resistance responses.

METHODS

Biological Material and Growth Conditions
A. thaliana plants were grown in sterilized soil as described previously (Hernández-Blanco et al., 2007). The following lines in the Col-0 background were used: agb1-1 (Lease et al., 2001), agb1-2 (Ullah et al., 2003), agg1-1 agg2-1 and agg1-1 (Trusov et al., 2007), agg2-1 and gpa1-4 (Jones et al., 2003). The irx1-6 and er-105 mutants have been previously described (Llorente et al., 2005; Hernández-Blanco et al., 2007). The sbg1-2 and agb1-2 sbg1-2 mutants and the 35S:SGB1 lines in agb1-2 background (Wang et al., 2006) were kindly provided by Dr Alan Jones (University of North Carolina, USA). The det3-1 and irx6-1 mutants (Brown et al., 2005) were provided by Dr Simon Turner (University of Manchester, UK) and the xyl1-2 mutant (Sampedro et al., 2010) by Dr Ignacio Zarra (Universidad de
Santiago, Spain). *P. cucumerina BMM* isolate was the gift of Dr B. Mauch-Mani (University of Neuchatel, Switzerland).

**Fungal Inoculation Assays**

Three-week-old *Arabidopsis* plants were inoculated with a spore suspension (4 × 10^6 spores ml^-1). Disease progression in the inoculated plants was estimated by an average disease rating (0–5), by trypsin blue staining and by relative quantification of fungal DNA (β-tubulin of *PcBMM*) by means of quantitative real-time PCR (qRT–PCR) as described (Sánchez-Rodriguez et al., 2010). For biomass quantification, the relative expression ratio of fungal β-tubulin was calculated as the differences between the Ct values (ΔΔct) and was determined using the equation 2^-ΔΔct (Rieu and Powers, 2009). The qRT-PCR results are mean values ± SD from at least two technical replicates. Differences in these parameters among *Arabidopsis* genotypes were analyzed by one-way analysis of variance using plant genotype as factor. To determine whether values of analyzed traits were significantly different among classes within each factor, the Bonferroni post hoc test was employed as reported (Sánchez-Rodriguez et al., 2009). For all the experiments, at least three independent assays were performed.

**Microarray and Gene Expression Analysis**

Leaves from 3-week-old Col-0 and *agb1-1* plants non-inoculated, mock-treated, or inoculated with a spore suspension of *P. cucumerina BMM* (4 × 10^6 spores ml^-1) were collected at 0 and 1 dpi. Each sample represented a pool of the rosettes from 25 plants grown under the growth conditions described before and inoculated as described. Four biological replicates were obtained. Total RNA was extracted from the plants using the method previously described (Berrocal-Lobo et al., 2002) and purified with Rneasy Kit (Qiagen, Germany). Three of the four biological replicates were independently hybridized for each transcriptomic comparison. RNA quality was tested with a Bioanalyzer 2100 (Agilent Technologies, USA). Biotinylated complementary RNA (20 μg) was prepared and the resulting complementary RNA was used to hybridize ATH1 *Arabidopsis* GeneChip (Affymetrix) following the manufacturer’s protocol, at the Genomic Unit of the CNB-CSIC (Madrid, Spain). The array images were analyzed with a GenePix 400B scanner (Molecular Devices) at 10-mm resolution. The images were quantified with GenePixPro 5.1. The expression levels of the genes were analyzed with Multixperiment viewer software (MeV 4.0). Differentially expressed genes in the mutants relative to wild-type samples or in the *P. cucumerina*-inoculated relative to the mock-treated samples were identified using two-way analysis of variance and critical p-value *p* = 0.01. Up- and down-regulated genes were selected using *n*-fold higher than 1.755 or lower than 0.625 relative to wild-type plants unless indicated. The microarray data have been deposited in the public repository Array-Express with the reference number E-MTAB-641.

For gene expressions analysis, RNA extractions from (*P. cucumerina*-infected or mock-treated) rosettes were performed as described (Llorente et al., 2005). qRT-PCR analyses were performed as previously reported (Hernández-Blanco et al., 2007) using the *UBQUITIN21* (At5g25760) expression to normalize the transcript level in each sample and calculate the ΔΔct value. Oligonucleotides used for cDNA amplification were designed with Primer Express (version 2.0; Applied Biosystems, Foster City, CA, USA) and their sequences are included in Supplemental Table 5. qRT-PCR reactions were performed in 20 μl containing 0.3 μM each of primer, 1/100 diluted cDNA, and SYBR Green PCR master mix (Roche). The PCR conditions were as described (Sánchez-Rodriguez et al., 2009). Data analysis was performed using the Relative Quantification Application of the Sequence Detector Software (version 1.4, Applied Biosystems, Foster City, USA), according to Rieu and Powers (2009). Differences in expression ratios (ΔΔct) among the samples were analyzed by ANOVA or t-test using the Statgraphics software.

**Extraction of Secondary Metabolites and Chromatographic Analysis**

Plant material was collected and frozen in liquid nitrogen. The tissue was extracted and obtained samples were analyzed an Agilent (Palo Alto, CA, USA) a 1100 high performance liquid chromatography (HPLC) system equipped with DAD and FLD detectors as described (Bednarek et al., 2009). 4MI3G peak was identified by comparing its retention time and spectral properties with those of a standard purified from plant tissue (Bednarek et al., 2009). Camalexin peak was identified by referring to a synthetic standard (Bednarek et al., 2009). The concentrations of the metabolites of interest were quantified on the basis of the comparison of their peak areas with those obtained during HPLC analyses of known amounts of the respective standards. The following chromatograms were used for quantifications: 4MI3G—UV absorption at 273 nm, camalexin—fluorescence (ex. 318 nm; em. 386 nm).

**Cell Wall Analyses**

For cell wall analysis, leaves of at least 30 individual 3-week-old plants were pre-cleaned (70% ethanol, methanol:chloroform (1:1, v:v) and acetone) and further air-dried and homogenized by wall milling. The resulting crude cell wall material of three independent biological replicates was used for Fourier Transform Infra Red (FTIR) and sugar composition analysis. For FTIR, the powder was dried, mixed with KBr, and pressed into 13-mm pellets. Fifteen FTIR spectra for each line were collected on a Thermo Nicolet Nexus 470 spectrometer (ThermoElectric Corporation, Chicago, USA) over the range 4000–400 cm^-1. For each spectrum, 32 scans were co-added at a resolution of 4 cm^-1 for Fourier transform processing and absorbance spectrum calculation by using OMNIC software (Thermo Nicolet, Madison, WI, USA). Using win-das software (Wiley, New York, USA), spectra were baseline-corrected and were
normalized and analyzed using the principal component (PC) analysis covariance matrix method (Kemsley, 1996).

Cell wall monosaccharides contained in 1 mg of cell wall material were assayed after hydrolysis with 2 M trifluoroacetic acid (TFA) as alditol acetate derivatives (Neumetzler, 2010; modified protocol from Stevenson et al., 1986) by gas chromatography performed on an Agilent 6890N GC System coupled with an Agilent 5973N Mass Selective Detector (Waldbronn, Germany). Myo-Inositol was added as an internal standard. Cellulose was determined on the fraction resistant to extraction with 2 M TFA (n = 3) by Seaman hydrolysis (Selvendran et al., 1979) using glucose equivalents as standard. The hexasaccharide content was determined with the anthrone assay (Dische, 1962). Uronic acids were colorimetrically quantified using the soluble 2 M TFA fraction (n = 3) using 2-hydroxydiphenyl as reagent (Vilim, 1985) using galacturonic acid as standard (Filisetti-Cozzi and Carpita, 1991). The data were analyzed using one-way ANOVA with the Bonferroni’s post-hoc test. All statistical analyses were performed using the statistical software package SPSS 13.0 (SPSS Inc., Chicago, USA).

SUPPLEMENTARY DATA

Supplementary Data are available at Molecular Plant Online.

FUNDING

Work in A. Molina’s group was supported by Spanish MICINN (Grants BIO2009-07161 and EUI2008-03728/BALANCE), in Dr Staffan Person’s group by the Max-Planck Gesellschaft, in Dr Paul Schulze-Lefert’s laboratory by BALANCE-Plant KBBE, and in Dr José M. Estevez’s group by PIP CONICET 2010-071.

ACKNOWLEDGMENTS

We thank Dr Roberto Solano and the Unidad de Genómica from the Centro Nacional de Biotecnología (CNB-CSIC, Madrid, Spain) for technical assistance with the transcriptomic analyses. The laboratory assistance of Gemma López is also acknowledged. M. Delgado-Cerezo, C. Sánchez-Rodríguez, C. Hernández-Blanco and A. Sánchez-Vallet were PhD Fellows from the MICINN (Ministerio de Ciencia e Innovacion, Spain), and Dr Eva Miedes was a JdC postdoctoral fellow from the MICINN. C. Sánchez-Rodríguez was a MICINN postdoctoral fellow (2008-0861) in SP lab. No conflict of interest declared.

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