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 *agb1* 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 *agb1* 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 spectratypes, which differed from that of wild-type plants. The data presented here suggest a canonical functionality of the Gβ and Gγ1/γ2 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, Gβ, 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 GTPase 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* Gβ and Gγ1/γ2 subunits (Marrari et al., 2007). However, some non-redundant and specific functions of the Gβγ1 and Gβγ2 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 Gβ 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 (RGS1) 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*, *Plectosphaerella cucumerina*, and *Alternaria brassicicola*, and to the vascular fungus *Fusarium oxysporum*, whereas the resistance of *agb1* plants to virulent and avirulent isolates of the biotrophic oomycete *Hyaloperonospora arabidopsidis* 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 fungi 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 *agg1 agg2* and *agg1* 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 Gγ1 single mutants and Gγ1γ2 double mutant indicated that the susceptibility of the *agg1* and *agg2* mutants was higher than that of wild-type plants, but lower than that of the *agg1 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 *agg1*, *agg2*, and *agg1 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. indolglucosinolates, 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 irr*egular *xyl*em) and primary (e.g. *prc1/ixr1/cev1*, *procuste1/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-Rodriguez 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; Llorente 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 *agb1* 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/Gβγ2 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 *agb1* alleles (*agb1-1/elk4* and *agb1-2*), and the *agg1-1 agg2-1* double mutant, with a spore suspension (4 × 10⁶ 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 *agb1* 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 *agb1* 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 *agb1* 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 (Llorente 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 *agb1* 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 *agb1* 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 *agb1* 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 *agb1-1* mutant allele. First, we studied the transcriptome of these genotypes before fungal inoculation (0 dpi) to determine whether the enhanced susceptibility to *PcBMM* of *agb1-1* mutant may be the result of the mis-regulation of constitutively expressed defensive genes. Also, we performed comparative transcriptomic analyses of Col-0 and *agb1-1* plants 1 d after water spraying (mock-treatment) or inoculation with *PcBMM*, to identify potential differences in the regulation of induced defense responses in *agb1* compared to Col-0 plants. Out of the 22000 genes tested in these transcriptomic analyses, 9 762 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 (PCC1, *At3g22231*), 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 methylesterase, 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
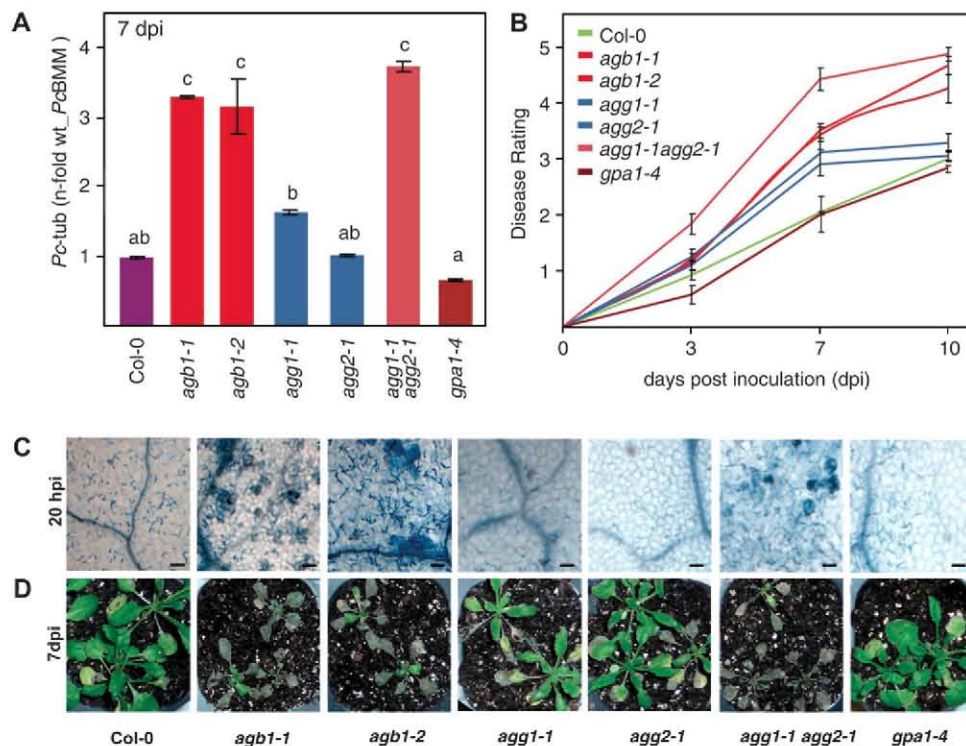
Figure 1. The Susceptibility of *agg1 agg2* Double Mutant to *P. cucumerina* Is Similar to that of *agb1*.
Plants of the showed genotypes were spray-inoculated with spores (4×10^6 sp ml^{-1}) of *P. cucumerina* BMM.
(A) Relative quantification of fungal DNA (*P. cucumerina* β-tubulin) by qRT–PCR at 7 d post inoculation (dpi). Values are represented as the average ($\pm$ SD) of the *n*-fold-increased expression compared with wild-type plants (Col-0). Letters indicate values statistically different among genotypes (ANOVA $p \leq 0.05$, Bonferroni Test).
(B) Average disease rating (DR $\pm$ 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.

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

Gene description	Locus	n-fold[1] expression *agb1-1*/Col-0	References[2]
Unknown protein	At3g22240	0.02	Kreps et al. (2002)
HAD superfamily, subfamily IIIB acid phosphatase	At4g29260	0.12	Liu et al. (2005)
AGB1; GTP binding protein beta 1	At4g34460	0.16	Weiss et al. (1994)
GDA1/CD39; nucleoside phosphatase family protein	At1g14230	0.56	Ascencio-Ibáñez et al. (2008)
ATPPCK1; phosphoenolpyruvate carboxylase kinase 1	At1g08650	0.57	Hartwell et al. (1999)
PCC1; pathogen and circadian controlled 1	At3g22231	0.61	Segarra et al. (2010)
Copper amine oxidase family protein	At1g31690	0.62	Fukao et al. (2003)
ATGCN1; transporter family protein	At5g60790	0.62	Sánchez-Fernández et al. (2001)
ACT7; actin 7	At5g09810	1.82	McDowell et al. (1996)
Unknown protein	At5g58570	1.86	Ascencio-Ibáñez et al. (2008)
Copper transport protein family	At5g52760	1.86	Taki et al. (2005)
AtPP2-A13; phloem protein 2-A13	At3g61060	2.01	Dinant et al. (2003)
PDF1.2A; plant defensin 1.2	At5g44420	2.18	Penninckx et al. (1996)
ATSEN1; rhodanese/cell cycle control phosphatase superfamily	At4g35770	2.19	Oh et al. (1996)
QQS; qua-quine starch	At3g30720	11.87	Li et al. (2009)

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.

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 *agg1-1 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/JA-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 *agg1-1 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 *agg1-1 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; Pfalz 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; Adjobo-Hermans et al., 2006). These data also demonstrate that G-protein-mediated resistance to necrotrophic fungi is not dependent on the
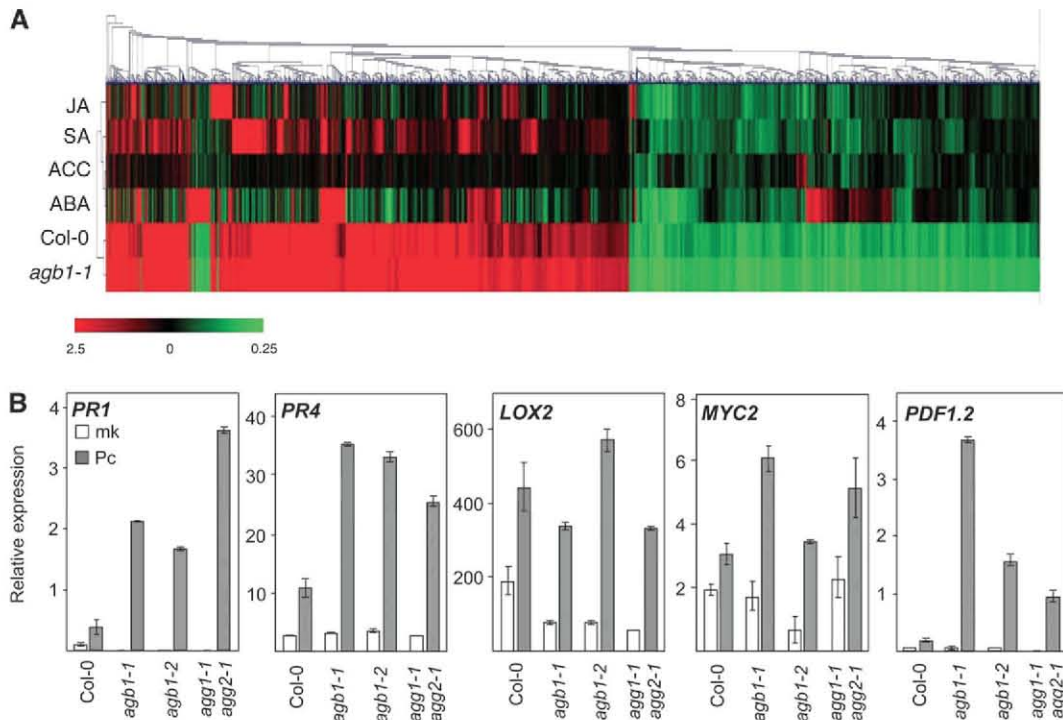
Figure 2. The SA-, ET-, JA-, and ABA-Pathways Are Not Impaired in *agb1* and *agg1 agg2* Mutants.

(A) Hierarchical cluster analysis of the probesets differentially expressed in *P. cucumerina*-inoculated *agb1-1* and Col-0 plants compared with mock-inoculated plants. Clustering was performed using a two-way analysis of variance and a Benjamini and Hochberg multiple testing correction ($P \leqslant 0.01$; Gene-Spring 7.2 software). Genes (rows) and samples from the different experiments (columns) were clustered with the multi-experiment viewer (MeV) using Pearson uncentered distance and average linkage.

(B) Expression of SA, ET, JA, and ABA defense-related genes is up-regulated in *agb1-1*, *agb1-2*, and *agg1 agg2* plants upon *PcBMM* infection. Expression of the indicated defense-related genes (*PR-1*, *PR-4*, *LOX2*, *MYC2*, and *PDF1.2a*) was determined by qRT–PCR at 3 dpi in mock-treated and *PcBMM*-inoculated samples. Values were normalized to *Arabidopsis* UBIQUITIN21 expression levels and represented as average (± SD) relative expression values. Data are from one out of three independent experiments, which gave similar results.

SA-, JA-, ET-, and ABA-signaling 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 *agb1* 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 *agb1-1* 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 *agb1-1* 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' cellular component category found among the genes differentially regulated in mock-treated *agb1-1* 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 *agb1-1* 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 *agb1-1* clustered in this category with *p*-values of 1.77×10^{-25} and 2.43×10^{-18}, respectively). These data indicate that, upon necrotrophic fungal infection, a substantial reprogramming of the cell wall architecture occurs in both wild-type and *agb1-1* plants.
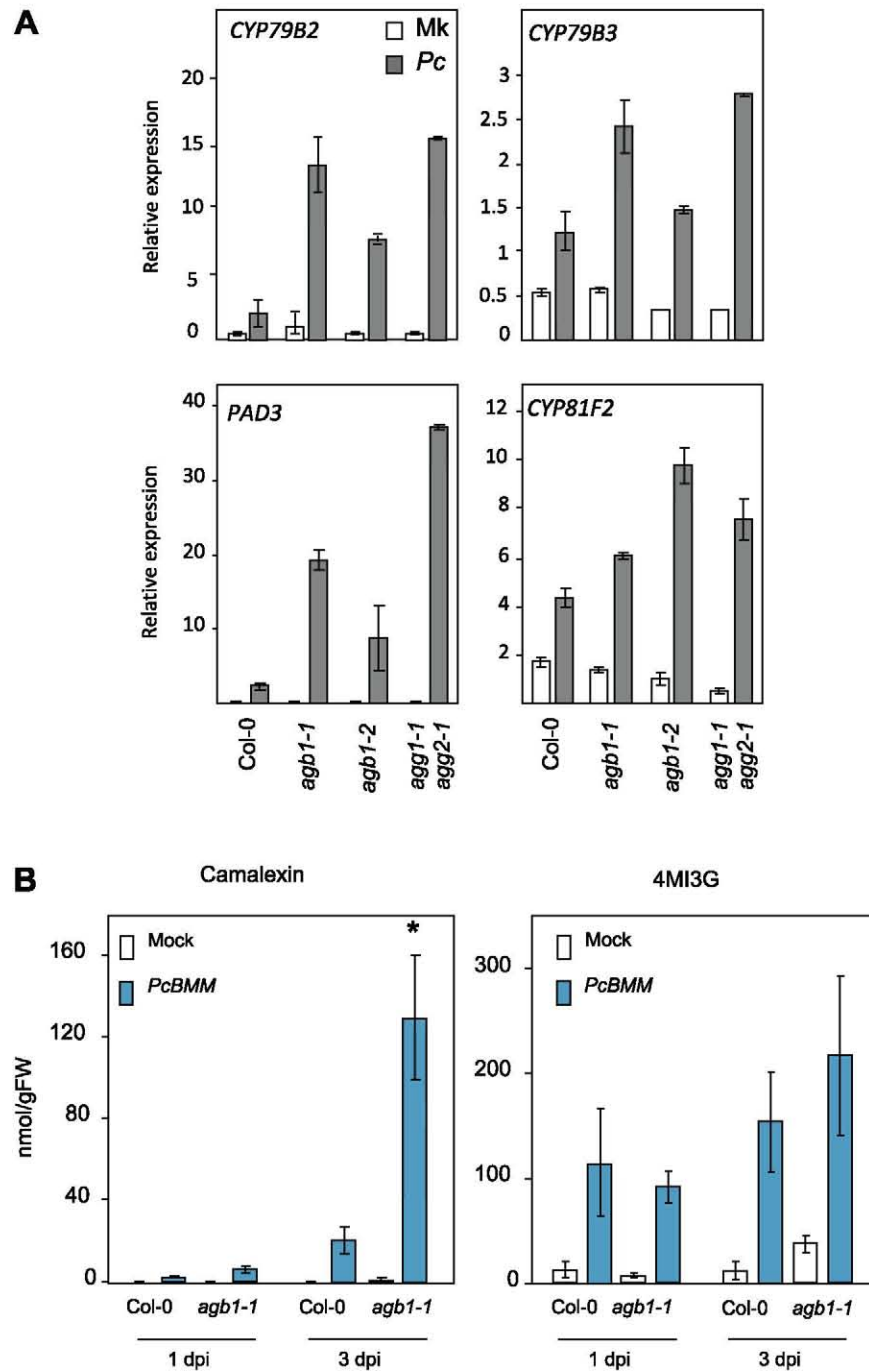
Figure 3. The Trp-Derived Biosynthetic Pathway Is Not Altered in the Heterotrimeric G-Protein Mutants.

(A) Wild-type plants (Col-0) and *agb1-1*, *agb1-2*, and *agg1 agg2* 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 $p \leqslant 0.05$, Bonferroni Test). The analyses were repeated three times and similar results were obtained.
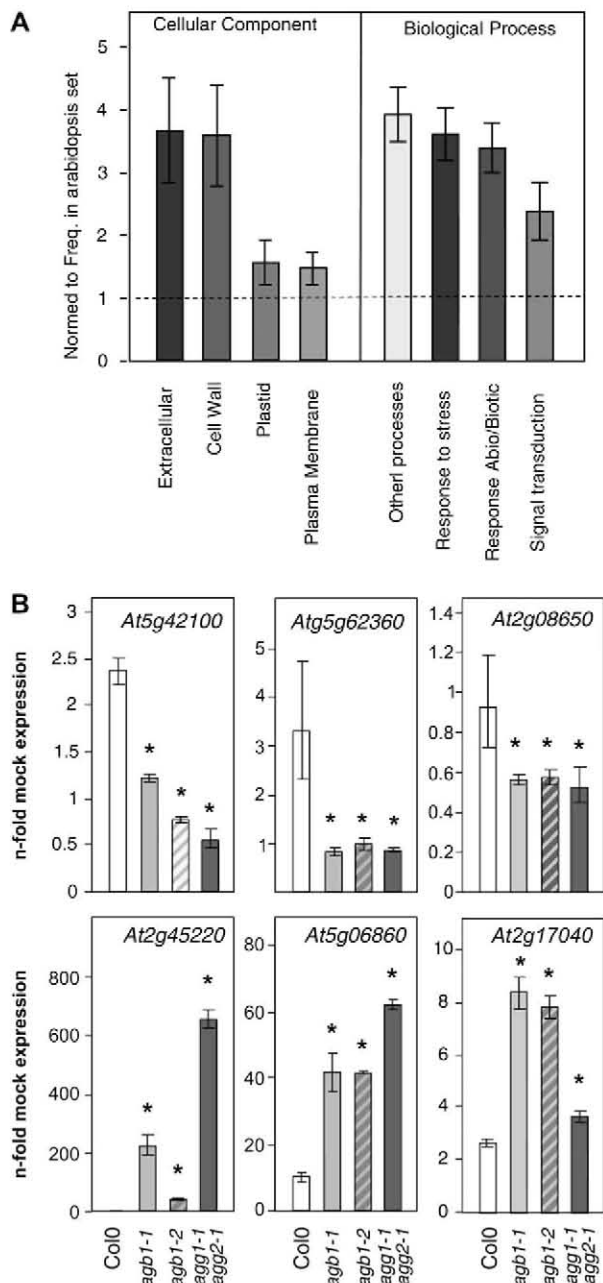
Figure 4. Cell Wall-Related Genes Are Mis-Regulated in *agb1* and *agg1 agg2* Mutants.

(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.utoron to.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 *agg1 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 *agg1 agg2* mutants. Values were normalized to *Arabidopsis UBIQUITIN21* expression levels and for

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 *agg1-1 agg2-1* double mutant (Figure 4B). Remarkably, the expression patterns of these genes in the *agg1-1 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-methylesterase (PME) inhibitors (PMEI: 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 *agg1-1 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/γ2 mutants.

The *agb1* and *agg1 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; Ramírez et al., 2011). Moreover, the characterization of the *SGB1* gene (suppressor of a*gb*1 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 *agg1-1 agg2-1* mutants (Figure 4 and Supplemental Tables 1 and 4), we decided to explore the cell wall composition of the *agb1* and *agg1-1 agg2-1* mutants.

Cell walls from non-inoculated leaves of 3-week-old plants from the *agb1-2* null allele, the *agg1-1*, *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 $p \leqslant 0.05$, Bonferroni Test). Data are from one out of three independent experiments, which gave similar results.
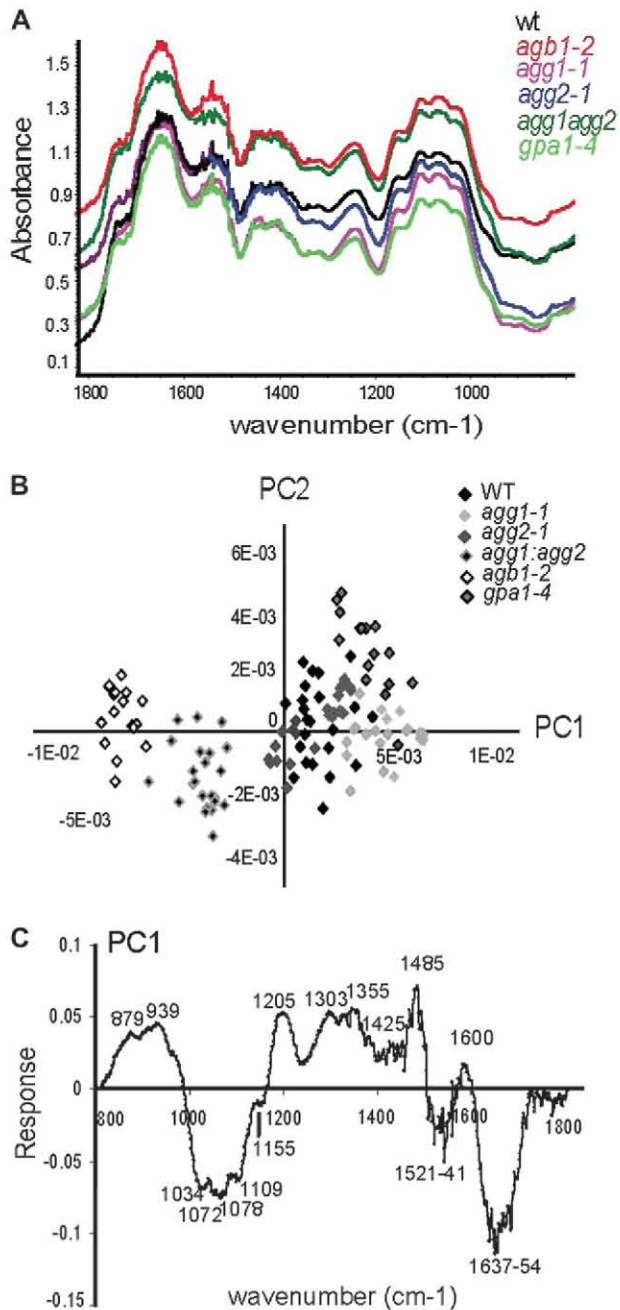
**Figure 5.** FTIR Analysis of the Cell Walls from Wild-Type Plant and Heterotrimeric G-Protein Mutants.

(A) FTIR average spectra (*n* = 15) from 3-week-old rosettes of Col-0 wild-type plants (WT), *agb1-2*, *agg1-1*, *agg2-1*, *agg1-1 agg2-1*, and *gpa1-4* mutants. Wavenumbers 1034, 1072, and 1155 cm^{-1} may correspond to galactan side-chains (with a (β-1→6)-D-Gal*p* backbone) in Rhammnogalacturonan I (RGI). The 1078 and 1106 cm^{-1} bands are common for 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} may correspond to unesterified pectins (COO− symmetric and anti-symmetric stretching, respectively (Kauráková et al., 2000; Wilson et al., 2000).

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 1 034, 1 072, and 1 155 cm^{-1}, which may correspond to galactan side-chains (with a (β-1→6)-D-Gal*p* 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 1 637–1 664 cm^{-1}), which might be associated 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 *gpa1-4* 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 *agb1-1* alleles (Blakeney et al., 1983; Reiter et al., 1993). Notably, the amount (% mol) of xylose was found to be lower in *agb1-2*, *agb1-1*, 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 *agb1* 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 Gβ (AGB1) or Gγ1γ2 (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 Gβ (AGB1) and Gγ (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 *irx6-1*; Brown et al., 2005; Rogers et al., 2005) or alterations in the structure of

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(B) Biplot analysis showing a clear separation of *agb1-2* and a partial segregation of *agg1-1 agg2-1* from WT, *agg1-1*, *agg2-2*, and *gpa1-4*. **(C)** Mid-infrared spectra were analyzed by the covariance-matrix approach for principal component analysis 1 (PC1).
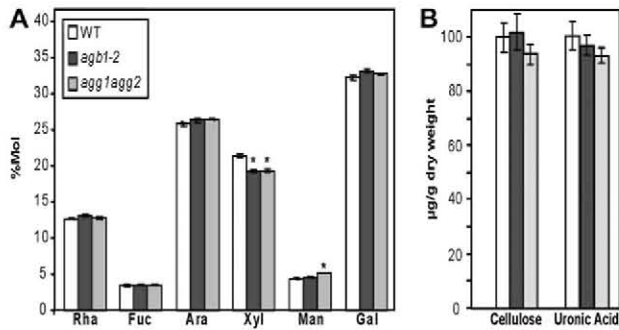
Figure 6. Biochemical Composition of the Cell Walls from Wild-Type, *agb1-1* and *agg1 agg2* Plants.

(A) Quantification of total and individual neutral sugars (% Mol) from the non-cellulosic carbohydrate fraction and **(B)** cellulose and total uronic acid content (μgr per gr of dry weight) from the cell walls of Col-0 wild-type plants (white bar) and *agb1-2* (dark bar) and *agg1 agg2* (gray bar) mutants. Data represent average values (± SE) of three replicates. Statistical analysis of the data was performed using ANOVA ($P \leqslant 0.05$) and the Bonferroni post-hoc test.

xyloglucan (*xyl1-2*; Sampedro et al., 2010) compared with their corresponding wild-type plants (Col-0 or Ws backgrounds). These mutants and the corresponding wild-type plants (Col-0 and Ws) were inoculated with a spore suspension (4×10^6 spores ml^{-1}) of *PcBMM* and their DRs were determined at different dpi. Remarkably, at latter stages of infection (10 dpi), we observed that disease symptoms (DR) were lower in the *det3*, *xyl1-2*, and *irx6-1* mutants that in the Col-0 or Ws wild-type plants (Supplemental Figure 3). These results suggest that the wall xylose content might be one of the determinants controlling *Arabidopsis* resistance to necrotrophic fungi.

The *agb1* 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-Rodriguez et al., 2009). The wall of the *er* mutant contains less xylose, like *agb1*, but more uronic acid than wild-type walls (Sánchez-Rodriguez et al., 2009). With these data in mind, we tested the genetic interaction between *er* and one of the *agb1* alleles (*agb1-1*) 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 *agb1-1* single mutants, and the *agb1-1 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 *agb1-1 er-105* double mutant was higher than that of the single *agb1-1* and *er-105* mutants. This indicates that ER and
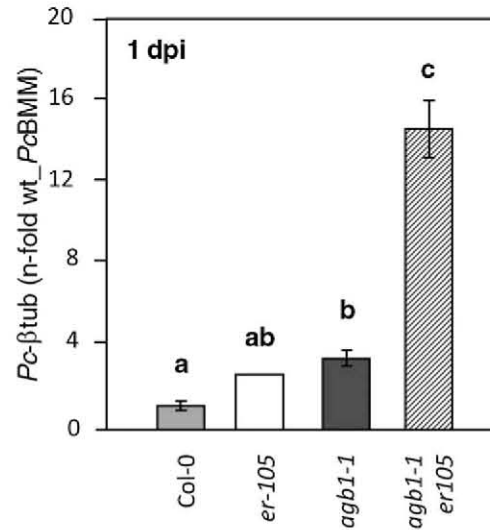
Figure 7. The Susceptibility to *P. cucumerina* of *agb1-1* Plants Can Be Enhanced by the *er-105* Mutation.

Wild-type plants (WT, Col-0), *agb1-1*, and *er-105* single mutants and the *agb1-1 er-105* double mutant were mock-treated or inoculated with 4×10^6 spores ml^{-1} of *PcBMM*. Fungal biomass quantification was determined at 1 dpi. Values were normalized to *Arabidopsis UBIQUITIN21* expression levels and represented as *n*-fold compared to the Col-0 plants. Bars represent the average (± SD) of two technical replicates. Letters indicate differences statistically significant among the genotypes (ANOVA $p \leqslant 0.05$, Bonferroni Test). Data are from one out of three independent experiments, which gave similar results.

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 *agb1-1 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 *agb1-1* 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 *agb1-1 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 *agb1-1* background led to the partial suppression of the *agb1-1* susceptibility phenotype, as the *agb1-1 irx1-6* double mutant supported more fungal growth than Col-0 plants, but less than *agb1-1*. These data indicate that inactivation of IRX1/CESA8 function in *agb1-1* 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 Gβ/γ subunits act as a functional mono-mer (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 Gγ1 and Gγ2 subunits display phenotypes that resemble those observed in the *agb1* mutant (Trusov et al., 2007, 2008b, 2010). However, specific functions of *Arabidopsis* Gγ1 and Gγ2 subu-nits have been also 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 discrepan-cies were found between some developmental-associated phenotypes of Gβ and Gγ1/Gγ2-deficient mutants, which led to the suggestion that additional Gγ subunits or some func-tional autonomy of G-protein subunits might exist in *Arabi-dopsis* (Trusov et al., 2008b). Some of these discrepancies can be explained by the functional contribution to these processes of the recently described atypical Gγ3 subunit (Chakravorty et al., 2011).

Here, we demonstrate that the Gβ/Gγ subunits act as a func-tional monomer and that the Gγ1 and Gγ2 subunits play redundant functions in the regulation of *Arabidopsis* defense response to the necrotrophic fungus *PcBMM*, as the suscepti-bility of *agg1-1 agg2-1* double mutant to this pathogen is similar to that of the *agb1* plants, but higher than that of the *agg1-1* 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 Gβγ 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 pat-tern in the *agg1-1 agg2-1* double mutant (Figures 3 and 4), which further indicates that inactivation of either Gβ or Gγ1γ2 subunits results in a similar transcriptional reprogram-ming of the immune response upon necrotrophic fungal infec-tion. These results contrast with the previously reported specific function of AGG1 in the control of *Arabidopsis* resis-tance to the necrotrophic fungus *A. brassicicola* (Trusov et al., 2006). However, our results are in agreement with the described canonical functionality of Gβγ in the control of *Arabidopsis* resistance to the vascular fungus *F. oxysporum* (Trusov et al., 2010). Additional analysis will be required to de-termine whether the Gβγ 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 *PBZ1*) upon fungal infection (Suharsono et al., 2002). In *Arabidopsis*, where the defense re-sponse is controlled by the Gβγ dimer (Figure 1), the accumu-lation 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 differ-ent roles in regulating resistance to *F. oxysporum* and *A. bras-sicicola*, revealed that AGB1-mediated resistance was independent of these pathways and that Gβ can act upstream of MYC2 and COI1, 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 *agg1-1 agg2-1* mutants (Figure 2A). Also, we show that, upon *PcBMM* infection, the expres-sion 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 *Arabi-dopsis* 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 reg-ulates 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. cichor-acearum* (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 *agg1-1 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 agg2-1* genotypes can not be assigned to a defect in just a single cell wall polymer. While FTIR analysis of *agb1* and *agg1-1 agg2-1* 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 and *agg1-1* and *agg2-1* single mutants (Figure 6A and Supplemental Figure 2). Remarkably, the *er* mutant, which is hypersusceptible to *PcBMM*-like *agb1* and *agg1-1 agg2-1* plants, also has some alteration in its cell wall structure/composition, including a reduced content of xylose, compared to wild-type plants (Sánchez-Rodriguez et al., 2009). The cell wall features of the *er* mutant, including its reduced xylose content, and its enhanced susceptibility to *PcBMM* are restored to wild-type levels by mutations in *SER1/SER2* genes (Sánchez-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 in least in part, the enhanced susceptibility to *PcBMM* observed in the Gβ/Gγ 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 mutants.

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 it 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 Höfte, 2008). The characterization of Gβ/Gγ-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 *sgb1-2* and *agb1-2 sgb1-2* 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 trypan 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-Vallet et al., 2010). For biomass quantification, the relative expression ratio of fungal β-tubulin was calculated as the differences between the Ct values (dCt) 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 form 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 Gene PixPro 5.1. The expression levels of the genes were analyzed with Multiexperiment 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 $p \leqslant 0.01$. 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-Expres 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 *UBIQUITIN21* (At5g25760) expression to normalize the transcript level in each sample and calculate the dCt 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 of each 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 (dCt) 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 hexose 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 (Vilím, 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*.

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