

Functional Interplay Between *Arabidopsis* NADPH Oxidases and Heterotrimeric G Protein

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The plant NADPH oxidases produce reactive oxygen species (ROS) in response to pathogens that have diverse functions in different cellular contexts. Distinct phenotypic outcomes may derive from the interaction of NADPH oxidase-dependent ROS with other signaling components that mediate defense activation. We analyze the interaction between NADPH oxidases *AtRbohD* and *AtRbohF* and the *Arabidopsis* heterotrimeric G protein. The G β subunit (*AGBI*) of the heterotrimeric G protein is required for full disease resistance to different *Pseudomonas syringae* strains. Genetic studies reveal that, upon *P. syringae* infection, *AGBI* and *AtRbohD* and *AtRbohF* can function in the same pathway, as the *agb1* null allele is epistatic to the NADPH oxidase null alleles, combinatorial mutants display the *agb1* phenotypes, and *agb1* suppresses some of the *atrbohD atrbohF* double mutant phenotypes. In contrast, increased susceptibility to the necrotrophic fungus *Plectosphaerella cucumerina* displayed by *agb1* and *atrbohD atrbohF* is enhanced in the *agb1 atrbohD atrbohF* triple mutant, suggesting that NADPH oxidase and heterotrimeric G proteins mediate different response pathways in response to this necrotrophic pathogen. The defense response mediated by *AGBI* is independent of pathogen-dependent salicylic acid accumulation and signaling, as the *agb1 sid2 (isochorismate synthase 2)* double mutant showed enhanced disease susceptibility to *P. syringae* and *Plectosphaerella cucumerina* as compared with both single mutants. This study exemplifies the complex interplay between signaling events mediating defense activation, depending on the type of plant-pathogen interaction.

The apoplastic production of reactive oxygen species (ROS) is among the fastest events observed ubiquitously in plants after pathogen attack (Lamb and Dixon 1997; Torres et al. 2006).

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These ROS, mainly the superoxide anion and its dismutated product hydrogen peroxide, are signals that orchestrate many responses, not only in response to pathogens but also to other biotic and abiotic stimuli and to developmental cues (Suzuki et al. 2011).

ROS have different cellular origins. Among these, ROS producing NADPH oxidases located at the cell plasma membrane are key early players in the control of many processes in response to both environmental and developmental stimuli (Suzuki et al. 2011). Plant NADPH oxidases, called RBOH for respiratory burst oxidase homologs, are a family of plasma membrane-localized enzymes with homology to mammalian NADPH NOX proteins (Sumimoto 2008). *Arabidopsis* contains a multigene family of 10 *AtRboh* members (Torres and Dangl 2005). Genetic studies revealed that, whereas some RBOH proteins perform specific functions, *AtRbohD* and *AtRbohF* can mediate multiple functions in the plant, from pathogen response to abiotic stress signaling, lignification, and stomatal closure, frequently with additive effects (Suzuki et al. 2011).

RBOH proteins are required for ROS production in response to pathogens, often in association with the hypersensitive response (HR), a type of programmed cell death (Pogany et al. 2009; Torres et al. 2002; Zhang et al. 2007). However, the extracellular superoxide produced by various RBOH proteins may serve different functions in defense. RBOH proteins can act as positive regulators of defense. For example, *NbRboh*-silenced plants displayed suppressed HR and were more susceptible to normally avirulent *Phytophthora infestans* (Yoshioka et al. 2003), and silencing of barley *HvRbohA* allowed increased penetration by *Blumeria graminis* (Trujillo et al. 2006). Also, *Arabidopsis AtRbohD* and *AtRbohF* are required for full HR in response to avirulent strains of the bacterium *Pseudomonas syringae* as well as for callose deposition in response to flg22 (Torres et al. 2002; Zhang et al. 2007). However, the enhanced disease resistance to a weakly virulent strain of the oomycete *Hyaloperonospora arabidopsidis* observed in *atrbohD* and *atrbohF* mutants suggested that RBOH proteins can function as negative regulators as well (Torres et al. 2002). Additionally, *AtRbohD* is required for cell death in cells that are damaged by the necrotrophic fungus *Alternaria brassicicola*, but it simultaneously inhibits cell death in neighboring cells (Pogany et al. 2009). Thus, while the RBOH proteins are required for pathogen-induced superoxide production, these ROS can apparently function in a context-dependent manner.

Interactions with other plant defense modulators may account for these context-dependent outcomes. RBOH activation affects different signal transduction pathways, such as calcium signaling, protein phosphorylation and lipid signaling. All plant RBOH proteins contain EF hands, calcium binding motifs in the N-terminal domain that are necessary for their functionality (Oda et al. 2010; Takeda et al. 2008). Thus, phosphorylation or binding of small Rac GTPases or phospholipase D α 1-derived phosphatidic acid to the N-terminal domain of RBOH activate its oxidase activity (Wong et al. 2007; Zhang et al. 2009). Moreover, RBOH-derived ROS activates calcium channels and the same phosphorylation pathways that mediate RBOH activation (Asai et al. 2008; Zhang et al. 2009).

Additionally, interaction between NADPH oxidases and plant hormone signaling modulates ROS function in defense. ROS interacts in different ways with salicylic acid (SA) signaling. Exogenously applied ROS acts synergistically in a signal-amplifying loop with SA to drive HR (Draper 1997; Shirasu et al. 1997). However, ROS produced via *AtRbohD* antagonizes SA to limit programmed cell death to the site of infection (Torres et al. 2005). Consistent with this observation, negative feedback regulation of SA and ethylene by *AtRbohD* may inhibit the spread of cell death induced by necrotrophic pathogens (Pogany et al. 2009). Also, coordinated production of ROS and nitric oxide (NO) lead to HR (Delledonne et al. 2001) and both signals act synergistically to regulate abscisic acid (ABA)-induced stomatal closure (Desikan et al. 2008). However, ROS antagonizes NO in basal defense to the necrotrophic pathogen *Botrytis cinerea* (Asai et al. 2008). ROS signaling is associated with growth regulating hormones such as gibberellin or ABA that can modulate ROS production following infection (Achard et al. 2008; Asselbergh et al. 2007). This interplay may help to fine-tune ROS signals and contribute to the multiplicity of functions of ROS in different plant-pathogen interactions.

Heterotrimeric G proteins constitute membrane-anchored complexes that couple extracellular signals to downstream effectors that mediate many signal transduction pathways (Temple and Jones 2007). Emerging evidence associates these regulatory proteins with signal transduction pathways mediating many processes in plants, including plant-pathogen interactions (Trusov et al. 2010). The canonical G-protein consists of three different units, G α , G β , and G γ . Upon activation, the G α subunit dissociates from the obligate G $\beta\gamma$ dimer to activate downstream elements of the signal transduction pathway (Temple and Jones 2007). *Arabidopsis* expresses single, canonical G α and G β proteins, and three G γ subunits (Jones and Assmann 2004; Offermanns 2003; Thung et al. 2012; Trusov et al. 2008). Studies with mutants in different subunits of the complex have confirmed their role in defense. For example, analysis of the rice *dwarf1* (*d1*) mutant in the single G α subunit indicates that G α is required for full HR and resistance to avirulent races of the rice blast fungus *Magnaporthe grisea* (Suharsono et al. 2002). However, in *Arabidopsis*, mutations in the G α subunit *GPA1* have minor or no effect on response; by contrast, the single canonical G β subunit *AGB1* is required for resistance to some fungal pathogens (Delgado-Cerezo et al. 2012; Llorente et al. 2005; Trusov et al. 2006, 2009). Thus, the *Arabidopsis agb1* mutant is more susceptible than wild-type plants to the necrotrophic fungi *Botrytis cinerea*, *Plectosphaerella cucumerina*, and *Alternaria brassicicola*, and to the vascular fungus *Fusarium oxysporum*. The response of *agb1* plants to virulent and avirulent isolates of the biotrophic oomycete *H. arabidopsidis* and the bacterium *P. syringae* did not differ from that of wild-type plants (Llorente et al. 2005, Trusov et al. 2006). Thus, the different heterotrimeric G protein subunits can function differently depending on the plant species and the particular

plant-pathogen interaction studied. AGB1 residues required for defense have recently been characterized (Jiang et al. 2012), and it has been demonstrated that AGB1 and G γ subunits (AGG1 and AGG2) function together, since the *agb1* and the *agg1 agg2* double mutant display comparable alterations in response to necrotrophic fungal infection (Delgado-Cerezo et al. 2012).

Genetic evidence indicates that heterotrimeric G proteins function with NADPH oxidase-derived ROS in several plant responses to the environment (Chen et al. 2004). High levels of exogenous ozone (O₃) drive a spatial and a temporal progression of ROS that is similar to the pathogen-induced oxidative burst (Joo et al. 2005). *AtRbohD* and *AtRbohF* were implicated in the intercellular signaling and ultimate cell death arising from high levels of O₃ exposure (Joo et al. 2005). Interestingly, heterotrimeric G proteins were shown to control this ROS production and sensitivity to O₃. In addition, heterotrimeric G proteins stimulate ROS production to activate guard-cell calcium channels (Chen et al. 2004). Pharmacological evidence suggests that, during plant defense responses, ROS production by the NADPH oxidases is mediated by heterotrimeric G proteins (Vera-Estrella et al. 1994). And genetic studies in rice showed that *OsRac1*, a small GTPase involved in the activation of the NADPH oxidase, acts downstream of the G α subunit in response to infection with *Magnaporthe grisea* (Suharsono et al. 2002). From these various studies, we hypothesized that heterotrimeric G proteins could interact with RBOH-derived ROS signaling in response to pathogen attack.

To test this hypothesis, we analyzed the defense response of *Arabidopsis* mutants in NADPH oxidase and in different subunits of the heterotrimeric G protein following challenge with two very different pathogens, either the hemibiotrophic bacterial pathogen *P. syringae* or the necrotrophic fungus strain, *Plectosphaerella cucumerina* BMM. Our results illustrate the complex relationship between the signals that mediate defense responses in which the relationship between signals varies depending on the type of plant-pathogen interaction.

RESULTS

agb1 displays increased susceptibility to *P. syringae*.

We examined whether mutants in the *Arabidopsis* heterotrimeric G protein subunits G β (*agb1-2*) (Ullah et al. 2003) and G β (*gpa1-4*) (Jones et al. 2003; Ullah et al. 2001) show differences compared with wild type Col-0 plants in response to the infection with the hemibiotrophic bacteria *P. syringae* pv. *tomato* DC3000 (Fig. 1A to D). After bacterial inoculation, *agb1* plants displayed increased chlorotic symptoms (Fig. 1A). These symptoms correlated with enhanced bacterial growth in *agb1* as compared with the wild type in response to both the virulent (*P. syringae* pv. *tomato* DC3000) and the avirulent [*P. syringae* pv. *tomato* DC3000(*avrRpm1*) and *P. syringae* pv. *tomato* DC3000(*avrRpt2*)] strains (Fig. 1B and C; Supplementary Fig. 1). Moreover, *agb1* displayed enhanced growth of *P. syringae* pv. *tomato* DC3000*hrcC*, a strain deficient in the type III secretion system (Fig. 1D). Thus, AGB1 is required for full basal defense against *P. syringae*. On the other hand, *gpa1* displayed the same level of bacterial growth as wild type. Bacterial growth in *agb1 gpa1* was similar to that determined in *agb1* (Fig. 1B and D). Together, these data indicate that AGB1 is required for basal disease resistance to *P. syringae*, whereas GPA1 has no effect on bacterial resistance.

We monitored both H₂O₂ production and the HR cell death associated with infection by avirulent bacteria. H₂O₂ accumulation appeared to be slightly diminished in the *agb1* mutant in response to *P. syringae* pv. *tomato* DC3000(*avrRpm1*) as revealed by diaminobenzidine (DAB) staining (Supplementary

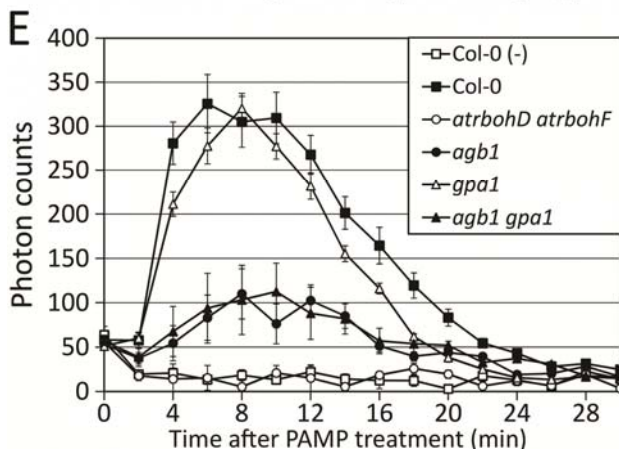
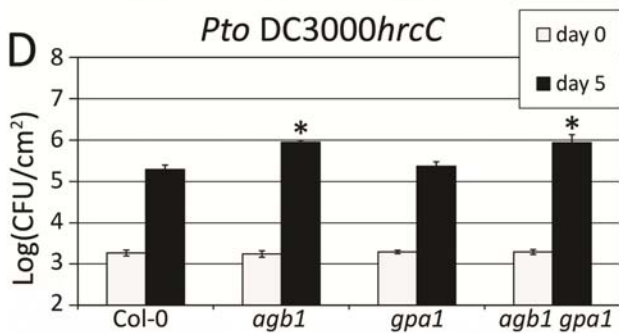
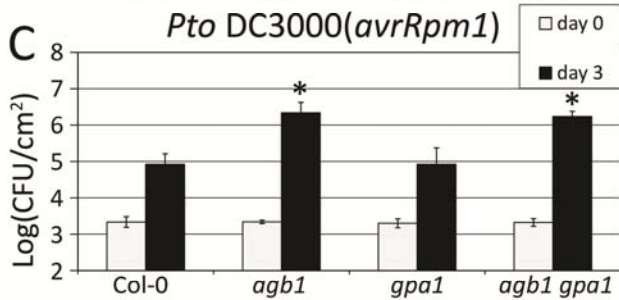
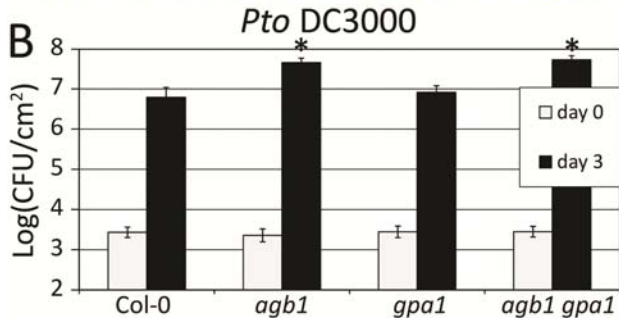
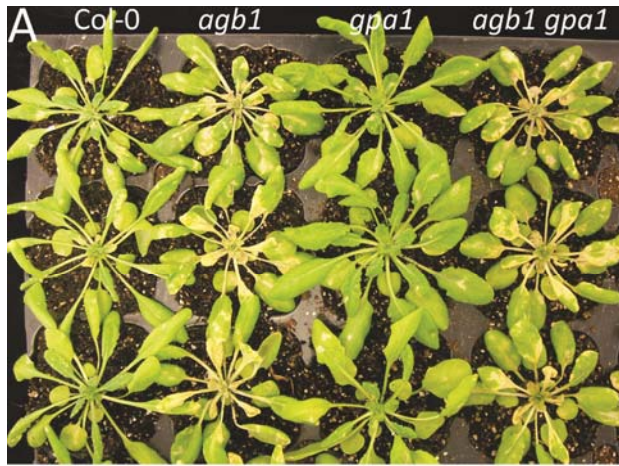


Fig. 2A). Similarly, *agb1* displayed a reduction of DAB stain after infection with *P. syringae* pv. *tomato* DC3000hrcC. To quantify the apparent reduction of ROS production in *agb1*, we monitored H₂O₂ production in response to flg22, the elicitor-active epitope of bacterial flagellin (Felix et al. 1999). Luminol assays following treatment with 100 nM flg22 confirmed that the production of total H₂O₂ in *agb1* was significantly reduced compared with that determined in Col-0 wild-type plants (Fig. 1E; Supplementary Fig. 3), as previously reported (Ishikawa 2009; Jiang et al. 2012). The double NADPH oxidase mutant *atrbohD atrbohF* fully abolished this ROS production (Fig. 1E). On the other hand, H₂O₂ accumulation in the heterotrimeric G protein mutants was not altered after infection with the necrotrophic fungus *Plectosphaerella cucumerina* (Lorente et al. 2005). Thus, AGB1 is required for full H₂O₂ production in response to hemibiotrophic bacteria but has no obvious effect on ROS produced in response to necrotrophic fungi. In addition, cell death after inoculation with *P. syringae* pv. *tomato* DC3000(*avrRpm1*), monitored by trypan blue staining, was not affected in these mutants. Thus, *Arabidopsis* heterotrimeric G proteins have no obvious effect on cell death occurring in response to pathogens, but AGB1 is required for full ROS accumulation in response to pathogen-associated molecular patterns (PAMPs) like flg22. Our data contrast previous reports of responses to acute ozone treatment (Joo et al. 2005), where *Rboh*-dependent ROS production and cell death is compromised in *gpa1* but not in *agb1*.

We also examined the response of *agb1*, *gpa1*, and *agb1 gpa1* to infection with the biotrophic oomycete *H. arabidopsidis*. No differences were observed in disease susceptibility between these heterotrimeric G protein mutants and Col-0 wt plants in response to virulent (*Noco2*) or avirulent (*Cala2*, *Emco5*) *H. arabidopsidis* isolates (Fig. 2A; Supplementary Fig. 4). Also, H₂O₂ production and cell death, monitored by DAB and trypan blue stains, respectively, were unaffected in these lines in response to *H. arabidopsidis* (data not shown).

agb1 suppresses some pathogen-associated phenotypes displayed by *atrbohD atrbohF*.

Epistasis analyses between the heterotrimeric G protein null alleles *gpa1* and *agb1* and the combined NADPH oxidase null alleles *atrbohD* and *atrbohF* showed that, late in development, the dwarf phenotype of *atrbohD atrbohF* was partially restored to wild type in *agb1 atrbohD atrbohF* (Fig. 2B). The *atrbohD atrbohF* plants displayed reduced HR in response to avirulent *P. syringae* pv. *tomato* DC3000(*avrRpm1*) and enhanced HR and disease resistance in response to a virulent *H. arabidopsidis* isolate (Torres et al. 2002). We found that *agb1* restored to wild-type levels the defective HR of *atrbohD atrbohF* in response to avirulent *P. syringae* pv. *tomato* DC3000(*avrRpm1*), as upon inoculation the conductivity (a measurement of ion

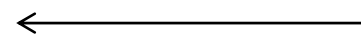


Fig. 1. Susceptibility is enhanced in the *agb1* mutant in response to *Pseudomonas syringae*. **A**, Symptoms on 4.5-week-old plants of the genotypes listed, 5 days after spray inoculation with a 5×10^7 CFU ml⁻¹ concentration of *P. syringae* pv. *tomato* DC3000. **B to D**, Quantification of bacterial growth on the heterotrimeric G protein mutants after injection with 10^5 CFU ml⁻¹ concentrations of the virulent bacterial strain *P. syringae* pv. *tomato* DC3000, the isogenic avirulent strain *P. syringae* pv. *tomato* DC3000(*avrRpm1*), or the type III secretion mutant *P. syringae* pv. *tomato* DC3000hrcC, respectively. Values represent the mean \pm standard error (SE) of four replicates. Asterisks indicate significant differences compared with wild-type Col-0 ($P < 0.01$, analysis of variance). Experiments were repeated three times with similar results. **E**, H₂O₂ production measured in a luminol-based assay using leaf discs from plants of the listed genotypes after treatment with 100 nM flg22 (except Col-0 (-) untreated). Values are means \pm SE ($n = 16$). The experiment was repeated three times with similar results.

leakage correlated with HR) was similar in *agb1 atrbohD atrbohF* and wild-type plants (Fig. 2C). *agb1* also abolished the enhanced disease resistance to the *H. arabidopsidis* isolate *Emco5* in the *atrbohD atrbohF* background (Fig. 2A). By contrast, *gpa1* did not affect the *atrbohD atrbohF* phenotypes. As expected, the *agb1 gpa1 atrbohD atrbohF* quadruple mutant exhibited the same phenotypes as *agb1 atrbohD atrbohF* (Fig. 2A to C). Thus, *agb1* suppresses the pathogen response phenotypes displayed by *atrbohD atrbohF*.

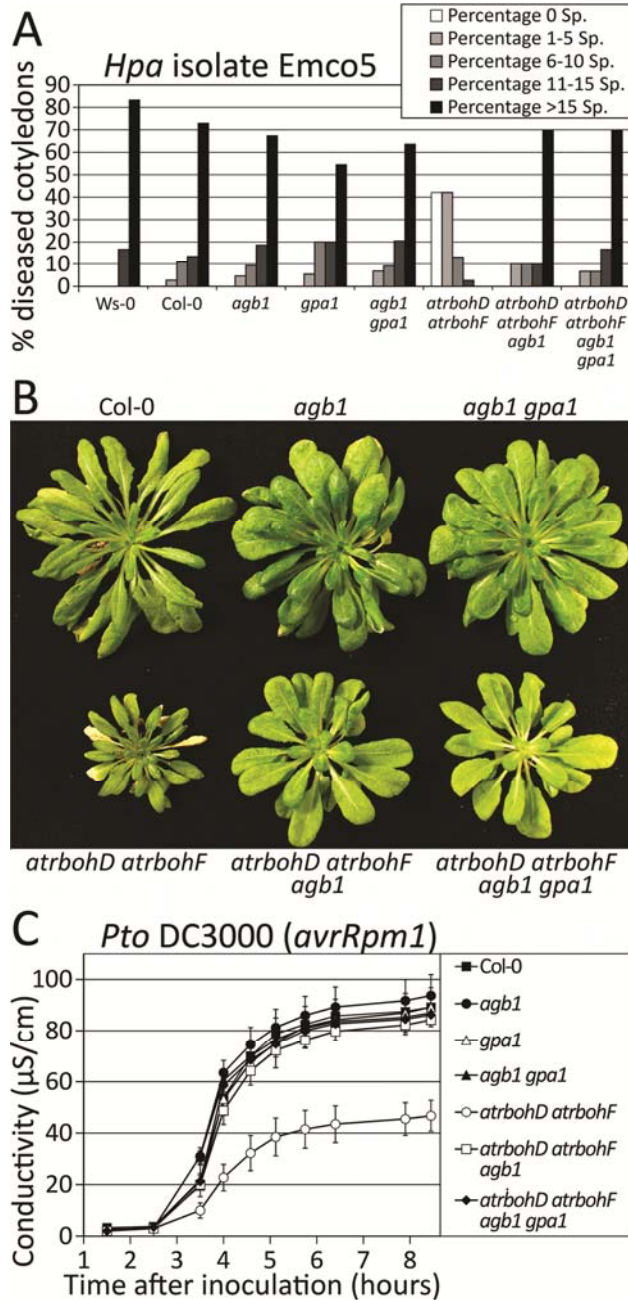


Fig. 2. *agb1* suppresses *atrbohD atrbohF* phenotypes. **A**, 10-day-old seedlings were inoculated with 5×10^{-4} ml $^{-1}$ sporangia of *Hyaloperonospora arabidopsidis* isolate *Emco5*. Sporangiophore (Sp) production was quantified at 5 days postinoculation on 40 to 80 cotyledons per genotype. **B**, Representative 6-week-old plants of the indicated genotypes. **C**, Conductivity measurement after injection of 4-week-old plants with 2.5×10^7 CFU ml $^{-1}$ of the avirulent strain *P. syringae* DC3000(*avrRpm1*). Data represented are means and standard error of conductivity ($\mu\text{S cm}^{-1}$) calculated from 6 ml of water containing four leaf discs (four repetitions per genotype) over time. Experiments were repeated three times with similar results.

Of note, the enhanced susceptibility of *agb1* to *P. syringae* pv. *tomato* DC3000 was not altered in the *atrbohD atrbohF* mutant background (Fig. 3). Similarly, the quadruple mutant *agb1 gpa1 atrbohD atrbohF* also displayed, like *agb1*, enhanced bacterial growth and chlorotic symptoms after *P. syringae* pv. *tomato* D3000 and *P. syringae* pv. *tomato* DC3000hrcC infection (Fig. 3). Thus, the *agb1* phenotype is epistatic to *atrbohD atrbohF* in response to *P. syringae* infection.

AGBI and the NADPH oxidases act independently to control resistance to the necrotrophic fungus *P. cucumerina*.

AGBI is required for full disease resistance to the necrotrophic fungus *Plectosphaerella cucumerina* (Delgado-Cerezo et al. 2012; Llorente et al. 2005). We sprayed spores of this

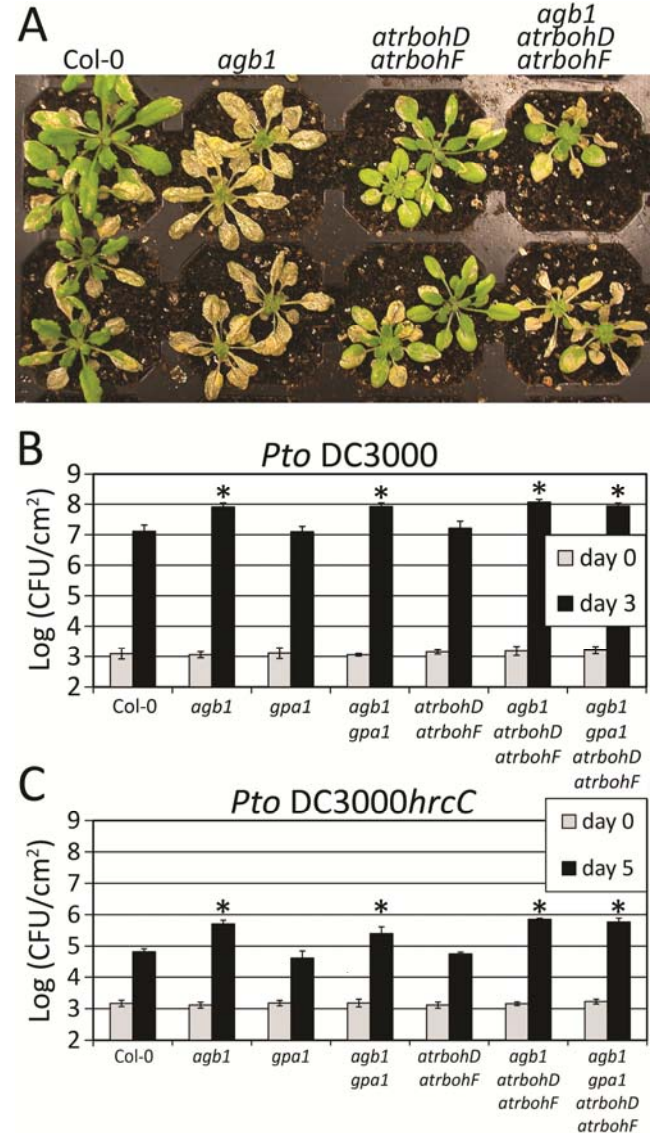


Fig. 3. *agb1* is epistatic to *atrbohD atrbohF* with respect to *Pseudomonas syringae* disease phenotypes. **A**, Symptoms of 4.5-week-old plants of the indicated genotypes, 8 days after spray inoculation with a 5×10^7 CFU ml $^{-1}$ concentration of *P. syringae* pv. *tomato* DC3000. **B** and **C**, Quantification of bacterial growth on 4.5-week-old plants after hand inoculation with concentrations of 10^5 CFU ml $^{-1}$ of the virulent bacterial strain *P. syringae* pv. *tomato* DC3000 and the type III secretion mutant *P. syringae* pv. *tomato* DC3000hrcC, respectively. Values are means \pm standard error (SE) calculated from four repetitions. Asterisks indicate significant differences compared with wild-type Col-0 ($P < 0.01$, analysis of variance). Experiments were repeated three times with similar results.

fungus on the *atrbohD atrbohF* mutant, on the heterotrimeric G protein mutants (*agb1*, *gpa1*, and *agb1 gpa1*) and on the combinatorial mutants. Analysis of the symptoms and quantification of the fungal biomass by quantitative polymerase chain reaction (qPCR) showed that *atrbohD atrbohF* were, like *agb1*, more susceptible to this fungus than the wild type (Fig. 4; Supplementary Fig. 5). Interestingly, *agb1 atrbohD atrbohF* displayed increased susceptibility compared with either *agb1* or *atrbohD atrbohF*. This result indicates that *AtRbohD*, *AtRbohF*, and *AGB1* are all required for full resistance to *Plectosphaerella cucumerina* and act independently. Thus, contrary to what we observed in response to the hemibiotrophic bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Figs. 1 to 3), the NADPH oxidase and the heterotrimeric G protein complex act independently to control basal defense in response to *Plectosphaerella cucumerina* infection.

Enhanced disease resistance mediated by *AGB1* is independent of SA signaling.

SA mediates many defense responses to biotrophic and hemibiotrophic pathogens. However, SA can antagonize or synergize with *AtRboh* function, depending on the context (Torres 2010). To assess the role of SA in the defense pathways mediated by the heterotrimeric G proteins and the

NADPH oxidase, we obtained combinatorial mutants between *agb1*, *atrbohD*, *atrbohF*, and the *sid2/eds16* mutant, which is impaired in the *isochorismate synthase 2* gene encoding a key SA biosynthetic enzyme (Wildermuth et al. 2001). Infection of these lines with virulent *P. syringae* pv. *tomato* DC3000 and DC3000*hrcC* revealed that the double mutant *agb1 sid2* displayed an increased susceptibility compared with both individual mutants, as measured by both disease symptoms and bacterial growth assays (Fig. 5A to C). This result indicates that heterotrimeric G proteins and SA act independently in basal defense to *P. syringae*. In contrast, the *atrbohD atrbohF sid2* mutant displayed the *sid2* enhanced disease susceptibility phenotype (Fig. 5A to C).

We inoculated, with *Plectosphaerella cucumerina* spores, *agb1*, *atrbohD atrbohF*, *sid2*, and combinatory lines, monitored disease symptoms, and quantified the fungal biomass by qPCR (Fig. 5D and E). Although the *sid2* mutant exhibited only a slight increase in susceptibility compared with Col-0, the double *agb1 sid2* displayed a large increased susceptibility compared with the individual mutants. The susceptibility of *agb1 sid2* to *Plectosphaerella cucumerina* was higher than that observed in the *agb1 atrbohD atrbohF* (Fig. 5D and E). These results suggest that heterotrimeric G proteins and SA act independently in defense against *Plectosphaerella cucumerina*. The triple *atrbohD atrbohF sid2* showed no increased disease susceptibility above that expressed by *atrbohD atrbohF*, suggesting that *AtRboh*-derived ROS and SA accumulation act in the same pathway (Fig. 5D and E).

DISCUSSION

The G β subunit of heterotrimeric G protein *AGB1* mediates basal resistance to *P. syringae*.

The interaction of ROS with diverse signaling components may explain the multiple physiological responses that they mediate. The *Arabidopsis* NADPH oxidases *AtRbohD* and *AtRbohF* account for the extracellular ROS produced in response to pathogens. Here, we explored whether ROS produced by these NADPH oxidases interacted with the heterotrimeric G protein in the response to different pathogens. Previous studies indicated that the G β subunit of the heterotrimeric G protein *AGB1* is important for resistance to several fungi, including the necrotroph *Plectosphaerella cucumerina* (Delgado-Cerezo et al. 2012; Llorente et al. 2005; Trusov et al. 2006, 2009). We have extended the relevance of *AGB1* in modulating *Arabidopsis* resistance to pathogens by demonstrating that *AGB1* also contributes to basal defense to the bacterium *P. syringae* pv. *tomato* DC3000. The *agb1* plants inoculated either by infiltration or by spray with different *P. syringae* pv. *tomato* DC3000 strains clearly showed increased bacterial growth and disease symptoms compared with those determined in wild-type plants (Figs. 1, 3, and 5). Previous studies reported no alterations of bacterial growth in the same *agb1* mutant allele after syringe inoculation with either virulent (DC3000) or avirulent (JL1065) strains of *P. syringae* (Trusov et al. 2006). We cannot rule out that these contradictory results could be due to slight differences in the experimental conditions that we used in comparison with those described previously (Trusov et al. 2006). However, the enhanced susceptibility of *agb1* mutant was corroborated in *agb1 gpa1*, which was as susceptible as *agb1* to all the *P. syringae* pv. *tomato* DC3000 strains tested, including DC3000*hrcC* (Figs. 1, 3, and 5), further demonstrating that *AGB1* contributes to basal defense to this bacterial pathogen. In line with our data, it has been previously shown that *AGB1* is also required for defense response against *Agrobacterium tumefaciens* (Ishikawa 2009), suggesting it mediates a common mechanism of response to infection by bacteria in

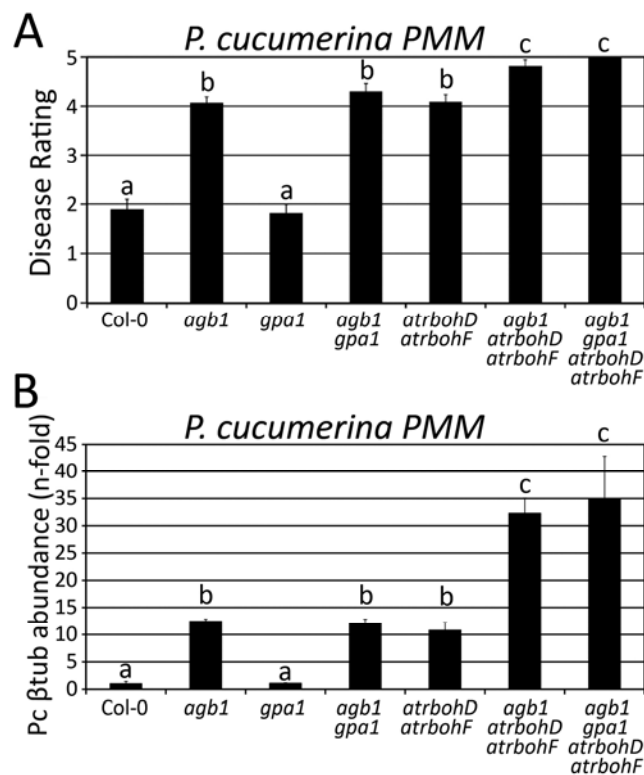


Fig. 4. *agb1* and *atrbohD atrbohF* display additive disease susceptibility to the necrotrophic fungus *Plectosphaerella cucumerina*. **A**, Average disease rating (\pm standard error [SE]) 7 days after spray inoculation with *P. cucumerina*: 1 = no symptoms; 2 = one chlorotic or necrotic leaf; 3 = two to three necrotic leaves; 4 = more than three leaves necrotic; 5 = plant dead. Data comes from one representative experiment out of three experiments carried out with at least 25 plants for each genotype. Letters indicate significant differences between classes at $P < 0.01$. **B**, Quantification of *P. cucumerina* biomass 3 days postinoculation by quantitative polymerase chain reaction analysis. Primers specific for β -tubulin from the fungus and ubiquitin from *Arabidopsis* were used. Values are representative as n -fold increased expression compared with wild-type Col-0. Data are the means (\pm SE) of three replicates. Statistical analyses were performed using analysis of variance 5%, corrected with Bonferroni test. Letters indicate significant differences. The experiment was repeated three times with similar results.

addition to necrotrophic and vascular fungi (Delgado-Cerezo et al. 2012; Llorente et al. 2005; Trusov et al. 2006, 2009). On the other hand and as previously noted (Llorente et al. 2005), we observed no differences between the heterotrimeric G protein mutants and wild-type plants in response to the biotrophic oomycete *H. arabidopsidis*. Thus, *AGB1* mediates defense responses to many but not all pathogens.

Our finding that *agb1 gpa1* displayed the same enhanced disease susceptibility to *P. syringae* pv. *tomato* and *Plectosphaerella cucumerina* as *agb1* further implies that *AGB1* is critical for the regulation of *Arabidopsis* defensive response to pathogens (Delgado-Cerezo et al. 2012; Trusov et al. 2006). However, in rice, $G\alpha$ was implicated in response to infection with the rice blast fungus *M. grisea*, as the rice *dl* mutant, defective in the $G\alpha$ subunit, displayed reduced HR and modest loss of resistance to avirulent rice blast (Suharsono et al. 2002). Interestingly, *AGB1*, but not *GPA1*, functions in *Arabidopsis* non-host resistance to the rice blast fungal pathogen *M. oryzae* (Maeda et al. 2009; Nakao et al. 2011). A double mutant impaired in *PEN2*, encoding a glycosyl hydroxylase involved in nonhost resistance (Lipka et al. 2005), and *AGB1* supported significantly increased penetration by this rice pathogen than the *pen2* plants, defective in nonhost resistance (Maeda et al. 2009; Nakao et al. 2011). Thus, although plants possess canonical $G\alpha$ and $G\beta$ subunits, the role of these subunits in defense may be different, depending on the plant species or the pathogen being studied. The diverse functions of these subunits may be accomplished by interaction with different downstream proteins in various contexts, as revealed by the interactome of the *Arabidopsis* heterotrimeric G proteins (Klopfleisch et al. 2011).

Heterotrimeric G proteins, ROS production, and cell death.

In the *Arabidopsis agb1* mutant the production of NADPH oxidase-dependent H_2O_2 upon inoculation with *P. syringae* or treatment with the PAMP flg22 was severely compromised (Fig. 1E), whereas H_2O_2 production (determined by DAB staining) in response to *Plectosphaerella cucumerina* or *H. arabidopsidis* infection was similar to that in wild-type plants, as previously described (Llorente et al. 2005). These data suggest that the *Arabidopsis* *AGB1* protein is required for the full oxidative burst in response to some pathogens, such as *P. syringae* bacteria, whereas it does not control H_2O_2 production in response to other pathogens, particularly necrotrophic fungi. This contrasts with the requirement of the rice $G\alpha$ subunit for ROS production in response to rice blast fungus (Suharsono et al. 2002). Activation of heterotrimeric G proteins requires the dissociation of the $G\alpha$ and the $G\beta\gamma$ subunits (Temple and Jones 2007). Accordingly, *Arabidopsis* $G\beta$ *AGB1* forms an obligate dimer with either one of the *Arabidopsis* $G\gamma$ subunits ($\gamma 1/AGG1$, $\gamma 2/AGG2$, or $\gamma 3/AGG3$). Thus, the $G\beta$ and $G\gamma 1+\gamma 2$ subunits were shown to function in the control of *Arabidopsis* immune responses (Delgado-Cerezo et al. 2012; Jiang et al. 2012). In line with this hypothesis, we found that the production of ROS upon flg22 treatment was compromised in *agg1 agg2* whereas, in either *agg1* or *agg2*, the level of ROS production was comparable to wild-type plants (Supplementary Fig. 6). Remarkably, ROS production impairment in *agg1 agg2* was similar to that observed in *atrbohD atrbohF*, disproving any significant contribution of *AGG3* to flg22-mediated ROS production. These results demonstrate that the $G\beta\gamma 1+\gamma 2$ heterodimer constitute the active complex that regulates ROS production, and are in line with the preeminent contribution of these subunits to the regulation of *Arabidopsis* innate immunity. These data also suggest that heterotrimeric G proteins could function downstream of some pattern recognition receptors, such as the flg22 receptor FLS2. In line with this hypoth-

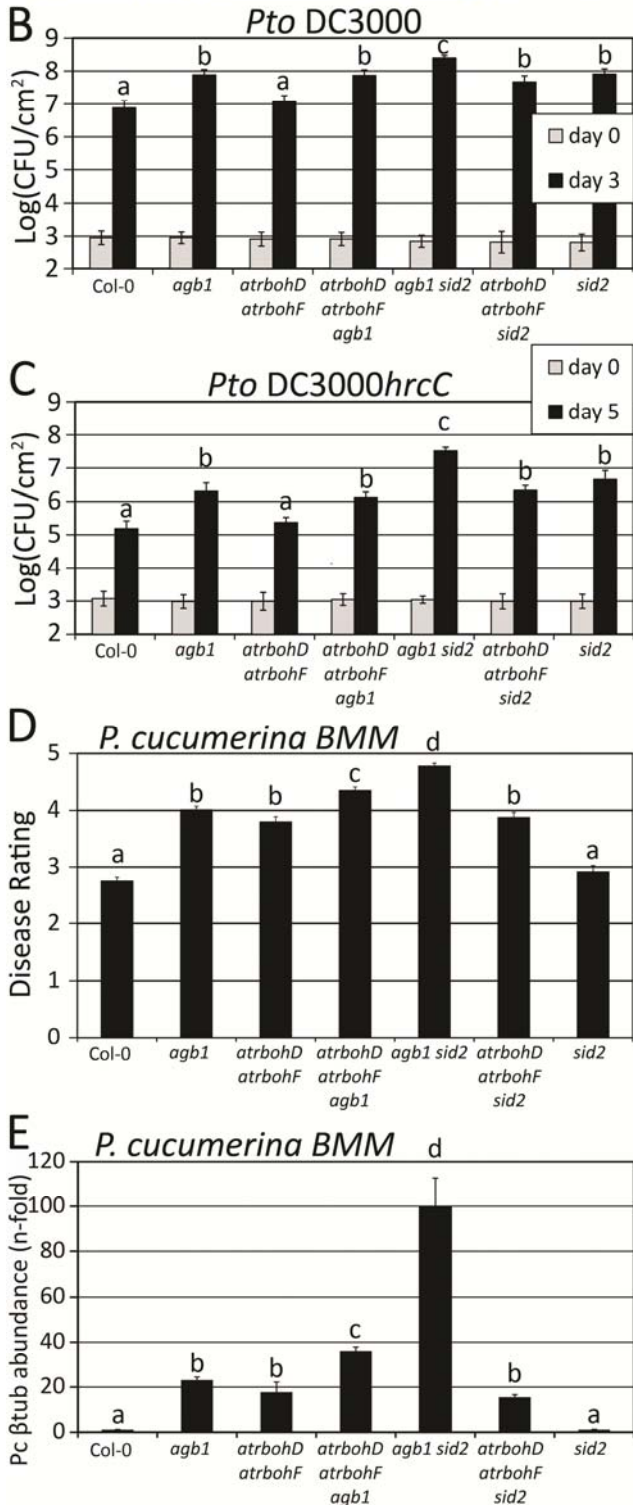
esis, a recent study reports that silencing of both $G\alpha$ and $G\beta$ subunits in *Nicotiana benthamiana* affects defense responses regulated by several microbial elicitors (Zhang et al. 2012).

In our studies, infected *gpa1* plants display wild-type accumulation of H_2O_2 (Fig. 1E). This is intriguing because the same *gpa1-4* mutant allele used in our experiments failed to activate ROS generation by NADPH oxidases in response to high levels of exogenous ozone (Joo et al. 2005). Thus, the interaction of heterotrimeric G protein signaling and ROS production can also vary depending on the stress. $G\beta\gamma$ downstream signaling would have a more preeminent role on regulating pathogen-induced ROS, whereas $G\alpha$ downstream signaling could regulate ROS production in response to abiotic stresses. In the heterotrimeric G protein mutants tested, we did not observe alterations in cell death, monitored either quantitatively (via conductivity assays) or qualitatively (via trypan blue staining) (Fig. 2). These results are in contrast to those reported following high levels of exogenous ozone or in the response to rice blast (Joo et al. 2005; Suharsono et al. 2002). However, the fact that impairment of *AGB1* restored cell death levels in the *atrbohD atrbohF* mutant after inoculation with avirulent bacteria (Fig 2B) indicates that *AGB1* can be a negative regulator of cell death in certain situations. Again, these data suggest that heterotrimeric G proteins can perform different signaling roles leading to cell death in different cellular contexts.

Interaction between *AGB1* and NADPH oxidase signaling.

Our epistasis analysis prompts us to suggest that *AGB1* works in the same pathway as NADPH oxidases *AtRbohD* and *AtRbohF* after *P. syringae* and *H. arabidopsidis* infection, since the phenotypes displayed by *atrbohD atrbohF* are suppressed in *atrbohD atrbohF agb1* (Fig. 2). The impairment of *AGB1* restored to wild-type levels the reduced cell death in response to avirulent *P. syringae* pv. *tomato* displayed by *atrbohD atrbohF* and reduced the enhanced cell death and disease resistance response to virulent *H. arabidopsidis* displayed by *atrbohD atrbohF*. Moreover, *atrbohD atrbohF agb1* displayed the same enhanced susceptibility to virulent *P. syringae* pv. *tomato* as *agb1* (Fig. 3). The fact that *agb1* displays reduced levels of ROS production in response to flg22 could indicate that the *AGB1/AGG1-AGG2* heterodimer works in concert with NADPH oxidases in some contexts to regulate ROS production. This is consistent with the situation in rice in response to *M. grisea* infection. Since ROS production is abolished in *dl* mutants, and since expression of a constitutive active form of *OsRac1* restored ROS production, *OsRac1* likely functions downstream of $G\alpha$ in ROS production (Suharsono et al. 2002). Interestingly, other phenotypes, such as dwarfing or pathogenesis-related protein expression in response to the *M. grisea* elicitor, were not restored in rice *dl* plants by expressing active *OsRac1*, indicating that the NADPH oxidase does not operate in all the pathways in which $G\alpha$ is involved.

AGB1 and NADPH oxidase can also work in different resistance pathways. We found that NADPH oxidases have a role in resistance to the necrotrophic fungus *Plectosphaerella cucumerina*, since *atrbohD atrbohF* displayed enhanced susceptibility to this pathogen, but this function is independent of *AGB1*, since *agb1 atrbohD atrbohF* plants showed an enhanced susceptibility to this fungal necrotroph compared with the individual mutants (Fig. 4). Thus, NADPH oxidases and *AGB1* have additional, independent functions in response to *Plectosphaerella cucumerina*. NADPH oxidase function has been linked to SA signaling, although synergism and antagonism has been observed in different cases (Torres 2010). However, we found that heterotrimeric G protein (*AGB1*) and SA signaling mediate independent responses against *Plectosphaerella cucumerina* and *P. syringae* pv. *tomato* (Fig. 5). Similarly, *AGB1*-mediated



responses against other necrotrophs such as *Fusarium oxysporum* and *Alternaria brassicicola* were described to be SA-independent (Trusov et al. 2006, 2009).

Our results indicate a complex relationship between the signaling roles of ROS produced by NADPH oxidase and the heterotrimeric G proteins, in which the functional interaction between these regulatory systems varies depending of the type of pathogen. This diversity of function may be based on the complexity of multiple, specific interactions of the G protein subunits with potential signaling components that exist in plants and that are different from interactors and effectors present in other metazoans (Klopffleisch et al. 2011). The characterization of these downstream components will ultimately contribute to clarification of the complex interaction between NADPH oxidases and heterotrimeric G proteins as they function in various responses to pathogens of diverse lifestyles.

MATERIALS AND METHODS

Arabidopsis plants and growth conditions.

Arabidopsis thaliana accession Col-0 and mutants derived from it were grown on a soil/vermiculite 3:1 mixture under a 10-h day and 14-h night schedule, with a daytime temperature of 24°C and a night temperature of 22°C, under relative humidity of 65% and light intensity of 120 $\mu\text{E m}^{-2} \text{s}^{-1}$. The mutant lines used in this study were *atrbohD* and *atrbohF* (Torres et al. 2002), *agb1* (*agb1-2*) (Ullah et al. 2003), *gpa1* (*gpa1-4*) (Jones et al. 2003), *agg1* and *agg2* (*agg1-1* and *agg2-1*, respectively) (Trusov et al. 2008), and *sid2* (*eds16-1*) (Wildermuth et al. 2001). Mutant combinations were generated by standard genetic crosses followed by the identification of homozygous mutant alleles by PCR (primers and conditions upon request).

Bacterial inoculation.

Bacterial strains used in this study were *P. syringae* pv. tomato DC3000, DC3000(*avrRpm1*), DC3000(*avrRpt2*), and DC3000*hrcC*. Four-week-old plants were inoculated using a 1-ml syringe (Debener et al. 1991) or were sprayed with a 0.1 optical density bacterial solution following standard procedures (Zipfel et al. 2004).

Conductivity measurements were performed as described (Torres et al. 2002). Four-week-old plants were injected with 2.5×10^7 CFU ml^{-1} of the avirulent *P. syringae* strain DC3000 (*avrRpm1*) in 10 ml MgCl_2 . Ten minutes after injection, 7.5-mm-diameter leaf discs were collected from the injected area and were washed extensively with water for 50 min. Then,

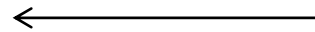


Fig. 5. *agb1 sid2* displays enhanced disease susceptibility compared with the individual mutants. **A**, Symptoms of 4.5-week-old plants, 5 days after spray inoculation with a concentration of 5×10^7 CFU ml^{-1} of *Pseudomonas syringae* pv. tomato DC3000. **B** and **C**, Quantification of bacterial growth on 4.5-week-old plants after spray inoculation with concentrations of 5×10^7 CFU ml^{-1} of the virulent bacterial strain *P. syringae* pv. tomato DC3000 and the type III secretion mutant *P. syringae* pv. tomato DC3000*hrcC*, respectively. Values are means \pm standard error (SE) calculated from four repetitions. Letters indicate significant differences at $P < 0.01$. **D**, Average disease rating (\pm SE) 7 days after spray inoculation with a concentration of *P. cucumerina* (4×10^6 spores per milliliter). Data derived from one representative experiment out of three experiments carried out with at least 30 plants for each genotype. Letters indicate significant differences between classes at $P < 0.05$. **E**, Quantification of *P. cucumerina* biomass 3 days postinoculation by quantitative polymerase chain reaction analysis. Primers specific for fungal β -tubulin and *Arabidopsis* ubiquitin were used. Values are representative as *n*-fold increased expression compared with wild-type Col-0. Data are the average (\pm SE) of three replicates. Statistical analyses were performed using analysis of variance 5%, corrected with Bonferroni test. Letters indicate significant differences. Experiments were repeated three times with similar results.

four discs were placed in a tube with 6 ml of water. Four replicates were taken per genotype. Conductivity measurements were taken from the tubes over time with an Orion (Boston) conductivity meter, model 130. The units of this measurement are microsiemens per centimeter ($\mu\text{S cm}^{-1}$).

Fungal and oomycete inoculation assays.

Sporangia from *H. arabidopsidis* isolates Emco5, Cala2, and Noco2 were suspended in water (5×10^4 spores ml^{-1}) and were sprayed on 10-day-old seedlings (Dangl et al. 1992). Inoculated plants were kept under a sealed lid to achieve high relative humidity, and sporangiophores were counted on cotyledons at 5 days postinoculation (dpi) (Emco5) or 7 dpi (Cala2, Noco2).

Spoore suspensions (4×10^6 spores ml^{-1}) from *Plectosphaerella cucumerina* BMM were sprayed on three-week-old plants. Subsequently, plants were kept under high relative humidity. Disease progression was estimated according to Hernandez-Blanco and associates (2007), by determining the average disease rating value: 1 = no symptoms; 2 = one chlorotic or necrotic leaf; 3 = two to three necrotic leaves; 4 = more than three leaves necrotic; 5 = plant dead. Relative quantification of fungal DNA was performed by qPCR with primers specific for β -tubulin from the fungus and ubiquitin from *Arabidopsis* as described (Sanchez-Rodriguez et al. 2009).

Histology.

To visualize H_2O_2 in situ, 3,3'-diaminobenzidine (Sigma, St. Louis) staining was performed on *Arabidopsis* plants after pathogen inoculation as described (Torres et al. 2002). Inoculated leaves were vacuum infiltrated with a 1-mg ml^{-1} DAB solution. Subsequently, leaves were placed in a plastic box under high humidity until brown precipitate was observed (5 to 6 h) and were then fixed with a solution of 3:1:1 ethanol/lactic acid/glycerol.

Dead cells were visualized by boiling leaves in lactophenol-trypan blue, followed by destaining in saturated chloral hydrate as described (Slusarenko et al. 1991).

H_2O_2 measurements.

Leaf discs (5 mm diameter; $n = 16$) were collected from fully expanded leaves from 4-week-old plants and were washed overnight with 150 ml of water. For H_2O_2 measurement, water was exchanged for a solution containing 10 μg horseradish peroxidase per milliliter (Sigma) and 100 μM luminol (Sigma). H_2O_2 production was triggered with 100 nM flg22. Luminescence was measured as relative light units with a TECAN Genius Pro luminescence reader over a period of 40 min. All H_2O_2 measurements were repeated three times with similar results.

Statistical analyses.

Statistical differences among *Arabidopsis* genotypes were determined by one-way analysis of variance and Bonferroni post hoc test, as previously reported (Sanchez-Rodriguez et al. 2009).

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