Transgenic Expression of a Fungal endo-Polygalacturonase Increases Plant Resistance to Pathogens and Reduces Auxin Sensitivity\cite{W}

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Polygalacturonases (PGs), enzymes that hydrolyze the homogalacturonan of the plant cell wall, are virulence factors of several phytopathogenic fungi and bacteria. On the other hand, PGs may activate defense responses by releasing oligogalacturonides (OGs) perceived by the plant cell as host-associated molecular patterns. Tobacco (\textit{Nicotiana tabacum}) and Arabidopsis (\textit{Arabidopsis thaliana}) plants expressing a fungal PG (PG plants) have a reduced content of homogalacturonan. Here, we show that PG plants are more resistant to microbial pathogens and have constitutively activated defense responses. Interestingly, either in tobacco PG or wild-type plants treated with OGs, resistance to fungal infection is suppressed by exogenous auxin, whereas sensitivity to auxin of PG plants is reduced in different bioassays. The altered plant defense responses and auxin sensitivity in PG plants may reflect an increased accumulation of OGs and subsequent antagonism of auxin action. Alternatively, it may be a consequence of perturbations of cellular physiology and elevated defense status as a result of altered cell wall architecture.

The plant cell wall possesses mechanical features determining strength and plasticity of a tissue and signaling properties affecting expansion, growth, and development (Carpita and McCann, 2000). The cell wall also represents a barrier against invading microorganisms. During early stages of infection, pathogens produce enzymes that degrade the various components of the wall, thus releasing compounds that are used as carbon sources. The major components of primary cell walls of higher plants are complex polysaccharides; increasing evidence indicates that these molecules contribute to disease resistance not just as mechanical barriers but also as sensors for incoming infections (Vorwerk et al., 2004). For example, partial degradation of homogalacturonan (HGA) by fungal endo-polygalacturonases (PGs) releases oligogalacturonides (OGs) with a degree of polymerization between 10 and 15 that show elicitor activity (Cervone et al., 1987a, 1987b, 1989). Treatment with OGs causes accumulation of reactive oxygen species, biosynthesis of phytoalexins (Hahn et al., 1981), and expression of pathogenesis-related (PR) proteins (Davis and Hahlbrock, 1987; Broekaert and Pneumah, 1988) in several plant species. In Arabidopsis (\textit{Arabidopsis thaliana}), OGs induce the expression of several defense genes and proteins (Ferrari et al., 2003b, 2007; Casasoli et al., 2008), including \textit{AIPGIP1} and \textit{PAD3}, which encode, respectively, an inhibitor of fungal PGs and the Cyt P450 \textit{CYP71B15} that catalyzes the last step of camalexin biosynthesis. Exogenous OGs protect Arabidopsis and grapevine leaves against the necrotrophic pathogen \textit{Botrytis cinerea} (Aziz et al., 2004; Ferrari et al., 2007). In analogy with the role of hyaluronan fragments in animal cells, OGs may be regarded as host-associated molecular patterns involved in the innate immunity (Stern et al., 2006; Taylor and Gallo, 2006).

Besides inducing defense responses, OGs can also affect several aspects of plant growth and development. In particular, a number of reports from our laboratory indicate that exogenously added OGs are able to antagonize the action of auxin, as in the case of pea (\textit{Pisum sativum}) stem elongation (Branca et al., 1988), tobacco (\textit{Nicotiana tabacum}) adventitious root formation (Bellincampi et al., 1993), and pericycle cell differentiation (Altamura et al., 1998). OGs prevent rhizogenesis in tobacco leaf explants expressing the \textit{Agrobacterium tumefaciens rolB} gene by inhibiting the auxin-induced expression of the transgene (Bellincampi et al., 1996). Furthermore, in tobacco, OGs inhibit the induction of the late auxin-responsive genes \textit{Nt1114}, \textit{rolB}, and \textit{rolD} (Mauro et al., 2002). In cucumber (\textit{Cucumis sativus}) seedlings, OGs allow for a more rapid recovery of root growth in auxin-treated roots (Spiro et al., 2002). To date, the mechanism underlying the antagonistic effect of OGs on auxin-induced responses is not known.

We have previously generated tobacco and Arabidopsis transgenic lines (hereafter referred to as PG plants)
expressing an attenuated version of PGII of *Aspergillus niger* (Capodicasa et al., 2004). PG plants of both species accumulate the enzyme in their tissues and display morphological alterations, such as dwarfism and slightly curled leaves, but no other severe aberrations (Capodicasa et al., 2004). These phenotypes are dependent on the enzyme activity because expression of an inactive form of *A. niger* PGII has no obvious impact on Arabidopsis development. Moreover, crossing tobacco PG plants with a line expressing high levels of the bean (*Phaseolus vulgaris*) PG-inhibiting protein PvPGIP2, an inhibitor of *A. niger* PGII, completely blocked PG activity and reverted the dwarf phenotype. The observed morphological alterations are associated to a reduced content of HGA in both tobacco and Arabidopsis, suggesting that the phenotype of PG plants is due to an enhanced degradation of HGA (Capodicasa et al., 2004). Interestingly, tobacco PG plants show no alterations of ion-mediated increase in xylem hydraulic conductivity (Nardini et al., 2007) and have a photosynthesis rate and stomatal conductance similar to those of wild-type plants (R. Galletti and D. Pontiggia, unpublished data). Therefore, PG plants, despite their growth defects, do not show alterations that are typical of plants suffering abiotic stresses, such as water stress (Flexas and Medrano, 2002).

Because the signaling potential of pectin and pectin-derived fragments may be critical for the outcome of a plant-pathogen interaction (Vorwerk et al., 2004), we have investigated whether the modifications caused by the expression of PG affect responses of plants to microbial pathogens. Here, we show that PG plants exhibit enhanced resistance to the necrotrophic fungal pathogen *B. cinerea* and to the virulent bacterial pathogen *Pseudomonas syringae*, and constitutive expression of defense responses. Notably, tobacco PG plants show a susceptibility to *B. cinerea* similar to wild-type plants when treated with auxin, as well as a reduced sensitivity to auxin in different bioassays. Furthermore, we show that auxin inhibits OG-induced resistance in wild-type tobacco plants. The relationship among pectin degradation, auxin responses, and defense against pathogens is discussed.

**RESULTS**

**PG Plants Are Less Susceptible to Pathogen Infection**

Tobacco PG plants were inoculated with *B. cinerea*, and their susceptibility to the fungus was compared to that of wild-type plants and plants expressing the bean PvPGIP2, which inhibits several fungal PGs, including *B. cinerea* PG (PGIP2 plants; Leckie et al., 1999; Capodicasa et al., 2004). Upon infection, wild-type plants exhibited typical soft rot symptoms with rapidly expanding water-soaked lesions, whereas PG plants displayed lesions with a reduced size (Fig. 1A). Furthermore, inoculation of PG plants resulted in dry lesions unable to develop beyond the inoculation site in a significant number of cases (Fig. 1B). PGIP2 plants also displayed reduced lesions that, unlike the lesions observed in PG plants, were typically water soaked (Fig. 1, A and B). Leaves from plants obtained by crossing line PG16 with the PGIP2 line (PG16×PGIP2 plants), expressing PGIP2 in great excess with respect to PG and showing no detectable PG activity in their tissues (Capodicasa et al., 2004), exhibited symptoms comparable to those developed on PGIP2 plants (Fig. 1, A and B).

Arabidopsis Wassilewskija (Ws) PG plants also displayed a significant reduction of lesion size after fungal inoculation (Fig. 1C). Line PG1, expressing the highest levels of PG (Capodicasa et al., 2004), developed smaller lesions (Fig. 1C) and showed a significant reduction of the number of spreading lesions compared to the other lines (Fig. 1D). Transcript levels of the *B. cinerea* actin gene *Bcact* were dramatically reduced in inoculated PG1 plants (Supplemental Fig. S1), confirming that the reduced lesion development mirrored a reduced fungal growth. On the other hand, leaves of a transgenic line (PG201), expressing an inactive form of *A. niger* PGII (van Santen et al., 1999; Federici et al., 2001), showed symptoms similar to those of wild-type plants (Fig. 1, C and D), indicating that enzymatic activity is required to confer enhanced resistance.

To determine whether the enhanced resistance observed in PG plants is specific for *B. cinerea* or is effective against other pathogens, leaves of tobacco wild-type and PG plants were inoculated with a virulent strain of the bacterial pathogen *P. syringae pv tabaci*. Infiltration with a bacterial suspension produced the collapse of the inoculated area in wild-type plants but no visible symptoms in line PG16 and only very mild symptoms in lines PG5 and 7 (Fig. 2). PGIP2 and PG16×PGIP2 plants showed symptoms similar to those observed in wild-type plants (Fig. 2). This indicates that PvPGIP2 has no effect on resistance to *P. syringae* and, more importantly, that inhibition of enzymatic activity of PG by PvPGIP2 completely blocks PG-mediated resistance. An active and free (uncomplexed) PG is therefore required to confer resistance to plants against organisms as different as the fungal pathogen *B. cinerea* and the bacterial pathogen *P. syringae*.

**PG Plants Have Enhanced Defense Responses**

PGs may act not only as virulence factors of phytopathogenic microorganisms but may also induce defense responses, by either releasing endogenous OG elicitors or affecting cell wall integrity (Hahn et al., 1981; Davis et al., 1986; Cervone et al., 1987a). Staining of leaves with 3-3′-diaminobenzidine (DAB) revealed accumulation of hydrogen peroxide (*H₂O₂*) in tobacco PG16 and PG7 and, to a lesser extent, PG5 leaves (Fig. 3A). No significant staining was observed in wild-type, PGIP2, and PG16×PGIP2 plants, indicating that accumulation of *H₂O₂* correlates with the level of active PG in the tissues. Similarly, constitutive accumulation of *H₂O₂* was observed in leaves of Arabidopsis
PG1 and, to a lesser extent, PG5 plants, whereas accumulation of H$_2$O$_2$ in Arabidopsis wild-type and PG201 plants was restricted to the severed petioles (Fig. 3B). Leaves of tobacco and Arabidopsis PG plants exhibited a bright fluorescence when illuminated with UV light, possibly due to the accumulation of secondary metabolites (Supplemental Fig. S2).

We also determined total peroxidase and 1,3-β-glucanase enzymatic activities, which are known to be regulated by biotic stress (Van Loon and van Strien, 1999). Total peroxidase activity was significantly higher in leaves of tobacco plants PG16 and PG7 as compared to control plants, whereas in PG5 as well as in PG16×PGIP2 plants it was only slightly, but still significantly, higher than in wild-type plants or plants expressing PvPGIP2 alone (Fig. 4A). Analysis of peroxidase activity in the intercellular washing fluids (IWFs) prepared from control and transgenic tobacco plants indicated that the increase of peroxidase activity of PG plants was mainly due to extracellular isoforms (Fig. 4B). Peroxidase activity was also significantly increased in leaves of Arabidopsis plants belonging to line PG1, compared to the other genotypes (Fig. 4C). Basal 1,3-β-glucanase activity was significantly higher only in tobacco and Arabidopsis lines expressing high levels of PG (Fig. 5, A and B).

Finally, we examined the expression of genes potentially involved in pathogen responses. Expression of two tobacco genes, EAS1/2, encoding a 5-epi-aristolochene synthase required for the biosynthesis of the phytoalexin capsidiol (Facchini and Chappell, 1992), and POX, a gene encoding an apoplastic anionic peroxidase (Diaz-De-Leon et al., 1993), was clearly induced by B. cinerea infection in both wild-type and PG16 plants (Fig. 6A). However, expression of POX was detectable also in uninfected PG16 plants and increased earlier and to a greater extent in response to fungal infection (Fig. 6A). EAS1/2 mRNA levels were not constitutively expressed in PG16 plants but increased earlier and to a greater extent in response to fungal infection (Fig. 6A). In Arabidopsis wild-type plants, expression of AtPGIP1, PR-1, and the defensin gene PDF1.2 steadily increased up to 2 d after infection with B. cinerea (Fig. 6, B–D). The crossing line PG16 with the line expressing PvPGIP2 (PG16×PGIP2) were inoculated with B. cinerea. Lesion area (A) and percentage of expanding lesions (B) were measured after 6 d. Bars in A indicate average lesion area ± se (n = 36); this experiment was repeated four times with similar results. Bars in B represent the average percentage of spreading lesions ± se of five experiments (n = 36 in each experiment). Adult rosette leaves from untransformed Arabidopsis plants (WT) and from transgenic plants expressing PG (PG1 and PG5) or an inactive version of the A. niger PGII (PG201) were inoculated with B. cinerea, and lesion size was determined after 2 d (C), whereas the number of expanding lesions was determined after 3 d (D). Bars in C indicate the average lesion area ± se (n = 12); this experiment was repeated three times with similar results. Bars in D represent the average percentage of spreading lesions ± se of three experiments (n = 12 in each experiment). Different letters represent data sets significantly different, according to ANOVA analysis followed by Tukey's test (P < 0.01).
basal mRNA levels of all three genes were higher in PG1 plants than in wild-type plants (Fig. 6, B–D). However, their expression differed after inoculation with B. cinerea. AtPGIP1 expression increased at 1 d postinfection (dpi) but returned to basal levels at 2 dpi (Fig. 6B). In contrast, transcripts of PR-1 and PDF1.2 showed only a mild increase after fungal infection of PG plants (Fig. 6, C and D). These findings are consistent with previous data correlating the expression levels of both genes to fungal lesion development rather than to basal resistance (Ferrari et al., 2003a).

A possible explanation for the enhanced expression of defense genes observed in healthy PG plants is the accumulation of OG elicitors released from the cell wall by the action of the fungal PG. We therefore determined whether the expression of these genes is up-regulated by exogenous OGs. Both POX and EASI/2 mRNA levels increased in tobacco leaf explants treated for 4 h with OGs (Fig. 7, A and B). Similarly, AtPGIP1 and PR-1 transcripts accumulated in response to OGs in Arabidopsis wild-type seedlings (Fig. 8, A and B), while no significant increase of PDF1.2 mRNA levels was observed (Fig. 8C). Taken together, these data support the hypothesis that the constitutive expression of at least some of the defense responses observed in PG plants is mediated by OGs released by the fungal enzyme expressed in the transgenic plants.

Increased Resistance of Tobacco PG Plants Is Abolished by Auxin

Because OGs have auxin-antagonistic activity and treatment with OGs leads to a decreased sensitivity to auxin in tobacco plants (Branca et al., 1988; Bellincampi et al., 1993, 1996; Altamura et al., 1998; Mauro et al., 2002), a support for the possible involvement of OGs in the phenotype of PG plants might be provided by the ability of auxin to revert some of the phenotypic features as well as by a decreased sensitivity to auxin of the PG plants.

We therefore investigated whether the increased resistance against B. cinerea of the tobacco PG plants could be reverted by exogenous auxin. Leaf discs from wild-type and PG16 tobacco plants were treated with 3-indoleacetic acid (IAA) and then inoculated with B. cinerea. Notably, auxin pretreatment of PG16 leaf discs restored their susceptibility to a level comparable to that of wild-type plants, whereas it did not significantly increase the susceptibility of wild-type leaf discs (Fig. 9A). Interestingly, whereas pretreatment of wild-type leaf discs with exogenous OGs reduced lesion development after B. cinerea inoculation, co-treatment with OGs and IAA did not produce any effect, and the susceptibility was comparable to that of untreated tissues (Fig. 9B).

We also evaluated the sensitivity to auxin of tobacco PG plants by analyzing the auxin-induced root formation in leaf explants. While a concentration of 0.57 μM IAA was sufficient to induce rhizogenesis in wild-type explants, no roots were observed in PG explants treated with up to 1.7 μM IAA; however, at higher concentrations, IAA induced root formation in PG explants in a dose-dependent fashion (Fig. 10A). The ability of PG plants to respond to auxin was also tested using a root growth inhibition assay. Wild-type plantlets showed a significant reduction of primary root growth in the presence of 10−7 M IAA, whereas concentrations of IAA up to 10−6 M were not effective in PG16 plants (Fig. 10B). However, when auxin concentration was increased to 10−5 M, a reduction of root length was observed in PG16 plants, consistent with the hypothesis that they are not completely resistant but only less sensitive to auxin. Taken together, these results indicate that auxin-induced developmental and growth responses are impaired in PG plants. This is not due to increased catabolism of IAA because no significant IAA oxidase activity could be detected in extracts from PG leaves (data not shown). We
therefore conclude that auxin signaling is partly compromised in PG plants.

Because high concentrations of IAA induce ethylene production in several plant species (Abeles, 1966) and ethylene-controlled responses play a role in both resistance against *B. cinerea* (Thomma et al., 1999; Diaz et al., 2002; Chague et al., 2006) and inhibition of root development (Ortega-Martinez et al., 2007; Stepanova et al., 2007), we investigated whether PG plants show increased ability to produce ethylene. Ethylene produced by wild-type and PG16 leaf explants was measured after water or IAA treatment. Water-treated wild-type or PG16 leaf explants did not release any detectable accumulation of ethylene; in contrast, 1-aminocyclopropane-1-carboxylic acid (ACC) induced high levels of ethylene in both genotypes, indicating that they have a similar ability of producing this hormone (Fig. 11). Furthermore, treatment with 100 μM IAA resulted in the accumulation of lower, but significant and comparable, levels of ethylene in wild-type and PG16 explants (Fig. 11). OGs had no significant impact on ethylene production in water- or IAA-treated wild-type tobacco explants (data not shown). A role of ethylene in the basal resistance, in the IAA-mediated reversion of the resistant phenotype, and in the reduced ability to form adventitious roots in response to IAA of tobacco PG plants is therefore unlikely.

**DISCUSSION**

In this article, we have shown that the expression of a fungal PG in Arabidopsis and tobacco increases plant resistance to microbial pathogens. The resistant phenotype is not exhibited by transgenic tobacco plants expressing both PG and its inhibitor PvPGIP2 or by Arabidopsis plants expressing a mutagenized and inactive AnPGII (PG201), indicating that resistance is dependent on the enzymatic activity of the expressed PG and is likely a consequence of the degradation of the host pectin accomplished by the enzyme. Interestingly, PG plants appear to have reduced sensitivity to exogenous auxin, and auxin treatments revert their resistant phenotype. The data presented here allow us to consider the reasons that might explain why heterologous expression of a fungal PG increases plant resistance, providing a link between HGA degradation, auxin perception, and activation of defense responses.

PG plants have an altered pectin composition with a reduced GalUA and HGA content (Capodicasa et al., 2004). A direct negative effect of the lack of consumable GalUA on pathogen growth appears unlikely, because we did not observe any significant reduction of growth when *B. cinerea* was cultivated in the presence of cell walls from PG plants as the sole carbon source (data not shown). On the other hand, PG plants constitutively express a number of defense responses, such as the accumulation of UV-fluorescent metabolites, H$_2$O$_2$, β-1,3-glucanase, and peroxidase, and expression of defense-related genes that are normally induced only in the presence of pathogens. High levels of H$_2$O$_2$ in PG plants are concomitant with an increased peroxidase activity, which, at least in tobacco, corresponds to an anionic and apoplastic enzyme possibly encoded by the constitutively expressed POX gene (see Fig. 6A). In the presence of H$_2$O$_2$, peroxidases may generate free radicals that have direct antimicrobial activity (Bolwell et al., 2002) or may modify the plant cell wall (Passardi et al., 2004). On the other hand, H$_2$O$_2$ itself may be responsible for the induction of PR proteins (Apostol et al., 1989; Chen et al., 1993). Whether the high peroxidase activity and elevated H$_2$O$_2$ levels in PG plants directly contribute to their increased resistance to pathogens still needs to be determined. Besides causing the constitutive activation of defense responses, PG expression makes plants more prone to respond to infection. Following *B. cinerea*
inoculation, tobacco PG plants accumulate more POX and EAS1/2 transcripts than wild-type plants, and Arabidopsis plants show enhanced expression of AtPGIP1.

The enhanced expression of diverse defense responses is likely the ultimate reason for their increased resistance to pathogens. We could not observe a perfect correlation between the levels of a specific defense response and the degree of resistance observed in independent PG lines, suggesting that multiple defense responses, activated to different extents in different PG lines, contribute to the final resistant phenotype. The observation that tobacco PG16×PGIP2 plants or Arabidopsis PG201 plants, which have no PG activity, display neither altered expression of defense responses nor increased resistance indicates that these phenotypes are mediated by HGA degradation in PG plants rather than by the recognition per se of the heterologous protein by plant cells. Enhanced expression of basal and inducible defense responses and increased resistance to pathogens were previously observed in plants expressing pectin-degrading enzymes. Basal levels of polyphenoloxidase activity and induction of Phe ammonia lyase upon wounding are enhanced in transgenic potato tubers expressing a bacterial pectate lyase (Wegener, 2002), and accumulation of β-1,3-glucanase and H2O2 is induced in tobacco leaves by the transient expression of a PG from Colletotrichum lindemuthianum (Boudart et al., 2003). Moreover, H2O2 accumulates in tomato plants that have increased PG activity upon antisense expression of the regulatory subunit of a

Figure 4. Peroxidase activity in PG plants. A, Peroxidase activity in total protein extracts from leaves of tobacco untransformed (WT) and transgenic PG5, PG7, PG16, PGIP2, and PG×PGIP2 plants. B, Peroxidase activity in IWFs (black bars) and intracellular proteins extracted after recovery of IWFs (white bars) from the same tobacco lines shown in A. C, Peroxidase activity in total protein extracts from leaves of Arabidopsis untransformed (WT) and transgenic PG201, PG5, and PG1 plants. Bars indicate the average activity, expressed as enzymatic units per milligram of total proteins, of three samples ± se. Different letters represent data sets significantly different, according to ANOVA analysis followed by Tukey’s test (P < 0.01).

Figure 5. Glucanase activity in PG plants. A, Levels of β-1,3-glucanase activity, expressed as enzymatic units per milligram of total proteins, in leaves of tobacco untransformed (WT) and transgenic PG5, PG7, PG16, PG×PGIP2, and PGIP2 plants. Bars indicate average activity of three independent samples ± se. B, Levels of β-1,3-glucanase activity, expressed as enzymatic units per milligram of total proteins, in leaves of Arabidopsis untransformed (WT) and transgenic PG201, PG5, and PG1 plants. Bars indicate the average activity of three independent samples ± se. Different letters represent data sets significantly different, according to ANOVA analysis followed by Tukey’s test (P < 0.01).
wound-inducible endogenous PG (PGβS); these plants exhibit constitutive expression of wound-inducible genes and enhanced resistance to insect attack (Orozco-Cardenas and Ryan, 2003).

One explanation for the observed phenotypes in the PG plants is that altered cell wall integrity may be perceived as a signal of pathogen attack or mechanical damage, leading to the induction of defense mechanisms (Humphrey et al., 2007). Links between pectin and cytoplasm, mediated by specific cell wall-associated transmembrane proteins, may allow the host cell to sense alterations caused by microbial pathogens and regulate the activation of defense responses. The observation that the Arabidopsis wall-associated kinase WAK1 (Decreux and Messiaen, 2005), which is able to bind polygalacturonic acid, is induced by bacterial infection and that this induction is required for survival upon treatments with chemical inducers of resistance supports this hypothesis (He et al., 1998). Apoplastic proteins that have domains interacting with pectin also include a peroxidase (Carpin et al., 2001) and PGIP (Spadoni et al., 2006); these proteins may participate to perceive alterations of pectin structure and transmit this information across the plasma membrane with mechanisms not yet investigated.

Indeed, increased disease resistance is observed in plants with alterations of cell wall structural components other than pectin. For example, mutations in an Arabidopsis cellulose synthase (CESA3) cause not only decreased cellulose content but also constitutive expression of defense responses (Ellis et al., 2002; Cano-Delgado et al., 2003). More recently, it has been reported that mutations in Arabidopsis cellulose synthase genes confer resistance to different pathogens through a mechanism that is independent of salicylic acid (SA), ethylene, and jasmonic acid signaling (Hernandez-Blanco et al., 2007). Defects in the cuticle cause complete resistance to B. cinerea, and this resistance is also independent of SA, ethylene, and jasmonic acid (Chassot et al., 2007). It is therefore likely that plants have evolved mechanisms to perceive the presence of a potential pathogen by monitoring the integrity of different components of their own cell wall. However, a direct mechanistic link between modifications of these cell wall components and activation of defense responses is still missing.

Another possible explanation for the enhanced resistance of plants with altered cell wall components is that plant cells perceive molecules released in the apoplast as a consequence of the hydrolysis of specific structural polymers and these molecules act as elicitors.

Figure 6. Expression of defense genes in PG plants after infection with B. cinerea. A, Fully expanded leaves from tobacco untransformed (WT) and transgenic PG16 (PG) plants were inoculated with B. cinerea and total RNA was extracted at the indicated time points (dpi). The RNA gel blot was hybridized with the indicated probes. Equal loading was verified by methylene blue staining of ribosomal RNA. B to D, Fully expanded leaves from Arabidopsis untransformed (WT, white bars) or transgenic PG1 (PG, black bars) plants were inoculated with B. cinerea, and total RNA was extracted at the indicated time points (dpi). The expression of AtPGIP1 (B), PR-1 (C), and PDF1.2 (D) was analyzed by real-time RT-PCR and normalized using the expression of UBQ5 in each sample. The insets in C and D show gene expression in untreated leaves (n.d., not detectable). Bars represent the average expression ± SD of two replicates.
of defense responses. In particular, OGs generated upon degradation of HGA by fungal PGs act as elicitors of defense responses in several plant systems (for review, see Ridley et al., 2001). It is possible that in planta expression of PG confers resistance to pathogens because of the release of OGs or other wall-derived elicitors. Circumstantial evidence supporting this hypothesis is presented here. First, PG plants show enhanced expression of defense responses similar to those observed in wild-type plants after addition of exogenous OGs. Reactive oxygen species accumulation is a hallmark of OG-mediated responses (Ridley et al., 2001; Aziz et al., 2004), and we detected a dramatic increase of H$_2$O$_2$ levels in both tobacco and Arabidopsis PG plants. Furthermore, we could demonstrate that expression of EAS1/2 and POX is induced by both OGs and B. cinerea in tobacco and that their expression is increased in PG plants. In Arabidopsis PG plants, high basal levels of three defense-related genes, AtPGIP1, PR-1, and PDF1.2, are also enhanced compared to the wild type. Expression of AtPGIP1 was previously demonstrated to be responsive to OGs (Ferrari et al., 2003b), and PR-1, a marker of SA-mediated defense responses (Delaney et al., 1994), is also up-regulated by OGs in the Ws ecotype, though at later time points than AtPGIP1. PDF1.2, which is not significantly regulated by OGs but is responsive to oxidative stress (Penninckx et al., 1996), may be high in PG plants because of the high levels of H$_2$O$_2$ in their tissues. The reduced mRNA levels of both PR-1 and PDF1.2 in inoculated PG plants reflect the fact that their expression correlates to fungal growth (reduced in PG plants) rather than to plant resistance (Ferrari et al., 2003a; Chassot et al., 2007). The observation that PG plants accumulate higher levels of mRNA corresponding to genes that are responsive to OGs suggests that HGA alterations cause the activation of a defense-related pathway that is also activated by
elicitors; this activation may be either directly mediated by pectin fragments or by other mechanisms. It should be noted that the levels of defense-related transcripts accumulating upon infection are dramatically higher than those observed in either OG-treated wild-type plants or in untreated PG plants. This may be due to the concomitant activation of multiple defense-related pathways during pathogen infection and/or the massive release of different elicitors in infected tissues.

Figure 9. Susceptibility to *B. cinerea* in tobacco PG plants treated with auxin. A, Leaf discs from tobacco untransformed (WT, white bars) or transgenic PG16 (PG, black bars) plants were incubated for 3 h in liquid medium containing water (control) or 100 μM IAA (IAA) and inoculated with *B. cinerea*. Lesion area was measured after 24 h. Bars indicate average area ± se of at least 10 lesions. Asterisks indicate statistically significant difference between lesions in wild-type and transgenic plants (***, P < 0.01). This experiment was repeated three times with similar results. B, Leaf discs from untransformed tobacco plants were incubated for 3 h in liquid medium containing water (control), 100 μM IAA (IAA), 200 μg mL⁻¹ OGs (OG), or both (OG + IAA) and inoculated with *B. cinerea*. Lesion area was measured after 24 h. Bars indicate the average area ± se of at least 10 lesions. Different letters indicate data sets significantly different, according to ANOVA followed by Tukey's test (*P*, <0.05). This experiment was repeated twice with similar results.

Figure 10. Auxin sensitivity of tobacco PG plants. A, Leaf explants from tobacco untransformed (WT, white squares) and transgenic PG16 (PG, black squares) plants were treated with the indicated concentrations of IAA for 15 d and the number of explants forming roots was measured. Each data point represents the average percentage of explants forming roots ± SD calculated in three independent experiments (*n* = 8 in each experiment). B, Length of primary roots of tobacco untransformed (WT) and transgenic PG16 (PG) plants grown for 12 d on solid medium containing the indicated IAA concentrations. Bars represent the average length of at least eight plants ± se. Asterisks indicate statistically significant difference between treated and control samples, according to the Student's *t* test (*P*, <0.01). This experiment was repeated twice with similar results.

et al., 1993, 1996; Mauro et al., 2002). In particular, tobacco PG plants show reduced sensitivity to IAA in experiments of rhizogenesis in leaf explants and inhibition of primary root growth. This reduced sensitivity does not appear to depend on IAA degradation, as suggested by the absence of IAA oxidase activity in PG plants.

We also show that tobacco PG plants do not differ from wild-type plants in their ability to produce ethylene, and, on the other hand, OGs do not induce significant production of ethylene in wild-type plants. This suggests that a role of this hormone in the basal resistance, as well as in the IAA-mediated reversion of the resistant phenotype and in the reduced ability to form adventitious roots in response to IAA, is unlikely.
of perception of auxin (Mayda et al., 2000). Importantly, it has been reported that the flagellin-derived peptide flg22 represses auxin signaling through the induction of a miRNA directed against the auxin receptors TIR1, AFB2, and AFB3, and this leads to an increased resistance to bacterial infection (Navarro et al., 2006). It is possible that release of the auxin-mediated inhibition of defense gene expression is a crucial step in the transcription pathway activated by general elicitors, including OGs. According to this model, OGs, by negatively regulating auxin signaling, allow for increased expression of defense responses. However, our observations imply that, if the phenotype of the PG plants is not due to accumulation of OGs but to the perception of a defective wall structure, this second mechanism also involves a cross-talk with auxin. Perception of cell wall-derived fragments and of alterations of cell wall biomechanical properties may all converge in a common signaling pathway (Humphrey et al., 2007) that features a negative cross-talk with the auxin response pathway.

In conclusion, we have shown that the expression of a fungal PG and the subsequent degradation of HGA increases resistance of plants to microbial pathogens, likely through a pre-activation of plant defense responses, and that auxin reverts the enhanced resistance to fungal infection. Our data also indicate that in muro pectin degradation by PG leads to decreased auxin sensitivity. This is reminiscent of the effects observed in untransformed plants treated with OGs, suggesting a possible role of these pectic fragments in the resistant phenotypes of PG plants. Alternatively, an altered cell wall architecture may be directly perceived by the plant cell as a signal of the presence of a pathogen, leading to an increased activation of defense responses. The molecular mechanisms linking pectin degradation, auxin signaling, and activation of defense responses still remain to be investigated. We believe that this line of research will provide new insights in the regulation of plant defense responses during plant-pathogen interactions.

**MATERIALS AND METHODS**

**Transgenic Lines, Plant Growth, and Treatments**

Generation of transgenic Arabidopsis (Arabidopsis thaliana) Ws and tobacco (Nicotiana tabacum) Petit Havana-SR1 plants expressing PG or PvPGIP2 and their cross were described previously (Capodicasa et al., 2004). The PG expressed in these plants had a point mutation (N178D) that has an estimated activity approximately 20-fold lower than the native Aspergillus niger PGII (data not shown). The expression of a PG with reduced enzymatic activity allowed us to generate viable plants. Arabidopsis line PG201 expresses an inactive version of A. niger PGII with a point mutation in the catalytic site (D201N) that causes complete loss of enzymatic activity (van Santen et al., 1999; Federici et al., 2001). These plants express a level of the inactive enzyme comparable to that observed in line PG5, as estimated by immunoblot analysis (data not shown), and show no obvious developmental defects (Capodicasa et al., 2004). Arabidopsis plants were grown in growth chambers at 22°C and 60% relative humidity, with a 12-h photoperiod (100 μmol m⁻² s⁻¹ of fluorescent light). Tobacco plants were grown in a greenhouse at 23°C and 60% relative humidity, with a 16-h photoperiod (130 μmol m⁻² s⁻¹).

For elicitor treatments on Arabidopsis seedlings, seeds were surface-sterilized and germinated in multiwell plates (approximately 10 seeds per
well) containing 1 mL per well of 1× Murashige and Skoog (MS) medium (Sigma; Murashige and Skoog, 1962) supplemented with 0.5% Suc. Plates were incubated at 22°C with a 16-h photoperiod and a light intensity of 200 μmol m⁻² s⁻¹. After 8 d, the medium was replaced, and after two additional days treatments with 100 mg L⁻¹ OGS or water were performed (Ferrari et al., 2003b). Tobacco leaf explants (5 × 10 mm) were obtained from apical leaves of 4-week-old SRI plants, washed six times for 30 min with 0.25× MS medium containing 2% Suc, and incubated in the same medium supplemented with 10 μg mL⁻¹ OGS. The OGS used in this work were with average degree of polymerization of 10 to 15, prepared as described previously (Bellincampi et al., 2000).

Ethylene Measurements
Approximately 20 to 30 tobacco leaf explants (approximately 500 mg fresh weight) were prepared from 7-week-old tobacco leaves, avoiding the midrib, and extensively washed with sterile distilled water. The explants were placed in sealed 500-mL flasks containing 100 mL of sterile water alone or supplemented with 250 μM ACC, 100 μM IAA sodium salt, or 200 mg L⁻¹ OGS. Gas samples were withdrawn from the flasks after 24 h and analyzed with a gas chromatograph equipped with a flame ionization detection system (Carlo Erba). Chromatographic separations were carried out on a Porapak Q 80/100 mesh column (4-mm i.d., 1.5 mL). The flow rate of the carrier gas (N₂ 0.80 kg cm⁻²) was 40 mL min⁻¹. Column, injector, and detector temperatures were 30°C, 50°C, and 120°C, respectively. Heating rate was 18°C min⁻¹ to 170°C. Air flow was 1.60 kg cm⁻² and hydrogen flow was 0.70 kg cm⁻². Ethylene concentration in each sample was normalized using dry weight of the leaf explants.

Pathogen Growth and Infection
Botrytis cinerea (a kind gift of J. Plotnikova, Massachusetts General Hospital) was grown for 7 to 10 d at 22°C under constant light on 20 g L⁻¹ malt extract, 10 g L⁻¹ mycological peptone, and 15 g L⁻¹ agar until sporulation. Conidia were collected with 10 mL of sterile water containing 0.05% Tween 20, filtered with sterile glass wool, and centrifuged for 5 min at 5,000 g. Before pathogen inoculation, fresh spores were resuspended in 24 g L⁻¹ potato dextrose broth and incubated for 3 h at room temperature to allow uniform germination. Inoculation of detached Arabidopsis leaves was performed as described previously (Ferrari et al., 2003b). Fully developed leaves of 8-week-old tobacco plants were detached and placed in large petri dishes, with the petiolar end immersed in 0.5% agar, and inoculated with a spore suspension in 24 g L⁻¹ potato dextrose broth (10⁵ spores mL⁻¹). Six droplets of spore suspension (10 μL each) were placed on the abaxial surface of each leaf. Plates containing the inoculated leaves were wrapped with a transparent plastic film and incubated in a growth chamber at 22°C under fluorescent light for a 16-h photoperiod. Disease progress was scored as described by ten Have et al. (1990).

Determination of H₂O₂ Content; Glucanase, Peroxidase, and IAA Oxidase Activity; and Visualization of UV-Fluorescent Compounds
For H₂O₂ visualization, leaves were cut from adult plants using a razor blade and dipped for 12 h in a solution containing 1 mg mL⁻¹ DAB, pH 5.0. Chlorophyll was extracted for 10 min with boiling ethanol and for 2 h with ethanol at room temperature prior to photography (Orozco-Cardenas and Ryan, 1999).
For total protein extraction, frozen leaves or stems were homogenized in 1× NaCl, 20 mM sodium acetate, pH 4.7, incubated under gentle shaking for 1 h, and centrifuged for 20 min at 10,000 g. IWFs were prepared from tobacco stems using 0.3 M NaCl and 20 mM sodium acetate, pH 4.7, buffer, as described previously (Terry and Bonner, 1980). After IWF extraction, stem sections were homogenized with 1× NaCl, 20 mM sodium acetate, pH 4.7, as described above for total protein extraction, to obtain intracellular proteins. Total protein concentration was determined by the Bradford method (Bradford, 1976).
Activity of β-1,3-glucanase was determined by incubating 50 μg and 150 μg of tobacco or Arabidopsis total proteins, respectively, at 37°C with 2 mg mL⁻¹ laminarin (Sigma) in 50 mM sodium acetate, pH 5.2. The produced reducing sugars were determined colorimetrically with dinitrosalicylic acid (Sigma; Miller, 1959). Peroxidase activity was measured using a modified version of the method described in Smith and Barker, 1988. Enzymatic activity was determined by the change in ΔA₂₅₄ due to the oxidation of amionoantipyrine and 3,5-dichloro-2-iodobenzenesulphonic acid. IAA oxidase activity was determined as described in Preiss, 1990.

The presence of UV-fluorescent compounds was visualized by photographing detached leaves under UV light (λ = 320 nm) using the “Image-Master” VDS (Pharmacia Biotech).

RNA Gel-Blot Analysis
Tobacco leaf discs of 5-mm radius around the site of inoculation were frozen in liquid nitrogen, homogenized, and total RNA was extracted with Tri-reagent (Sigma). RNA was separated on agarose-formaldehyde gel and transferred to a nylon membrane as described previously (Ferrari et al., 2003b). Equal RNA loading and transfer were verified by staining the membrane with a solution containing 0.05% methylene blue (w/v) and 0.3 mM sodium acetate, pH 4.7. Prehybridization was performed for 6 h at 42°C in 50% formamide, 6× sodium chloride/sodium phosphate/EDTA, 1× Denhardt’s solution, 0.1% SDS, 50 μg mL⁻¹ denatured herping DNA, and 100 ng of denatured probe. 32P-labeled probes were prepared by PCR as templates for the tobacco probes, two DNA fragments, corresponding respectively to a region of a tobacco POX gene (GeneBank accession no. L02124; Daza-De-Leon et al., 1993) conserved also in different anionic peroxidase genes, and to the EASI and EAS2 genes (Facchini and ChapPELL, 1992), were amplified from cDNA of infected tobacco leaves, using the following primers: 5′-TAAGTACAGTGTGG-CAGGAGG-3′ (EASI/2 forward), 5′-CTAGAATATCATATTACCC-3′ (EASI/2 reverse), 5′-ACATGCTATTCGCGCATG-3′ (POX forward), 5′-GTTCG-AATGTTCCCCTC-3′ (POX reverse). Probes were purified using ProbeQuant G-50 microcolumns (Amersham Pharmacia Biotech). Hybridized blots were washed once with 2× SSC, 0.1% SDS at room temperature and twice with 0.1× SSC, 0.1% SDS at 65°C. Images were taken after overnight exposure using a phosphor imager (Typhoon 9200; Amersham).

Real-Time Reverse Transcription-PCR Analysis
Total RNA was extracted with Tri-reagent (Sigma) and treated with DNase I (Ambion). First-strand cDNA was synthesized using ImProm-II Reverse Transcriptase (Promega). Real-time PCR analysis was performed using an iQ-Cycler (Bio-Rad) according to the manufacturer’s guide. A total of 2 μL of cDNA (corresponding to 120 ng of total RNA) was amplified in 30 μL of reaction mix containing iQ SYBR Green Supermix (Bio-Rad) and 0.4 μM each primer. Primer sequences for POX and EASI/2 are described above. The tobacco actin gene (Tob66, accession no. L000491) was amplified using the following primers: 5′-CTGCATCTAGTATGTCTGATT-3′ and 5′-AGTCCCA- ATTATCCCAT-3′. The primers for PDF1.2, PDF1.1, APOX2, and UBQ5 genes were described previously (Pennicknics et al., 1996; Rogers and Ausubel, 1997; Ferrari et al., 2006). The primers utilized for the amplification of the B. cinerea Bact actin gene (accession no. A0003035) were the following: 5′-AAGTGTG-AATGGTATGGTCC-3′ and 5′-CTTGTTAAGGAAGTAGACAAGA-3′. Relative expression of the reverse transcription (RT)-PCR products was determined using a modified version of the Pfaffi method (Pfaffl, 2001; Ferrari et al., 2006).

Auxin Responses in Tobacco Leaf Explants and Seedlings
For rhizogenesis of leaf explants, the second apical leaves from tobacco plants grown for 4 weeks on soil were harvested and surface sterilized. Ten explants of about 0.4 × 0.8 cm were excised in correspondence with the midrib vein and placed, abaxial side down, in petri dishes containing 10 mL of sterile
MS medium, pH 5.7, supplemented with 2% Suc, 0.8% plant agar, and IAA at the indicated concentrations. Leaf explants were incubated at 25°C under low intensity light (60 μE m⁻² s⁻¹) for 15 d. For seedling assays, seeds were sterilized and germinated in 0.5X MS liquid medium supplemented with 2% Suc, pH 5.8. After 1 week, seedlings were transferred to full-strength MS medium, pH 5.7, supplemented with 2% Suc, 1.5% plant agar, and IAA. Primary root length was measured after 12 d of growth at 22°C with a photoperiod of 16 h.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number L02124.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of the B. cinerea BacT gene in PG plants after infection.

Supplemental Figure S2. Accumulation of UV-fluorescent compounds in PG plants.

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LITERATURE CITED


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lates stem cell division in the Arabidopsis thaliana root. Science 317: 507–510