

The AtrbohD-Mediated Oxidative Burst Elicited by Oligogalacturonides in Arabidopsis Is Dispensable for the Activation of Defense Responses Effective against *Botrytis cinerea*^{1[W][OA]}

Roberta Galletti, Carine Denoux, Stefano Gambetta, Julia Dewdney, Frederick M. Ausubel, Giulia De Lorenzo, and Simone Ferrari*

Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza," 5-00185 Rome, Italy (R.G., G.D.L., S.F.); Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova, 35020 Legnaro, Italy (S.G.); and Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114 (C.D., J.D., F.M.A.)

Oligogalacturonides (OGs) are endogenous elicitors of defense responses released after partial degradation of pectin in the plant cell wall. We have previously shown that, in *Arabidopsis* (*Arabidopsis thaliana*), OGs induce the expression of *PHYTOALEXIN DEFICIENT3* (*PAD3*) and increase resistance to the necrotrophic fungal pathogen *Botrytis cinerea* independently of signaling pathways mediated by jasmonate, salicylic acid, and ethylene. Here, we illustrate that the rapid induction of the expression of a variety of genes by OGs is also independent of salicylic acid, ethylene, and jasmonate. OGs elicit a robust extracellular oxidative burst that is generated by the NADPH oxidase AtrbohD. This burst is not required for the expression of OG-responsive genes or for OG-induced resistance to *B. cinerea*, whereas callose accumulation requires a functional AtrbohD. OG-induced resistance to *B. cinerea* is also unaffected in *powdery mildew resistant4*, despite the fact that callose accumulation was almost abolished in this mutant. These results indicate that the OG-induced oxidative burst is not required for the activation of defense responses effective against *B. cinerea*, leaving open the question of the role of reactive oxygen species in elicitor-mediated defense.

Plants need to recognize invading pathogens in a timely manner to mount appropriate defense responses. Specific molecules associated with different microbial pathogens can be perceived by plant cells at early stages of infection and trigger inducible defenses that include phytoalexin accumulation, expression of pathogenesis-related proteins, production of reactive oxygen species (ROS), and, at least in some cases, programmed cell death. Many of these molecules, traditionally called general elicitors, are secreted or are present on the surface of all strains of a given microbial taxonomic group and activate defense responses ef-

fective against a wide range of pathogens (Nurnberger et al., 2004). For this reason, they are also referred to as microbe-associated molecular patterns or pathogen-associated molecular patterns (PAMPs; Parker, 2003; He et al., 2007). PAMPs (for review, see Nurnberger and Brunner, 2002) are often structural components of the pathogen cell wall (e.g. chitin, glucan) or other macromolecular structures (e.g. bacterial flagellin).

Hahn and colleagues (1981) first showed that structural components of the plant cell wall, released during pathogen infection as a consequence of microbial enzymatic activities, can also induce defense responses. In particular, oligogalacturonides (OGs) with a degree of polymerization (DP) between 10 and 15 can accumulate when fungal polygalacturonases (PGs) degrade the homogalacturonan component of plant pectin (Hahn et al., 1981). OGs elicit a variety of defense responses, including accumulation of phytoalexins (Davis et al., 1986), glucanase, and chitinase (Davis and Hahlbrock, 1987; Broekaert and Pneumas, 1988). Exogenous treatment with OGs protects grapevine (*Vitis vinifera*) and *Arabidopsis* (*Arabidopsis thaliana*) leaves against infection with the necrotrophic fungus *Botrytis cinerea* (Aziz et al., 2004; Ferrari et al., 2007), suggesting that production of this elicitor at the site of infection, where large amounts of PGs are secreted by the fungus, may contribute to activate defense responses. For these reasons, OGs can be considered as danger

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* Corresponding author; e-mail simone.ferrari@uniroma1.it.

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signals derived from an altered self (host-associated molecular patterns).

A prominent feature of the plant defense response is the oxidative burst, a common early response of plant cells to pathogen attack and elicitor treatment (Lamb and Dixon, 1997). ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are toxic intermediates resulting from reduction of molecular O_2 . ROS are important signals for defense responses and phytoalexin accumulation in several species. It is generally thought that ROS contribute to plant resistance by directly exerting a cytotoxic effect against pathogens, by participating in cell wall reinforcement (cross-linking of structural protein and lignin polymers), or by inducing hypersensitive cell death, expression of defense genes, or the accumulation of antimicrobial compounds (Levine et al., 1994). Generation of ROS can be induced by a variety of elicitors (Apostol et al., 1989; Legendre et al., 1993; Bolwell et al., 2002; Aziz et al., 2003; Kasparovsky et al., 2004; Pauw et al., 2004; Xu et al., 2005) and in many plant systems ROS production is biphasic (e.g. Dorey et al., 1999; Yoshioka et al., 2001).

O_2^- -generating NADPH oxidases are generally considered to be a major enzymatic source of ROS in the oxidative burst of plant cells challenged with pathogens or elicitors (Torres and Dangl, 2005; Torres et al., 2006). Two different NADPH oxidase genes in potato (*Solanum tuberosum*) are responsible for the elicitor-induced biphasic oxidative burst (Yoshioka et al., 2001). In Arabidopsis, several genes encoding proteins with high similarity to the mammalian NADPH oxidase gp91^{phox} subunit have been characterized. Among them, *AtrbohD* is required for the production of ROS during infection with different bacterial and fungal pathogens, including *B. cinerea* (Torres et al., 2002, 2005). Besides NADPH oxidases, other enzymes appear to be important in the elicitor-mediated oxidative burst, including apoplastic oxidases, such as oxalate oxidase (Dumas et al., 1993), amine oxidase (Allan and Fluhr, 1997), and pH-dependent apoplastic peroxidases (Bolwell et al., 1995; Frahry and Schopfer, 1998), which generate either O_2^- or H_2O_2 .

We have recently shown that OGs and an unrelated elicitor, the synthetic 22-amino acid peptide flg22 derived from bacterial flagellin (Felix et al., 1999), activate defense responses against *B. cinerea* both in wild-type Arabidopsis and in mutants impaired in salicylic acid (SA), jasmonate (JA)-, or ethylene (ET)-mediated signaling (Ferrari et al., 2007). Elicitor-induced protection against *B. cinerea* requires the *PHYTOALEXIN DEFICIENT3* (*PAD3*) gene (Ferrari et al., 2007). *PAD3* encodes the cytochrome P450 CYP71B15, which catalyzes the last step of the biosynthesis of the phytoalexin camalexin (Schuhegger et al., 2006). Camalexin is known to contribute to Arabidopsis basal resistance to *B. cinerea* (Ferrari et al., 2003a; Kliebenstein et al., 2005). Notably, the expression of *PAD3*, as well as that of another defense-related gene, *AtPGIP1*, which encodes a PG-inhibiting protein effective against *B. cine-*

rea, is induced by OGs independently of SA-, JA-, and ET-mediated signaling (Ferrari et al., 2003b, 2007). It is therefore likely that multiple defense responses are induced by OGs independently of SA, ET, and JA.

Transient accumulation of extracellular H_2O_2 was previously observed in tobacco (*Nicotiana tabacum*) leaf explants and grapevine cells treated with OGs (Bellincampi et al., 1996; Aziz et al., 2004). Because *PAD3* expression and camalexin accumulation can be induced by chemicals that generate oxidative stress (Zhao et al., 1998; Denby et al., 2005), we have investigated the hypothesis that H_2O_2 mediates the induction of defense responses effective against *B. cinerea* in Arabidopsis plants treated with OGs. Here, we show that OGs induce an oxidative burst in Arabidopsis that is *AtrbohD*-dependent; however, we also show that H_2O_2 -dependent responses are not required for OG-induced resistance against *B. cinerea*.

RESULTS

Early Activation of Genes in Response to General Elicitors Is Independent of SA, ET, and JA Signaling

To establish the degree of specificity of early gene expression in response to OGs and other general elicitors, we monitored the expression of *AtPGIP1*, *PAD3*, and several other early elicitor-induced genes (Ferrari et al., 2007; Denoux et al., 2008) in response to a pool of OGs with a DP between 10 and 15 (hereafter referred to as OGs), to purified oligodecagalacturonic acid (DP10), to flg22, and to a β -glucan elicitor from *Phytophthora megasperma* f. sp. *Glya* (Cheong et al., 1991). In addition to *AtPGIP1* and *PAD3*, we tested the expression of *AtWRKY40* (At1g80840), encoding a transcription factor that acts as a negative regulator of basal defense (Xu et al., 2006; Shen et al., 2007); *CYP81F2* (At5g57220), encoding a cytochrome P450 with unknown function; and *RetOx* (At1g26380), encoding a protein with homology to reticuline oxidases, a class of enzymes involved in secondary metabolism and in defense against pathogens (Dittrich and Kutchan, 1991; Carter and Thornburg, 2004). These genes were selected because they are rapidly and strongly up-regulated upon exposure to elicitors, as previously demonstrated by whole-genome transcript profiling and real-time quantitative PCR analyses (Ferrari et al., 2007; Denoux et al., 2008). As negative controls, we treated seedlings with water or α -1,4-trigalacturonic acid (DP3; Hahn et al., 1981; Cervone et al., 1989; Bellincampi et al., 2000; Navazio et al., 2002).

As shown in Figure 1, OGs, DP10, flg22, and β -glucan activated the expression of all tested genes in Arabidopsis seedlings, whereas water and DP3 failed to induce the expression of any of the genes analyzed. The expression of *PAD3*, *RetOx*, *CYP81F2*, *AtWRKY40*, and *AtPGIP1* was also compared across a set of 322 publicly available Arabidopsis microarray datasets using the Arabidopsis Coexpression Tool (Manfield

et al., 2006). The Pearson correlation coefficient between *PAD3* and *RetOx* expression was the highest ($r = 0.78$) among the tested genes (Supplemental Fig. S1), followed by *RetOx* and *CYP81F2* ($r = 0.71$). The *AtWRKY40* expression pattern appeared to correlate moderately with that of *PAD3* and *RetOx* ($r = 0.58$ in both cases), whereas no significant correlation between *AtPGIP1* and any of the other genes was observed, suggesting that the expression of this gene is regulated differently from that of *PAD3*, *RetOx*, and *CYP81F2*. Despite the fact that *AtPGIP1* does not significantly correlate with any other analyzed gene, it was included in subsequent analyses because of its established role in plant defense (Ferrari et al., 2003b, 2006). Transient expression of *PAD3*, *RetOx*, *CYP81F2*, and *AtWRKY40* was also observed in rosette leaves infiltrated with OGs (Supplemental Fig. S2) with kinetics comparable to those occurring in seedlings, indicating that these genes can be considered markers of early elicitor-induced responses both in seedlings and in adult plants.

To determine whether *RetOx*, *CYP81F2*, and *AtWRKY40* are expressed after elicitor treatment independently of SA, ET, or JA, as previously shown for *AtPGIP1* and *PAD3* (Ferrari et al., 2003b, 2007), we analyzed their expression in the *npr1ein2jar1* (*nej*) genetic background harboring mutations in the *NON-PR1 EXPRESSOR1* (*NPR1*; Cao et al., 1997), *JASMONATE RESISTANT1* (*JAR1*; Staswick et al., 1992), and *ETHYLENE INSENSITIVE2* (*EIN2*; Guzman and Ecker, 1990) genes, and therefore impaired in all three signaling pathways (Clarke et al., 2000). No major difference in expression of *RetOx*, *CYP81F2*, and *AtWRKY40* was observed, either in terms of kinetics of induction or in transcript levels, in wild-type or *nej* plants treated with OGs (Fig. 2, A–C), or in *npr1*, *ein2*, and *jar1* single mutants (Supplemental Fig. S3A). Expression of *AtPGIP1*, that was previously shown to be independent of *JAR1*, *EIN2* or *NPR1*, based on data obtained with single mutants (Ferrari et al., 2003b), was also unaffected in the triple mutant (Fig. 2D).

Because some reports have suggested that the *jar1-1* mutation is leaky (Staswick et al., 1998; Kariola et al.,

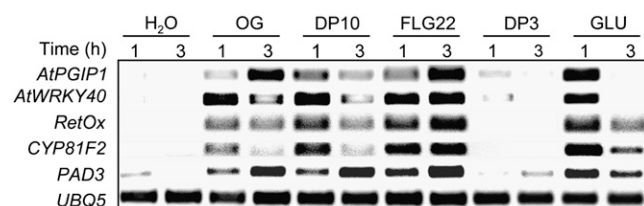


Figure 1. Expression analysis of marker genes in response to elicitors. Arabidopsis seedlings were treated at the indicated time (h) with water (H_2O), OGs, purified oligodecagalacturonic acid (DP10), flg22, trigalacturonic acid (DP3), or β -glucan (GLU). Expression of the indicated genes was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

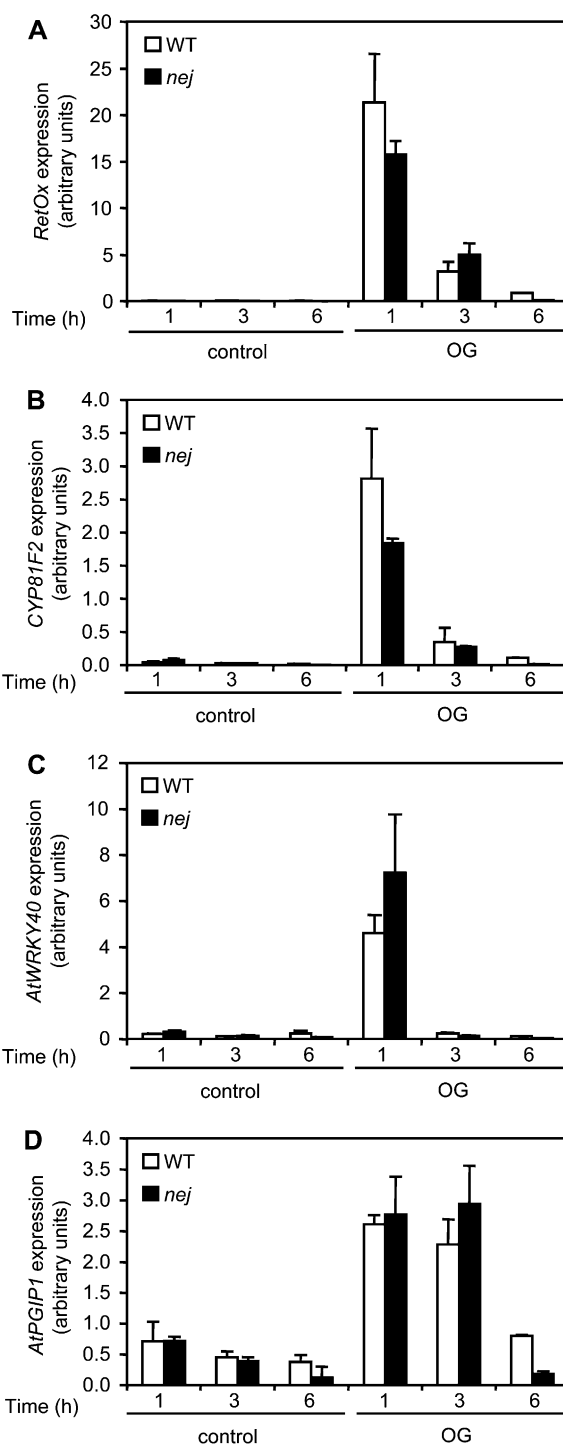


Figure 2. Expression of elicitor-responsive genes in the *nej* triple mutant. Arabidopsis wild-type (white bars) or *nej* triple mutant (black bars) seedlings were treated with water (control) or OGs for 1, 3, or 6 h. Expression of *RetOx* (A), *CYP81F2* (B), *AtWRKY40* (C), and *AtPGIP1* (D) was analyzed by real-time quantitative PCR and normalized using the expression of the *UBQ5* gene. Bars indicate average expression \pm SD of three replicates. This experiment was repeated three times with similar results.

2003), we also analyzed the *coronatine insensitive1 (coi1)* mutant, which is severely impaired in JA-mediated responses (Xie et al., 1998). Induction of *RetOx* and *CYP81F2* by OGs in wild-type and *coi1* seedlings was indistinguishable, whereas *AtWRKY40* expression was slightly reduced in *coi1* (Supplemental Fig. S3B), in accordance with a previous report indicating that *AtWRKY40* gene can be induced by JA in a *COI1*-dependent manner (Wang et al., 2008). Similarly, to further rule out an effect of SA on OG-induced gene expression, we treated *sid2-2* seedlings, which carry a mutation in the isochorismate synthase gene *ICS1* required for pathogen-activated biosynthesis of SA (Wildermuth et al., 2001). Also, in this case, no significant reduction of OG-induced gene expression was observed compared to the wild type (Supplemental Fig. S3C). These results indicate that expression of the OG-induced marker genes tested is independent of SA, ET, and JA.

Production of H₂O₂, But Not Gene Expression, in Response to OGs Is Mediated by AtrbohD

Analysis of the publicly available expression data using Genevestigator (<https://www.genevestigator.ethz.ch>) indicates that *PAD3*, *RetOx*, *AtWRKY40*, and *CYP81F2* transcript levels increase after treatment with H₂O₂, suggesting that their expression may be mediated by ROS (data not shown). Transient accumulation of extracellular H₂O₂ was previously observed in tobacco leaf explants and grapevine cells treated with OGs (Bellincampi et al., 1996; Aziz et al., 2004). To investigate whether OGs are also able to induce an apoplastic oxidative burst in Arabidopsis, we measured the release of H₂O₂ in the culture medium of seedlings treated with these elicitors. A significant oxidative burst was observed in response to OGs and DP10, whereas H₂O₂ accumulated to a much smaller extent in response to flg22, β -glucan, or DP3 (Fig. 3A).

We then investigated the source of H₂O₂ generated after treatment with OGs. Previous reports suggest that the oxidative burst observed after inoculation with virulent and avirulent pathogens is generated in Arabidopsis by the NADPH oxidase AtrbohD (Torres et al., 2005). To determine whether this enzyme is also the source of the extracellular burst observed in response to OGs, we analyzed an Arabidopsis knockout (KO) line containing a T-DNA insertion in the *AtrbohD* gene (Torres et al., 2002). This line failed to accumulate extracellular H₂O₂ after elicitation (Fig. 4A), indicating that AtrbohD is necessary for the OG-induced oxidative burst.

To determine the role of the oxidative burst in OG-triggered early gene expression, we analyzed the expression of *PAD3*, *RetOx*, *CYP81F2*, and *AtWRKY40* in elicited wild-type and *atrbohD* mutant seedlings. Strikingly, despite the absence of a functional *AtrbohD* gene and of an oxidative burst, no significant differences in the mRNA levels of all tested marker genes

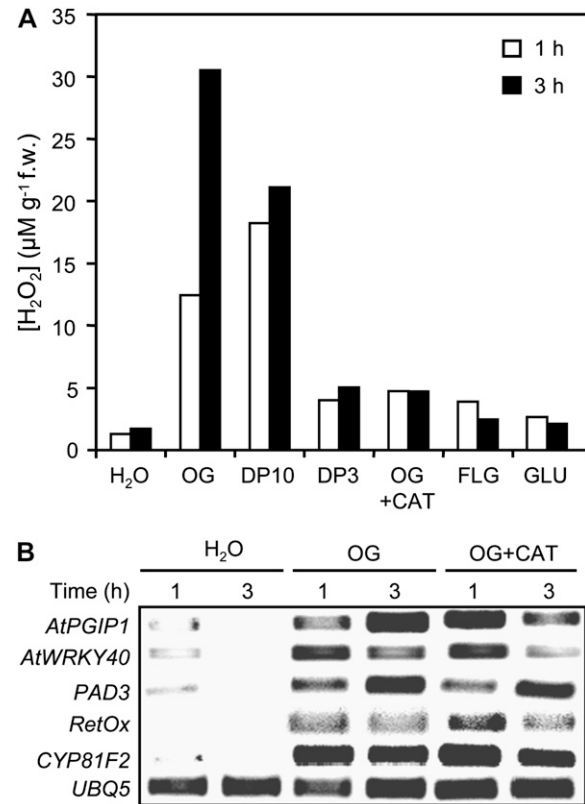


Figure 3. Oxidative burst and gene expression in response to elicitors. A, Arabidopsis seedlings were treated with water (H₂O), OGs alone, or in the presence of catalase (OG + CAT), purified oligodecagalacturonic acid (DP10), flg22 (FLG), trigalacturonic acid (DP3), or β -glucan (GLU). H₂O₂ accumulation in the culture medium, expressed as $\mu\text{M g}^{-1}$ fresh weight, was measured after 1 (white bars) or 3 h (black bars). This experiment was repeated twice with similar results. B, Arabidopsis seedlings were treated for 1 or 3 h with water (H₂O) or with OGs alone or in presence of catalase (OG + CAT). Expression of the indicated genes was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

could be detected (Fig. 5). Similar results were obtained in wild-type and *atrbohD* adult plants infiltrated with OGs (Supplemental Fig. S2). To conclusively rule out a role of NADPH oxidases in OG-induced marker gene expression, before application of OGs, we treated seedlings with diphenylene iodonium (DPI), which, at low concentrations, specifically inhibits this class of enzymes (Bolwell et al., 1995; Frahry and Schopfer, 1998). DPI completely blocked the OG-induced oxidative burst (Fig. 6A), but had no effect on the expression of *PAD3*, *RetOx*, *CYP81F2*, and *AtWRKY40* (Fig. 6B), confirming that NADPH oxidases are not required for early OG-induced transcriptional changes.

To conclusively demonstrate that extracellular H₂O₂ is not involved in OG-induced gene expression, we elicited Arabidopsis seedlings in the presence of catalase at a concentration that almost completely abolished the oxidative burst (Fig. 3A). Coincubation of

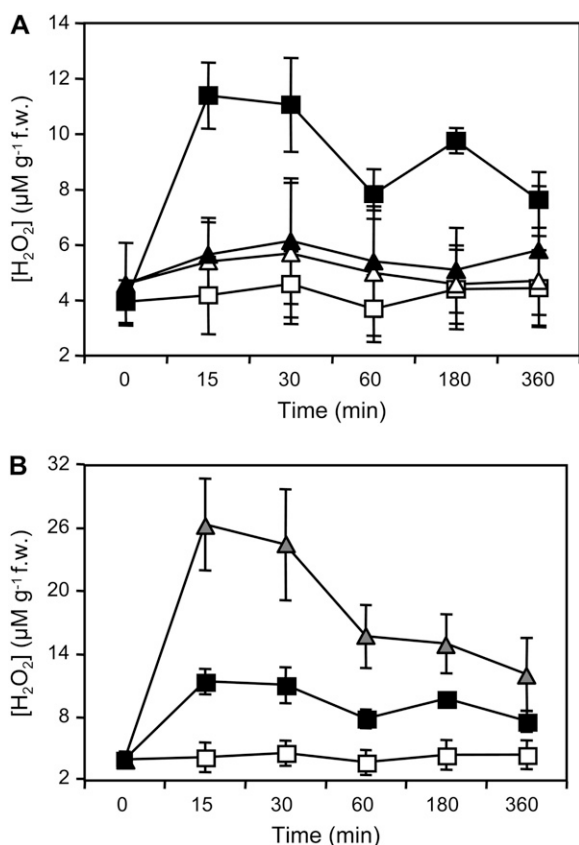


Figure 4. Accumulation of extracellular H₂O₂ in response to OGs or G/GO in Arabidopsis seedlings. A, Arabidopsis wild-type and *atrbohD* seedlings were treated with water (H₂O) or OGs for the indicated time (min). Arabidopsis wild-type (squares) and *atrbohD* (triangles) seedlings were treated with water (white symbols) or OGs (black symbols). B, Arabidopsis seedlings were treated with water (H₂O, white squares), OGs (black squares), or G/GO (gray triangles). H₂O₂ accumulation in the culture medium, expressed as $\mu\text{M g}^{-1}$ fresh weight, was measured at the indicated times (min). Values are means of three samples \pm SD.

OGs with catalase had no significant effect on the expression of *PAD3*, *AtPGIP1*, *RetOx*, *CYP81F2*, and *AtWRKY40* (Fig. 3B), confirming that H₂O₂ is not required for OG-induced marker gene expression. Furthermore, treatment of seedlings with Glc and Glc oxidase (G/GO) at concentrations that induced H₂O₂ levels in the same order of magnitude observed after OG treatments (Fig. 4B), failed to induce the expression of the same set of genes (Fig. 5).

Taken together, our results indicate that OG-mediated early gene expression is independent of the extracellular oxidative burst.

Basal and OG-Induced Resistance to *B. cinerea* Infection Are Independent of *AtrbohD* and of *PMR4/GSL5*

To determine whether defense responses that occur relatively late after treatment with OGs are also independent of H₂O₂, we analyzed callose deposition and

induced resistance in wild-type and *atrbohD* KO plants. Callose is a high-*M_r* β -1,3-glycan deposited at the site of infection by pathogens, probably acting as a physical barrier against colonization of the intercellular space (Ryals et al., 1996; Donofrio and Delaney, 2001). It was previously shown that flg22 induces callose deposition in Arabidopsis seedlings (Gomez-Gomez et al., 1999) and that callose accumulation induced by flg22 is impaired in leaf strips of *atrbohD* KO plants (Zhang et al., 2007). Similarly, infiltration of OGs in wild-type rosette leaves resulted in a significant accumulation of callose (Denoux et al., 2008), which was reduced of about 50% in *atrbohD* leaves (Fig. 7A), indicating that the oxidative burst contributes to callose synthesis also in response to OGs. As expected, infiltration of leaves of the powdery mildew resistant4 (*pmr4*) mutant, which has a mutation in the callose synthase gene *GLUCAN SYNTHASE-LIKE5* (*GSL5*; Nishimura et al., 2003), resulted in a dramatic decrease of callose deposition (Fig. 7B).

We have previously observed that OGs induce protection of Arabidopsis plants against *B. cinerea* and that this protection requires *PAD3* expression (Ferrari et al., 2007). To determine the role of *AtrbohD* in induced resistance, we treated wild-type, *atrbohD*, and, as a negative control, *pad3* plants with OGs, and subsequently inoculated them with *B. cinerea*. As expected, *pad3* plants showed increased basal susceptibility, and OG pretreatment did not reduce lesion development (Fig. 8A). In contrast, no significant difference in basal susceptibility and in OG-induced resistance between wild-type and *atrbohD* plants was observed either in detached leaves (Fig. 8A) or in intact plants (Fig. 9). This indicates that OG-induced activation of defense responses effective against *B. cinerea* does not require *AtrbohD*.

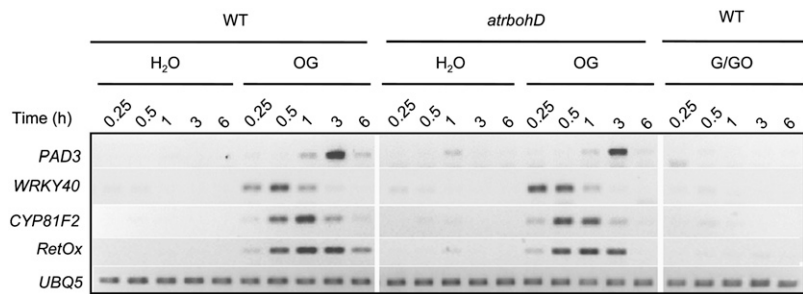
Furthermore, we investigated the role of callose in OG-elicited resistance to *B. cinerea*. As shown in Figure 8B, lesion development in *pmr4* plants inoculated with *B. cinerea* was unaffected or, in some experiments, slightly reduced, compared to wild-type plants. Moreover, OG treatment of the *pmr4* mutant resulted in protection against *B. cinerea* infection (Fig. 8B), indicating that callose does not play a major role in either basal or elicitor-induced resistance against this pathogen.

Finally, we infiltrated adult rosette leaves with G/GO at concentrations that in seedlings induced production of H₂O₂ levels in the same order of magnitude observed after OG treatments. G/GO caused significant accumulation of H₂O₂ in infiltrated tissues (Fig. 10A), but did not alter basal resistance to *B. cinerea* (Fig. 10B). These data indicate that a moderate extracellular oxidative burst, comparable to that observed after OG treatment, is not sufficient to induce defense responses effective against *B. cinerea*.

DISCUSSION

One of the earliest responses observed in plants inoculated with a pathogen or treated with an elicitor

Figure 5. Effects of endogenous and exogenous H_2O_2 on OG-responsive genes. Arabidopsis wild-type and *atrbohD* seedlings were treated with water (H_2O) or OGs for the indicated time (h). Wild-type seedlings were also treated with G/GO. Expression of the indicated genes was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.



is the oxidative burst, characterized by a rapid and transient production of ROS. OGs induce a strong extracellular oxidative burst, initially suggesting that ROS might play an important role in mediating responses to OGs. We therefore adopted both pharmacological and genetic approaches to investigate both the genesis and the role of the oxidative burst elicited by OGs in Arabidopsis plants.

There are a number of potential sources of ROS generated upon pathogen or elicitor perception. Increasing evidence points to superoxide-generating NADPH oxidases as the main sources of extracellular ROS produced during pathogen infection or elicitation (Yoshioka et al., 2001, 2003; Torres et al., 2002; Kobayashi et al., 2006; Nuhse et al., 2007). O_2^- generated by NADPH oxidases is rapidly dismutated into H_2O_2 , which is much more stable and can accumulate in tissues. Extracellular H_2O_2 can also be generated by other sources, most notably apoplastic peroxidases (Bolwell et al., 2002), making it sometimes difficult to discern the involvement of specific sources of ROS in the oxidative burst. The data presented here clearly indicate that the NADPH oxidase *AtrbohD* is necessary for the extracellular burst induced in Arabidopsis by OGs, as previously shown for *flg22* (Nuhse et al., 2007). H_2O_2 produced after OG treatment is therefore likely released by dismutation of O_2^- directly generated by *AtrbohD* in accordance with the observation that OGs induce the accumulation of O_2^- in Arabidopsis leaves (Song et al., 2006). In addition to the extracellular oxidative burst, protoplasmic sources of ROS emanating from mitochondrial, chloroplastic, or peroxisomal generating systems have also been documented (Bolwell et al., 2002). However, intracellular generation of ROS has mainly been studied in relation to abiotic stress (Asada, 1999; del Río et al., 2002). There are reports of intracellular accumulation of ROS in response to elicitors, such as cryptogein (Ashtamker et al. 2007), although its role in plant defense response has not been assessed.

OGs activate a very strong extracellular oxidative burst; surprisingly, however, this burst has a minor, if any, role in several downstream responses, based on the following evidence: (1) under our experimental conditions, there is significantly less H_2O_2 accumulation in response to *flg22* and β -glucan than in response to OGs, but the effect of *flg22* and β -glucan on the

expression of early molecular marker genes is comparable to that observed with OGs; (2) H_2O_2 generated by G/GO at levels comparable to those observed in OG-treated plants fails to activate the expression of elicitor-activated marker genes or to induce resistance to *B. cinerea*; (3) scavenging of H_2O_2 accumulation by catalase or inhibition of the OG-induced oxidative burst either by DPI or by the *atrbohD* mutation did not affect

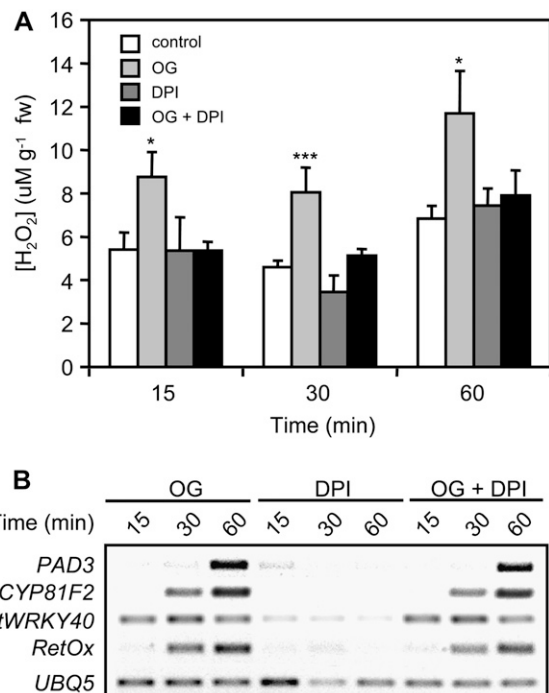


Figure 6. Effect of DPI on the expression of elicitor-responsive genes. A, Arabidopsis seedlings were treated with water (control, white bars), OGs (light gray bars), DPI (dark gray bars), or OGs + DPI (black bars). H_2O_2 accumulation in the culture medium, expressed as $\mu M g^{-1}$ fresh weight, was measured at the indicated times (min). Values are means of three samples \pm SD. Asterisks indicate statistically significant differences between control and OG-treated seedlings, according to Student's *t* test (*, $P < 0.05$; ***, $P < 0.01$). The experiment was repeated twice with similar results. B, Arabidopsis seedlings were treated with DPI, OGs alone, or in the presence of DPI (OG + DPI) for the indicated time (min). Gene expression was analyzed by semiquantitative RT-PCR, using the *UBQ5* gene as internal standard. This experiment was repeated twice with similar results.

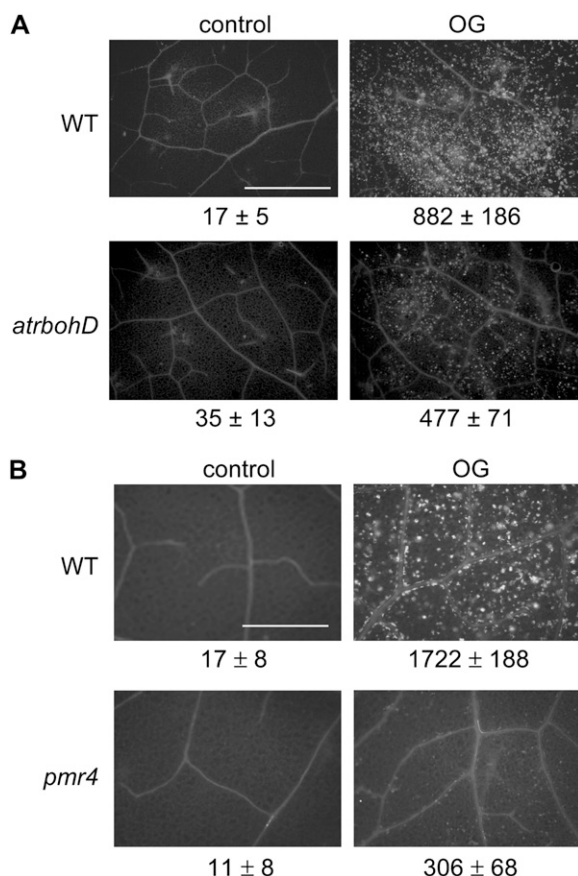


Figure 7. Callose accumulation in *atrbohD* and *pmr4* plants. Arabidopsis wild-type and *atrbohD* (A) or *pmr4* (B) leaves were infiltrated with water (control, left) or OGs (right) for 24 h and stained with aniline blue for callose visualization. The number below each image indicates the average number of callose deposits \pm SE of eight different leaf samples from at least five independent plants (three microscopic fields of 0.1 mm² for each leaf). Images show representative leaves for each treatment. All images are at the same scale; scale bar = 1 mm (10 \times magnification). This experiment was repeated twice with similar results.

early gene expression. Taken together, these results indicate that early changes in gene expression activated by OGs independently of SA, ET, and JA do not require the oxidative burst generated by *AtrbohD*. Furthermore, OG-triggered resistance against *B. cinerea*, which is also independent of SA, ET, and JA, occurs in the absence of *AtrbohD*.

In contrast to OGs, flg22 and β -glucan elicited very low levels of H₂O₂ under our experimental conditions. An extracellular oxidative burst, peaking at about 10 to 15 min, was previously observed using a H₂O₂-dependent luminescence assay in Arabidopsis leaf explants treated with 1 μ M flg22 (Gomez-Gomez et al., 1999). It is possible that the xylenol orange-based system used here is not sensitive enough to detect the burst induced by flg22, although previous work indicates the equivalence of this xylenol orange and the luminescence assays (Bindschedler et al., 2001). It is possible that the

different levels of H₂O₂ that we observed after treatment with OGs or flg22 could be ascribed to different concentrations of the elicitors. However, at the doses used in this work, flg22 induced the expression of marker genes to levels comparable to OGs, indicating that the gene-activation response does not directly correlate to H₂O₂ accumulation. The observation that catalase, DPI treatments, or the *atrbohD* mutation block the oxidative burst, but have no significant impact on the expression of the early marker genes, confirms that the induction of these genes is uncoupled to ROS production.

The fact that none of the analyzed marker genes changed expression in response to H₂O₂ generated by G/GO was unexpected. Previous work showed that *PAD3* expression and camalexin accumulation can be up-regulated by ROS-generating chemicals (Zhao et al., 1998; Denby et al., 2005) and the expression of *CYP81F2*, *RetOx*, and *AtWRKY40* has been shown to be induced

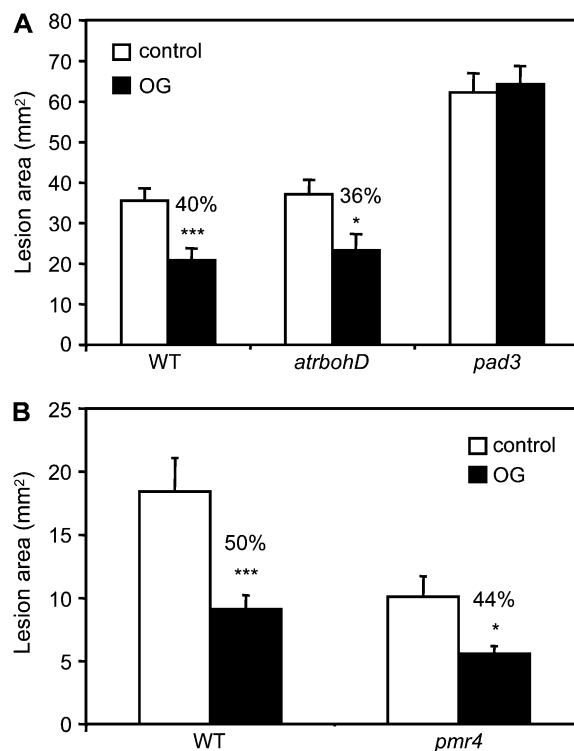


Figure 8. OG-induced resistance to *B. cinerea* is independent of *AtrbohD* and *PMR4*. A, Arabidopsis Col-0 (wild type), *atrbohD*, and *pad3* plants were treated with a control solution (white bars) or OGs (black bars) and inoculated with *B. cinerea* 24 h after treatment. B, Arabidopsis Col-0 (wild type) and *pmr4* plants were treated with a control solution (white bars) or OGs (black bars) and inoculated with *B. cinerea* 24 h after treatment. Lesion areas were measured 48 h after inoculation. Values are means \pm SE of at least 14 lesions. Asterisks indicate statistically significant differences between control and OG-treated plants, according to Student's *t* test (*, *P* < 0.05; ***, *P* < 0.01). Numbers above bars represent the average reduction of lesion size (%) of OG-treated plants with respect to control-treated plants. The experiments were repeated at least twice with similar results.

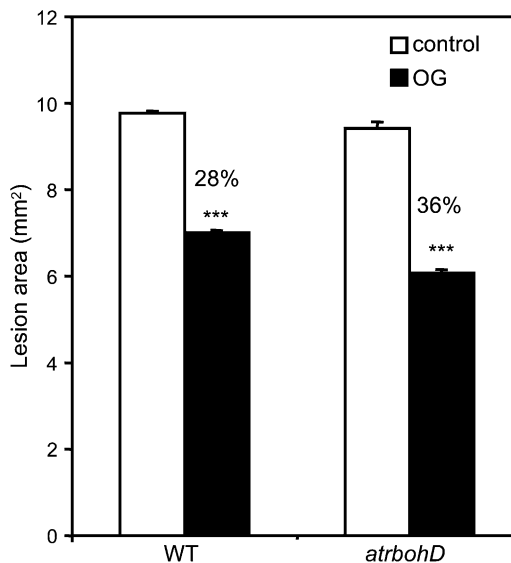


Figure 9. Basal and OG-induced resistance to *B. cinerea* in whole plants. Arabidopsis Col-0 (wild type) and *atrbohD* plants were treated with a control solution (white bars) or OGs (black bars) and leaves were inoculated with *B. cinerea* 24 h after treatment. Lesion areas were measured 48 h after inoculation. Values are means \pm SE of at least 12 lesions. Asterisks indicate statistically significant differences between control and OG-treated plants, according to Student's *t* test (***, $P < 0.01$). Numbers above bars represent the average reduction of lesion size (%) of OG-treated plants with respect to control-treated plants. The experiments were repeated at least twice with similar results.

by millimolar concentrations of H_2O_2 (Davletova et al., 2005). However, the concentration of H_2O_2 measured in our experiments with G/GO was in the same order of magnitude as the concentration measured after elicitation with OGs (in the range of 10–30 $\mu M g^{-1}$ fresh weight), which is comparable to the concentrations measured in leaves of different plant species under natural conditions (Cheeseman, 2006). This suggests that the relatively high concentrations of H_2O_2 used in previous expression analyses might be nonphysiological. Similarly, basal resistance to *B. cinerea* was not affected by treatment with G/GO at the same concentrations used in the seedling experiments. This result is apparently in contrast with a previous report indicating that G/GO infiltration of Arabidopsis leaves increases susceptibility to this pathogen (Govrin and Levine, 2000). However, the concentration of GO used by Govrin and Levine was 10^4 -fold higher than in our work, suggesting that only very high levels of H_2O_2 , which are not normally induced by elicitors, can affect basal resistance to *B. cinerea*.

Whereas OG-induced early gene expression and protection against *B. cinerea* occur independently of *AtrbohD*, callose accumulation is reduced in *atrbohD* KO plants. A similar result was obtained in *atrbohD* leaf strips treated with flg22 (Zhang et al., 2007). Callose deposition is required for β -amino butyric acid-induced resistance against the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina*

(Ton and Mauch-Mani, 2004). Our observation that induced resistance to *B. cinerea* is unaffected in *atrbohD* plants, despite a reduction in callose accumulation, suggests that callose contributes only marginally to restrict *B. cinerea* in Arabidopsis. This hypothesis is confirmed by the observation that both basal and OG-induced resistance against *B. cinerea* are not impaired in the *pmr4* mutant, which accumulates very little callose.

Besides callose accumulation, other responses induced by OGs and other elicitors may be dependent on the oxidative burst. Previous reports suggest the existence of both oxidative burst-dependent and independent signaling pathways linking elicitor perception to downstream responses. Treatment of parsley (*Petroselinum crispum*) cells with DPI blocked both Pep-13-induced phytoalexin production and accumulation of transcripts encoding enzymes involved in their synthesis. In contrast, DPI had no effect on Pep-13-induced *PR* gene expression (Kroj et al., 2003). In grapevine, the expression of six out of nine defense-related genes responsive to OGs is blocked by DPI (Aziz et al., 2004), and in Arabidopsis Landsberg *erecta* seedlings treated with OGs, DPI blocks the expression of several defense genes (Hu et al., 2004). It is possible

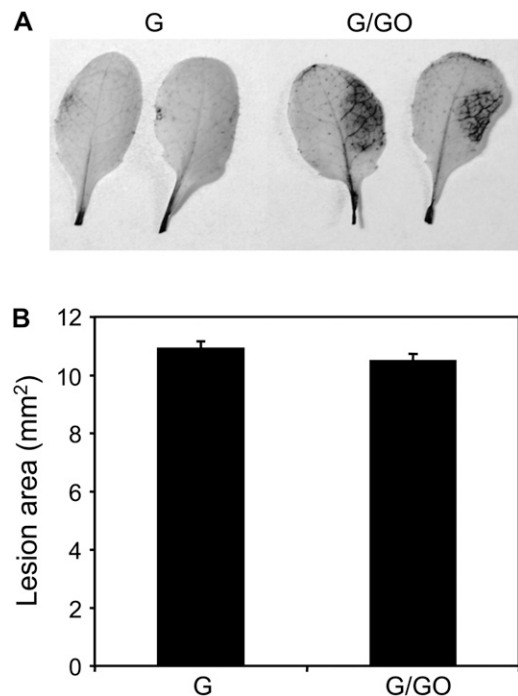


Figure 10. Basal resistance to *B. cinerea* after treatment with G/GO. A, Arabidopsis wild-type plants were infiltrated with Glc (G) alone or G/GO and, 24 h after treatment, stained with 3,3'-diaminobenzidine (DAB) for in vivo H_2O_2 visualization. B, Arabidopsis wild-type plants were infiltrated with Glc or G/GO and inoculated with *B. cinerea* 24 h after treatment. Lesion areas were measured 48 h after inoculation. Values are means \pm SE of at least 12 lesions. No statistically significant differences between Glc- and G/GO-treated plants were observed, according to Student's *t* test ($P > 0.7$).

that the activation of a subset of late, secondary responses to elicitors is dependent, or at least is amplified by the earlier production of ROS.

CONCLUSION

In this work, we investigated the role of the extra-cellular oxidative burst in the induction of early and late responses to OGs in Arabidopsis plants. Our results indicate that OGs induce a transient, but robust, production of H₂O₂ that is dependent on the NADPH oxidase AtrbohD. This oxidative burst does not have a major role in the induction of several early OG-responsive marker genes and in the induced protection against *B. cinerea*. It was previously observed that early gene expression, in contrast to callose deposition, in response to the bacterial PAMP flg22, is independent of AtrbohD (Zhang et al., 2007). Here, we show that OGs, which are host-associated molecular patterns of a completely different chemical nature, behave in a similar fashion. However, we have demonstrated that defense responses that require the oxidative burst, such as callose deposition, are not involved in OG-induced resistance to *B. cinerea*. In contrast, flg22-induced resistance against *Pseudomonas syringae* infection is dependent on the NADPH oxidase AtrbohD (Zhang et al., 2007). Taken together, these results indicate that the signaling pathway activated by elicitors bifurcates: activation of one branch requires the oxidative burst and is important against bacterial pathogens, whereas the oxidative burst-independent branch regulates defense responses effective against necrotroph fungi.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) wild-type seeds were purchased from Lehle Seeds. *pad3-1* (Glazebrook and Ausubel, 1994) and *eds16-1/sid2-2* (Wildermuth et al., 2001) mutant lines were previously described. Seeds of *ein2-1* and *jar1-1* were obtained from the Arabidopsis Biological Resource Center. The *npr1-1* line and the triple mutant *nej* were a kind gift from Xinnian Dong (Duke University). Heterozygous *coi1-1/COI1-1* seeds were a kind gift from John Turner (University of East Anglia). The *atrbohD* KO line was kindly provided by Jonathan G.D. Jones (Sainsbury Laboratory, John Innes Centre). The *pmr4-1* mutant line was kindly provided by Shauna C. Somerville (Carnegie Institution). All mutant lines used in this work are in the Col-0 background.

Growth Conditions and Plant Treatments

Plants were grown on a 3:1 mixture of soil (Einheitserde) and sand (Compo Agricoltura) at 22°C and 70% relative humidity under a 16-h light/8-h dark cycle (approximately 120 μmol m⁻² s⁻¹). For OG treatments, leaves from 4-week-old plants were infiltrated with water or 200 μg mL⁻¹ OGs using a needleless syringe and harvested at the indicated times. Generation of H₂O₂ in adult plants was obtained by infiltrating rosette leaves of 4-week-old plants with 0.25 mM Glc and 0.01 unit mL⁻¹ Glc oxidase (Sigma). As a negative control, plants were infiltrated with 0.25 mM Glc alone.

For seedling treatments, seeds were surface sterilized and germinated in multiwell plates (approximately 10 seeds/well) containing 1 mL per well of Murashige and Skoog medium (Sigma; Murashige and Skoog, 1962) supple-

mented with 0.5% Suc. Plates were incubated at 22°C with a 12-h light/12-h dark cycle and a light intensity of 120 μmol m⁻² s⁻¹. After 8 d, the medium was replaced and treatments were performed after two additional days. For treatment of *coi1* seedlings, heterozygous *coi1/COI1* seeds were first germinated on agar plates containing 30 μM methyl jasmonate, and, after 8 d of growth, homozygous JA-resistant seedlings were transferred to liquid Murashige and Skoog medium and treated with OGs 2 d later. As a control, wild-type seedlings were grown for 8 d on agar plates and then transferred to liquid Murashige and Skoog medium.

OG pools with an average DP of 10 to 15 (OGs) and purified decagalacturonic acid (DP 10) were kindly prepared by Gianni Salvi (Università di Roma "La Sapienza") as previously described (Bellincampi et al., 2000). Trigalacturonic acid (DP3) was purchased from Sigma. Matrix-assisted laser desorption/ionization time-of-flight MS was used to verify the DP of OG preparations. *Phytophthora megasperma* f. sp. *Glya* β-glucan elicitor was a kind gift of Michael G. Hahn (Complex Carbohydrate Research Center, University of Georgia). The flg22 peptide was synthesized by Maria Eugenia Schininà (Università di Roma "La Sapienza"). Lyophilized elicitors or chemicals were dissolved in double-distilled water and added to the culture medium at the following final concentrations: 100 μg mL⁻¹ (approximately 54 μM) OG; 52 μg mL⁻¹ (approximately 54 μM) DP10; 29 μg mL⁻¹ (approximately 54 μM) DP3, 1 μM flg22 and 50 μg mL⁻¹ β-glucan. H₂O₂ was removed from the culture medium by adding bovine catalase (Sigma) at the same time as OG at a final concentration of 600 units mL⁻¹. Generation of H₂O₂ was obtained by adding 0.25 mM Glc and 0.01 unit mL⁻¹ Glc oxidase (Sigma) to the culture medium. DPI was prepared as a 1 mM stock in 20% dimethyl sulfoxide. DPI was added to the seedling growth medium at a final concentration of 10 μM, 15 min before OG treatment. As a control, dimethyl sulfoxide was added to the medium at a final concentration of 0.2%.

Botrytis cinerea growth and protection assays on detached leaves were performed as previously described (Ferrari et al., 2007). Infection of intact plants was performed by inoculating about three leaves per plant (at least four plants per genotype) with two 5-μL droplets of a *B. cinerea* spore suspension. Plants were subsequently covered with a plastic dome to keep humidity high as previously described (Ferrari et al., 2003a).

Determination of H₂O₂

The H₂O₂ concentration in the incubation medium of treated seedlings (about 100–120 mg in 1 mL of medium) was measured by the FOX1 method (Jiang et al., 1990), based on the peroxide-mediated oxidation of Fe²⁺, followed by the reaction of Fe³⁺ with xylenol orange dye (*o*-cresolsulfophthalein 3',3''-bis[methylimino] diacetic acid, sodium salt; Sigma). This method is extremely sensitive and used to measure low levels of water-soluble H₂O₂ present in the aqueous phase. To determine H₂O₂ concentration, 500 μL of the incubation medium were added to 500 μL of assay reagent (500 μM ammonium ferrous sulfate, 50 mM H₂SO₄, 200 μM xylenol orange, and 200 mM sorbitol). Absorbance of the Fe³⁺-xylenol orange complex (A₅₆₀) was detected after 45 min of incubation. The specificity for H₂O₂ was tested by eliminating H₂O₂ in the reaction mixture with catalase. Standard curves of H₂O₂ were obtained for each independent experiment. Data were normalized and expressed as micromolar H₂O₂/g fresh weight of seedlings.

For in vivo H₂O₂ visualization, leaves were cut from infiltrated adult plants using a razor blade and dipped for 12 h in a solution containing 1 mg mL⁻¹ of 3,3'-diaminobenzidine-HCl, 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).

Gene Expression Analysis

Treated seedlings or leaves were frozen in liquid nitrogen, homogenized with a mortar and pestle, and total RNA was extracted with Tri-Reagent (Sigma) according to the manufacturer's protocol. RNA was treated with RQ1 DNase (Promega) and first-strand cDNA was synthesized using ImProm-II reverse transcriptase (Promega) according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed using an I-Cycler (Bio-Rad). Two microliters of a 1:5 dilution of cDNA (corresponding to 20 ng of total RNA) were amplified in a 30-μL reaction mix containing 1× IQ SYBR Green Supermix (Bio-Rad) and 0.4 μM of each primer. Expression levels of each gene, relative to *UBQ5*, were determined using a modification of the Pfaffl method (Pfaffl, 2001) as previously described (Ferrari et al., 2006). Semiquantitative reverse transcription (RT)-PCR analysis was performed in a

50- μ L reaction mix containing 1 μ L of cDNA, 1 \times buffer (Bioline), 3 mM MgCl₂, 100 μ M of each dNTP, 0.5 μ M of each specific primer, and 1 unit Taq DNA Polymerase (Bioline). Twenty-five, 30, and 35 PCR cycles were performed for each primer pair to verify linearity of the amplification. Primer sequences are shown in Supplemental Table S1. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Pearson correlation coefficients between the expression pattern of selected genes in 322 Affymetrix ATH1 microarray datasets obtained from different Arabidopsis tissues and after different treatments and available in the Genomic Arabidopsis Resource Network/Nottingham Arabidopsis Stock Centre microarray database (Craigon et al., 2004) and scatter plots of the correlation coefficient values were obtained using the Arabidopsis Coexpression Tool (<http://www.arabidopsis.leeds.ac.uk/act/index.php>; Manfield et al., 2006). The scatter plot allows users to visualize the correlation of all probe sets against two selected probe sets simultaneously. Every probe set is plotted on a scatter graph, where the two axes are the Pearson correlation coefficients against two different query probe sets. Analysis of the expression of single genes in publicly available microarray experiments was performed using Genevestigator (<https://www.genevestigator.ethz.ch>; Zimmermann et al., 2004).

Callose Deposition

Leaves from 4-week-old plants were infiltrated with water or 200 μ g mL⁻¹ OGs using a needleless syringe. After 24 h, for each treatment, about eight leaves, from at least five independent plants, were cleared and dehydrated with 100% ethanol. Leaves were fixed in an acetic acid:ethanol (1:3) solution for 2 h, sequentially incubated for 15 min in 75% ethanol, in 50% ethanol, and in 150 mM phosphate buffer, pH 8.0, and then stained for 1 h at 25°C in 150 mM phosphate buffer, pH 8.0, containing 0.01% (w/v) aniline blue. After staining, leaves were mounted in 50% glycerol and examined by UV epifluorescence using an Axioskop 2 plus microscope (Zeiss). Images were taken with a ProgRes C10 3.3 MegaPixel digital color camera (Jenoptik). Callose quantification was performed by using ImageJ software.

Supplemental Data

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

Supplemental Figure S1. Co-correlation between expression pattern of *PAD3* and *RetOx*.

Supplemental Figure S2. Expression of elicitor-responsive genes in adult Arabidopsis wild-type and *atrbohD* plants.

Supplemental Figure S3. Expression of selected marker genes in mutants impaired in SA, JA, and ET signaling.

Supplemental Table S1. Primers used in this article.

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