Dual Roles of Reactive Oxygen Species and NADPH Oxidase RBOHD in an Arabidopsis-*Alternaria* Pathosystem[1][W]

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Arabidopsis (*Arabidopsis thaliana*) NADPH oxidases have been reported to suppress the spread of pathogen- and salicylic acid-induced cell death. Here, we present dual roles of RBOHD (for respiratory burst oxidase homolog D) in an Arabidopsis-*Alternaria* pathosystem, suggesting either initiation or prevention of cell death dependent on the distance from pathogen attack. Our data demonstrate that a *rbohD* knockout mutant exhibits increased spread of cell death at the macroscopic level upon inoculation with the fungus *Alternaria brassicicola*. However, the cellular patterns of reactive oxygen species accumulation and cell death are fundamentally different in the *AtrbohD* mutant compared with the wild type. Functional RBOHD causes marked extracellular hydrogen peroxide accumulation as well as cell death in distinct, single cells of *A. brassicicola*-infected wild-type plants. This single cell response is missing in the *AtrbohD* mutant, where infection triggers spreading-type necrosis preceded by less distinct chloroplastic hydrogen peroxide accumulation in large clusters of cells. While the salicylic acid analog benzothiadiazole induces the action of RBOHD and the development of cell death in infected tissues, the ethylene inhibitor aminoethoxyvinylglycine inhibits cell death, indicating that both salicylic acid and ethylene positively regulate RBOHD and cell death. Moreover, *A. brassicicola*-infected *AtrbohD* plants hyperaccumulate ethylene and free salicylic acid compared with the wild type, suggesting negative feedback regulation of salicylic acid and ethylene by RBOHD. We propose that functional RBOHD triggers death in cells that are damaged by fungal infection but simultaneously inhibits death in neighboring cells through the suppression of free salicylic acid and ethylene levels.

An apoplastic oxidative burst with accumulation of reactive oxygen species (ROS) in the extracellular space of plant tissues is characteristic of plant cells exposed to abiotic stress (Joo et al., 2005), herbivores (Leitner et al., 2005), symbiotic microorganisms (Santos et al., 2001), or pathogens (Doke, 1983; Wojtaszek, 1997). Plasma membrane-bound NADPH oxidases and cell wall peroxidases are considered as main sources of an oxidative burst in the apoplast. Newly generated knockout mutants or sense and antisense transgenic lines provided impressive genetic evidence for the significance of these enzymes in the response of plants to pathogens. Cell wall peroxidases have been reported to function as resistance factors conferring defense to plants against various invading pathogens and also as positive regulators of cell death (Bindschedler et al., 2006; Choi et al., 2007). Interestingly, results about the role of NADPH oxidases in the interaction of plants with invading pathogens have presented a less uniform, more complex picture.

Plasma membrane NADPH oxidases in plants have been discovered on the basis of their sequence similarity to the mammalian respiratory burst NADPH oxidase subunit $gp91^{phox}$ (Groom et al., 1996; Keller et al., 1998; Torres et al., 1998). They are encoded by a 10-member gene family in Arabidopsis (*Arabidopsis thaliana*; *rbohA* to *rbohJ*, for respiratory burst oxidase homolog).

Some reports about plant NADPH oxidases indicated that RBOH proteins possess a resistance function and/or trigger cell death in inoculated plants. An *AtrbohD AtrbohF* Arabidopsis double mutant displayed reduced cell death compared with wild-type plants after infiltration with an avirulent bacterium strain without showing any effect on the in planta growth of the bacterium (Torres et al., 2002). Virus-induced gene silencing of two $gp91^{phox}$ orthologs in *Nicotiana benthamiana* (*NbrbohA* and *NbrbohB*) compromised the resistance of plants against the oomycete pathogen *Phytophthora infestans* and also reduced cell

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death response of the leaves (Yoshioka et al., 2003). A potato (*Solanum tuberosum*) NADPH oxidase (*StrbohA*) has been implicated in the wound healing and resistance to natural microbial infections of potato tubers (Kumar et al., 2007).

Other experiments yielded contrasting results indicating that plant NADPH oxidases promoted pathogen susceptibility or that they operated as cell death suppressors. The Arabidopsis *AtrbohF* mutant was more resistant to a weakly virulent strain of the oomycete *Hyaloperonospora parasitica* and exhibited enhanced cell death (Torres et al., 2002). Transient silencing of a barley (*Hordeum vulgare*) *gp91^{phox}* ortholog (*HvrbohA*) decreased the penetration efficiency of the barley powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*), revealing that the RBOHA barley protein facilitates fungal accessibility and hence promotes the development of powdery mildew (Trujillo et al., 2006).

Using *lsd1 AtrbohD* and *lsd1 AtrbohF* double mutants, Torres et al. (2005) reported that RBOHD and RBOHF activity antagonized salicylic acid (SA)-induced prodeath signals in Arabidopsis. The presence of the *rbohD* or *rbohF* mutant allele (resulting in dysfunctional RBOHD or RBOHF protein) in the *lsd1* background did not suppress the spreading cell death phenotype, which is a typical characteristic of *lsd1* plants. On the contrary, SA- or pathogen-induced cell death was even enhanced in *lsd1 AtrbohD* and *lsd1 AtrbohF* double mutants in comparison with the *lsd1* single mutant. Thus, this study concluded that RBOHD/F functioned as suppressors of cell death in neighboring cells around infection sites with elevated levels of SA.

SA acts as a local and systemic signal molecule in plant defense against pathogen attack, and cells around infection sites accumulate particularly high levels of SA (Raskin, 1992; Durner et al., 1997). Many Arabidopsis lesion-mimic mutants showed elevated SA and ROS production as well as continuous expression of defense mechanisms (Lorrain et al., 2003). SA was also necessary for the constitutive transcriptional activation of the Arabidopsis NADPH oxidase *rbohD* in the *hrl1* lesion-mimic mutant (Devadas et al., 2002). A mutation in the isochorismate synthase gene of the SA biosynthetic pathway (*eds16*; identical with *ics1* or *sid2*) suppressed uncontrolled cell death in the *lsd1 AtrbohD* double mutant. The *lsd1 AtrbohD eds16* triple mutant (which is not able to accumulate SA), unlike the *lsd1 AtrbohD* double mutant, did not exhibit spreading cell death, suggesting that SA promoted, while RBOHD and LSD1 inhibited, the spread of cell death in Arabidopsis (Torres et al., 2005). These results indicated that SA and NADPH oxidases performed a complex interplay in the regulation of cell death. It should be noted that other plant-derived, characterized regulators of cell death, such as jasmonic acid, ethylene (ET), and nitric oxide (NO), seem to be involved in disease development and pathogen resistance as well (Hofius et al., 2007).

While a growing body of knowledge is accumulating regarding the Arabidopsis-*Pseudomonas* species pathosystem, less is known about Arabidopsis responses to fungal infection. *Alternaria brassicicola* is a toxin-producing, necrotrophic fungal pathogen that causes black spot disease of cruciferous plants (Lawrence et al., 2008). There are no examples of gene-for-gene resistance against *A. brassicicola* in Arabidopsis (Glazebrook, 2005). It is interesting that Arabidopsis possesses a LysM-type receptor-like kinase (CERK1) that operates as a microbe-associated molecular pattern perception protein. It is required for chitin signaling and contributes to the basal defense of Arabidopsis against *A. brassicicola* (Miya et al., 2007; Wan et al., 2008).

In sum, using *AtrbohD* and *AtrbohF* Arabidopsis T-DNA insertion lines and *A. brassicicola* as a model fungus, we present results about the mechanisms of action of RBOH-derived ROS during fungal pathogenesis in Arabidopsis. We are particularly interested in the questions of how ROS produced by RBOH proteins affect fungal resistance or what role they play in the regulation of plant cell death elicited by a necrotrophic fungus.

RESULTS

Characterization of *AtrbohD* and *AtrbohF* T-DNA Insertion Mutants

Homozygous *AtrbohD* (SALK_070610) and *AtrbohF* (SALK_059888) knockout lines were isolated by genomic PCR-based screening and DNA sequencing (for details, see "Materials and Methods"). The T-DNA insertion in SALK_07610 was located in the seventh exon (2,193 bp downstream of the ATG translation start site), and that in SALK_059888 was located in the third intron 2 bp downstream of the junction of the third exon and the third intron (Fig. 1A). As analyzed by reverse transcription (RT)-PCR, accumulation of *rbohD* or *rbohF* transcripts was absent in the corresponding mutants (Fig. 1B). *AtrbohD* and *AtrbohF* both exhibited normal morphology, although *AtrbohF* was slightly smaller than the wild type (Fig. 1C).

A. brassicicola Infection Induces Spreading Necrosis in *AtrbohD*

A. brassicicola is incompatible on the Arabidopsis ecotype (Columbia [Col-0]) we used (Thomma et al., 1998; Narusaka et al., 2005). Our experiments, however, were conducted under conditions that were overwhelmingly favorable for an *A. brassicicola* strain, CBS 125088, used throughout this work (for details, see "Materials and Methods"). These conditions enabled some fungal growth and sporulation, particularly on older leaves of plants (see Figs. 5 and 6 and Supplemental Fig. S4 below). *Alternaria* species are known to be more virulent on weakened plant tissues (Thomma, 2003).

In the leaves of the *AtrbohD* mutant, necrotic symptoms of *A. brassicicola* infection developed faster and

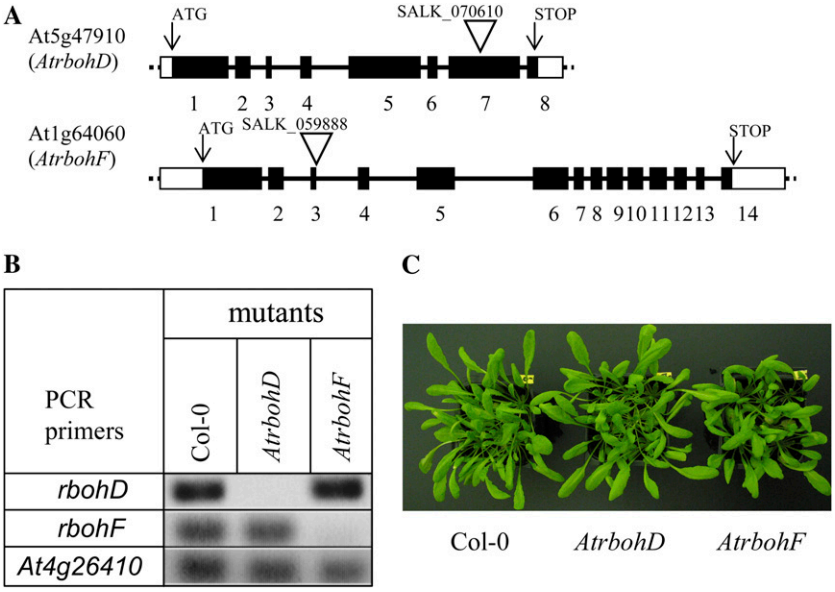

Figure 1. Characterization of *AtrbohD* and *AtrbohF* knockout mutants. Confirmation of T-DNA insertions and lack of *rbohD* and *rbohF* transcripts in the insertion lines. A, Schematic presentation of the gene structures and T-DNA insertion sites of *AtrbohD* (SALK_070610) and *AtrbohF* (SALK_059888). Exons are indicated as black boxes and introns as lines, and 5′ and 3′ untranslated regions are shown as white boxes. Triangles mark the positions of the T-DNA insertions. B, Transcripts of *rbohD* and *rbohF* do not accumulate in the corresponding mutants. Wild-type plants (Col-0) show normal *rbohD* and *rbohF* mRNA expression. An *At4g26410* cDNA fragment served as a loading control. C, Phenotypes of 5-week-old wild-type, *AtrbohD*, and *AtrbohF* plants.

the sizes of the necroses were larger than in the wild type (Fig. 2). The *AtrbohF* mutant responded similarly to the fungal infection like the wild type. Cell death was quantified by trypan blue staining, scanning of the stained leaves, and a subsequent Adobe Photoshop image analysis. These results also confirmed that cell death was enhanced in the *AtrbohD* mutant (Fig. 3).

The reliability of our pathogen results was further demonstrated by four additional experiments. First, an independent set of *AtrbohD* and *AtrbohF* mutants (described by Torres et al., 2002) was tested for its susceptibility to *A. brassicicola* strain CBS 125088 (Supplemental Fig. S1). Second, a drop-inoculation method with our Salk Arabidopsis lines was introduced as a comparison with spray inoculation (Supplemental Fig. S2). The results of these two experiments fully supported our original findings, proving that functional RBOHD is necessary for control of the spread of cell death in *A. brassicicola*-infected Arabidopsis leaves. In a third set of control experiments, two more isolates of *A. brassicicola*, MUCL 20297 and Abra 43, were included, and symptom development after inoculation with the three fungal strains was evaluated on all three Arabidopsis lines used in this work (Supplemental Fig. S3). The symptoms caused by strain MUCL 20297 were similar to those caused by strain CBS 125088, while those exhibited after inoculation with strain Abra 43 were less striking. These observations were valid for all three Arabidopsis genotypes tested. Interestingly, strains CBS 125088 and MUCL 20297 originated from cabbage (*Brassica oleracea capitata*) leaves, while strain Abra 43 was isolated from radish (*Raphanus sativus*). In a fourth confirmatory experiment, we tested whether colonization of *A. brassicicola* strain CBS 125088 on Arabidopsis was age dependent or not. It was previously observed that *A. brassicicola*-induced lesion diameters positively correlated with the age of leaves (Supplemental Figs. S2 and S3). *A. brassicicola* biomass was assayed by real-time PCR from leaves representing different physiological states. Older leaves contained significantly higher fungal biomass levels 9 d after inoculation, indicating that senescence of leaves indeed supported colonization by *A. brassicicola* (Supplemental Fig. S4).

A. brassicicola Infection Up-Regulates Expression of *rbohD*

Transcript abundance of the genes *rbohD* and *rbohF* were analyzed in *A. brassicicola* strain CBS 125088-

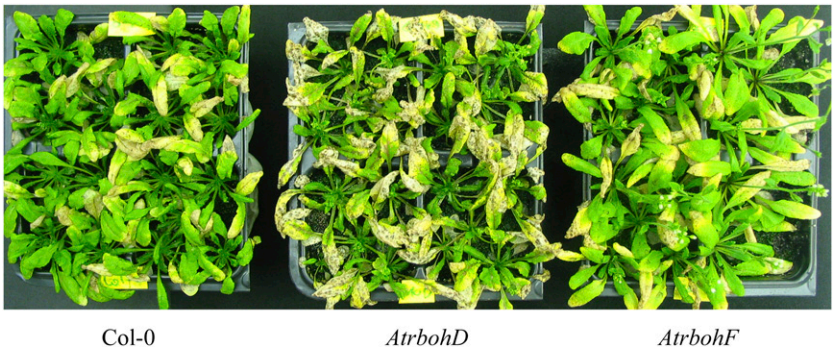

Figure 2. Necrotic symptoms of *A. brassicicola* strain CBS 125088 infection are enhanced in the Arabidopsis mutant *AtrbohD*. Plants were inoculated 12 d before the photograph was taken. The experiment was repeated several (>10) times with the same results.

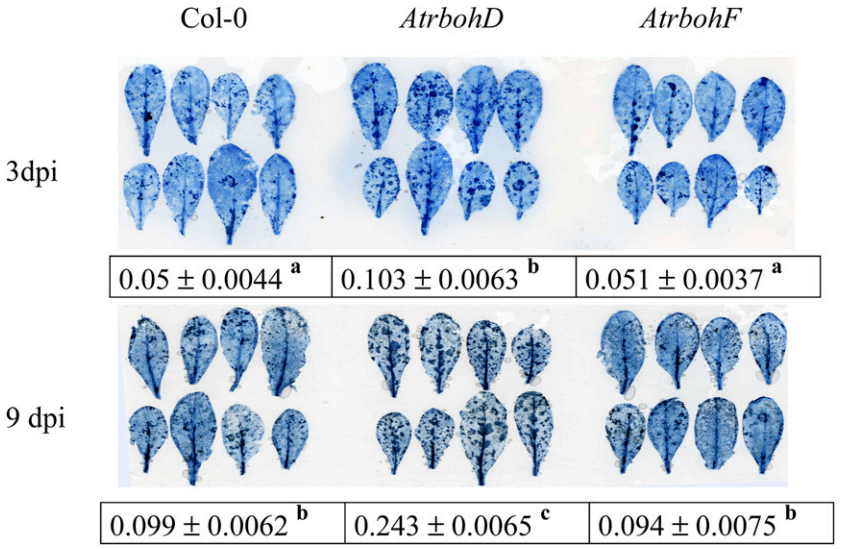

Figure 3. Trypan blue staining for the detection of cell death in wild-type (Col-0) and *rboh* mutant Arabidopsis genotypes 3 and 9 d after inoculation (dpi) with *A. brassicicola* strain CBS 125088. Spread of cell death is accelerated in *AtrbohD*. Plants were stained with trypan blue and decolorized, and randomly selected leaves were used for quantification. Results are presented as necrotized leaf area compared with the total surface of leaf blades analyzed by Adobe Photoshop (1 is equal to100% leaf surface) and represent means ± SE of 15 Arabidopsis leaves per genotype. The experiment was repeated three times. Different letters indicate statistically significant differences between genotypes and the two time points using Tukey's posthoc test for pairwise comparisons ($\alpha = 0.01$).

infected wild-type plants by real-time RT-PCR assays between 6 and 48 h after inoculation. Expression of the *rbohD* gene was clearly induced by the fungal infection in comparison with corresponding mock-inoculated plants. This provides further evidence that RBOHD may have an important role in the interaction of the plant with the fungus. The transcript level of *rbohF*, on the contrary, was not elevated as a result of the infection (Fig. 4).

Enhanced Spread of Cell Death Is Not Accompanied by Faster Fungal Colonization in *AtrbohD*

It was a crucial question to answer whether accelerated cell death was a consequence of faster fungal growth (i.e. weakened plant resistance) or was a result of disturbed cell death regulation in *AtrbohD*. In order to answer this question, we extracted DNA from the three Arabidopsis genotypes 3 and 9 d after inoculation and determined the biomass of *A. brassicicola* strain CBS 125088 in the samples by real-time PCR. It was found that, in spite of the enhanced cell death phenotype, fungal colonization in *AtrbohD* was not more intense in comparison with the wild type or *AtrbohF*. On the contrary, *AtrbohD* actually contained less fungal biomass than the wild type or *AtrbohF* 9 d after inoculation (Fig. 5). These data were confirmed when we examined older infected leaves from the three genotypes with bright-field microscopy and observed substantially reduced *A. brassicicola* conidia formation on the surface of *AtrbohD* leaves (Fig. 6, A–C). At a late stage of the infection, differences in fungal colonization became obvious even when we examined leaves macroscopically (Fig. 6D). Therefore, we conclude from these results that RBOHD is most likely not a resistance factor in Arabidopsis to the necrotrophic fungus *A. brassicicola* but rather a regulator of programmed cell death.

AtrbohD Is Lacking the Function to Perform Oxidative Burst and Cell Death in Single, Distinct Foliar Cells

In addition to macroscopic evaluation of the *A. brassicicola*-induced disease symptoms, microscopic studies were also conducted on the *rboh* mutants searching for cellular and intercellular ROS and cell death responses that were dependent on functional RBOHs. Hydrogen peroxide (H_2O_2) was detected by fluorescent (2′,7′-dichlorodihydrofluorescein diacetate [carboxy-H_2DCFDA]) and nonfluorescent (3,3′-diaminobenzidine [DAB]) methods, cell death was detected by trypan blue staining, and H_2O_2 and cell death together were visualized by a combination of DAB and Evans blue staining methods (Fig. 7).

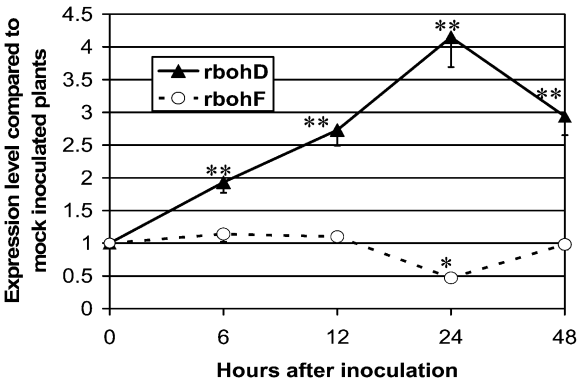

Figure 4. mRNA expression of *rbohD* and *rbohF* genes in wild-type Arabidopsis plants following inoculation with *A. brassicicola* strain CBS 125088 detected by real-time RT-PCR. Each time point was statistically analyzed individually (time points were not compared with one another). Data points represent means ± SE of three independent biological samples (a pool of five plants per sample) analyzed in triplicate by real-time RT-PCR assays using SYBR Green as a fluorescent reporter. Asterisks indicate statistically significant differences between *A. brassicicola*-infected and mock-inoculated plants using Tukey's posthoc test for pairwise comparisons (* $\alpha = 0.05$, ** $\alpha = 0.01$).

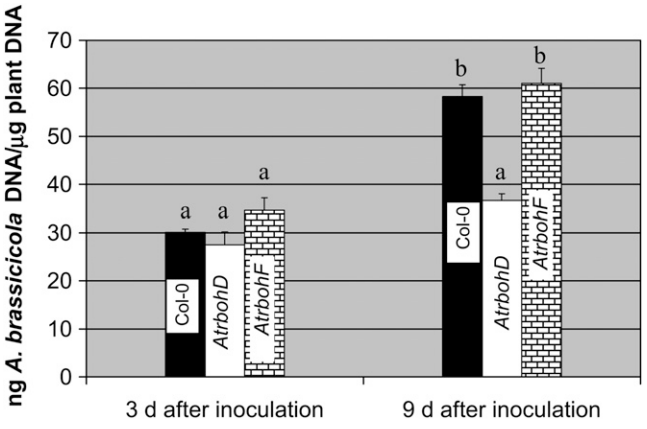

Figure 5. *A. brassicicola* strain CBS 125088 biomass content in wild-type (Col-0) and *rboh* mutant Arabidopsis genotypes measured by real-time PCR from leaf total DNA extracts 3 and 9 d after inoculation. Biomass of the fungus is shown as the ratio between fungal and plant DNA. Presented values are means ± SE of three independent biological samples (a pool of five plants per sample) analyzed in triplicate by real-time PCR assays using SYBR Green as a fluorescent reporter. Different letters indicate statistically significant differences between genotypes and the two time points using Tukey's posthoc test for the statistical analysis ($\alpha = 0.01$).

These histological experiments revealed that *A. brassicicola* strain CBS 125088 infection induced H_2O_2 bursts in single cells of wild-type and *AtrbohF* Arabidopsis leaves (in both epidermal and mesophyll cells) and that this response was abolished in *AtrbohD*. In the *AtrbohD* mutant, H_2O_2 accumulated only in chloroplasts of the inoculated leaves, but this phenomenon occurred in large groups of cells compared with the single cell H_2O_2 bursts observed in wild-type plants (Fig. 7, A, B, E, F, I, and J). The appearance of cell death in *A. brassicicola*-infected Arabidopsis leaves showed a similar pattern as the distribution of H_2O_2. In wild-type and *AtrbohF* plants, cell death appeared in distinct, single cells, while *AtrbohD* plants developed spreading-type cell death that affected a higher number of cells. Dead cells in *AtrbohD* greatly overlapped with cells exhibiting chloroplastic H_2O_2 accumulation (Fig. 7, C, G, and K). Double staining detecting both H_2O_2 and cell death confirmed that cellular H_2O_2 accumulation and cell death colocalized and that H_2O_2 accumulation preceded cell death (Fig. 7, D, H, and L). These results clearly demonstrated that the loss of RBOHD function makes Arabidopsis incapable of executing single foliar cells after inoculation with *A. brassicicola*.

Transcriptome Analysis of *A. brassicicola*-Infected *rboh* Mutants

A transcriptome analysis using a custom-designed microarray platform was carried out on the *rboh* mutants inoculated with *A. brassicicola* strain CBS 125088 (24 h after inoculation). Our cDNA microarray slides comprised approximately 1,400 different cDNA clones, including 1,164 stress- or defense-related Arabidopsis genes and also some other genes associated with primary metabolism or housekeeping (Zeidler et al., 2004; Raacke et al., 2006). Microarray results were verified by real-time RT-PCR analyses, which revealed that our microarray method consistently underestimated the levels of transcriptional changes. Transcript abundance of 55 genes showed induction and 38 genes were repressed as a result of the fungal infection (Supplemental Table S1). The three geno-

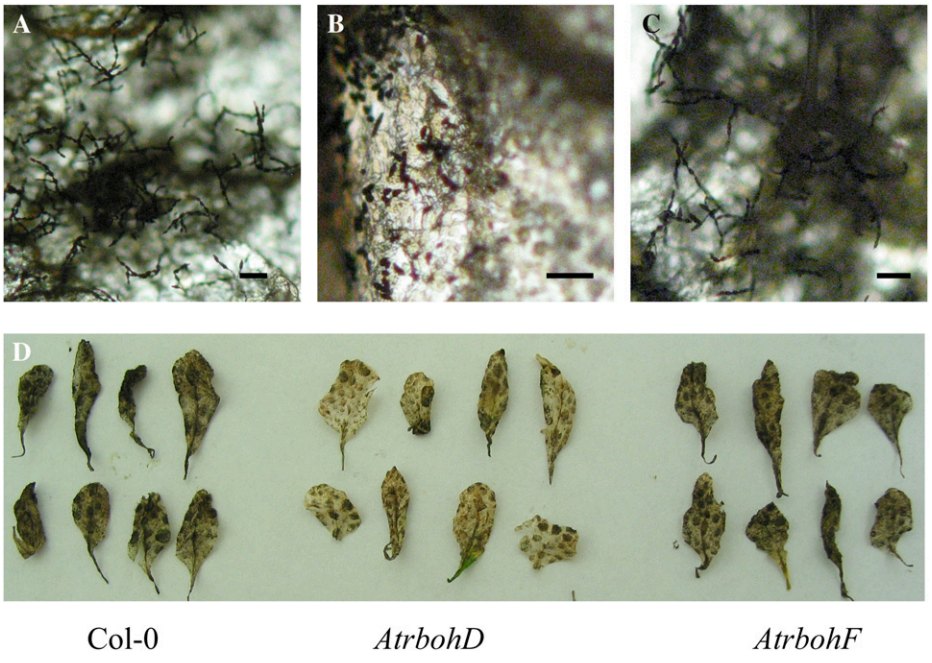

Figure 6. A to C, Sporulation of *A. brassicicola* strain CBS 125088 on older Col-0 (A), *AtrbohD* (B), and *AtrbohF* (C) Arabidopsis leaves 12 d after inoculation. Conidia in the image that shows the leaf surface of *AtrbohD* (B) were derived from the original inoculum. Newly formed conidia (appearing in chains) are missing, indicating that conidia formation is inhibited in *AtrbohD*. Bars = 100 µm. D, Decayed Arabidopsis leaves collected at a late stage of *A. brassicicola* infection (21 d after inoculation). Leaves in the center were harvested from *AtrbohD* plants and exhibit an altered necrosis phenotype and reduced fungal colonization as compared with wild-type (Col-0) and *AtrbohF* leaves on the left and right sides, respectively.

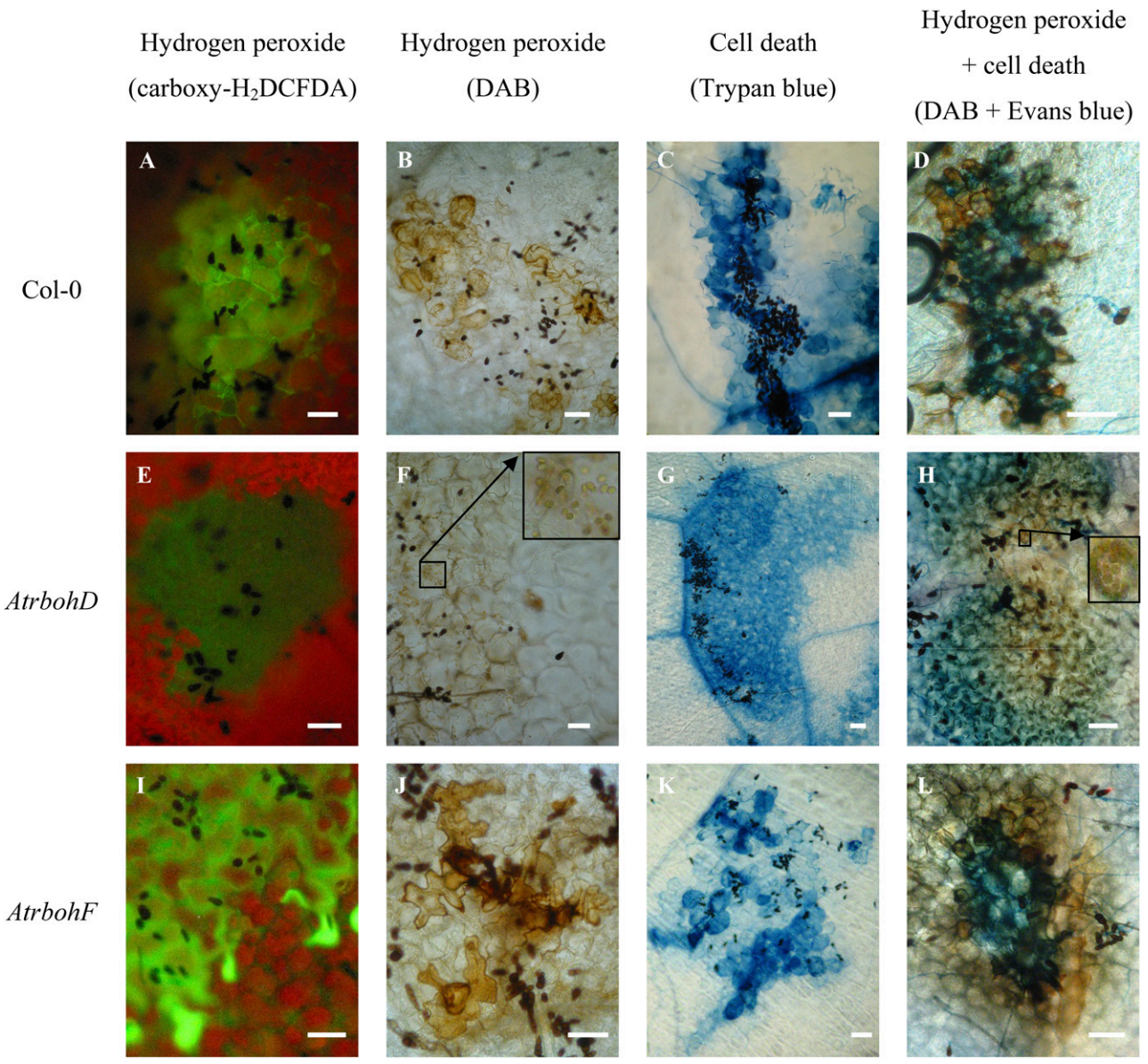

Figure 7. Microscopic detection of H_2O_2 accumulation (carboxy-H_2DCFDA and DAB) and cell death (trypan blue) and double staining for H_2O_2 accumulation and cell death together (DAB + Evans blue) in wild-type (Col-0) and *rboh* mutant Arabidopsis lines 3 d after inoculation with *A. brassicicola* strain CBS 125088. A, E, and I, Detection of H_2O_2 with the fluorescent dye carboxy-H_2DCFDA. Col-0 (A) and *AtrbohF* (I) leaf cells exhibit H_2O_2-specific green fluorescence, and cells of a leaf from *AtrbohD* (E) show only dark green autofluorescence characteristic of dead cells. B, F, and J, H_2O_2 accumulation detected with DAB. Col-0 (B) and *AtrbohF* (J) perform H_2O_2 bursts in single epidermal or mesophyll cells, while *AtrbohD* cells (F) accumulate H_2O_2 only in the chloroplasts (see enlarged inset of the image), although in a spreading-type manner. C, G, and K, Cell death visualized with trypan blue. *A. brassicicola* infection induces cell death in leaves of Col-0 (C) and *AtrbohF* (K) in a single-cell-type manner. *AtrbohD* (G), on the contrary, exhibits spreading necrosis. D, H, and L, Combination of H_2O_2-specific staining (DAB) and cell death detection (Evans blue). H_2O_2 bursts and cell death colocalize in cells of *A. brassicicola*-infected Arabidopsis leaves, and the accumulation of H_2O_2 precedes cell death. *AtrbohD* cells (H) accumulate H_2O_2 in the chloroplasts (enlarged inset). Bars = 50 μm.

types (*AtrbohD*, *AtrbohF*, and the wild type) yielded very similar transcriptional profiles. It is noteworthy that the lack of RBOHD or RBOHF activity in the mutants did not result in the elimination of transcriptional responses induced by *A. brassicicola*. In fact, transcriptional changes in the *AtrbohF* mutant plants were almost identical to those observed in the wild-type plants. A dozen genes in the *AtrbohD* mutant, however, responded more intensively to *A. brassicicola* infection than in the wild type. Some of these differentially expressed genes are implicated in pathogen responses (Table I).

Table I. *List of genes exhibiting altered transcript abundance in AtrbohD in comparison with the wild type or AtrbohF after inoculation with A. brassicicola strain CBS 125088*

Top values show results of microarray experiments, and bottom values in parentheses display the corresponding real-time RT-PCR data derived from independent biological material. Microarray results present means ± SE after $\log_2$ transformation generated from signals of eight hybridization spots. Data show transcript abundance ratios between infected and mock-inoculated samples. FDR values calculated by the Rank Products method reveal differentially expressed genes that were induced or repressed by the fungal infection. *P* values of the ANOVA test highlight altered transcriptional responses between genotypes. Results of real-time RT-PCR measurements are indicated as fold induction or repression values (after $\log_2$ transformation) representing means ± SE gained from two independent biological samples analyzed in three technical replicates. Different letters indicate statistically significant differences between genotypes using Tukey's posthoc test ($\alpha = 0.01$, except for two fold induction results [b*], which are different from a only at $\alpha = 0.05$).

Description (Arabidopsis Genome Initiative Code)	Col-0	FDR	*AtrbohD*	FDR	*AtrbohF*	FDR	*P*
ACC synthase 6 (ACS6; At4g11280)	0.511 ± 0.184 (2.160 ± 0.062) a	0.465	1.695 ± 0.217 (4.053 ± 0.219) b	0.002	0.289 ± 0.161 (2.362 ± 0.086) a	0.690	<0.0001
Glutathione *S*-transferase 2 (At4g02520)	2.654 ± 0.082	0	3.350 ± 0.146	0	2.772 ± 0.158	0	0.0029
Glutathione *S*-transferase 6 (At1g02930)	2.313 ± 0.101	0	4.162 ± 0.225	0	3.416 ± 0.177	0	<0.0001
Glutathione *S*-transferase 7 (At1g02920)	3.381 ± 0.109 (4.398 ± 0.090) a	0	4.097 ± 0.125 (5.133 ± 0.201) b	0	3.522 ± 0.086 (3.987 ± 0.129) a	0	0.0004
WRKY40 transcription factor (At1g80840)	−0.460 ± 0.177 (2.358 ± 0.221) a	0.121	1.317 ± 0.280 (3.918 ± 0.140) b	0.013	−0.170 ± 0.262 (2.773 ± 0.134) a	0.723	<0.0001
WRKY6 transcription factor (At1g62300)	0.001 ± 0.176 (3.208 ± 0.030) b	0.872	1.362 ± 0.269 (4.063 ± 0.244) c	0.006	−0.272 ± 0.263 (2.370 ± 0.068) a	0.602	0.0002
Copper/zinc superoxide dismutase (At1g08830)	0.553 ± 0.234 (1.120 ± 0.061) a	0.228	1.920 ± 0.164 (2.502 ± 0.049) b	0.002	0.460 ± 0.285 (0.982 ± 0.072) a	0.635	0.0003
Anthranilate phosphoribosyl transferase (At5g17990)	0.386 ± 0.266 (2.135 ± 0.080) b	0.617	1.618 ± 0.196 (3.020 ± 0.082) c	0.002	0.211 ± 0.205 (1.555 ± 0.089) a	0.211	0.0004
Pathogenesis-related protein 1 (PR1; At2g14610)	0.362 ± 0.293 (1.233 ± 0.143) a	0.703	2.075 ± 0.190 (2.774 ± 0.211) b*	0.003	0.465 ± 0.246 (0.627 ± 0.497) a	0.623	<0.0001
Tryptophan synthase (At5g54810)	1.712 ± 0.145 (3.397 ± 0.072) b*	0.002	3.135 ± 0.145 (4.183 ± 0.242) c	0	2.183 ± 0.261 (2.657 ± 0.093) a	0.007	0.0001
Response regulator 4 (ARR4; At1g10470)	−1.070 ± 0.282 (−2.057 ± 0.220) a	0.003	−2.552 ± 0.565 (−3.002 ± 0.147) b	0	−1.336 ± 0.260 (−1.913 ± 0.071) a	0.036	0.033
Expansin 5 (At3g29030)	−0.181 ± 0.128 (−2.790 ± 0.037) a	0.724	−1.226 ± 0.210 (−3.690 ± 0.167) b	0.058	−0.052 ± 0.378 (−2.882 ± 0.100) a	0.771	0.0051

RBOHD Interplays with SA and ET in the Execution of *A. brassicicola*-Elicited Cell Death

Torres et al. (2005) concluded that RBOHD antagonizes the development of SA-induced cell death. In addition to that, one of the genes that showed differential expression in the *AtrbohD* mutant (versus Col-0 or *AtrbohF*) after fungal infection was *ACC synthase* (*At4g11280*; Table I). Thus, we suspected that ET, another stress-signaling molecule in plants, might also interplay with RBOHD in the response of Arabidopsis to *A. brassicicola* strain CBS 125088 infection. A pharmacological approach, therefore, was conducted targeting the potential roles of SA and ET in the regulation of RBOHD and cell death during *A. brassicicola*-induced pathogenesis in Arabidopsis. Treatment of Arabidopsis with benzothiadiazole (BTH; a SA analog) stimulated both the RBOHD-dependent (apoplastic) and the RBOHD-independent (chloroplastic) H_2O_2 bursts. This treatment also enhanced cell death in the wild type as well as in the *AtrbohD* mutant. Foliar application of aminoethoxyvinylglycine (AVG; an inhibitor of ET biosynthesis) had the opposite effect on H_2O_2 accumulation and cell death: it reduced the activity of both the RBOHD-dependent and -independent H_2O_2-generating mechanisms and suppressed cell death in Col-0 and also in *AtrbohD* (Fig. 8). BTH and AVG working solutions (in 0.35 and 0.1 mM, respectively) sprayed onto mock-inoculated wild-type or *AtrbohD* Arabidopsis leaves did not cause any visible symptoms on the leaves; neither did they possess any direct antimicrobial effect against *A. brassicicola* when mixed directly with the fungal medium (data not shown). These observations indicate that SA and ET might be two factors that positively regulate the cell death machinery (including RBOHD) during *A. brassicicola* infection of Arabidopsis.

This hypothesis was strongly supported by the results of ET and SA measurements in Col-0, *AtrbohD*, and *AtrbohF*. Infection by *A. brassicicola* induced a 6- to 8-fold increase in the ET formation of Col-0 and *AtrbohF* compared with the corresponding mock-inoculated plants. Interestingly, *AtrbohD* performed an even more remarkable increase in its ET formation, producing about twice as much ET as Col-0 or *AtrbohF* following inoculation with the fungus (Fig. 9). Free (unconjugated) SA levels in *A. brassicicola*-infected Col-0, *AtrbohD*, and *AtrbohF* plants showed a similar pattern

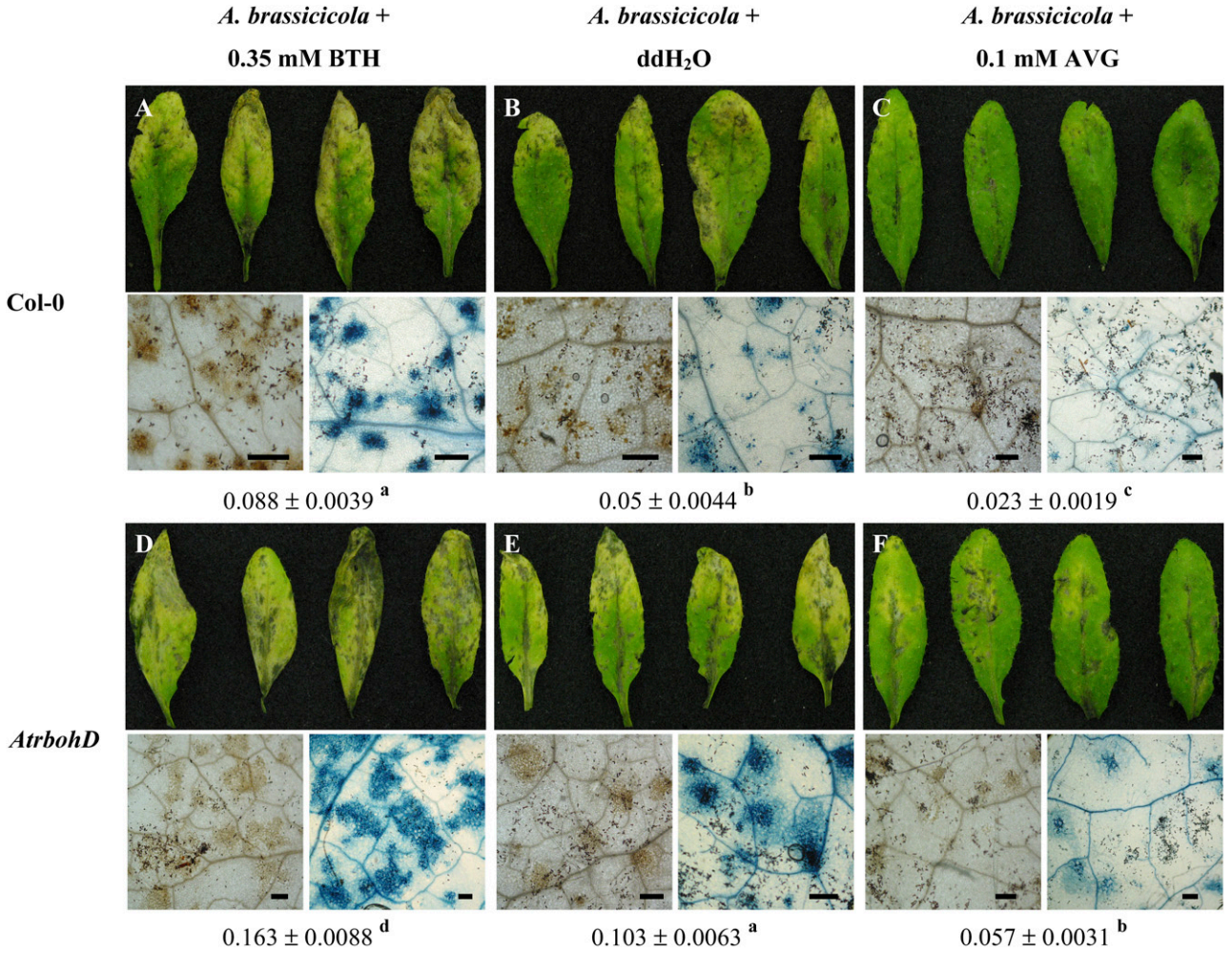

Figure 8. A, C, D, and F, The SA analog BTH (0.35 mM) accelerates (A and D) and the ET biosynthesis inhibitor AVG (0.1 mM) inhibits (C and F) *A. brassicicola* strain CBS 125088-induced cell death in both the wild type (Col-0) and *AtrbohD*. B and E, Wild-type and *AtrbohD* leaves treated with water (instead of BTH or AVG) after the fungal inoculation. All leaf images were taken 9 d following inoculation. Microscopic images (under the leaf panels) exhibit the effects of corresponding chemical treatments on the accumulation of H_2O_2 (detected by DAB staining) and the development of cell death (visualized by trypan blue staining) 3 d after inoculation. Bars = 200 μm. Numbers under the microscopic images show results of cell death quantification as described for Figure 3. Values represent means ± SE of 15 Arabidopsis leaves per genotype and per treatment. Different letters indicate statistically significant differences between genotypes and treatments using Tukey's posthoc test for pairwise comparisons (α = 0.01).

like ET. Infection by the fungus elevated the free SA content in both the wild type and *AtrbohF*. The increase of the level of free SA in *AtrbohD* upon *A. brassicicola* infection, however, significantly exceeded even those levels observed in the other two genotypes (Fig. 10).

DISCUSSION

The Necrotrophic Fungus *A. brassicicola* Triggers Accelerated Cell Death in *AtrbohD*

Phytopathogenic *Alternaria* species are well known for their ability to produce phytotoxins as pathogenicity or virulence factors (Thomma, 2003). Accordingly, germinating *A. brassicicola* conidia release a proteinaceous, host-specific AB toxin on leaves of host plants (Otani et al., 1998), and they also synthesize an array of potentially phytotoxic fusicoccane-like diterpenoids (MacKinnon et al., 1999). A recent systematic investigation of phytotoxic compounds produced by *A. brassicicola* revealed the presence of the host-specific toxin brassicicolin A and three mildly phytotoxic metabolites with fusicoccane structure in the culture broth of the fungus (Pedras et al., 2009). Cutinases and lipases secreted by *A. brassicicola* might contribute to the establishment of infection as well (Trail and Köller, 1993; Berto et al., 1999). Nevertheless, the release of toxic proteins and metabolites seems to be the primary

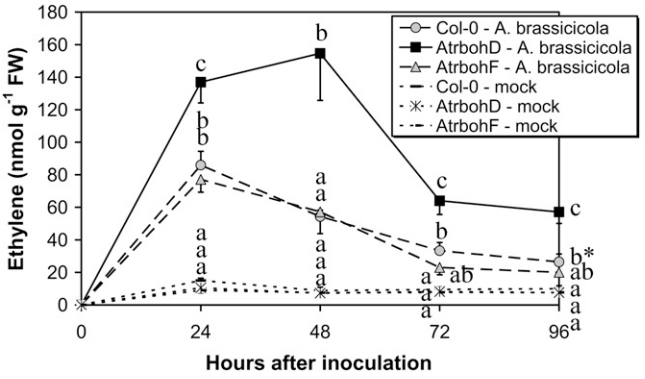

Figure 9. ET formation of *A. brassicicola* strain CBS 125088-infected Col-0, *AtrbohD*, and *AtrbohF* Arabidopsis lines measured by gas chromatography. The fungal infection stimulates ET production in all three genotypes, but *AtrbohD* responds more intensively than Col-0 or *AtrbohF*. Data points show means ± SE of two independent experiments (n = 4 in each experiment), and different letters indicate statistically significant differences between genotypes and treatments using Tukey's posthoc test for pairwise comparisons (α = 0.01, except for one data point [b*], which is different from a only at α = 0.05). Each time point was statistically analyzed individually (time points were not compared with one another). FW, Fresh weight.

reason for the fungus-induced cell death/tissue damage in the host plant (Lawrence et al., 2008).

Here, we describe that an *AtrbohD* Arabidopsis mutant displayed enhanced cell death at the macroscopic level compared with wild-type plants following inoculation with *A. brassicicola* strain CBS 125088, whereas an *AtrbohF* mutant responded to the fungus infection like the wild type (Figs. 2 and 3). These results suggested that RBOHD is an important factor in the Arabidopsis-*A. brassicicola* interaction, whereas RBOHF is probably less important or even insignificant. The increased cell death response of *AtrbohD* to *A. brassicicola* infection is in accordance with the results of Torres et al. (2005), who characterized the function of Arabidopsis RBOHD as a suppressor of the spread of cell death based on pathological and pharmacological experiments. RBOHF was reported to be less able to contribute to the ROS accumulation and suppression of cell death in comparison with RBOHD. The latter was also found to be more significant than RBOHF as a source of *Erwinia chrysanthemi*-elicited ROS production and as a suppressor of *E. chrysanthemi*-induced soft rot in Arabidopsis (Fagard et al., 2007). Interestingly, *AtrbohD* knockout plants inoculated with another necrotrophic fungus, *Botrytis cinerea*, did not exhibit altered cell death response in comparison with wild-type plants (Torres et al., 2005; Galletti et al., 2008), which observation may indicate remarkable differences between fungal species that are normally considered similar as a result of their lifestyles.

In our hands, *A. brassicicola* infection activated transcription of the *rbohD* gene in the wild-type plants as early as 6 h after inoculation, with maximum expression 24 h post inoculation (Fig. 4). Expression of the *rbohF* gene was not induced by the infection, providing additional evidence that RBOHD plays a more significant role in the response of Arabidopsis to a necrotrophic fungus attack than RBOHF. We find it interesting that RBOHD is not only regulated at the level of transcription but also is under tight posttranslational control through synergistic activation by phosphorylation or Ca^{2+} binding (Benschop et al., 2007; Nühse et al., 2007; Ogasawara et al., 2008). Conformational changes regulated by two Ca^{2+}-binding EF-hand motifs and induction of the $S^{343/347}$ phosphorylation sites are both necessary for a full RBOHD-dependent oxidative burst.

To find out whether RBOHD-derived ROS operate as resistance factors capable of controlling a necrotrophic fungus, it seemed to be important to determine how accelerated cell death in *AtrbohD* correlated with the colonization of plants by *A. brassicicola*. Enhanced spread of cell death in *AtrbohD* was not accompanied by faster fungal growth; in fact, the opposite was true: *A. brassicicola* biomass was significantly lower in *AtrbohD* than in the wild type or *AtrbohF* (Figs. 5 and 6). Consequently, ROS produced by RBOHD probably do not possess a specific antimicrobial function, nor do they operate as resistance factors in our plant-fungus interaction; rather, they act as regulators of plant cell death. This result is another example where resistance and the process of cell death can be clearly separated from each other in plants attacked by various pathogens (Király et al., 1972; Bendahmane et al., 1999; Cole et al. 2001). Similarly, altered cell death responses in *AtrbohD* and *AtrbohF* Arabidopsis mutants inoculated with virulent and avirulent bacterial strains or with the necrotrophic fungus *B. cinerea* were not accompanied by enhanced pathogen growth either (Torres et al., 2002, 2005).

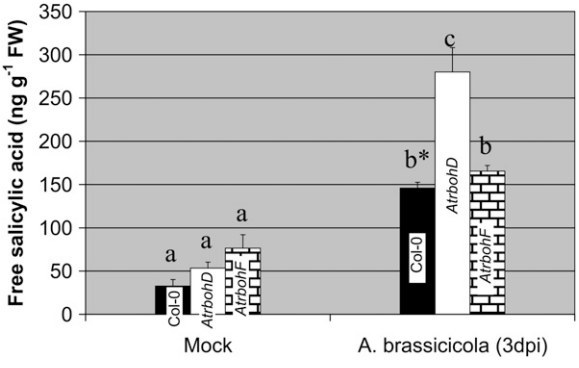

Figure 10. Free SA levels in *A. brassicicola* strain CBS 125088-infected Arabidopsis lines analyzed by HPLC. The fungal infection elevates foliar free SA contents in all three genotypes, but *AtrbohD* accumulates more free SA than Col-0 or *AtrbohF* 3 d after inoculation (dpi). Presented values are means ± SE of two independent experiments (n = 3 in each experiment). Each sample comprised the pool of five Arabidopsis rosettes. Different letters indicate statistically significant differences between genotypes and treatments using Tukey's posthoc test (α = 0.01, except for one data point [b*], which is different from a only at α = 0.05). FW, Fresh weight.

We did not try to elucidate the reason for reduced pathogen growth on *AtrbohD* in comparison with the wild type. Our most important goal was to determine whether RBOHD functions as a resistance factor against *A. brassicicola* or not. The reduced fungal biomass content in *AtrbohD*, however, was surprising because one would expect more intense colonization by a necrotrophic fungus on a mutant that exhibits increased cell death (Govrin and Levine, 2000). One interesting point we noticed in our experiments was that this type of accelerated cell death deriving from the lack of RBOHD activity was always accompanied by relatively fast dehydration of inoculated leaves, even if plants were incubated in a humid environment. It might be possible that the fungus needs a sufficient amount of moisture in the attacked tissue for early steps of colonization and it was not available in *AtrbohD* leaves.

AtrbohD Does Not Respond at the Level of a Single Cell

NADPH oxidases produce superoxide anion (O_2^-), which dismutates to H_2O_2 enzymatically (catalyzed by superoxide dismutases) or spontaneously. However, in agreement with Torres et al. (2002, 2005), we were not able to detect extracellular O_2^- production in Arabidopsis leaves after inoculation using nitroblue tetrazolium stain (data not shown). We observed O_2^- accumulation only in the chloroplasts. On the other hand, we did notice marked qualitative differences between wild-type and *AtrbohD* plants in the cellular morphology of H_2O_2 accumulation and cell death. Leaves from wild-type plants performed characteristic extracellular H_2O_2 bursts by single epidermal or mesophyll cells beginning 2 d after inoculation with the fungus (Fig. 7, A and B). This phenomenon was strictly associated with a subsequent cell death response, which shared the same morphological (single-cell) pattern as the H_2O_2 bursts (Fig. 7C). Single-cell H_2O_2 accumulation followed by cell death was reported in various plant-fungus interactions (Thordal-Christensen et al., 1997; Asselbergh et al., 2007; Vorwerk et al., 2007). Cells of inoculated *AtrbohD* leaves, on the contrary, did not exhibit extracellular H_2O_2 accumulation but showed H_2O_2-specific DAB polymer precipitation in the chloroplasts. This chloroplastic H_2O_2 accumulation in *AtrbohD* leaves did not follow a single-cell pattern but always occurred in clusters of large numbers of cells (Fig. 7F). The pattern of cell death in *AtrbohD* leaves was of a spreading type and correlated well with the pattern of cells exhibiting chloroplastic H_2O_2 accumulation (Fig. 7G). Microscopic H_2O_2 and cell death responses in the *AtrbohF* mutant were indistinguishable from those in the wild type, confirming previous conclusions about this genotype (Fig. 7, I–K). Double staining specific for H_2O_2 and cell death in one leaf tissue specimen revealed that the accumulation of H_2O_2 (either extracellular in the wild type or chloroplastic in *AtrbohD*) greatly overlapped with cell death and that the formation of H_2O_2 preceded the appearance of cell death (Fig. 7, D, H, and L). It should be noted that we were also able to detect NO in response to *A. brassicicola* infection (data not shown); however, we did not try to specify a role for NO in ROS-induced cell death, as suggested by Delledonne et al. (2001). A detailed analysis would require a defined NO underproducer, which currently is not available (Zemojtel et al., 2006). It is obvious that the lack of functional RBOHD makes Arabidopsis plants incapable of performing single-cell H_2O_2 bursts and also to knock single cells out from the integral texture of a leaf blade. On the basis of these microscopic results, we infer that RBOHD-dependent ROS accumulation is directly or indirectly responsible for the death of single cells in *A. brassicicola*-infected Arabidopsis tissues. Soybean (*Glycine max*) cells challenged with an avirulent strain of *Pseudomonas syringae* pv *Glya* (*avrA*) responded with a diphenylene iodonium (DPI)-sensitive H_2O_2 burst, which in turn triggered death of the elicited cells (Levine et al., 1994). Similarly, a DPI-sensitive, oxidase-dependent burst was necessary for the induction of cell death in a catalase-deficient transgenic tobacco (*Nicotiana tabacum*) line following irradiation with high light. In addition, transcript accumulation of a tobacco $gp91^{phox}$ homolog (*NtrbohD*) was observed in the same catalase antisense plants after exposure to high light (Dat et al., 2003). DPI efficiently inhibits the activity of Arabidopsis RBOHD in a heterologous expression system (Ogasawara et al., 2008).

It is noteworthy that even in a plant-pathogen interaction that we consider to be necrotrophic (Arabidopsis-*A. brassicicola*), plant cell death development is under the control of the host. RBOHD activity is an excellent illustration for that. Van Breusegem and Dat (2006) pointed out that in contrast to humans and animals, where necrosis and programmed cell death are well defined, in plants there seems to be much more overlap between the phenotypic and molecular hallmarks of necrosis and programmed cell death. Arabidopsis and tobacco plants infected with the necrotrophic fungus *B. cinerea* also showed ROS accumulation and hallmarks of programmed cell death (Govrin and Levine, 2000).

RBOHD and RBOHF Do Not Seem to Operate as Inducers of Defense Genes in *A. brassicicola*-Elicited Arabidopsis Leaves

Transcriptional profiling of the *rboh* mutants performed by a custom-designed microarray chip indicated that ROS produced by RBOHD and RBOHF did not have dramatic effects on transcription in our plant-pathogen interaction. Most of the 93 genes that exhibited differential expression as a result of *A. brassicicola* strain CBS 125088 infection showed similar transcriptional changes in the wild type, just as in *AtrbohD* or *AtrbohF*. A dozen genes in *AtrbohD*, nevertheless, responded more intensively to the fungal infection than in the wild type or *AtrbohF* (Table I).

Verification of the microarray results by real-time RT-PCR assays allowed more precise analysis of original cDNA levels in the RT reaction mixtures and revealed that all genes showing altered expression in *AtrbohD* were actually induced or repressed in the wild type as well, although to a lesser extent. We conclude from this result that active RBOHD negatively regulates the transcription of a limited number of genes and that this regulatory function is rather quantitative. In a recent work, RBOHD-derived ROS accumulation was excluded as an important regulator of oligogalacturonide-induced transcriptional responses in Arabidopsis (Galletti et al., 2008).

It should be noted that a comprehensive survey of *Alternaria*-induced transcriptional changes was beyond the scope of our paper. We focused on different responses between the wild type and the insertion lines. However, comparison of our microarray data with an extensive expression profiling published by van Wees et al. (2003) confirmed the validity of our results, showing that 62% of our *Alternaria*-induced Arabidopsis genes were also found to be up-regulated in that previous work.

RBOHD Interplays with SA and ET in the Regulation of Cell Death

Resistance of Arabidopsis to *A. brassicicola* depends on a jasmonic acid-inducible signaling pathway (Thomma et al., 1998), but *R* gene-mediated resistance, SA signaling, and ET signaling do not appear to play important roles (Glazebrook, 2005). Our results revealed that SA and ET do participate in the regulation of *A. brassicicola*-elicited cell death in Arabidopsis. First, we presented evidence that the SA analog BTH stimulated both the RBOHD-dependent and the RBOHD-independent H_2O_2 bursts in the wild type and *AtrbohD*, respectively. BTH also enhanced cell death both in the wild type and in *AtrbohD* (Fig. 8). SA applied to soybean cell suspensions in physiological concentrations stimulated H_2O_2 accumulation and cell death if an avirulent strain (*avrA*) of *P. syringae* pv *Glya* bacterium was present in the medium (Shirasu et al., 1997). Exogenous application of SA also induced H_2O_2 formation and oxidative damage in detached Arabidopsis leaves (Rao et al., 1997).

In addition to SA, ET is another plant hormone identified as a key player in plant defense to pathogens and regulation of cell death (Wang et al., 2002). We observed that the ET biosynthesis inhibitor AVG suppressed H_2O_2 production and cell death in the wild type and *AtrbohD* (Fig. 8). This finding suggested that not only SA but also ET positively regulated the cell death machinery (including RBOHD) during *A. brassicicola* strain CBS 125088 infection. Similar to our results, AVG treatment drastically suppressed the ozone-induced accumulation of H_2O_2 and lesion development in tomato (*Solanum lycopersicum*) leaves (Castagna et al., 2007). ET synthesis and perception were required for ROS accumulation and cell death in ozone-exposed tomato plants (Moeder et al., 2002), and ET acted in concert with SA for ozone-induced lesion formation in Arabidopsis (Rao et al., 2002). In tomato cell suspension, ET production was necessary for camptothecin-induced programmed cell death and for the preceding release of ROS (de Jong et al., 2002). ET was also required for the constitutive induction of the *rbohD* gene in the *vad1* Arabidopsis lesion-mimic mutant (Bouchez et al., 2007), and treatment of tomato plants with the ET precursor 1-aminocyclopropane-1-carboxylic acid resulted in significantly higher levels of RBOH proteins assayed by protein gel blot analysis (Sagi et al., 2004). Determination of endogenous ET and SA levels in our *A. brassicicola*-infected Arabidopsis genotypes disclosed that *AtrbohD* responded to the fungal infection with ET hyperproduction and hyperaccumulation of foliar free SA as compared with wild-type or *AtrbohF* plants (Figs. 9 and 10).

The idea that SA, ET, and ROS intimately interplay in the control of plant cell death is widely accepted (Overmyer et al., 2003; Dat et al., 2007). Veinal necrosis and preceding accumulation of H_2O_2 in turnip mosaic virus-infected Landsberg *erecta* Arabidopsis plants were dependent on the increased formation of SA and ET (Kim et al., 2008). SA and ET are also necessary for constitutive transcriptional activation of the *rbohD* gene in two different Arabidopsis lesion-mimic mutants (Devadas et al., 2002; Bouchez et al., 2007). Torres et al. (2005) reported dramatically enhanced cell death in the *lsd1 AtrbohD* double mutant as a result of SA or BTH treatment in comparison with SA- or BTH-treated *lsd1* or *AtrbohD* single mutant. A mutation in the isochorismate synthase SA biosynthetic pathway (*eds16*), on the other hand, suppressed the enhanced cell death phenotype in the *lsd1 AtrbohD eds16* triple mutant, suggesting that LSD1 and RBOHD synergistically inhibit but SA induces the spread of cell death in Arabidopsis. Our SA and ET results seem to be analogous to those reported by Diara et al. (2005) that H_2O_2 accumulation and ozone sensitivity of two ozone-exposed poplar (*Populus* spp.) clones correlated with the kinetics and magnitude of their SA and ET productions. Both the tolerant and sensitive clones responded to ozone fumigation with elevated SA and ET levels, but optimal concentrations of these two signaling molecules were required to achieve the maximum stimulation of defense with minimal induction of cell death.

When we integrate our data on ROS, cell death, SA, and ET with the results of others as described above, the following model becomes apparent: *A. brassicicola* infection leads to elevated levels of free SA and ET in Arabidopsis tissues, and this physiological transition induces RBOHD activity and contributes to cell death. This is the case, as we propose, in single cells that are primarily affected (i.e. damaged) as a result of the fungal infection (Fig. 11, C and D). In adjacent cells, on the other hand, mild RBOHD activity triggers a feedback regulation, partially suppressing the accumulation of free SA and ET, which in turn inhibits cell death

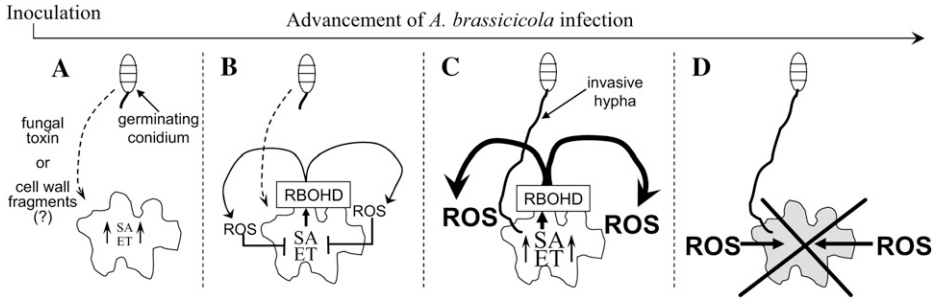

Figure 11. Schematic model showing the interplay of RBOHD-derived ROS, SA, and ET in the regulation of *A. brassicicola*-induced cell death in Arabidopsis. A, An Arabidopsis cell spatially distant from a germinating *A. brassicicola* conidium is elicited either by low concentration of fungal toxin or by oligosaccharide chitin fragments (Miya et al., 2007; Wan et al., 2008), leading to elevated endogenous SA and ET levels. B, Increasing cellular SA and ET concentrations induce moderate RBOHD activity (Devadas et al., 2002; Bouchez et al., 2007). The resulting mild generation of ROS inhibits the accumulation of SA and ET and suppresses cell death. C and D, When a developing fungal hypha reaches the Arabidopsis cell and causes considerable cellular damage, RBOHD is no longer able to control the increase of SA and ET. This transition induces drastic RBOHD activity, accompanied by a marked burst of extracellular H_2O_2, which triggers death of the cell. Arabidopsis leaves attacked by the necrotrophic fungus *A. brassicicola* exhibit all four different cellular responses (A–D) simultaneously.

and serves cell survival (Fig. 11, A and B). If RBOHD is not functional, free SA and ET hyperaccumulate and cells perform unlimited spread of cell death. This model gives us an answer to the question of why cell death is accelerated in the *AtrbohD* mutant when functional RBOHD triggers death in single cells. There are several signs indicating that RBOHD activity is not limited only to those Arabidopsis cells that exhibit marked apoplastic H_2O_2 burst or cell death. (1) Extracellular, DAB-detectable H_2O_2 accumulation in single cells develops 2 d after the inoculation with *A. brassicicola* or later. Expression of *rbohD* mRNA, on the contrary, is induced as early as 6 h after the inoculation and reaches its maximum 24 h after inoculation. (2) Different transcriptional responses between the wild type and *AtrbohD* are detected 24 h after the inoculation with the fungus. (3) The *AtrbohD* mutant produces significantly more ET than the wild type even 24 h after the inoculation. (4) Lesion-free leaves of the *hrl1* lesion-mimic mutant exhibit higher transcriptional *rbohD* induction than leaves actually displaying lesions (Devadas et al., 2002). (5) Most interestingly, as has been pointed out in two recent papers, pathogen- or host-associated molecular pattern elicitors such as flagellin (flg22) or oligogalacturonides do induce a rapid, transient, RBOHD-dependent oxidative burst in Arabidopsis leaves detected by chemiluminescence or xylenol orange methods (Zhang et al., 2007; Galletti et al., 2008). When we reproduced flg22 treatment as described by Zhang et al. (2007), DAB staining in our hands was not sensitive enough to detect this ROS response, which may indicate that early ROS accumulation occurs in a lower concentration range (M. Pogány and J. Durner, unpublished data).

Our findings and model concerning the roles of RBOHD-derived ROS in the regulation of *A. brassicicola*-induced plant cell death represent a refinement of a concept suggested previously (Levine et al., 1994; Rao and Davis, 1999; Vandenabeele et al., 2003). ROS produced by plant cells under stress possess dual, concentration-dependent functions. They either act as signals leading to cell survival or they trigger cell death. One characteristic of Arabidopsis RBOHD is that it does not seem to act directly as an inducer of defense genes but rather modulates plant hormone (SA and ET) levels in order to control plant cell death.

We do not see a contradiction between our results on the involvement of SA and ET in *A. brassicicola*-elicited cell death in Arabidopsis and the generally accepted view that SA and ET are not significant players with regard to the resistance to *A. brassicicola* (Thomma et al., 1998; Glazebrook, 2005). Enhanced necrosis is not a sign of dampened plant resistance in *AtrbohD* but rather a consequence of altered cell death regulation. Similarly, BTH and AVG were applied to Arabidopsis following inoculation with the fungus, and leaves were analyzed soon after that (2 d after spraying with BTH or AVG), indicating that the goal of these experiments was not an induction or suppression of pathogen resistance but an approach to manipulate cell death. As already mentioned, RBOHD seems to participate only in the regulation of cell death and not in resistance against *A. brassicicola* in leaves of Arabidopsis.

In our work, we provide further insight into the regulatory mechanism of RBOHD in cell death, including informative microscopic images of RBOHD activity in cells of intact Arabidopsis leaves elicited by *A. brassicicola* strain CBS 125088. As a conclusion of our data presented here, we propose that ROS produced by RBOHD operate as precision factors of the programmed cell death machinery in *A. brassicicola*-infected Arabidopsis tissues, presumably in a concentration-dependent manner. RBOHD-derived ROS trigger death in cells damaged by fungal infection

but simultaneously down-regulate ET and free SA levels and inhibit the spread of cell death in neighboring cells.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) T-DNA insertion lines SALK_070610 (*AtrbohD*; seventh exon insertion) and SALK_059888 (*AtrbohF*; third intron insertion) were ordered from the Salk collection. DNA was extracted from leaf samples, and homozygous mutant segregants were isolated by PCR screening using a left-border T-DNA primer (5′-GGTTCACGTAGTGGGCCATC-3′) together with gene-specific primers as follows: SALK_070610 (*AtrbohD*), forward (5′-TGTTTACCCCGGGAACGTGTTGTCTCTA-3′) and reverse (5′-TTGCATCACCTTCCTCGTACACACTCGT-3′); SALK_059888 (*AtrbohF*), forward (5′-GCTCCGATTTCGCTCAATGCAT-3′) and reverse (5′-CCAAATCCCCGCCTAACTTTGATT-3′). Lack of *rbohD* or *rbohF* transcript accumulation in the two identified homozygous mutants was confirmed by RT-PCR as described below.

AtrbohD-3 (European Arabidopsis Stock Centre code N9555) and *AtrbohF-3* (European Arabidopsis Stock Centre code N9557) transposon mutant Arabidopsis lines (Torres et al., 2002) were ordered from the European Arabidopsis Stock Centre and used as references for our Salk insertion lines.

Wild-type (Col-0) and mutant Arabidopsis plants were grown at 22°C in growth chambers programmed for a 14-h-light/10-h-dark cycle with 85 μmol m^{-2} s^{-1} irradiation. Plants were used 4 to 6 weeks after sowing, and they developed approximately 12 true leaves under our growing conditions.

Cultivation of the Fungus and Inoculation

A strain of *Alternaria brassicicola* was isolated from leaves of cabbage (*Brassica oleracea capitata*) in Hungary. The species identification of this strain relied on conidia and colony morphology of cultures cultivated on three different media (potato dextrose agar, dichloran rose Bengal yeast extract Suc agar, and water agar; Ellis, 1971; Vörös, 1985; Simmons, 1995). Species-specific random amplified polymorphic DNA and microsatellite analyses (Avenot et al., 2005a; Dongó et al., 2005) have also supported the identity of the isolate. The internal transcribed spacer (ITS) region of the ribosomal DNA of this strain was determined as described by Szentiványi et al. (2005) and deposited in GenBank under the accession number GQ496082. This strain, used in all of the experiments described in our work, was deposited in the public culture collection of the Centraalbureau voor Schimmelcultures, The Netherlands, under the accession number CBS 125088. In addition, two more strains of *A. brassicicola* were used to confirm the reliability of our inoculations done on Arabidopsis. One of them, MUCL 20297, isolated from cabbage, was included in previous works on Arabidopsis (Thomma et al., 1998), while another one, Abra 43, isolated from radish (*Raphanus sativus*), was obtained from Angers University, France (Avenot et al., 2005b).

Conidial suspensions (5×10^5 conidia in 1 mL of distilled water) for the inoculation were prepared from 10- to 14-d-old *A. brassicicola* cultures grown on potato dextrose agar medium at 22°C in the dark. Fungal susceptibility of transposon mutant *AtrbohD-3* and *AtrbohF-3* Arabidopsis lines (Supplemental Fig. S1) was tested with spore suspensions containing 10^6 *A. brassicicola* conidia in 1 mL of distilled water. The inoculum was sprayed evenly onto the adaxial surface of Arabidopsis leaves. Inoculated plants were incubated in nearly 100% relative humidity throughout the whole experiment (they were covered with plexiglass boxes) under similar temperature and light conditions as they were kept in before inoculation. Conidial suspension drops on the leaf surface were rigorously protected from drying until an experiment was finished by sprinkling distilled water into the atmosphere of containers used for plant incubation. Mock-inoculated plants were sprayed with distilled water and incubated under the same conditions as inoculated ones.

An alternative inoculation method was also introduced with the purpose of confirming the reliability of our spray inoculation. In these experiments, 10-μL drops of spore suspension (5×10^5 conidia in 1 mL of distilled water) were transferred with a pipette onto the adaxial surface of leaves. Lesion diameters corresponding to consecutive leaf levels were evaluated 9 d after inoculation.

RNA Extraction, First-Strand cDNA Synthesis, and RT-PCR

Arabidopsis leaves frozen in liquid nitrogen and stored at −80°C were homogenized, and 200 mg of material was used for total RNA extraction. The RNA isolation protocol supplied to the TRIzol Reagent (Invitrogen) was followed according to the manufacturer's instructions. Samples were treated with DNase, and the RNA content was analyzed by UV photometry and separation on ethidium bromide-stained formaldehyde agarose gels (Mukhopadhyay and Roth, 1998). RNA extracts were used for *rbohD* and *rbohF* transcript accumulation RT-PCR assays, for real-time RT-PCR analysis of *rbohD* and *rbohF* gene expression in wild-type plants inoculated with *A. brassicicola*, for microarray experiments, and for real-time RT-PCR verification of microarray results.

First-strand cDNA synthesis was performed in a 20.5-μL reaction volume containing 5 μg of total RNA, 250 ng of Random Hexamer (GE Healthcare), 122 μM of each deoxyribonucleotide triphosphate (Fermentas), 1× first-strand buffer (Invitrogen), 10 mM dithiothreitol, 20 units of RNase inhibitor (Invitrogen), and 200 units of SuperScript II RNase H^- reverse transcriptase (Invitrogen) by incubating for 75 min at 42°C. The reaction was stopped by a subsequent incubation step at 70°C for 15 min.

Lack of *rbohD* or *rbohF* transcripts in the two Arabidopsis insertion lines was confirmed by RT-PCR carried out from the cDNA samples described above.

Two microliters of RT reaction mixture was used for PCR in a 25-μL reaction volume containing 2.5 units of Taq DNA polymerase (MP Biomedicals), 50 μM of each deoxyribonucleotide triphosphate (Fermentas), 1× incubation buffer (MP Biomedicals), 0.4 μM of each forward and reverse gene-specific primer (see primer sequences below in "Real-Time RT-PCR Assays"), and 2 mM $MgCl_2$. Amplification conditions were as follows: 2 min of denaturation at 94°C; 30 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s; followed by 7 min at 72°C. Expression of an Arabidopsis *At4g26410* gene served as a loading control (Czechowski et al., 2005; see oligonucleotide sequences below in "Real-Time RT-PCR Assays"). 15-μL aliquot of each PCR mixture was separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Microarray Analysis

Transcriptional profiling of our three Arabidopsis genotypes inoculated with *A. brassicicola* strain CBS 125088 was carried out by a custom-designed cDNA microarray platform as described earlier by Raacke et al. (2006). The fold change result of each specific gene (for each genotype) was generated from signals of eight hybridization spots: two independent sets of *A. brassicicola*-infected and mock-inoculated biological samples were collected (24 h after inoculation), two technical replicates per sample were performed using dye-swap design, and each cDNA clone was spotted twice onto one slide. Every sample contained the whole rosette of 10 5-week-old Arabidopsis plants. RT of RNA extracts in the presence of Cy3-dUTP or Cy5-dUTP (GE Healthcare), hybridization, and scanning were performed according to von Rad et al. (2005) and Raacke et al. (2006). The photomultiplier tube voltage value of the scanner (Axon GenePix 4000A; Molecular Devices) was adjusted to yield a Cy3/Cy5 signal intensity as close to 1.0 as possible. Differentially expressed genes were identified by GenePix Pro 6.0, Acuity 4.0 (Molecular Devices, MDS Analytical Technologies), and Rank Products (GlaMA; Glasgow Microarray Analysis) software packages. Global linear normalization of microarray data was performed using normalization factors based on overall median intensity. Outlier features were not used to calculate normalization factors, but all features were normalized. Features were considered to be outliers if (1) more than 3% of the pixels were saturated; (2) background uniformity [Rgn r^2(635/532)] was lower than 0.5; (3) the signal was weak (sum of medians > 500); and (4) more than 45% of the pixels in both signals (635 and 532 nm) did not exceed background intensity plus SD. Replicate data were combined, $\log_2$ transformed, and filtered on the basis of their coefficient of variance values. We addressed the problem of multiple testing associated with simultaneous analysis of over 1,000 features by calculating false discovery rate (FDR) values using the Rank Products method (Breitling et al., 2004; Brown et al., 2005). Transcriptional differences between the wild type and the NADPH oxidase mutants were identified by taking fold change results of a specific gene (gained from the eight hybridization spots) and making a second statistical comparison between genotypes by one-way ANOVA with $P \leq 0.05$. Tables displaying microarray data include the average ($\log_2$-transformed) fold

induction or repression ratios, the FDR values, which reveal significant differences (in all three genotypes) caused by the fungal infection, and the *P* values of the ANOVA test that highlight altered transcriptional responses between genotypes.

Real-Time RT-PCR Assays

Real-time RT-PCR analyses were performed from Arabidopsis cDNA samples (reverse transcribed as described above) for assaying *rbohD* and *rbohF* transcript accumulation in wild-type plants upon inoculation with *A. brassicicola* strain CBS 125088 and also for verifying microarray results. All of the reactions were carried out in an Applied Biosystems 7500 Real Time PCR System, using SYBR Green Low Rox Mix (ABgene) as a fluorescent reporter. Ten microliters of 20 times-diluted RT reaction mixture was used as a template for amplification in a 25-μL total reaction volume containing 12.5 μL of SYBR Green mix and 0.2 μM of each specific primer. Cycling parameters were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The specificity of the PCR was confirmed by melting-curve analysis. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and *At4g26410* as a reference gene (Czechowski et al., 2005). Each cDNA sample was run in triplicates of three independent biological samples for measuring *rbohD* and *rbohF* expression and of two independent biological samples for microarray verification. Each biological sample comprised the pool of five Arabidopsis plants.

We used the following gene-specific primer pairs: *rbohD* forward (5′-CTGGACACGTAAGCTCAGGA-3′) and reverse (5′-GCCGAGACCTACGAGGAGTA-3′); *rbohF* forward (5′-TCACAAATCAACGACGAGAGTT-3′) and reverse (5′-CCCATCTTCATTCTTGTCCA-3′); ACS6 forward (5′-CCGAGTTCATAAGTGTTGCG-3′) and reverse (5′-CCAGGCAGACCCATATCTTT-3′); GSTF7 forward (5′-CTAGCCAAGGTCCTCGATGT-3′) and reverse (5′-AAGTGATGTCAGCAACCCAA-3′); WRKY40 forward (5′-CAAGTGCTTTGGTGGAAGAA-3′) and reverse (5′-ATCCCTCTCGGTTATGTTGC-3′); WRKY6 forward (5′-CTGTTCTTCCTTGCTCCACA-3′) and reverse (5′-GAGGTGAGTGGGTGAGGTCT-3′); CSD1_G forward (5′-ACAGCAGTGAGGGTGTTACG-3′) and reverse (5′-GTGTCACCAAGAGCATGGAC-3′); PAT1 forward (5′-CAAGACGCTTGATTCTTGGA-3′) and reverse (5′-AGCTTCTTCCTTTGGCACAT-3′); PR1 forward (5′-GTGCAATGGAGTTTGTGGTC-3′) and rev (5′-TCACATAATTCCCACGAGGA-3′); Trp synthase forward (5′-CAGAGAGGCTTACGGAGCAT-3′) and reverse (5′-AACCGCTTAGCAAGAAGAGC-3′); ARR4 forward (5′-ATCGGAGGAATTGAATCTGC-3′) and reverse (5′-ATCTACCGCCGTAACTTTGC-3′); EXP5 forward (5′-AATCTCGCTTCTCGTGGTTC-3′) and reverse (5′-GTTGATCCAAGGTCCCATGT-3′); and *At4g26410* forward (5′-GAGCTGAAGTGGCTTCCATGAC-3′) and reverse (5′-GGTCCGACATACCCATGATCC-3′).

Determination of Fungal Biomass

Genomic DNA was isolated from spray-infected Arabidopsis leaves 3 and 9 d after inoculation with *A. brassicicola* strain CBS 125088 as described by Bahnweg et al. (1998), except that an initial extraction step (with methanol and benzyl chloride) was omitted from the original protocol. Samples were treated with RNase A (Invitrogen), and the DNA content was measured by UV photometry. Fungal biomass content in the DNA extracts was determined by quantitative PCR analysis using the same platform and conditions as for real-time RT-PCR assays above. Ten microliters of DNA extract (containing 0.5 μg of genomic DNA) was used as template for the PCR. Primer sequences specific for our *A. brassicicola* isolate were derived from the ribosomal ITS region of the fungus: forward (5′-TCTCCAGTTTGCTGGAGACT-3′) and reverse (5′-GGATGCTGACCTTGGCTGGA-3′). Plant biomass in the samples was monitored by independent real-time PCR amplifications where the reaction mixtures contained the primer pair designed for the Arabidopsis gene *At4g26410*.

Calibration curves were obtained using genomic DNA extracted from *A. brassicicola* and Arabidopsis. Results are presented as a proportion between fungal and plant DNA in the various genotypes. Each data point represents the mean of three independent biological samples analyzed in triplicate by real-time PCR.

Comparison of *A. brassicicola* biomass in leaves representing different physiological states was performed by following a modified method. Twelve true leaves of fully developed plants were divided into three age groups: leaves between positions 1 and 4 (first group), 5 and 8 (second group), and 9 and 12 (third group). Each leaf was drop inoculated with the fungus as described earlier, and leaf discs (5 mm in diameter) centered on the lesion were excised 9 d after inoculation using a cork borer. Discs collected from six plants composed a biological sample. DNA extractions and real-time PCR were performed as described above. Three independent biological samples were analyzed in triplicate. Data points represented the ratio between the quantitative PCR signals of the *A. brassicicola*-specific ribosomal DNA ITS region and the Arabidopsis-specific gene *At4g26410*.

Staining Methods and Microscopy

Cellular and extracellular H_2O_2 accumulation was visualized by H_2DCFDA and DAB staining methods. A 10 mM stock solution of H_2DCFDA (Molecular Probes, Invitrogen) was prepared by dissolving H_2DCFDA in anhydrous dimethyl sulfoxide, and after a subsequent dilution step with distilled water, a 10 μM working concentration was set. Infected leaves were detached before staining, incubated in the working solution for 5 min, and washed with distilled water. Cells exhibiting green fluorescence on the adaxial side of leaves were examined and photographed.

Whole Arabidopsis plants (without the root system) were vacuum infiltrated with DAB, decolorized, and mounted in 50% glycerol solution as described before (Pogány et al., 2004). For the detection of cell death, lactophenol-trypan blue staining was performed. A trypan blue stock solution was prepared by mixing 10 g of phenol, 10 mL of glycerol, 10 mL of lactic acid, 10 mL of distilled water, and 0.02 g of trypan blue (Sigma). This stock solution was further diluted with ethanol (96%; 1:2, v/v) to obtain trypan blue working solution. Arabidopsis leaves soaking in trypan blue working solution were boiled in a water bath for 1 min, incubated in the working solution for 1 d, and cleared with saturated chloral hydrate solution (1 kg of chloral hydrate dissolved in 400 mL of distilled water). Fifteen decolorized leaves per treatment were scanned, and cell death was quantified by the histogram tool of Adobe Photoshop measuring the pixel number of trypan blue-stained areas and the number of pixels covering the total leaf surface. The level of cell death was presented as a ratio between the two pixel number values.

Simultaneous detection of H_2O_2 accumulation and cell death in one Arabidopsis leaf specimen was accomplished by a combination of DAB and Evans blue staining methods. DAB-infiltrated leaves were incubated in light for 2 h and vacuum infiltrated for a second time with 1.3 mM Evans blue (Fluka) solution (0.125 g of Evans blue dissolved in 100 mL of distilled water). After 15 min of incubation in the dark, leaves were transferred to DAB clearing solution and handled as DAB-stained tissues.

The *A. brassicicola*-infected and stained Arabidopsis leaf samples were examined and photographed with a Zeiss Axioskop microscope and digital camera.

Treatments with BTH and AVG

The SA analog BTH (Ciba-Geigy) and the ET biosynthesis inhibitor AVG (Sigma) were sprayed onto *A. brassicicola*-infected Arabidopsis plants 1 d after inoculation in 0.35 and 0.1 mM concentrations (dissolved in distilled water), respectively. Plants sprayed with distilled water served as controls. Microscopic images presenting the effects of BTH and AVG treatments on H_2O_2 accumulation and cell death were captured 3 d after inoculation. Cell death was quantified as described above. Leaf photographs exhibiting altered *A. brassicicola* symptoms as a result of BTH or AVG treatments in wild-type and *AtrbohD* plants were taken 9 d after inoculation.

ET Measurement

Four-week-old Arabidopsis plants were carefully taken from their pots, the root systems were removed with scissors, and the plants were weighed. Rosettes were inoculated with the fungus using the same spray-inoculation protocol as for intact plants and were gently placed into 20-mL glass vessels. The vessels contained 1 mL of distilled water and were sealed with gas-tight rubber stoppers. Mock-inoculated samples were sprayed with distilled water. Plants in the vessels were incubated under the same temperature and light conditions as they were kept in before. ET formation analysis was carried out by gas chromatography as described earlier (Pogány et al., 2004). Data points represent means of two independent experiments (each experiment consisting of four samples per treatment), and ET production was monitored between 24 and 96 h after inoculation.

Determination of SA

Free (unconjugated) SA levels in *A. brassicicola*-infected and mock-inoculated Arabidopsis leaves were determined 3 d after inoculation according to Meuwly and Métraux (1993) with modifications. Plant material was ground in liquid nitrogen, and 200 mg of tissue powder was extracted with 3.5 mL of methanol/2% formic acid (1:2, v/v) in a 15-mL Falcon tube on ice. A 25-μL aliquot of *ortho*-anisic acid (500 μg mL^{-1}) was added to each sample as an internal standard. Samples were sonicated for 1 min and centrifuged for 6 min (16,000 rpm, 4°C). The supernatant was removed and kept on ice, and the pellet was subjected to a second round of extraction with 2 mL of methanol/2% formic acid following the same procedure as for the first extraction. Supernatants deriving from the two extraction steps were combined and filled up with cold methanol/2% formic acid to a final volume of 6 mL, and then 30 μL of 32% HCl was added. The free SA was isolated from leaf extracts using Oasis HLB solid-phase extraction cartridges (WAT 094225; Waters). In each isolation step, the flow of liquids through the cartridges was accelerated by a gentle stream of nitrogen gas. Cartridges were conditioned by passing through 1 mL of cold methanol and then 1 mL of 2% formic acid. Each leaf extract was passed through a conditioned cartridge in 1-mL aliquots. The cartridges were washed with 500 μL of 2% formic acid and then with 200 μL of distilled water. SA was eluted from the cartridges with 500 μL of cold elution buffer containing 20% methanol and 0.28% NH_4OH. Finally, 25 μL of concentrated formic acid was added to the purified SA extracts, and samples were transferred into HPLC vials. HPLC separation of SA and *ortho*-anisic acid was performed on an HPLC system comprising an autosampler, a model L6200A Hitachi pump, a reverse-phase column (Nucleosil 120-5-C18; Macherey-Nagel) with 200-mm length, 4.6-mm diameter, and 5-μm particle size parameters, and a Shimadzu RF 535 fluorescence detector. For fluorescence detection, an excitation wavelength of 305 nm and an emission wavelength of 405 nm were used. Presented results are means of two independent experiments comprising three samples per treatment (each sample contained the pool of five whole Arabidopsis rosettes).

Statistical Analysis

Experimental data were statistically analyzed by one-way ANOVA and subsequent Tukey's honestly significant difference tests for pairwise comparisons.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number GQ496082.

Supplemental Data

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

Supplemental Figure S1. Necrotic symptoms of *A. brassicicola* strain CBS 125088 infection on an independent set of Arabidopsis NADPH oxidase mutants.

Supplemental Figure S2. Evaluation of *A. brassicicola* strain CBS 125088 drop infection on wild type, *AtrbohD*, and *AtrbohF* Arabidopsis plants.

Supplemental Figure S3. Evaluation of necrotic symptoms caused by three different *A. brassicicola* strains, CBS 125088, Abra 43, and MUCL 20297, on Arabidopsis.

Supplemental Figure S4. Effect of leaf age on the colonization success of *A. brassicicola* strain CBS 125088.

Supplemental Table S1. List of *A. brassicicola* strain CBS 125088-induced or -repressed genes, including expression ratios in Col-0, *AtrbohD*, and *AtrbohF* Arabidopsis genotypes detected by cDNA microarray analysis.

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