

# Short-Term Exposure to Nitrogen Dioxide Provides Basal Pathogen Resistance<sup>1[OPEN]</sup>

Dörte Mayer,<sup>a</sup> Axel Mithöfer,<sup>b</sup> Erich Glawischnig,<sup>c,2</sup> Elisabeth Georgii,<sup>a</sup> Andrea Ghirardo,<sup>d</sup> Basem Kanawati,<sup>e</sup> Philippe Schmitt-Kopplin,<sup>e</sup> Jörg-Peter Schnitzler,<sup>d</sup> Jörg Durner,<sup>a</sup> and Frank Gaupels<sup>a,3,4</sup>

<sup>a</sup>Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

<sup>b</sup>Max Planck Institute for Chemical Ecology, Department Bioorganic Chemistry, D-07745 Jena, Germany

<sup>c</sup>Department of Plant Sciences, Technical University of Munich, D-85354 Freising, Germany

<sup>d</sup>Research Unit Environmental Simulation, Institute of Biochemical Plant Pathology, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

<sup>e</sup>Analytical BioGeoChemistry, Helmholtz Zentrum München, German Research Center for Environmental Health, D-85764 Neuherberg, Germany

ORCID IDs: 0000-0002-5186-7013 (D.M.); 0000-0001-5229-6913 (A.M.); 0000-0001-9280-5065 (Er.G.); 0000-0002-7511-5510 (El.G.); 0000-0003-1973-4007 (A.G.); 0000-0002-9825-867X (J.-P.S.); 0000-0002-6079-6800 (F.G.)

Nitrogen dioxide (NO<sub>2</sub>) forms in plants under stress conditions, but little is known about its physiological functions. Here, we explored the physiological functions of NO<sub>2</sub> in plant cells using short-term fumigation of *Arabidopsis thaliana* for 1 h with 10 μL L<sup>-1</sup> NO<sub>2</sub>. Although leaf symptoms were absent, the expression of genes related to pathogen resistance was induced. Fumigated plants developed basal disease resistance, or pattern-triggered immunity, against the necrotrophic fungus *Botrytis cinerea* and the hemibiotrophic bacterium *Pseudomonas syringae*. Functional salicylic acid and jasmonic acid (JA) signaling pathways were both required for the full expression of NO<sub>2</sub>-induced resistance against *B. cinerea*. An early peak of salicylic acid accumulation immediately after NO<sub>2</sub> exposure was followed by a transient accumulation of oxophytodienoic acid. The simultaneous NO<sub>2</sub>-induced expression of genes involved in jasmonate biosynthesis and jasmonate catabolism resulted in the complete suppression of JA and JA-isoleucine (JA-Ile) accumulation, which was accompanied by a rise in the levels of their catabolic intermediates 12-OH-JA, 12-OH-JA-Ile, and 12-COOH-JA-Ile. NO<sub>2</sub>-treated plants emitted the volatile monoterpene  $\alpha$ -pinene and the sesquiterpene longifolene (syn. junipene), which could function in signaling or direct defense against pathogens. NO<sub>2</sub>-triggered *B. cinerea* resistance was dependent on enhanced early callose deposition and *CYTOCHROME P450 79B2* (*CYP79B2*), *CYP79B3*, and *PHYTOALEXIN DEFICIENT3* gene functions but independent of camalexin, *CYP81F2*, and 4-OH-indol-3-ylmethylglucosinolate derivatives. In sum, exogenous NO<sub>2</sub> triggers basal pathogen resistance, pointing to a possible role for endogenous NO<sub>2</sub> in defense signaling. Additionally, this study revealed the involvement of jasmonate catabolism and volatiles in pathogen immunity.

Plants face many challenges from phytopathogenic bacteria, fungi, and oomycetes. These pathogenic organisms have evolved various feeding strategies.

<sup>1</sup>E.G. was supported by a Deutsche Forschungsgemeinschaft Heisenberg Fellowship (GL346/5) and the TUM Junior Fellow Fund. E.Ge. was supported by the German Plant Phenotyping Network funded by the German Federal Ministry of Education and Research (DPPN, no. 031A053C).

<sup>2</sup>Current address: Chair of Chemistry of Biogenic Resources, Technical University of Munich-Campus Straubing for Biotechnology and Sustainability, Schulgasse 16, D-94315 Straubing, Germany.

<sup>3</sup>Author for contact: frank.gaupels@helmholtz-muenchen.de.

<sup>4</sup>Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors ([www.plantphysiol.org](http://www.plantphysiol.org)) is: Frank Gaupels (frank.gaupels@helmholtz-muenchen.de).

D.M., F.G., J.D., P.S.-K., and J.-P.S. planned and designed the research; D.M., A.M., E.G., A.G., B.K., and F.G. performed the research; E.Ge., D.M., and B.K. analyzed the data; F.G., D.M., and J.D. wrote the article with contributions from all the authors.

[OPEN]Articles can be viewed without a subscription.

[www.plantphysiol.org/cgi/doi/10.1104/pp.18.00704](http://www.plantphysiol.org/cgi/doi/10.1104/pp.18.00704)

Biotrophic pathogens such as powdery mildew nourish on nutrients from living cells, while necrotrophic pathogens such as *Botrytis cinerea* kill the host to feed on dead cell contents (Glazebrook, 2005; Mengiste, 2012). Hemibiotrophs including *Pseudomonas syringae*, on the other hand, can pursue both feeding strategies (Glazebrook, 2005).

The plant perceives the invading pathogen by recognizing conserved pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), including the bacterial flagellin, fungal chitin, and oligogalacturans derived from damaged plant cell walls (Boller and Felix, 2009; Heil and Land, 2014). The binding of such elicitors to specific pattern-recognition receptors initiates PAMP-triggered immunity (PTI), also referred to as basal pathogen resistance (Boller and Felix, 2009; Couto and Zipfel, 2016). Immediate cellular responses upon PAMP recognition are the rapid influx of calcium ions into the cytosol and the production of reactive oxygen species such as superoxide or hydrogen peroxide (Boller and Felix, 2009; Bigeard et al., 2015). Additionally, reactive nitrogen species (RNS), such as nitric

oxide (NO), are crucial for pathogen-induced signal transduction (Gaupels et al., 2011; Mur et al., 2013).

The phytohormones salicylic acid (SA), jasmonic acid (JA), and the bioactive JA-Ile conjugate are considered to be major mediators of plant defense (Browse, 2009; Vlot et al., 2009; Pieterse et al., 2012; Wasternack and Hause, 2013). NONEXPRESSOR OF PR GENES1 (NPR1) and CORONATINE INSENSITIVE1 (COI1) are central transcriptional regulators of SA- and JA-responsive genes, respectively. The SA and JA/ethylene pathways are interconnected via complex regulatory networks and commonly antagonize each other, with SA being a potent antagonist of JA signaling (Robert-Seilaniantz et al., 2011; Caarls et al., 2015). Several NPR1-regulated TGA and WRKY transcription factors have been implicated in SA/JA cross talk (Pieterse et al., 2012; Caarls et al., 2015). The JA pathway also is controlled on the level of jasmonate catabolism. In response to wounding and pathogen attack, excess JA and JA-Ile are inactivated by hydroxylation and carboxylation, forming tuberonic acid (12-OH-JA), hydroxyl-JA-Ile (12-OH-JA-Ile), and dicarboxy-JA-Ile (12-COOH-JA-Ile; Heitz et al., 2016; Caarls et al., 2017; Smirnova et al., 2017). The jasmonate catabolism pathway is inducible by JA in the course of a negative feedback regulation (Caarls et al., 2017).

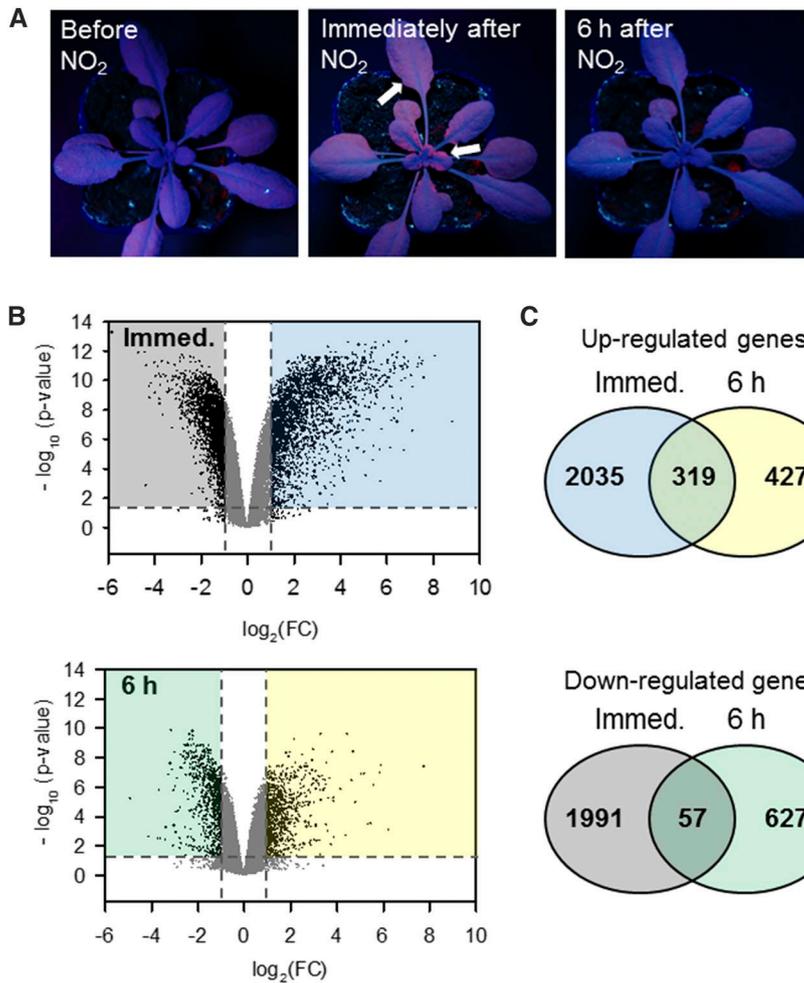
Pathogens can be prevented from spreading by PAMP-triggered formation of the (1,3)- $\beta$ -glucan polymer callose, which is deposited between the plasma membrane and cell wall at infection sites (Luna et al., 2011; Ellinger and Voigt, 2014). Callose deposition is induced after *B. cinerea* infection of Arabidopsis (*Arabidopsis thaliana*; García-Andrade et al., 2011). POWDERY MILDEW RESISTANT4 (PMR4) is the predominant callose synthase during pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003; Ellinger et al., 2013). Other well-studied components of the plant's arsenal against pathogens are indole glucosinolates and the phytoalexin camalexin (3-thiazol-2'-yl-indole) found in Arabidopsis (Glawischnig, 2007). In planta, camalexin is synthesized upon the detection of various PAMPs and DAMPs (Kliebenstein et al., 2005; Rauhut et al., 2009; Ahuja et al., 2012), and its antimicrobial activity against *P. syringae* and *B. cinerea* has been confirmed in vitro (Rogers et al., 1996; Kliebenstein et al., 2005). Indole glucosinolates such as 4-OH-indol-3-ylmethylglucosinolate (4-OH-I3M) have important functions in antifungal defense after activation by the P450 monooxygenase CYP81F2 and the atypical myrosinase PENETRATION RESISTANCE2 (PEN2; Bednarek et al., 2009; Clay et al., 2009).

The RNS nitrogen dioxide (NO<sub>2</sub>) arises during stress-induced signaling by the oxidation of NO, reduction of nitrite, or decomposition of peroxyxynitrite (Pryor et al., 2006; Groß et al., 2013). Chloroplastic nitrite reductase activity utilizes electrons diverted from photosynthesis for the multistep reduction of nitrite to ammonia (Beever and Hageman, 1969). Accordingly, treatment of soybean (*Glycine max*) with a photosynthesis-inhibiting herbicide or incubation in darkness

leads to the accumulation of nitrite and the subsequent emission of NO<sub>2</sub> that is derived from nitrite by an unknown mechanism (Klepper, 1979, 1990). In vitro experiments demonstrate that the heme-containing horseradish peroxidase can produce NO<sub>2</sub> through one-electron reduction of nitrite in the presence of hydrogen peroxide (Shibata et al., 1995; Sakihama et al., 2003). Additionally, HEMOGLOBIN1 of Arabidopsis and alfalfa (*Medicago sativa*) can produce NO<sub>2</sub> mechanistically similar to horseradish peroxidase (Sakamoto et al., 2004; Maassen and Hennig, 2011).

NO<sub>2</sub> is a highly reactive compound that can exert specific physiological functions by the nitration (-NO<sub>2</sub> group) of nucleophiles such as fatty acids (FAs), nucleotides, and proteins. The nitration of FAs (nitro-FAs) has been observed in Arabidopsis exposed to abiotic stresses (Mata-Pérez et al., 2016b), and nitro-FAs are proposed to act as signaling molecules (Schopfer et al., 2011; Mata-Pérez et al., 2016b). The nitration of cyclic guanosine monophosphate (cGMP) to give 8-nitro-cGMP triggers stomatal closure, whereas unmodified cGMP mediates stomatal opening (Joudoi et al., 2013). Moreover, increased protein Tyr nitration is a common event during plant defense responses (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Gaupels et al., 2011; Mata-Pérez et al., 2016a; Kolbert et al., 2017). This protein modification is mediated directly by NO<sub>2</sub> or via the decomposition of peroxyxynitrite to NO<sub>2</sub>, which subsequently binds to accessible protein Tyr residues (Pryor et al., 2006; Groß et al., 2013; Radi, 2013; Kolbert et al., 2017). NO<sub>2</sub>-modified proteins often are inhibited irreversibly, as described for several antioxidant enzymes and the abscisic acid receptor PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (Gaupels et al., 2011; Groß et al., 2013; Castillo et al., 2015; Mata-Pérez et al., 2016a). Together, these examples illustrate how NO<sub>2</sub> can participate in defense signaling. On the other hand, high endogenous levels of RNS also can result in excessive oxidation and nitration of biomolecules, severe metabolic perturbations, and even structural injuries of cells (Corpas and Barroso, 2013; Groß et al., 2013). Dependent on the severity of the inflicted nitro-oxidative stress, cells either trigger defense and repair mechanisms or die (Thomas et al., 2008; Groß et al., 2013). In this scenario, NO<sub>2</sub> and other RNS would act as inducers of defense signaling rather than signals themselves.

To date, the investigation of NO<sub>2</sub> in vivo is hampered by the fact that no specific dyes and donors are commercially available. For this reason, nothing is known about endogenous levels of NO<sub>2</sub> under stress conditions. Nevertheless, the functions of NO<sub>2</sub> in plants have been explored frequently by fumigations with gaseous NO<sub>2</sub> as a donor treatment. After stomatal uptake, the lipophilic NO<sub>2</sub> and its more water-soluble dimer N<sub>2</sub>O<sub>4</sub> readily penetrate cell membranes and diffuse into the cytosol (Wellburn, 1990). In the aqueous environment of the leaf, NO<sub>2</sub> disproportionates to nitrite and nitrate that are reduced further to ammonia by nitrite



**Figure 1.** NO<sub>2</sub> triggers a rapid and transient defense response. Arabidopsis Col-0 plants were fumigated with 10 μL L<sup>-1</sup> NO<sub>2</sub> or air for 1 h. A, NO<sub>2</sub> caused no visible leaf damage (Supplemental Fig. S1) but a transient increase in red chlorophyll autofluorescence under UV light (white arrows) indicative of stress-induced photoprotective energy dissipation. B, Leaf material was harvested in quadruplicates for microarray analysis immediately or 6 h after fumigation. Volcano plots visualize the changes in gene expression at 0 and 6 h after fumigation by plotting the adjusted *P* value over the fold change. Horizontal dashed lines mark *P* = 0.05; vertical dashed lines indicate log<sub>2</sub> fold change [log<sub>2</sub>(FC)] ± 1. Data points represent the expression of individual genes. The expression of genes appearing in the colored left sections was significantly down-regulated [*P* < 0.05, log<sub>2</sub>(FC) < -1], whereas the expression of genes within the colored right sections showed significant up-regulation [*P* < 0.05, log<sub>2</sub>(FC) > 1]. C, Venn diagrams illustrate the number of genes that were significantly up-regulated (top) or down-regulated (bottom) after NO<sub>2</sub> exposure with *P* < 0.05 and log<sub>2</sub>(FC) ± 1. The color code is consistent in B and C, indicating genes down-regulated immediately (gray) and 6 h (green) after fumigation or up-regulated immediately (blue) and 6 h (yellow) after fumigation.

and nitrate reductases (Beever and Hageman, 1969; Zeevaart, 1976; Sparks, 2009). Nitrite levels are correlated positively with NO<sub>2</sub>-induced leaf damage in a number of plant species (Zeevaart, 1976; Kasten et al., 2016). Plants generally accumulate high nitrite levels and show strong leaf damage after NO<sub>2</sub> fumigation in the dark (Zeevaart, 1976; Yoneyama and Sasakawa, 1979; Shimazaki et al., 1992) because, as mentioned above, nitrite reductase activity is dependent on photosynthesis. However, nitrite levels also increase strongly in pea (*Pisum sativum*) and Arabidopsis after NO<sub>2</sub> fumigation in the light, probably because they exceed the enzymatic capacity of nitrite reductase (Zeevaart, 1976; Kasten et al., 2016).

Long-term exposure to nL L<sup>-1</sup> levels of NO<sub>2</sub> has beneficial effects on plant growth and development (Srivastava et al., 1994; Takahashi et al., 2014), whereas NO<sub>2</sub> concentrations in the μL L<sup>-1</sup> range cause the induction of antioxidant defense and other stress responses (Xu et al., 2010; Liu et al., 2015; Kasten et al., 2016). In this work, Arabidopsis was exposed to 10 μL L<sup>-1</sup> NO<sub>2</sub> for 1 h, which did not cause visible leaf symptoms or ion leakage as a measure of membrane damage (Kasten et al., 2016). Responses of Arabidopsis to NO<sub>2</sub> were investigated by microarray analysis, pathogen assays,

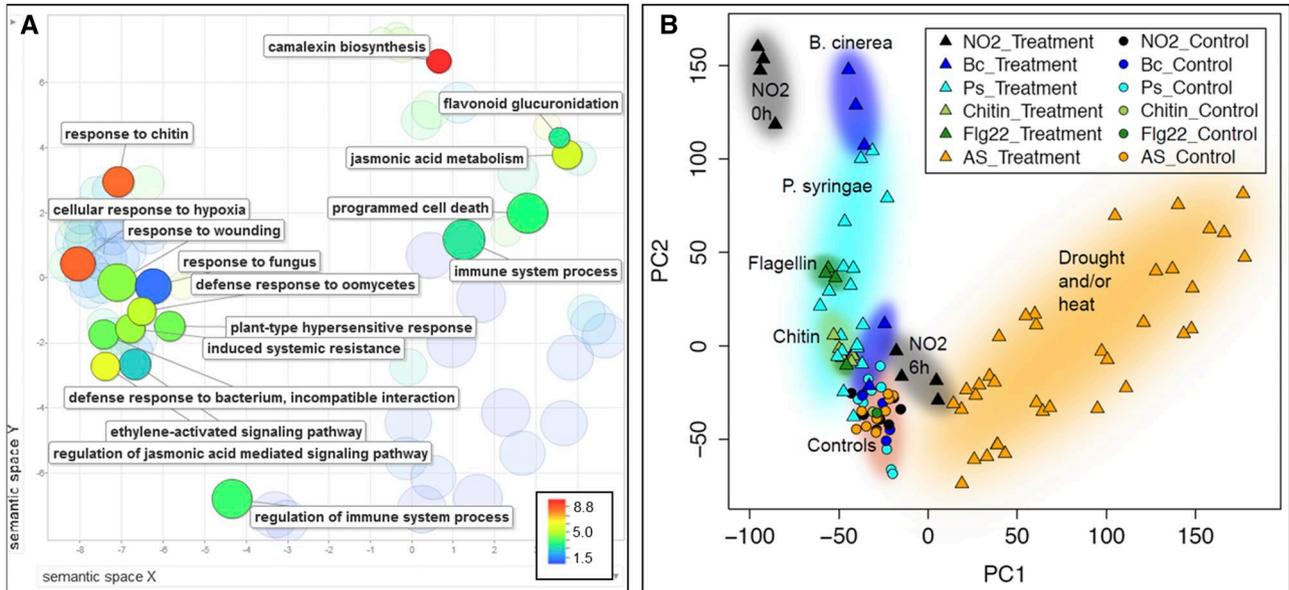
and measurements of phytohormones, volatiles, camalexin, and callose.

## RESULTS

### NO<sub>2</sub> Triggers the Expression of Genes Related to Pathogen Defense

Exposure of Arabidopsis Columbia-0 (Col-0) plants to 10 μL L<sup>-1</sup> NO<sub>2</sub> for 1 h did not cause visible symptoms (Supplemental Fig. S1), which is in agreement with previous data showing that ion leakage as a measure of membrane damage does not increase after this treatment (Kasten et al., 2016). However, close examination under UV light revealed the emission of red chlorophyll fluorescence immediately after fumigation that faded at the 6-h time point (Fig. 1A), indicative of photoprotective energy dissipation due to a transient stress-induced metabolic perturbation (Lichtenthaler and Miehe, 1997; Chaerle and Van Der Straeten, 2000).

Microarray analysis was performed with leaf material sampled immediately or 6 h after fumigation with air or 10 μL L<sup>-1</sup> NO<sub>2</sub> for 1 h (Supplemental Data Set S1).



**Figure 2.** NO<sub>2</sub>-induced genes are related to pathogen defense. A, GO term enrichment of genes up-regulated directly (0 h) after fumigation. Enriched GO terms ( $P < 0.05$ ) were identified using the PANTHER 11.0 overrepresentation test and visualized in scatterplots using the REVIGO tool. Each circle represents a GO term, and circle size represents the number of genes encompassed. The color code depicts the fold enrichment of the respective GO term within the data set compared with the PANTHER Arabidopsis reference list. Circles are clustered according to the distance of the respective GO terms within the GO hierarchical tree. Highly enriched or interesting GO terms were labeled. B, Principal component analysis of Arabidopsis gene expression responses to NO<sub>2</sub> fumigation, biotic stress, and abiotic stress. Data from microarray analysis after NO<sub>2</sub> fumigation were combined with previously published data sets representing responses to different stresses and elicitors (115 samples in total). The overall expression response similarities between samples of the combined data set are visualized using the top two principal components (PC1 and PC2), capturing 22% and 14% of the total variation, respectively. NO<sub>2</sub>, NO<sub>2</sub> fumigation; Bc, *B. cinerea* infection, ArrayExpress accession number E-GEOD-5684; Ps, *P. syringae* infection, E-GEOD-6176; Chitin, chitin treatment, E-GEOD-2538; Flg22, flagellin epitope 22 treatment, E-GEOD-17382; AS, abiotic stress treatment study, E-MTAB-4867. For each study, treated samples are marked by triangles and controls by circles.

Volcano plots illustrated that both the up- and down-regulation of gene expression was more pronounced directly after NO<sub>2</sub> fumigation than after 6 h (Fig. 1B). Approximately 4,400 genes were significantly regulated immediately after fumigation, whereas 6 h later, only 1,430 genes were differentially regulated (Fig. 1C). The regulated genes scarcely overlapped among both time points (Fig. 1B). Only 11.5% of all up-regulated genes and 2.1% of all down-regulated genes were affected at both time points, which suggested discrete time-dependent responses of the plant to NO<sub>2</sub>.

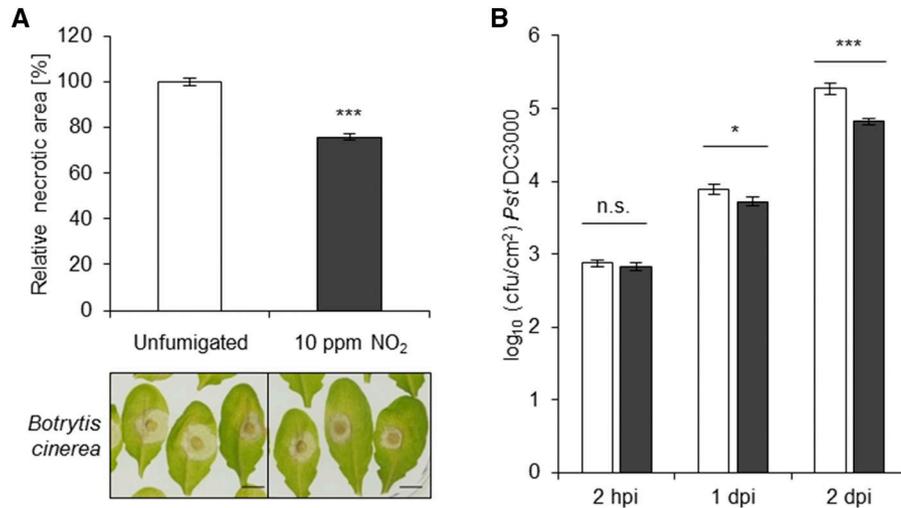
Gene Ontology (GO) term enrichment analysis (Supplemental Fig. S2) was applied to assess biological processes underlying the observed NO<sub>2</sub>-induced gene regulation. Directly after fumigation, 122 GO terms were enriched significantly in the up-regulated gene set (Supplemental Data Set S1). The majority of GO terms were related to plant defense, including responses to wounding, the fungal elicitor chitin, and fungal as well as bacterial pathogen attacks (Fig. 2A). NO<sub>2</sub> also activated genes involved in the JA and ethylene signaling pathways, camalexin biosynthesis, flavonoid glucuronidation, and programmed cell death. Principal component analysis was used for comparison of

the NO<sub>2</sub>-regulated genes with previously published microarray data sets obtained after treatment of plants with *B. cinerea* (Ferrari et al., 2007), *P. syringae* (Lewis et al., 2015), chitin (Ramonell et al., 2005), the bacterial elicitor flg22 (Zipfel et al., 2004; Boudsocq et al., 2010), and exposure to the abiotic stresses drought and/or heat (Georgii et al., 2017; Fig. 2B). Although the biotic stress studies were conducted on a different microarray platform (Affymetrix ATH1), their posttreatment expression samples are closer to the NO<sub>2</sub>-fumigated samples directly after treatment than to the abiotic stress samples sharing the same platform with the NO<sub>2</sub> study (Agilent At8x60K; identifier 29132).

In summary, the microarray analysis revealed that exposure to 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> specifically up-regulated the expression of genes associated with defense against fungal and bacterial pathogens.

### NO<sub>2</sub> Triggers Basal Pathogen Resistance

To investigate whether NO<sub>2</sub> induces resistance against necrotrophic fungi, as suggested by the gene expression data, NO<sub>2</sub>-fumigated plants were infected with *B. cinerea*. Arabidopsis Col-0 plants were fumigated



**Figure 3.** NO<sub>2</sub> induces resistance against *B. cinerea* and *P. syringae*. A, Col-0 plants were fumigated or not (control) with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h, followed by droplet infection of detached leaves with approximately 1,000 spores of *B. cinerea* 6 h after fumigation. Necrotic lesion area was measured 3 d later using ImageJ. Columns represent means of 18 independent experiments  $\pm$  SE ( $n = 624\text{--}640$ ). Asterisks indicate significant differences from the control according to the Mann-Whitney rank-sum test (\*\*\*,  $P < 0.001$ ). Representative photographs of necrotic lesions 3 d after droplet infection with *B. cinerea* are shown. Bars = 5 mm. B, Col-0 plants were fumigated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h and syringe infiltrated with  $1 \times 10^5$  cfu mL<sup>-1</sup> *P. syringae* pv *tomato* DC3000 4 h after fumigation. Leaf discs from infected leaves were obtained 2 h or 1 and 2 d after infection to determine the bacterial titer (cfu cm<sup>-2</sup> leaf material). Columns represent means  $\pm$  SE from seven independent experiments ( $n$  [2 h post infection] = 26–27,  $n$  [1 dpi] = 72, and  $n$  [2 dpi] = 66). Asterisks indicate significant differences of all pairwise comparisons via two-way ANOVA plus the Holm-Sidak posthoc test (\*,  $P < 0.05$  and \*\*\*,  $P < 0.001$ ); n.s., not significant. White columns, unfumigated; black columns, 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub>.

with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h, followed by droplet infection of detached leaves with *B. cinerea* 6 h later. The areas of the developing necrotic lesions were then analyzed to assess if NO<sub>2</sub> provides resistance against this pathogen. In Figure 3A, representative examples of the necrotic lesions formed on NO<sub>2</sub>-fumigated and nontreated plants are illustrated. Quantification of the necrotic areas revealed that the average sizes of necrotic lesions formed on NO<sub>2</sub>-fumigated leaves were reduced significantly by  $\sim 30\%$  when compared with unfumigated leaves (Fig. 3A). Therefore, these results confirmed that NO<sub>2</sub> induces resistance against the necrotrophic fungus *B. cinerea*.

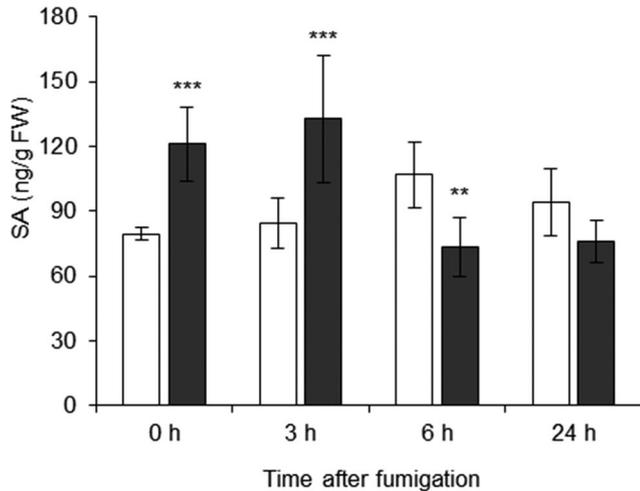
The GO term enrichment analysis and principal component analysis suggested that NO<sub>2</sub> also elicits defense responses effective against bacterial pathogens. Therefore, plants were fumigated with NO<sub>2</sub> followed by syringe infiltration with  $1 \times 10^5$  colony-forming units (cfu) mL<sup>-1</sup> *P. syringae* pv *tomato* DC3000 4 h later. The bacterial titers in the infected leaves were determined 2 h, 1 d, and 2 d post infection (dpi) to determine if NO<sub>2</sub> fumigation influenced bacterial growth. As shown in Figure 3B, infected leaves pretreated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> harbored fewer bacteria than their unfumigated counterparts. Therefore, it can be concluded that NO<sub>2</sub>-induced signaling also decreased the susceptibility of *Arabidopsis* to the hemibiotrophic bacterium *P. syringae*.

Together, the findings above imply that NO<sub>2</sub> initiated the onset of basal pathogen resistance similar to the induction of PTI by PAMPs such as chitin and flagellin.

#### NO<sub>2</sub> Triggers Signaling by SA and Oxophytodienoic Acid, While JA and JA-Ile Are Catabolized

SA biosynthesis and signaling genes were enhanced following NO<sub>2</sub> exposure (Supplemental Data Set S1; Supplemental Fig. S3). Therefore, levels of this hormone were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> (Fig. 4). SA levels were approximately 90 ng g<sup>-1</sup> fresh weight in air-fumigated leaves when averaged across time points but increased to 121 and 133 ng g<sup>-1</sup> fresh weight directly or 3 h after fumigation with NO<sub>2</sub>, respectively. At the 6-h time point, the SA content declined rapidly again to 73 ng g<sup>-1</sup> fresh weight, resulting in a significant decrease of 31% when compared with the concentration in the respective air-fumigated control. This is in line with the observation that transcript levels of the biosynthetic genes declined at this time point as well (Supplemental Data Set S1). In summary, exposure to 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> provoked a rapid, but transient, accumulation of SA.

Jasmonates derive from the FA linolenic acid, which undergoes oxidation via lipoxygenases, dehydration



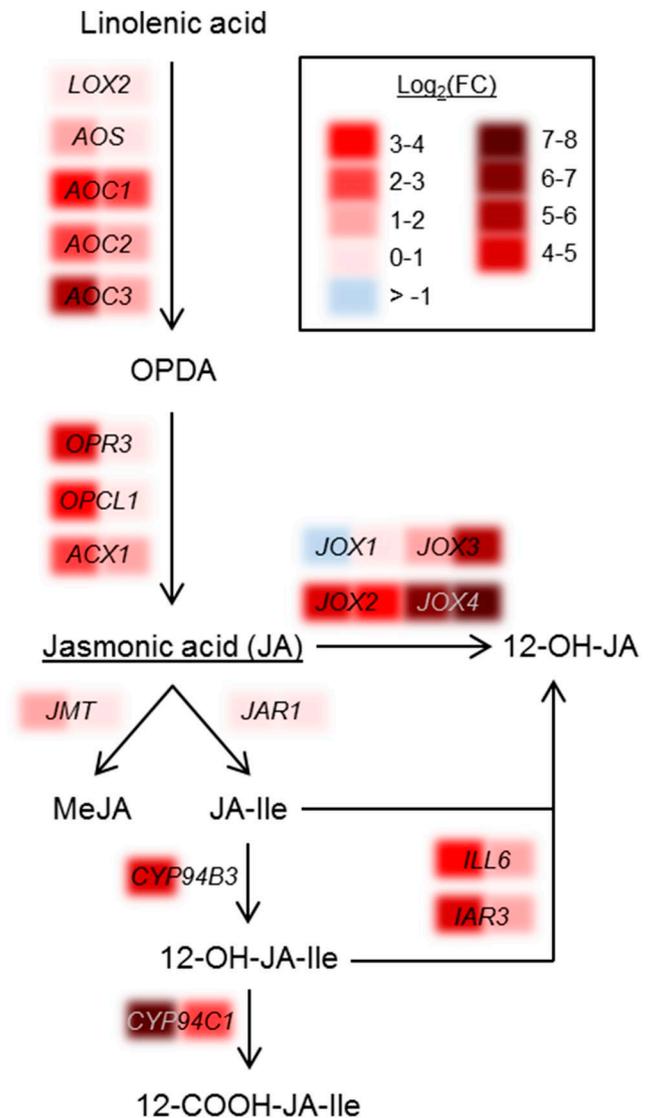
**Figure 4.** NO<sub>2</sub> induces signaling by SA. SA levels at different time points after fumigation with air or 10 µL L<sup>-1</sup> NO<sub>2</sub> were measured via LC-MS/MS and normalized to the samples' fresh weight (FW). Columns represent means ± SD (*n* = 5). Asterisks indicate significant differences within the time points as determined by two-way ANOVA plus the Holm-Sidak posthoc test (\*\*, *P* < 0.01 and \*\*\*, *P* < 0.001). White columns, air; black columns, 10 µL L<sup>-1</sup> NO<sub>2</sub>.

via the allene oxide synthase (AOS), followed by subsequent cyclization to oxophytodienoic acid (OPDA) via the allene oxide cyclases (AOC). After cis-OPDA is reduced by OPDA-reductase (OPR3), three rounds of β-oxidation (e.g. via acyl-CoA oxidase and OPC-8:0 CoA ligase) are necessary to form JA. JA, in turn, can be modified to JA-Ile or methyl JA via jasmonate-amido synthetase and JA-carboxyl methyltransferase, respectively (Browse, 2009; Wasternack and Hause, 2013). This biosynthetic pathway is outlined in Figure 5. The majority of depicted genes were significantly up-regulated directly after fumigation, with a log<sub>2</sub>(FC) of up to 5.9 for AOC3, whereas 6 h after fumigation, the expression levels generally declined.

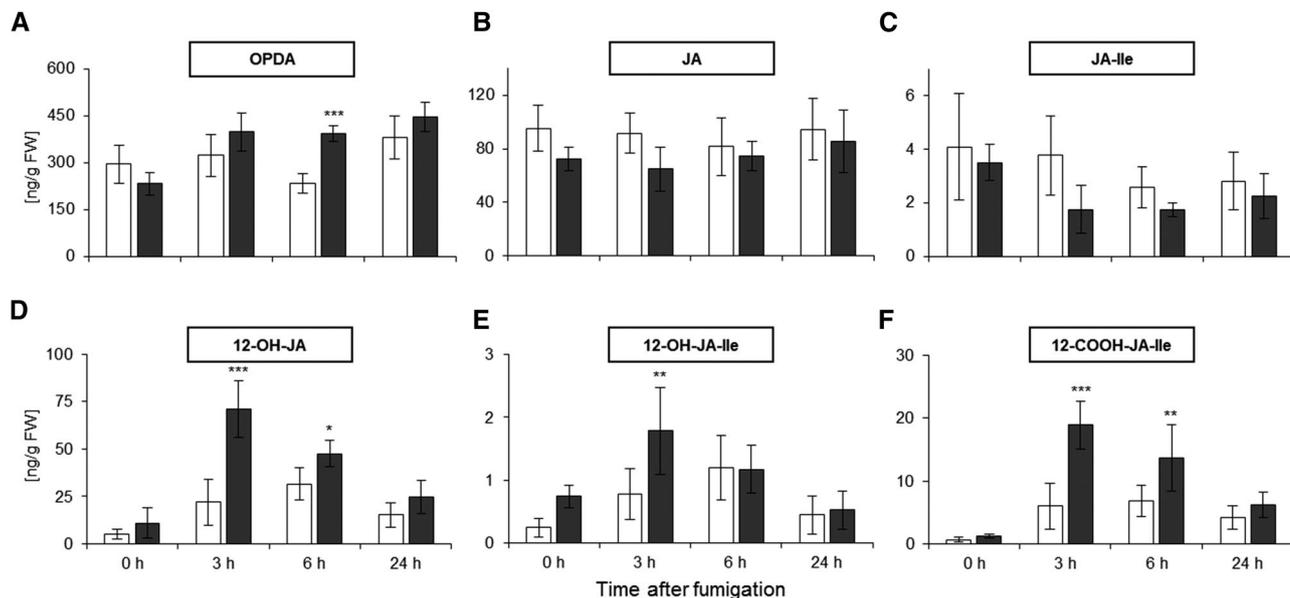
A major step during the catabolic turnover of active jasmonates is the oxidation of JA-Ile by members of the cytochrome P450 94 (CYP94) family (Fig. 5), resulting in biologically inactive 12-OH-JA-Ile and 12-COOH-JA-Ile (Kitaoka et al., 2011; Koo et al., 2011; Heitz et al., 2012). JA-Ile and its hydroxylated form can be further catabolized to tuberonic acid (12-OH-JA) by the amidohydrolases IAA-ALANINE RESISTANT3 and IAA-LEUCINE RESISTANT-LIKE6 (Widemann et al., 2013). Moreover, jasmonate-induced oxygenases (JOXs) hydroxylate JA to its inactive 12-OH-JA derivative (Caarls et al., 2017; Smirnova et al., 2017), which represents yet another pathway of jasmonate catabolism. Directly after fumigation, the majority of genes involved in these catabolic reactions were highly up-regulated, with fold changes to the respective air controls ranging from log<sub>2</sub>(FC) 1.3 to 7.4. The genes encoding for the CYP94 enzymes and members of the JOXs were highly induced. The transcript levels of

JOXs were still elevated significantly up to a log<sub>2</sub>(FC) of 7.8 at 6 h after NO<sub>2</sub> treatment. Besides the JOXs, the gene transcripts of most of the above-mentioned catabolic enzymes also were still highly abundant at this time point after fumigation. All expression levels of the depicted genes can be found in Supplemental Data Set S1.

The gene expression data suggested the simultaneous induction of jasmonate biosynthesis and catabolism. LC-MS/MS revealed that OPDA levels were elevated by 69% at 6 h after fumigation compared with



**Figure 5.** JA biosynthesis and degradation pathways are up-regulated simultaneously in response to NO<sub>2</sub>. The schematic pathway of jasmonate metabolism illustrates the change in expression levels [log<sub>2</sub>(FC)] of the respective genes obtained from the microarray analysis immediately (0 h, left part of the colored sections) or 6 h (right part of the colored sections) after fumigation with 10 µL L<sup>-1</sup> NO<sub>2</sub>. Expression levels of all depicted genes can be found in Supplemental Table S1. JAR1, Jasmonate-amido synthetase; JMT, JA-carboxyl methyltransferase; MeJA, methyl jasmonate



**Figure 6.** JA degradation products accumulate in response to NO<sub>2</sub>. Various jasmonates were measured by LC-MS/MS at different time points after fumigation with air or 10 µL L<sup>-1</sup> NO<sub>2</sub>. Concentrations were normalized to the leaf sample fresh weight (FW). A, OPDA. B, JA. C, JA-Ile. D, 12-OH-JA. E, 12-OH-JA-Ile. F, 12-COOH-JA-Ile. A to C show products of the JA biosynthesis pathway, and D to F show JA catabolism products. Columns represent means ± SD (n = 5). Asterisks indicate significant differences within the time points according to two-way ANOVA plus the Holm-Sidak posthoc test (\*, P < 0.05; \*\*, P < 0.01; and \*\*\*, P < 0.001). White columns, air; black columns, 10 µL L<sup>-1</sup> NO<sub>2</sub>.

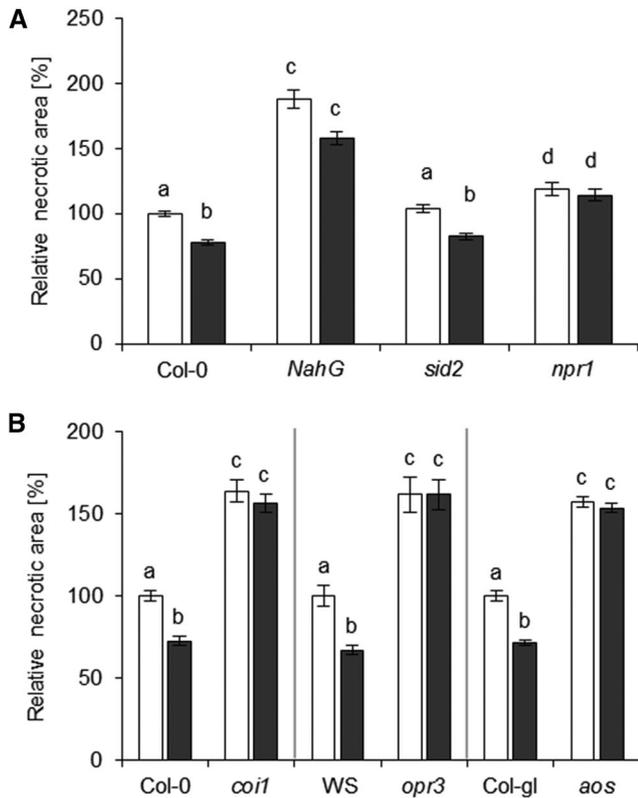
leaf extracts from air-fumigated plants (Fig. 6A), which was associated with the enhanced expression of defense-coding genes, including *PLANT DEFENSIN1.2A* (Supplemental Data Set S1). By contrast, significant changes were not detected for JA or JA-Ile (Fig. 6, B and C). The rapid and extensive NO<sub>2</sub>-induced transcription of genes whose products are necessary for jasmonate catabolism encourages the hypothesis that NO<sub>2</sub> stimulates rapid jasmonate turnover. Accordingly, all catabolic intermediates of JA and JA-Ile increased and peaked in their concentrations at 3 h after NO<sub>2</sub> fumigation. 12-OH-JA increased significantly by a factor of 3.2 from 22 ng g<sup>-1</sup> fresh weight in air-fumigated plants to 71 ng g<sup>-1</sup> fresh weight after NO<sub>2</sub> treatment (Fig. 6D), while 12-OH-JA-Ile levels increased significantly by 2.3-fold at 3 h after fumigation when compared with the air-fumigated control (Fig. 6E). A 3.1-fold increase was observed for 12-COOH-JA-Ile from 6 ng g<sup>-1</sup> fresh weight (air) to 19 ng g<sup>-1</sup> fresh weight (NO<sub>2</sub>; Fig. 6F). After the concentrations of all intermediates peaked at 3 h after treatment, their accumulation declined gradually to baseline levels 24 h after NO<sub>2</sub> treatment.

Together, these results suggest that exposure to NO<sub>2</sub> triggered consecutive peaks of SA and OPDA. The simultaneous induction of jasmonate production and catabolism pathways resulted in the accumulation of 12-OH-JA, 12-OH-JA-Ile, and 12-COOH-JA-Ile. The latter process might be controlled by genes involved in SA/JA antagonism cross talk that were strongly

up-regulated upon NO<sub>2</sub> exposure (Supplemental Data Set S1).

#### The SA and JA Signaling Pathways Are Both Crucial for NO<sub>2</sub>-Induced *B. cinerea* Resistance

Since SA biosynthesis was up-regulated upon NO<sub>2</sub> fumigation, the role of SA in the NO<sub>2</sub>-induced resistance against *B. cinerea* was examined by utilizing mutants defective in *SALICYLIC ACID INDUCTION DEFICIENT2 (SID2)* and plants expressing the *Pseudomonas putida NahG* gene. The *sid2* mutant is impaired in the main SA biosynthesis pathway and, therefore, does not accumulate SA upon pathogen infection (Nawrath and Métraux, 1999; Wildermuth et al., 2001), whereas *NahG* transgenic plants express a bacterial SA hydroxylase that degrades SA to catechol (Delaney et al., 1994). The *B. cinerea* infection assay after NO<sub>2</sub> fumigation revealed that, in Col-0 plants, the lesion size was reduced by 22% upon NO<sub>2</sub> pretreatment (Fig. 7A). The relative necrotic area of NO<sub>2</sub>-fumigated *sid2* also was reduced by 18.4% when compared with the lesion size of its nonfumigated counterpart. However, NO<sub>2</sub> pretreatment provoked only a 14% reduction of the relative necrotic area of *NahG*-expressing plants. This decrease was not significantly different (P > 0.05) from the average lesion size measured in unfumigated *NahG* plants (Fig. 7A), indicating that the NO<sub>2</sub>-induced resistance against *B. cinerea* was compromised. Furthermore, the SA-insensitive *npr1* mutant was included



**Figure 7.** SA and JA function in NO<sub>2</sub>-induced resistance against *B. cinerea*. Mutants were subjected to *B. cinerea* droplet infection 6 h after fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h. Necrotic areas were measured 3 d later and were normalized to the mean necrotic area of the respective unfumigated wild type. A, SA-deficient (*NahG* and *sid2*) or SA-signaling (*npr1*) mutants and the corresponding Col-0 wild type. Columns represent means of at least three independent experiments  $\pm$  SE ( $n = 95\text{--}331$ ). B, JA-deficient (*aos* and *opr3*) or JA-signaling (*coi-1*) mutants and corresponding wild-types (Col-gl for *aos*, Wassilewskija [WS] for *opr3*, and Col-0 for *coi-1*). Columns represent means of three independent experiments  $\pm$  SE ( $n = 66\text{--}126$ ). Letters indicate significant differences of all pairwise comparisons via the Kruskal-Wallis test plus Dunn's posthoc test ( $P < 0.05$ ). White columns, unfumigated; black columns, 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub>.

in the *B. cinerea* infection assay after NO<sub>2</sub> fumigation. Interestingly, NO<sub>2</sub> pretreatment of *npr1* plants did not result in a reduction of *B. cinerea*-induced necrotic lesions. The basal resistance of unfumigated plants was not strongly altered in *sid2* and *npr1* (+18.7% relative necrotic area compared with Col-0 in untreated trials) but was markedly compromised in *NahG* transgenic plants (+88% relative necrotic area). Similar results have been reported for these plant lines (Ferrari et al., 2003). Taken together, these results suggest that the NO<sub>2</sub>-induced resistance against *B. cinerea* is mediated by NPR1. However, it did not require SA synthesis via SID2, whereas the degradation of SA by bacterial *NahG* partially abolished the NO<sub>2</sub>-induced resistance phenotype.

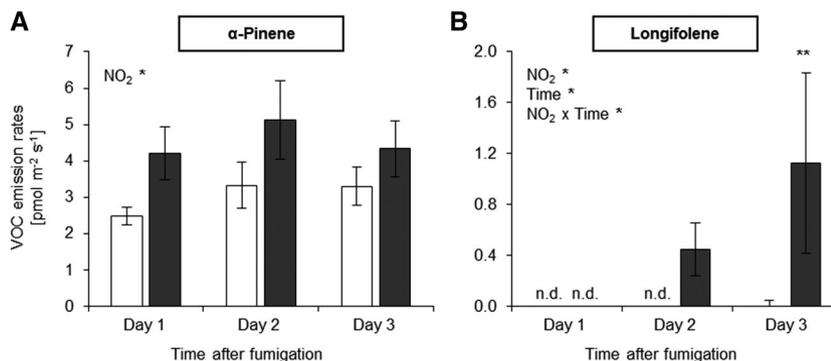
NO<sub>2</sub> exposure caused a strong rearrangement of jasmonate metabolism. To investigate whether jasmonates

or components of the JA signaling pathway were implicated in the NO<sub>2</sub>-induced pathogen resistance, several Arabidopsis knockout mutants impaired in JA biosynthesis and signaling were subjected to the *B. cinerea* infection assay. The *aos* and *opr3* knockout mutants were utilized, since they are impeded in JA accumulation upon wounding or *B. cinerea* infection (Stintzi and Browse, 2000; Park et al., 2002). As shown in Figure 7B, the size of the *B. cinerea*-induced lesions was not affected by NO<sub>2</sub> treatment in both JA-deficient mutants, whereas the necrotic lesions on NO<sub>2</sub>-treated Col-gl (the *aos* parental line) were reduced by 28.6% and Wassilewskija (the wild type of *opr3*) displayed a 33.1% reduction in lesion size upon NO<sub>2</sub> fumigation. Knockout mutants that were impaired in JA signaling also were examined for their NO<sub>2</sub>-induced resistance phenotype. The JA-insensitive *coi1* mutant did not show any significant differences in the size of the necrotic lesions that developed on NO<sub>2</sub>-fumigated or untreated leaves upon *B. cinerea* infection. These results indicated that the NO<sub>2</sub>-induced resistance against *B. cinerea* is dependent on JA accumulation and COI1-mediated signaling. It is noteworthy that the three tested JA mutants all were more susceptible than the respective wild-type lines, confirming the importance of JA in basal resistance against *B. cinerea* (Thomma et al., 1998).

Collectively, the results above argue for a crucial role of SA and jasmonates during NO<sub>2</sub>-induced pathogen resistance. However, further phytohormone measurements revealed that the levels of SA, JA, and JA-Ile at 16, 24, and 48 h after *B. cinerea* infection were not influenced by NO<sub>2</sub> pretreatment (Supplemental Fig. S4). This would suggest that SA, OPDA, and possibly the accumulating JA/JA-Ile catabolites function in the NO<sub>2</sub>-mediated induction of defense responses before but not during *B. cinerea* infection.

### The Volatile Organic Compounds $\alpha$ -Pinene and Longifolene Are Induced after NO<sub>2</sub> Exposure

Under stress conditions, plants emit a wide range of volatile organic compounds (VOCs; Niinemets, 2010). Among several detected VOCs (Supplemental Fig. S5), only the emission of the monoterpene  $\alpha$ -pinene and the sesquiterpene longifolene were increased significantly after exposing plants to NO<sub>2</sub> (Fig. 8; Supplemental Figs. S5 and S6).  $\alpha$ -Pinene acts as a signal in the plant-to-plant communication of systemic acquired resistance (SAR; Riedlmeier et al., 2017), while sesquiterpenes often are released after the occurrence of abiotic/biotic stress (Ghirardo et al., 2012, 2016). NO<sub>2</sub> induced the emission of  $\alpha$ -pinene between 1 and 9 h (day 1) after NO<sub>2</sub> fumigation (Fig. 8A), although the expression of the monoterpene biosynthetic gene *GERANYLGERANYL REDUCTASE* (*GGR*) was not enhanced immediately or 6 h after NO<sub>2</sub> exposure (Supplemental Data Set S1). By comparison, increases of longifolene (syn. junipene) were not detectable until the day after NO<sub>2</sub> exposure



**Figure 8.** NO<sub>2</sub> exposure induces volatile emissions. A, Emission of the monoterpene  $\alpha$ -pinene. B, Emission of the sesquiterpene longifolene. After 1 h of fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub>, Arabidopsis Col-0 plants were enclosed in a flow-through cuvette system and volatile emissions were collected and analyzed successively by thermal desorption-gas chromatography-mass spectrometry and multivariate data analysis (Supplemental Figs. S5 and S6). Columns represent means  $\pm$  SE ( $n = 10$ –12). Significant main effects (NO<sub>2</sub> and Time) and interactions (NO<sub>2</sub>  $\times$  Time) are shown (two-way ANOVA, all pairwise multiple comparison Holm-Sidak posthoc test). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ ; and n.d., not detected. White columns, control (air); black columns, 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub>.

(day 2) and increased significantly ( $P < 0.05$ ) the following day (day 3; Fig. 8B). The sesquiterpene-related gene *CYP81D11* was found to be up-regulated immediately after the NO<sub>2</sub> treatment (Supplemental Data Set S1). Similar to  $\alpha$ -pinene, longifolene emission rates were very low.

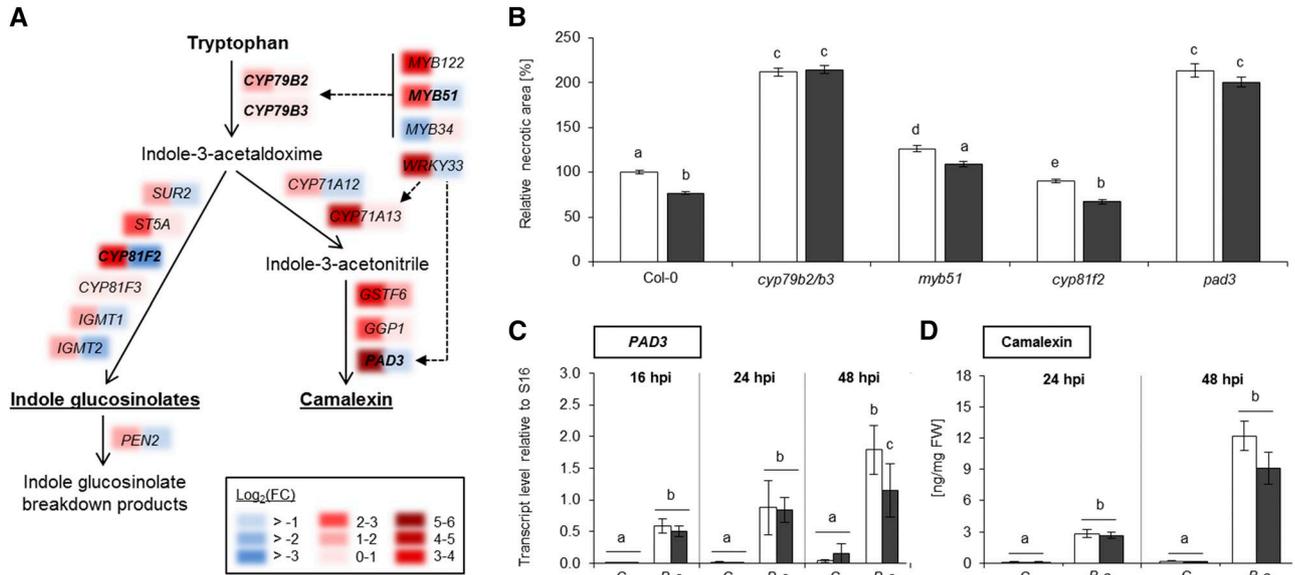
#### NO<sub>2</sub>-Induced *B. cinerea* Resistance Involves CYP79B2/B3 and PHYTOALEXIN DEFICIENT3 But Not Camalexin

NO<sub>2</sub> exposure triggered the expression of genes involved in the biosynthesis of Trp-derived secondary metabolites (Fig. 9A; Supplemental Data Set S1). In the initial step of this pathway, CYP79B2 and CYP79B3 convert Trp into indole-3-acetaldoxime, which serves as a precursor for both indole glucosinolates as well as camalexin (Hull et al., 2000; Glawischnig et al., 2004). The expression of *CYP79B2* increased immediately after NO<sub>2</sub> fumigation, with a log<sub>2</sub>(FC) of 1.6, but was not strongly altered at the 6-h time point, while *CYP79B3* was generally less responsive to NO<sub>2</sub> (Fig. 9A). In the indole glucosinolate pathway, CYP81F2 and CYP81F3 catalyze the hydroxylation of indol-3-ylmethylglucosinolate (I3M; glucobrassicin) to 4-OH-I3M. The expression of *CYP81F2* was up-regulated, with a log<sub>2</sub>(FC) of 3.3, immediately after NO<sub>2</sub> fumigation but was down-regulated at the 6-h time point. By comparison, *CYP81F3* expression was altered only marginally after the NO<sub>2</sub> treatment. Camalexin biosynthesis is dependent on the enzyme PHYTOALEXIN DEFICIENT3 (*PAD3*), which synthesizes both camalexin and the precursor dihydrocamalexin (Schuhegger et al., 2006; Böttcher et al., 2009). *PAD3* expression was enhanced, with a log<sub>2</sub>(FC) of 5.5, immediately after NO<sub>2</sub> fumigation but dropped to wild-type levels at the 6-h time point.

The above-mentioned genes all function in plant resistance against fungal pathogens; therefore, their pos-

sible involvement in NO<sub>2</sub>-induced resistance against *B. cinerea* was investigated further using appropriate mutants. Upon *B. cinerea* infection, the *cyp79b2/b3* double mutant displayed a 112% increase in necrotic area formation compared with wild-type plants, which was not influenced by pretreatment with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> 6 h before inoculation (Fig. 9B). Hence, CYP79B2/B3 play an important role in basal and NO<sub>2</sub>-induced resistance against *B. cinerea*. This conclusion was corroborated by the fact that *myb51* mutant plants lacking the MYB51 positive regulator of *CYP79B2/B3* expression were significantly more susceptible to *B. cinerea* than Col-0 wild-type plants. Upon NO<sub>2</sub> fumigation, the necrotic area was reduced only by 12% as compared with 23% in Col-0 plants, suggesting that the NO<sub>2</sub>-induced resistance was partially compromised (Fig. 9B).

Additional experiments with the *cyp81f2* and *pad3* mutants were aimed at determining the specific contributions of indole glucosinolates and camalexin to basal and NO<sub>2</sub>-induced pathogen immunity. *B. cinerea* infection of the *cyp81f2* mutant caused necrotic lesions with 10% smaller areas than in wild-type plants. Pretreatment with NO<sub>2</sub> before infection resulted in a 33% reduction in lesion size, demonstrating that the *cyp81f2* mutant was capable of establishing NO<sub>2</sub>-induced pathogen resistance (Fig. 9B). By contrast, the camalexin-deficient *pad3* mutant displayed an enhanced susceptibility toward *B. cinerea*, as reported earlier (Ferrari et al., 2003). This became apparent by the 113% increase in necrotic lesion size that developed on unfumigated *pad3* plants compared with unfumigated wild-type plants (Fig. 9B). At 3 dpi, the necrotic lesions on NO<sub>2</sub>-pretreated leaves of *pad3* did not differ significantly in their size in comparison with their unfumigated control, suggesting that *pad3* did not develop NO<sub>2</sub>-induced *B. cinerea* immunity (Fig. 9B). Regarding the compromised basal and NO<sub>2</sub>-induced *B. cinerea*



**Figure 9.** NO<sub>2</sub>-induced resistance against *B. cinerea* is dependent on *CYP79B2*, *CYP79B3*, and *PAD3* but independent of camalexin. A, The expression of genes related to the biosynthesis of Trp-derived indole glucosinolates and camalexin was strongly up-regulated after fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h. Colored areas indicate gene expression [ $\log_2(\text{FC})$ ] immediately (left) or 6 h (right) after the NO<sub>2</sub> treatment. Genes that were investigated further are highlighted in boldface letters. Gene regulation by transcription factors is indicated by dashed-line arrows. B, The *cyp79b2/b3* double mutant and the *myb51*, *cyp81f2*, and *pad3* mutants were subjected to *B. cinerea* droplet infection 6 h after fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h. Necrotic areas were measured 3 d later and were normalized to the mean necrotic area of the unfumigated Col-0 wild type. Columns represent means of three independent experiments  $\pm$  SE ( $n = 81\text{--}418$ ). Letters indicate significant differences of all pairwise comparisons via the Kruskal-Wallis test plus Dunn's posthoc test ( $P < 0.01$ ). C, NO<sub>2</sub>-exposed or control (unfumigated) Col-0 plants were spray infected with  $2 \times 10^5$  *B. cinerea* spores 6 h after fumigation. *PAD3* transcript levels were quantified 16, 24, or 48 h after infection relative to housekeeping gene expression via RT-qPCR. Columns represent means of two independent experiments  $\pm$  SD ( $n = 5$ ). Letters indicate significant differences of all pairwise comparisons within the time points via two-way ANOVA plus the Holm-Sidak posthoc test ( $P < 0.01$ ). D, Plants were spray infected with *B. cinerea* at 6 h after NO<sub>2</sub> or air fumigation. Camalexin levels were measured by HPLC-MS 24 and 48 h after infection. Columns represent means  $\pm$  SE ( $n = 12$ ). Letters indicate significant differences of all pairwise comparisons within the time points via two-way ANOVA plus the Holm-Sidak posthoc test ( $P < 0.01$ ). FW, Fresh weight.

resistance, *pad3* had a similar phenotype to the *cyp79b2/b3* mutant.

These findings led us to conclude that the induction of camalexin biosynthesis genes contributed to the NO<sub>2</sub>-induced resistance against *B. cinerea*. Surprisingly, however, no accumulation of camalexin was observed upon NO<sub>2</sub> exposure (Supplemental Fig. S7). Moreover, the NO<sub>2</sub> treatment did not alter *PAD3* expression or camalexin levels upon *B. cinerea* infection (Fig. 9, C and D). Reverse transcription quantitative PCR (RT-qPCR) analysis 16 and 24 h after infection demonstrated that *PAD3* transcript levels increased significantly to the same extent upon *B. cinerea* infection in unfumigated and NO<sub>2</sub>-treated Col-0 plants (Fig. 9C). Accordingly, no statistical differences in the camalexin content of air- and NO<sub>2</sub>-treated Col-0 plants were detected after *B. cinerea* infection (Fig. 9D), although *B. cinerea* infection led to a significant gradual increase in camalexin from basal 0.1 to 12.2 ng mg<sup>-1</sup> fresh weight after 48 h in NO<sub>2</sub>-treated plants.

Taken together, these results indicated that NO<sub>2</sub> fumigation rapidly induced the expression of camalexin

biosynthesis genes but did not result in camalexin accumulation. It was shown further that NO<sub>2</sub>-induced *B. cinerea* resistance was dependent on *CYP79B2/B3* and *PAD3* but independent of camalexin, *CYP81F2*, and indole glucosinolates.

#### Trp-Derived Secondary Metabolites Accumulate after NO<sub>2</sub> Fumigation

Nontargeted Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) was employed to identify Trp-derived secondary metabolites that could function as signals or defensive compounds in NO<sub>2</sub>-induced *B. cinerea* resistance. To this end, Arabidopsis Col-0 plants were exposed to 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h, and leaves were sampled 6 h later because this was the time point at which plants were usually inoculated with *B. cinerea* spores. Leaf extracts from untreated *cyp79b2/b3* plants served as a zero reference because they are devoid of Trp-derived indole glucosinolates and camalexin (Hull et al., 2000; Glawischnig et al., 2004). The following criteria were applied to filter the

**Table 1.** CYP79B2/B3-dependent accumulation of metabolites 6 h after fumigation with  $10 \mu\text{L L}^{-1} \text{NO}_2$ 

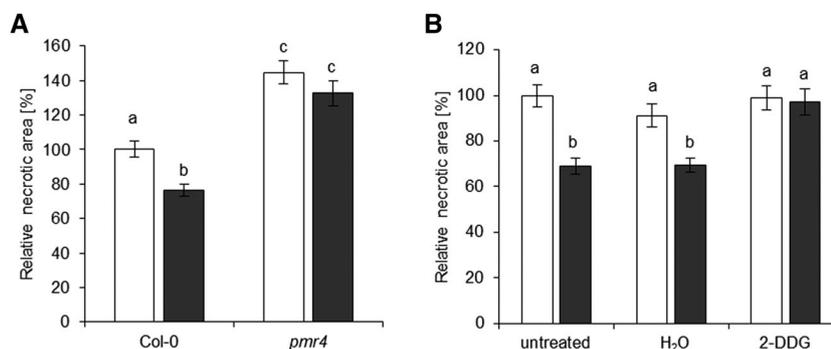
Metabolites were not detected in the *cyp79b2/b3* double mutant.  $\text{NO}_2$ -induced up-regulation in wild-type plants is given as fold change of median spectral count. See Supplemental Table S1 for the complete data set, including statistics. Formulae and tentative annotations were deduced from the exact masses as determined by FT-ICR-MS.

Mass $m/z$ [M-H] <sup>-</sup>		Up-Regulation by $10 \mu\text{L L}^{-1} \text{NO}_2$	Formula [M-H]	Tentative Annotation
Measured	$\Delta \mu\text{L L}^{-1}$			
447.0537	-0.05	1.8	$\text{C}_{16}\text{H}_{20}\text{N}_2\text{O}_9\text{S}_2$	Glucobrassicin, indol-3-ylmethylglucosinolate
367.0783	$\pm 0.00$	3.0	$\text{C}_{15}\text{H}_{16}\text{N}_2\text{O}_9$	Unknown CYP79B2/B3-dependent metabolite
364.1038	$\pm 0.00$	2.7	$\text{C}_{17}\text{H}_{19}\text{NO}_8$	1,4-Dimethoxyindol-3-ylmethylascorbate
304.0826	-0.04	2.8	$\text{C}_{15}\text{H}_{15}\text{NO}_6$	Ascorbigen, indol-3-ylmethylascorbate
232.0463	$\pm 0.00$	2.2	$\text{C}_8\text{H}_{11}\text{NO}_7$	Unknown CYP79B2/B3-dependent metabolite

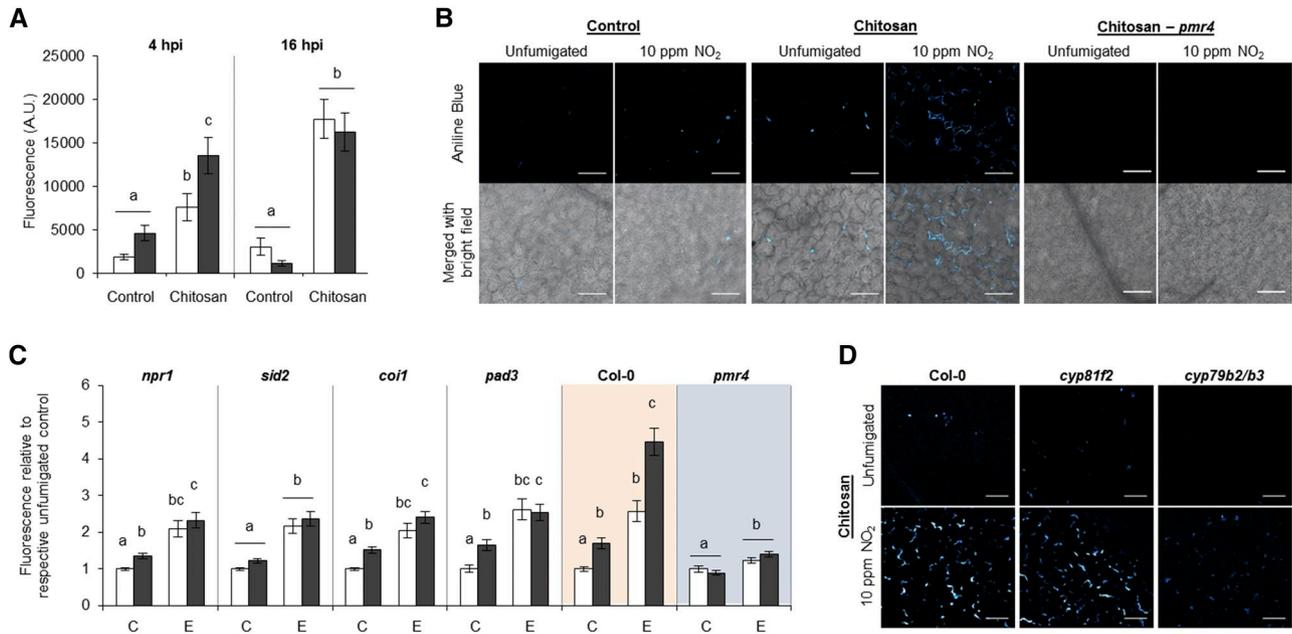
FT-ICR-MS results for candidate CYP79B2/B3-dependent metabolites involved in  $\text{NO}_2$ -induced pathogen immunity: (1) metabolites were not detected in *cyp79b2/b3* extracts but in all 10 leaf extracts from wild-type plants; and (2) metabolites showed significantly up-regulated levels at 6 h after  $\text{NO}_2$  fumigation. Three of nine identified metabolites had exact masses corresponding to the indole glucosinolate glucobrassicin (I3M), its degradation product ascorbigen, and the methoxylated ascorbigen derivative 1,4-dimethoxyindol-3-ylmethylascorbate (Table 1; Supplemental Table S1), confirming that the experimental approach identified Trp-derived compounds. Two other metabolites contained no sulfur atom but at least one nitrogen atom and, thus, could represent indolic compounds. Further experiments are needed to specify if and how glucobrassicin, ascorbigen, 1,4-dimethoxyindol-3-ylmethylascorbate, and the other identified CYP79B2/B3-dependent metabolites are involved in  $\text{NO}_2$ -induced *B. cinerea* immunity.

### Enhanced Callose Formation Is Essential for $\text{NO}_2$ -Induced *B. cinerea* Resistance

Callose deposition at infection sites is an effective plant defense mechanism against various pathogens (Ellinger and Voigt, 2014). The *pmr4* mutant is defective in the *GLUCAN SYNTHASE-LIKE5* gene and does not deposit callose upon pathogen infection (Jacobs et al., 2003; Nishimura et al., 2003). This mutant was subjected to the *B. cinerea* infection assay after  $\text{NO}_2$  fumigation (Fig. 10A). The size of necrotic lesions did not differ between  $\text{NO}_2$ -treated and unfumigated *pmr4* plants, whereas the  $\text{NO}_2$ -treated Col-0 wild type exhibited a 23.7% reduction of the necrotic area. Additionally, Col-0 leaves were infiltrated with the callose deposition inhibitor 2-deoxy-D-glucose (2-DDG; Bayles et al., 1990) or water 24 h before  $\text{NO}_2$  treatment, followed by *B. cinerea* droplet infection 6 h after fumigation (Fig. 10B). Water-infiltrated plants developed a 31% lesion size reduction when compared with the lesions formed on unfumigated, noninfiltrated leaves.



**Figure 10.** Plants impaired in callose formation display a loss in  $\text{NO}_2$ -induced resistance against *B. cinerea*. A, Col-0 and callose-deficient *pmr4* plants were subjected to *B. cinerea* droplet infection 6 h after fumigation with  $10 \mu\text{L L}^{-1} \text{NO}_2$  for 1 h. Necrotic areas formed on fumigated leaves after 3 d were normalized to the mean necrotic areas of the respective unfumigated leaves. Columns represent means of four independent experiments  $\pm$  SE ( $n = 135$ – $145$ ). B, Relative necrotic areas determined on Col-0 plants that were infiltrated with 1.2 mM of the callose synthesis inhibitor 2-DDG (water as a control) 24 h before fumigation followed by *B. cinerea* infection. Columns represent means  $\pm$  SE ( $n = 70$ – $130$ ). Letters indicate significant differences of all pairwise comparisons via the Kruskal-Wallis test plus Dunn's posthoc test ( $P < 0.05$ ). White columns, unfumigated; black columns,  $10 \mu\text{L L}^{-1} \text{NO}_2$ .



**Figure 11.** NO<sub>2</sub> pretreatment enhances early callose deposition upon treatment with the fungal elicitor chitosan. Plants were fumigated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> for 1 h and infiltrated with 500  $\mu\text{g mL}^{-1}$  chitosan (0.04% acetic acid as a control) 4 h after fumigation. Leaf discs were obtained for callose quantification with Aniline Blue 4 or 16 h after chitosan treatment. A, Callose quantification in Col-0. Columns represent means  $\pm$  SE ( $n = 34\text{--}44$  from 10 plants per time point and treatment). A.U., Arbitrary units; hpi, hours after infection. B, Detection of Aniline Blue-stained callose by confocal laser scanning microscopy. Fluorescence and bright-field channels were merged using ImageJ software. Representative photographs were taken of NO<sub>2</sub>-fumigated or unfumigated Col-0 or *pmr4* (right section) 4 h after treatment with chitosan. Bars = 100  $\mu\text{m}$ . C, Callose quantification in mutants impaired in SA synthesis (*sid2*), SA signaling (*npr1*), JA signaling (*coi1*), camalexin synthesis (*pad3*), and callose deposition (*pmr4*). Columns represent means  $\pm$  SE ( $n = 103\text{--}159$  for Col-0 and *pmr4*;  $n = 57\text{--}65$  for other mutants). White columns, unfumigated; black columns, 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub>. C, Infiltration control; E, elicitor chitosan. Letters indicate significant differences of all pairwise comparisons within time points via the Kruskal-Wallis test plus Dunn's posthoc test ( $P < 0.05$ ). White columns, unfumigated; black columns, 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub>. D, Detection of Aniline Blue-stained callose in NO<sub>2</sub>-fumigated or unfumigated *cyp81f2* and *cyp79b2/b3* mutant plants 4 h after treatment with chitosan. Col-0 stained in the same experiment is shown for comparison. Bars = 100  $\mu\text{m}$ .

Importantly, NO<sub>2</sub>-induced resistance was suppressed in NO<sub>2</sub>-fumigated, 2-DDG-treated leaves (Fig. 10B). Hence, PMR4-mediated callose deposition is essential for NO<sub>2</sub>-induced resistance.

The autofluorescence of *B. cinerea* interfered with the Aniline Blue staining of callose. Therefore, chitosan was employed as a potent elicitor of callose deposition (Köhle et al., 1985). Arabidopsis Col-0 plants were fumigated with NO<sub>2</sub> followed by leaf infiltration of 500  $\mu\text{g mL}^{-1}$  chitosan 4 h later (Fig. 11). The photometric Aniline Blue assay revealed that chitosan triggered a 4- to 6-fold increase in fluorescence at 4 and 16 h after the elicitor treatment, respectively (Fig. 11A). Exclusively at the earlier time point, the callose-dependent fluorescence was enhanced further in NO<sub>2</sub>-pretreated plants. Aniline Blue fluorescence was localized in the extracellular space but was absent in *pmr4*, confirming the specificity of the callose detection (Fig. 11B). The NO<sub>2</sub>-enhanced callose formation at 4 h after chitosan treatment was suppressed in the SA mutants *npr1* and *sid2* and in the JA signaling mutant *coi1*, although the chitosan-induced callose formation was

observed in all mutants (Fig. 11C). As expected, almost no chitosan-induced callose formation was detected in the *pmr4* mutant. The NO<sub>2</sub>-enhanced early callose formation upon elicitor treatment was strongly diminished in the camalexin-deficient *pad3* mutant and in the *cyp79b2/b3* double mutant but was unaffected in the indole glucosinolate mutant *cyp81f2* (Fig. 11, C and D). Only in the *cyp79b2/b3* double mutant were no chitosan-triggered callose depositions detected by microscopy (Fig. 11D). In many experiments, NO<sub>2</sub> alone already stimulated weak callose deposition, which also was seen in the tested mutants except for *sid2* and *cyp79b2/b3*.

Two lines of evidence support an important role of callose in NO<sub>2</sub>-induced pathogen resistance. (1) The resistance was suppressed in the callose-deficient *pmr4* mutant and in plants treated with the callose inhibitor 2-DGG. (2) Mutants that did not exhibit NO<sub>2</sub>-induced resistance also were impaired in NO<sub>2</sub>-enhanced callose deposition upon chitosan elicitation, with the exception of *sid2*, which exhibited NO<sub>2</sub>-induced pathogen immunity but impaired callose formation.

## DISCUSSION

Under physiological conditions, NO<sub>2</sub> can arise from the oxidation of NO, reduction of nitrite, or decomposition of peroxyxynitrite (Pryor et al., 2006; Groß et al., 2013). Although the formation of NO<sub>2</sub> under stress conditions is well supported by direct and indirect evidence, less is known about the physiological functions of NO<sub>2</sub>. To address this issue, we fumigated Arabidopsis plants with μL L<sup>-1</sup> levels of NO<sub>2</sub> as a donor treatment. Previous experiments showed that 1 h of exposure of Arabidopsis to 30 μL L<sup>-1</sup> NO<sub>2</sub> triggered rapid cell death, whereas 10 μL L<sup>-1</sup> NO<sub>2</sub> did not cause visible leaf symptoms or ion leakage as a marker of cell damage (Kasten et al., 2016). However, immediately after NO<sub>2</sub> exposure, plants displayed the enhanced chlorophyll autofluorescence (Fig. 1A; Supplemental Fig. S1) indicative of photoprotective energy dissipation in the course of a transient defense response (Lichtenthaler and Miehe, 1997; Chaerle and Van Der Straeten, 2000). In this study, the NO<sub>2</sub>-induced defense response was investigated in detail.

Short-term exposure to 10 μL L<sup>-1</sup> NO<sub>2</sub> induced an up-regulation of more than 2,300 genes immediately after the 1-h treatment period. The number of up-regulated genes decreased to approximately 750 at 6 h after fumigation, indicating that many genes were rapidly and transiently induced by NO<sub>2</sub> (Fig. 1, B and C). GO term enrichment and cluster analysis revealed that predominantly genes involved in pathogen resistance were expressed strongly after NO<sub>2</sub> fumigation (Fig. 2). Many of these genes are induced by flg22 (Zipfel et al., 2004), chitin (Ramonell et al., 2005), *B. cinerea* (Ferrari et al., 2007), and *P. syringae* (Lewis et al., 2015; Fig. 2), suggesting that NO<sub>2</sub> triggered basal pathogen resistance or PTI.

Accordingly, NO<sub>2</sub>-pretreated plants showed resistance against the fungal pathogen *B. cinerea* and the bacterial pathogen *P. syringae* (Fig. 3). The fact that plants could fend off pathogens of distinct life styles confirmed that NO<sub>2</sub> conferred PTI. How the rather simple molecule NO<sub>2</sub> can specifically evoke pathogen resistance is not yet known. NO<sub>2</sub> might activate signaling cascades by the nitration of electrophiles. Particularly, nitro-FAs such as nitrolinolenic acid have reported functions in defense and antiinflammatory signaling (Schopfer et al., 2011; Mata-Pérez et al., 2016b). Alternatively, endogenous elicitors possibly derived from NO<sub>2</sub>-induced cell wall or membrane modifications are formed within the leaf. For instance, NO<sub>2</sub> can cause both the oxidation as well as nitration of lipids (Pryor et al., 2006; Schopfer et al., 2011), which could lead to membrane damage and the subsequent formation of DAMPs. Such nitro signals and endogenous elicitors also could arise when NO<sub>2</sub> is formed under natural stress conditions.

Plant defense responses to pathogen assaults often are orchestrated by SA and JA, although the exact interactions of these phytohormones in PTI are not fully understood (Couto and Zipfel, 2016). SA levels increased

0 to 3 h after NO<sub>2</sub> fumigation, which was accompanied by the increased expression of genes involved in the early SA response (Fig. 4; Supplemental Fig. S3; Blanco et al., 2009) and SAR, including *METHYL ESTERASE9*, *FLAVIN-DEPENDENT MONOOXYGENASE1*, *AZELAIC ACID INDUCED1 (AZI1)*, *AZI2*, *DEFECTIVE IN INDUCED RESISTANCE1 (DIR1/AZI6)*, and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (Supplemental Data Set S1). NO<sub>2</sub> also activated the jasmonate biosynthesis pathway. The accumulation of OPDA 6 h after fumigation could be responsible for the enhanced expression of 13 genes coding for antimicrobial defensins at this time point (Figs. 5 and 6; Supplemental Data Set S1), as reported earlier (Stintzi et al., 2001).

Notably, NO<sub>2</sub> not only initiated jasmonate biosynthesis but also JA and JA-Ile catabolism (Fig. 5). As a result, the levels of JA and its bioactive derivative JA-Ile were unchanged, whereas their degradation products 12-OH-JA, 12-OH-JA-Ile, and 12-COOH-JA-Ile increased up to 3-fold after fumigation (Figs. 5 and 6). Several genes involved in jasmonate catabolism, including *JOX1* to *JOX4*, are inducible by jasmonates as a means of terminating the defense response (Caarls et al., 2017; Smirnova et al., 2017). However, jasmonate-induced JA catabolism is not a likely scenario after NO<sub>2</sub> exposure because neither JA nor JA-Ile levels were altered significantly under these conditions. 12-OH-JA has reported functions in the down-regulation of JA biosynthesis and JA-mediated defense responses (Miersch et al., 2008; Patkar et al., 2015; Caarls et al., 2017; Smirnova et al., 2017), whereas the biological activities of other jasmonate hydroxylation and carboxylation products are yet unexplored. Genes coding for proteins involved in SA/JA cross talk, such as several WRKY transcription factors, *GLUTAREDOXIN480*, *UDP-DEPENDENT GLYCOSYLTRANSFERASE76B1*, and jasmonate-zim-domain transcriptional repressors, were strongly up-regulated (Supplemental Data Set S1; von Saint Paul et al., 2011; Caarls et al., 2015). Therefore, it is tempting to speculate that the NO<sub>2</sub>-induced SA peak and proteins functioning in SA/JA cross talk control both the repression of JA-responsive genes as well as JA/JA-Ile degradation, but this remains to be elucidated.

NO<sub>2</sub> fumigation triggered SA and OPDA signaling and defense gene expression. Further experiments were aimed at detailing the role of phytohormones during the NO<sub>2</sub>-induced basal pathogen immunity. NO<sub>2</sub>-induced *B. cinerea* resistance was compromised in plants expressing the SA hydrolase NahG and in the SA signaling mutant *npr1* but was not altered in the SA biosynthesis mutant *sid2* (Fig. 7). These results are in accordance with previous findings showing that SA produced by Phe ammonia lyase but not the SID2 pathway contributes to the establishment of *B. cinerea* resistance in Arabidopsis (Ferrari et al., 2003). Further mutant analyses revealed that JA biosynthesis and signaling via the COI1 transcriptional activator was essential for NO<sub>2</sub>-induced resistance against *B. cinerea*, as reported earlier (Thomma et al., 1998). Ethylene is well

known for its role in PTI (Boller and Felix, 2009; Couto and Zipfel, 2016). The GO term enrichment of genes involved in ethylene-activated signaling pathways indicated that this gaseous defense hormone contributes to NO<sub>2</sub>-induced immunity. However, this will need to be proven by future experiments.

The emission of the monoterpene  $\alpha$ -pinene and the sesquiterpene longifolene was increased significantly after exposing plants to NO<sub>2</sub> (Fig. 8). It has been demonstrated that monoterpenes, including  $\alpha$ -pinene, play an essential role in the SA-dependent establishment of SAR (Riedlmeier et al., 2017). Likewise, the transient NO<sub>2</sub>-induced SA peak was followed by the emission of  $\alpha$ -pinene, which might trigger SAR within and between plants, as shown recently (Riedlmeier et al., 2017).  $\alpha$ -Pinene production was not regulated at the transcriptional level because NO<sub>2</sub> exposure had no effect on the expression of *GGR1*, which codes for an enzyme involved in the biosynthesis of the monoterpene precursor geranyl diphosphate (Tholl and Lee, 2011; Supplemental Data Set S1). NO<sub>2</sub>-dependent increases of terpenoid emissions might originate from changes of metabolic pool size and fluxes (Ghirardo et al., 2014) and enzyme activities (Ghirardo et al., 2010). These results suggest the induction of local and systemic pathogen resistance by NO<sub>2</sub> analogous to the induction of a local PTI and the subsequent establishment of SAR following the treatment with pathogen-derived elicitors (Mishina and Zeier, 2007).

Longifolene occurs commonly in plant species of the genus *Pinus*, where it is produced by longifolene synthases and stored in (oleo)resin (Martin et al., 2004). Treatment with methyl JA caused an enhanced accumulation of longifolene in Douglas fir (*Pseudotsuga menziesii*) stem and root samples (Huber et al., 2005). In the resin, longifolene could act as an antimicrobial compound (Himejima et al., 1992). The biosynthesis and functions of longifolene remain undocumented in *Arabidopsis*. However, it was reported that *CYP81D11*-overexpressing *Arabidopsis* lines emitted the sesquiterpene isolongifolene in the context of cis-jasmone-regulated tritrophic interactions between plants, aphids, and parasitoids (Bruce et al., 2008). Noteworthy, *CYP81D11* was strongly induced by NO<sub>2</sub> (Supplemental Data Set S1).

The indole alkaloid camalexin and indole glucosinolates are both derived from Trp, and their biosynthesis pathways are closely interconnected (Glawischnig et al., 2004). NO<sub>2</sub> fumigation triggered the expression of several genes involved in the production of these compounds. *CYP79B2*, *CYP79B3*, *MYB51*, *CYP81F2*, and *PAD3* were investigated further for their possible functions in NO<sub>2</sub>-induced *B. cinerea* resistance because these genes have been associated previously with immunity against fungal pathogens. The *cyp79b2/b3* mutant is deficient in both camalexin as well as indole glucosinolates (Bednarek et al., 2009). Experiments with this mutant confirmed the reported high susceptibility to *B. cinerea* (Nafisi et al., 2007) and additionally revealed a complete loss of NO<sub>2</sub>-induced resistance

(Fig. 9B). Moreover, *myb51* plants, which have reduced levels of indolic compounds (Frerigmann et al., 2016), were partially compromised in basal and NO<sub>2</sub>-induced *B. cinerea* resistance. Together, these results suggest an essential role of indolic secondary metabolites in NO<sub>2</sub>-induced *B. cinerea* immunity. *cyp81f2* mutant plants produce glucobrassicin but not 4-OH-I3M and its derivatives, which are essential for the basal resistance of *Arabidopsis* against biotrophic powdery mildews and the necrotrophic fungal pathogen *Plectosphaerella cucumerina* (Bednarek et al., 2009). However, in this study, *cyp81f2* plants did not show a resistance-related phenotype, indicating that CYP81F2-dependent indole glucosinolates are dispensable for basal and NO<sub>2</sub>-induced resistance against *B. cinerea* (Fig. 9B).

Camalexin inhibits the growth of *B. cinerea* (Kliebenstein et al., 2005; Glawischnig, 2007). NO<sub>2</sub> exposure triggered the induction of the camalexin biosynthesis gene *PAD3*, and the *pad3* mutant did not develop NO<sub>2</sub>-induced resistance against *B. cinerea* (Fig. 9). Regarding the compromised basal and NO<sub>2</sub>-induced *B. cinerea* resistance, *pad3* had a similar phenotype to the *cyp79b2/b3* mutant. Therefore, it was hypothesized that the *cyp79b2/b3* phenotype was likely caused by camalexin deficiency rather than a defect in indole glucosinolate biosynthesis. Unexpectedly, during *B. cinerea* infection, neither *PAD3* expression nor camalexin production was influenced by NO<sub>2</sub> pretreatment. These findings resemble a previous study showing that *PAD3* expression but not camalexin levels were increased strongly upon elicitor treatment with plant cell wall-derived oligogalacturans (Ferrari et al., 2007). Thus, rather than camalexin itself, a downstream metabolite (Böttcher et al., 2009) with so far obscure physiological functions might be involved in NO<sub>2</sub>-induced *B. cinerea* resistance.

In a pioneering attempt to identify such Trp-derived metabolites, we analyzed leaf extracts from NO<sub>2</sub>-treated plants using nontargeted direct infusion FT-ICR-MS. Nine candidate metabolites accumulated significantly at 6 h after NO<sub>2</sub> fumigation but were not detectable in leaf samples from *cyp79b2/b3* plants that are devoid of indolic compounds (Supplemental Table S1). Five metabolites could represent indole derivatives because they contained at least eight carbon atoms and one nitrogen atom (Table 1). Neither camalexin nor known camalexin-related metabolites (Böttcher et al., 2009) were found among the NO<sub>2</sub>-regulated CYP79B2/B3-dependent metabolites. Instead, measured exact masses were annotated as the indole glucosinolate glucobrassicin (I3M), its degradation product ascorbigen, and the methoxylated ascorbigen derivative 1,4-dimethoxyindol-3-ylmethylascorbate. If and how these candidate metabolites are linked to NO<sub>2</sub>-induced *B. cinerea* resistance will be defined by future FT-ICR-MS runs with leaf extracts from air- and NO<sub>2</sub>-exposed mutants including *pad3* and *cyp81f2*.

Cell wall fortification by callose deposition is a frequently used readout of PTI induction (Boller and Felix, 2009). In response to pathogens and pathogen-derived elicitors, callose is mostly synthesized by the callose

synthase PMR4 (Jacobs et al., 2003; Nishimura et al., 2003; Clay et al., 2009; Ellinger and Voigt, 2014). NO<sub>2</sub>-induced pathogen resistance was compromised in *pmr4* and in wild-type plants treated with a callose synthase inhibitor, implying a major role of callose in NO<sub>2</sub>-induced *B. cinerea* immunity (Fig. 10). The fungal elicitor chitosan triggers callose formation (Köhle et al., 1985; Ramonell et al., 2005) and was used here to mimic the infection by a fungal pathogen. NO<sub>2</sub> alone already induced a slight increase in callose formation, which was increased further 4 h after chitosan application as compared with unfumigated plants (Fig. 11). Hence, preformed and more rapidly occurring callose deposition contributed to the NO<sub>2</sub>-induced resistance against *B. cinerea*. The stimulatory effect of NO<sub>2</sub> on chitosan-induced callose formation was not seen in *npr1*, *sid2*, and *coi1*, indicating that SA and JA signaling were essential for the induction of callose formation. However, given that the *sid2* mutant was capable of establishing NO<sub>2</sub>-induced *B. cinerea* resistance (Fig. 7), this form of immunity is not based solely on callose depositions but can be compensated by other defense mechanisms.

It was reported that, in *Arabidopsis*, a yet uncharacterized CYP81F2- and PEN2-dependent 4-OH-I3M breakdown product functions as a signal or coactivator for Flg22-induced callose deposition (Clay et al., 2009). However, in this study, chitosan-triggered callose formation was not altered in *cyp81f2*, which is in line with the previous finding that Flg22- but not chitosan-triggered callose synthesis was affected in the *pen2* mutant (Luna et al., 2011). Hence, the regulation of callose buildup is specific to the perceived elicitor. Flg22-induced callose formation was not compromised in the *pad3* mutant, suggesting that this defense response was not dependent on camalexin (Clay et al., 2009). Accordingly, Aniline Blue fluorescence was enhanced significantly in *pad3* after chitosan treatment (Fig. 11C). By contrast, the enhancing effect of NO<sub>2</sub> on the early chitosan-triggered callose deposition was suppressed in *pad3* and *cyp79b2/b3* (Fig. 10, C and D). The latter mutant showed a reduced ability to produce callose in response to chitosan, although this has to be confirmed by quantitative measurements. Together, these findings argue for a role of PAD3-produced metabolites other than camalexin in the NO<sub>2</sub>-enhanced early callose deposition. These results also suggest that chitosan-induced callose formation and NO<sub>2</sub>-enhanced callose formation are controlled by different signaling pathways.

The lack of NO<sub>2</sub>-enhanced callose formation 4 h after chitosan treatment in *pad3* and all tested phytohormone mutants (except *sid2*) was associated with the inability of these mutants to establish NO<sub>2</sub>-induced *B. cinerea* resistance. Callose synthesis in response to NO<sub>2</sub> alone was not altered in most of the tested mutants, suggesting that only the NO<sub>2</sub>-enhanced callose formation upon the perception of pathogen-derived elicitors was decisive for the NO<sub>2</sub>-induced immunity against *B. cinerea*.

## CONCLUSION

Donor treatments of *Arabidopsis* with 10 μL L<sup>-1</sup> NO<sub>2</sub> triggered basal disease resistance against *B. cinerea* and *P. syringae*. Transcriptomics suggested that NO<sub>2</sub> fumigation led to the onset of phytohormone signaling and the biosynthesis of indolic compounds such as camalexin. Therefore, these biological processes were investigated in more detail. The NO<sub>2</sub>-induced resistance was dependent on SA and jasmonate signaling. An early peak of SA immediately after the NO<sub>2</sub> treatment was followed by the transient accumulation of OPDA and JA catabolites. Particularly interesting was the finding that the activation of JA catabolism represents a mechanism for the complete suppression of JA signaling, presumably in the course of SA/JA antagonism. Mutants impaired in SA or jasmonate signaling were compromised in NO<sub>2</sub>-induced *B. cinerea* resistance, confirming that the coordinated action of both signaling pathways is required for this form of pathogen immunity.

The *cyp79b2/b3* double mutant that is deficient in indole phytoalexins did not establish NO<sub>2</sub>-induced *B. cinerea* resistance. Further investigations of the *pad3* mutant combined with biochemical measurements specified that unknown camalexin-derived metabolites but not camalexin itself function in the resistance induction by NO<sub>2</sub>. The SA and jasmonate signaling mutants as well as the camalexin-deficient mutants all were more susceptible to *B. cinerea*, suggesting that basal resistance in untreated plants and NO<sub>2</sub>-induced resistance likely are mediated by similar defense mechanisms. The inability of these mutants to establish NO<sub>2</sub>-induced immunity was associated with the loss of NO<sub>2</sub>-enhanced callose formation upon perception of the fungal elicitor chitosan. Therefore, timely callose deposition seems to be an essential defense mechanism during the NO<sub>2</sub>-induced *B. cinerea* resistance. Further defense mechanisms could be related to the observed emission of α-pinene and longifolene from NO<sub>2</sub>-fumigated plants.

The exact mechanism by which NO<sub>2</sub> triggers PTI remains to be investigated. NO<sub>2</sub> might function as a dedicated signal (e.g. via the nitration of electrophilic target molecules). However, NO<sub>2</sub> also could act more indirectly as a defense elicitor by causing nitrooxidative stress.

## MATERIALS AND METHODS

### Plant Material and NO<sub>2</sub> Fumigation

The utilized *Arabidopsis* (*Arabidopsis thaliana*) genotypes and their descriptions and origins are summarized in Supplemental Table S2. Plants were grown and fumigated for 1 h with 10 μL L<sup>-1</sup> NO<sub>2</sub> as described previously (Kasten et al., 2016). A fumigation system was used as detailed in Supplemental Figure S8 (Kasten et al., 2017). The only difference was the installation of a NO<sub>2</sub> mixing cylinder (1.5 L) containing Raschig rings, in which 15% (v/v) NO reacted with 100% oxygen to give NO<sub>2</sub>. Up to 100 plants were fumigated with NO<sub>2</sub> in parallel. The light conditions within the fumigation chamber were

adjusted to the settings in the growth chamber (65–85  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity) where the plants were raised to avoid any light artifacts on nitrogen metabolism (Beavers and Hageman, 1969) and plant-pathogen interactions (Roden and Ingle, 2009).

### Autofluorescence Detection

UV autofluorescence was detected using a hand-held UV lamp (Blak-Ray B-100AP; UVP) and documented with a Nikon DC300 camera. Camera settings were kept consistently at an exposure time of 2 s at ISO-3200 with an aperture of F/18.

### Statistics

SigmaPlot 12.0 (Systat Software) was used for the statistical evaluation of all data sets as described earlier (Kasten et al., 2016). When comparing two independent groups, Student's *t* test was used, in cases where the Shapiro-Wilk normality test ( $P > 0.05$ ) was passed. If the normality test failed, the analysis was done with the nonparametric Mann-Whitney rank-sum test. The comparison of more than two independent groups that passed the Shapiro-Wilk normality test ( $P > 0.05$ ) was done by one-way ANOVA and subsequent Holm-Sidak posthoc tests for all pairwise comparisons or comparisons against a control group. When the normality assumption of ANOVA failed on original or log-transformed data (Shapiro-Wilk test), the nonparametric Kruskal-Wallis test with subsequent Dunn's method posthoc test was performed to test for differences between the groups.

### *Pseudomonas syringae* pv *tomato* DC3000

*P. syringae* pv *tomato* (*Pst*) DC3000 was cultivated at 28°C for 2 d on selective nutrient-yeast extract glycerol agar (0.5% [w/v] bacto-tryptone pepton, 0.3% [w/v] yeast extract, 2% [v/v] glycerol, and 1.8% [w/v] agar) supplemented with rifampicin and kanamycin (50  $\mu\text{g mL}^{-1}$ ). Five-week-old plants were fumigated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> (unfumigated plants as a control) and inoculated 4 h after fumigation with  $1 \times 10^8$  cfu mL<sup>-1</sup> *Pst* DC3000 in 10 mM MgCl<sub>2</sub>. Three to four leaves per plant were infiltrated with the bacterial suspension from their abaxial side using a 1-mL needleless syringe. The *Pst* DC3000 bacterial titer within the leaves was determined 2 h (bacterial load control) or 1 and 2 d after infection. At the indicated time points, 6-mm leaf discs were obtained from each infected leaf and, at the 2-h time point, surface sterilized for 30 s in 80% ethanol. Three leaf discs from different plants were merged into one biological replicate, which was then homogenized for 20 s in 200  $\mu\text{L}$  of 10 mM MgCl<sub>2</sub> using a Silamat S6 Tissue Homogenizer (Ivoclar Vivadent) and 1.7- to 2-mm glass beads. The resulting bacterial suspension was diluted in 10 mM MgCl<sub>2</sub> in a serial logarithmic dilution (10-fold) ranging from 10<sup>0</sup> to 10<sup>5</sup>. Subsequently, 20  $\mu\text{L}$  of each dilution was spotted onto selective nutrient-yeast extract glycerol agar before incubating them for 2 d at 28°C. Bacterial colonies were counted in spots containing between 10 and 100 colonies, and the bacterial titer (cfu cm<sup>-2</sup>) per biological replicate was calculated as follows: cfu cm<sup>-2</sup> = colony count  $\times$  dilution factor  $\times$  volume total/volume spotted  $\times$  1.18 cm<sup>-1</sup> (leaf disc factor).

### *Botrytis cinerea*

*B. cinerea* (strain SAS 56) was cultivated on halves of canned apricots (*Prunus armeniaca*) that were soaked for several hours in distilled, deionized water to reduce their sugar content. After cultivating *B. cinerea* on the apricots for approximately 1 week, the spores were used for infection experiments. Leaves of 4-week-old Arabidopsis plants were harvested 6 h after fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> and placed with their abaxial side down onto 0.8% (w/v) agar. Droplets (maximum of 10  $\mu\text{L}$ ) containing a maximum of 1,000 spores of *B. cinerea* in one-half-strength grape juice were spotted onto the leaves, avoiding the middle vein. After a 3-d incubation in a long-day climate chamber, the necrotic lesions were documented with a camera and their areas were determined via ImageJ 1.49m. The areas of the necrotic lesions developed on fumigated leaves were normalized to those formed on unfumigated leaves. NO<sub>2</sub>-treated and untreated wild-type plants were always included during the evaluation of mutants. For the phytohormone, camalexin, and RT-qPCR analyses, entire plants were infected with *B. cinerea* 6 h after fumigation with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> (controls as indicated) by spraying a one-half-strength grape juice suspension containing  $2 \times 10^8$  fungal spores mL<sup>-1</sup> and 0.01% (v/v) Silwet

L-77 (Lehle Seeds) onto the plants until runoff. The negative spray control contained no fungal spores. The infected plants were covered with a clear lid to ensure high humidity for proper infection.

### Phytohormone Measurements

To quantify SA, JA, cis-OPDA, OH-JA, OH-JA-Ile, and COOH-JA-Ile, approximately 250 mg of leaf material of 4-week-old Col-0 plants that were fumigated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> or air was harvested 0, 3, 6, and 24 h after fumigation. Similarly, leaf material from plants that were spray infected with *B. cinerea* 6 h after fumigation was collected 16, 24, and 48 h after infection. The LC-MS/MS analyses were performed as described previously (Vadassery et al., 2012; Kasten et al., 2016).

### Camalexin Measurements

Four-week-old Col-0 plants were fumigated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> or air, and approximately 100 mg of leaf material was collected and frozen in liquid nitrogen 6 h after fumigation. At the same time, the remaining plants were spray infected with *B. cinerea* and harvested 24 and 48 h after infection as described above. Camalexin extraction and quantification were performed as described previously (Frerigmann et al., 2015; Müller et al., 2015).

### FT-ICR-MS

A total of 450 mg of leaf material was frozen in liquid nitrogen, homogenized using a Silamat S6 tissue homogenizer (Ivoclar Vivadent) and 1.7- to 2-mm glass pearls, and incubated subsequently in 1.5 mL of extraction buffer (2% acetic acid and 80% ethanol) for 30 min at 4°C. After centrifugation for 20 min at 15,000g and 4°C, the supernatant was collected and the pellet was extracted again with 1.5 mL of extraction buffer. An Oasis WAX 6cc solid-phase extraction column (Waters) was rinsed with 1 mL of methanol and 1 mL of water before addition of the 3-mL pooled leaf extract. The column was then washed with 2 mL of 2% acetic acid. Metabolites were recovered from the solid-phase extraction columns by consecutive elutions with 2 mL of methanol and 2 mL of 5% NH<sub>4</sub>OH in methanol. Samples were dried under vacuum, dissolved in 1 mL of 70% methanol, centrifugated, and 200-fold diluted in 70% methanol before the MS run.

A Solarix FT-ICR mass spectrometer (Bruker Daltonics) coupled to a 12-Tesla magnet (Magnex) with an Infinity ICR cell was used for the experimental study. A time-domain transient was obtained with 4M Words size (4 million 32-integers) and was Fourier transformed to obtain a frequency spectrum, which was then converted by the Solarix Control program (Bruker Daltonics) into a mass spectrum. All ion excitations were performed in broadband mode (frequency sweep radial ion excitation). Three hundred scans were accumulated for each mass spectrum. Ions were accumulated in the collision cell for 300 ms for thermalization and enrichment prior to ICR ion detection. The base pressure in the ICR vacuum chamber was  $7 \times 10^{-10}$  mbar. The electrospray ionization source (Apollo II; Bruker Daltonics) was used in the negative ionization mode to ionize the studied analytes in 70% methanolic solution (Lichrosolv; Sigma-Aldrich). The sample solutions were injected directly to the ionization source by the use of a microliter pump at a flow rate of 2  $\mu\text{L min}^{-1}$ . A source heater temperature of 200°C was maintained, and no nozzle-skimmer fragmentation was performed in the ionization source. The instrument was calibrated previously by the use of Arg negative cluster ions starting from a methanolic Arg solution of 5 mg L<sup>-1</sup>.

Results of the FT-ICR-MS runs were subjected to normalization. Wilcoxon rank-sum tests for differential analysis between samples from NO<sub>2</sub>-fumigated plants and samples from air-fumigated plants were performed in R (version 3.3.3) using wilcox.test (R Core Team, 2014). Accurate masses corresponding to regulated metabolites were searched against public databases with Metlin (Smith et al., 2005) and MassTRIX (Suhre and Schmitt-Kopplin, 2008).

### Chitosan Elicitation and Callose Quantification

Four- to 5-week-old plants were treated with the fungal elicitor chitosan (medium M<sub>1</sub>; Sigma-Aldrich) 4 h after they were fumigated with 10  $\mu\text{L L}^{-1}$  NO<sub>2</sub> (unfumigated plants as a control). Here, three to four leaves per plant were infiltrated from their abaxial side with 500  $\mu\text{g mL}^{-1}$  chitosan in 0.04% acetic acid using a 1-mL needleless syringe. As a negative control, plants were treated with 0.04% acetic acid.

Leaf discs (6 mm) were obtained from treated leaves with a cork borer at the indicated time points and incubated overnight in 96% ethanol to remove chlorophyll. The destained leaf discs were gently dried off and then incubated for 1 h in 150 mM  $K_2HPO_4$  buffer (pH 9.5) at room temperature and with mild agitation. Meanwhile, an Aniline Blue (Sigma-Aldrich) staining solution (0.01% [w/v] Aniline Blue in 150 mM  $K_2HPO_4$  buffer, pH 9.5) was prepared and stirred until decolorized while protecting it from light. The samples were stained overnight in the dark at room temperature and with gentle agitation. After rinsing the leaf discs in the  $K_2HPO_4$  buffer, they were transferred into wells of a black flat-bottom 96-well plate containing 50  $\mu$ L of the same buffer. Callose was quantified by measuring the Aniline Blue fluorescence (mean of nine reads per leaf disc) with the Infinite M1000 Pro plate reader (Tecan) after adjusting the Z-positioning of the fluorescence top optics. Aniline Blue fluorescence was excited with 405 nm (5-nm bandwidth), and the emission wavelength was set to 490 nm (20-nm bandwidth). To minimize the noise of potential autofluorescence, the fluorescence of leaf discs that were incubated overnight in 150 mM  $K_2HPO_4$  buffer (pH 9.5) without Aniline Blue was subtracted from the values of stained samples for each treatment.

For the microscopic inspection of callose depositions, Aniline Blue-stained leaf discs were mounted in 50% glycerol and analyzed with the TCS SP8 X confocal laser scanning microscope (Leica) using the HC PL APO CS2 20 $\times$ /0.75 IMM objective. The samples were excited with a Diode 405 Laser (laser line UV 405 nm) at 0.1% laser intensity. The emitted fluorescence was detected with a photomultiplier at 480 to 500 nm (gain 800), whereas bright-field micrographs were taken at gain 400 using the transmission photomultiplier.

In some experiments, leaves of 4-week-old Col-0 plants were syringe infiltrated with 1.2 mM of the callose synthesis inhibitor 2-DDG (Sigma-Aldrich) or distilled, deionized water 24 h before fumigation with 10  $\mu$ L  $NO_2$  (unfumigated as a control). Six hours after fumigation, the infiltrated leaves were detached, placed on 0.8% agar, and droplet infected with *B. cinerea*, and the necrotic lesions were analyzed after 3 d. The necrotic areas were compared with those formed on unfumigated and noninfiltrated leaves (= 100%).

## VOC Collection and Analysis

Three to four biological replicates were collected in each of the three independent experiments ( $n_{total} = 10-12$ ). For each replicate, 50 Arabidopsis plants were enclosed in glass cuvettes flushed continuously with 200 mL  $min^{-1}$  VOC-free synthetic air containing 400  $\mu$ L  $L^{-1}$   $CO_2$  and  $\sim 9,000$   $\mu$ L  $L^{-1}$  water. The dynamic cuvette system and the experimental procedure have been described in detail previously (Riedlmeier et al., 2017). Sample collection was 8 h at 60 mL  $min^{-1}$ . To ensure the collection of plant volatiles under steady-state conditions of net assimilation (Ghirardo et al., 2014), sampling started 1 h after plants were exposed to  $NO_2$  and 2 h after the light was switched on in the morning for the days following the  $NO_2$  fumigation. An overflow of  $\sim 140$  mL  $min^{-1}$  was maintained during the VOC collection to avoid any contaminations. Background measurements were performed for  $NO_2$  and control samples separately, in exactly the same way as the collection of samples, but plants were removed immediately after  $NO_2$  or air fumigation for the treated and control plants, respectively. Quantitative and qualitative analyses of VOCs were achieved by thermal desorption-gas chromatography-mass spectrometry following established methods (Ghirardo et al., 2011, 2012, 2016; Weikl et al., 2016). The breaking of VOCs through the polydimethylsiloxane adsorbent was negligible ( $0.08\% \pm 0.06\%$  [SD];  $n = 8$ ) at  $\sim 10$  nL  $L^{-1}$  of an 11-VOC standard mixture containing  $\alpha$ -pinene (Apel-Riemer Environmental). Longifolene was quantified using isolongifolene as a pure standard. Fluxes of plant volatiles were calculated after background correction and normalized to biomass dried weight (dw) of leaves. Successively, dw was converted in leaf area (la) by using the factor of 26.6 g  $m^{-2}$  (dw  $la^{-1}$ ) calculated from previous experiments (Riedlmeier et al., 2017).

## Microarray Analysis

Four-week-old Arabidopsis Col-0 plants were fumigated with 10  $\mu$ L  $L^{-1}$   $NO_2$  or air. Approximately 50 mg of pooled leaf material sampled from at least two different plants was harvested immediately and 6 h after fumigation and frozen in liquid nitrogen. Four biological replicates per treatment were collected. The samples were homogenized twice for 10 s using a Silmat S6 Tissue Homogenizer (Ivoclar Vivadent) and 1.7- to 2-mm glass beads. RNA was extracted, and any potentially remaining DNA was digested using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The gene expression measurements were performed using Agilent one-color microarrays as

described recently (Riedlmeier et al., 2017). The Agilent Feature Extraction software was used with the template GE1\_1100\_Jul11. Gene expression levels were determined by the limma software (version 3.18.13; Smyth, 2005) using the TAIR10 genome annotation (Berardini et al., 2015). The differential expression between  $NO_2$  and air treatments for each time point was computed using the limma software (version 3.18.13) with a nested interaction model (Ritchie et al., 2015). Genes with adjusted  $P < 0.05$  (based on the false discovery rate method for adjustment) and absolute  $\log_2(FC) > 1$  were selected for further analysis. The differential expression results were visualized via volcano plots generated by SigmaPlot 12.0 (Systat Software) and Venn diagrams created with jvenn (Bardou et al., 2014). Differentially expressed genes were subjected to GO term overrepresentation analysis with PANTHER 11.0 (release date, July 15, 2016) using the annotation from the GO database (release date, December 28, 2016) and the Arabidopsis reference list from PANTHER (Mi et al., 2017). The obtained enriched GO terms ( $P < 0.05$ ) were visualized in semantic similarity-based scatterplots generated with the REVIGO tool (Supek et al., 2011).

For the meta-analysis of stress-related expression responses, raw data and sample annotation from five Arabidopsis experiments (accession nos. E-GEOD-5684, E-GEOD-6176, E-GEOD-2538, E-GEOD-17382, and E-MTAB-4867) were downloaded from the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>; Kolesnikov et al., 2015). The abiotic stress data set (E-MTAB-4867) was selected because it was measured with the same microarray platform as the  $NO_2$  fumigation data (Agilent At8x60K one-color microarrays; design identifier 29132). The pathogen and pathogen elicitor data sets (E-GEOD-5684, E-GEOD-6176, E-GEOD-2538, and E-GEOD-17382) were found by keyword search. Due to the unavailability of Agilent one-color microarray measurements, Affymetrix ATH1-121501 data sets were chosen for these conditions. The combined Affymetrix data were preprocessed using the R package affy (version 1.40.0; Gautier et al., 2004). The combined Agilent data were preprocessed as stated for the  $NO_2$  data set. Based on TAIR10 annotation (Berardini et al., 2015), average  $\log_2$  gene expression levels were computed and subsequently centered for each experiment relative to the mean of its controls to focus on treatment responses (log fold changes relative to the mean of controls). Principal component analysis across all expression response profiles was performed in R (version 3.0.3) using prcomp (R Core Team, 2014).

## RT-qPCR

RNA was isolated from approximately 100 mg of leaf material using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. If necessary, samples were subjected to the RNA Clean Up Protocol of the RNeasy Mini Kit (Qiagen). Reverse transcription of 1  $\mu$ g of total RNA to cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. cDNA was diluted 1:16 in distilled, deionized water prior to RT-qPCR, which was performed using the SensiMix SYBR Low-ROX Kit (Bioline) and the following primers: *RIBOSOMAL PROTEIN S16* housekeeping gene; *S16*fwd, 5'-TTTACGCCATCCGTCAGAGAT-3'; *S16*rev, 5'-TCTGTGAACGAGAACGAGCAC-3'; *PAD3*fwd, 5'-TACTTGTGAGATGGCATTGTTGAA-3'; and *PAD3*rev, 5'-CTTCCTCCTCCTCGCCAAT-3'. The annealing temperature was 60°C for all primers.

## Accession Numbers

The microarray data have been deposited in the ArrayExpress database at EMBL-EBI (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6522>).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.**  $NO_2$  fumigation does not cause visible leaf symptoms.

**Supplemental Figure S2.** GO term enrichment analysis of genes regulated by 10  $\mu$ L  $L^{-1}$   $NO_2$ .

**Supplemental Figure S3.** Venn diagram showing that the expression of early SA response genes is induced after  $NO_2$  fumigation.

**Supplemental Figure S4.** *B. cinerea*-induced SA and jasmonate accumulation is not altered by  $NO_2$  pretreatment.

- Supplemental Figure S5.** Overview of volatiles emitted from *Arabidopsis* following the fumigation experiment.
- Supplemental Figure S6.** Effects of NO<sub>2</sub> fumigation on the volatile emissions.
- Supplemental Figure S7.** NO<sub>2</sub> treatment does not alter camalexin levels.
- Supplemental Figure S8.** NO<sub>2</sub> fumigation system.
- Supplemental Table S1.** Candidate Trp-derived metabolites involved in the plant response to NO<sub>2</sub>.
- Supplemental Table S2.** *Arabidopsis* mutant lines used in this study.
- Supplemental Data Set S1.** Microarray analysis of gene expression after fumigation of *Arabidopsis* for 1 h with 10 μL L<sup>-1</sup> NO<sub>2</sub>.

## ACKNOWLEDGMENTS

We thank Michael Reichelt for support with the phytohormone measurements. We acknowledge the donation of the *cyp81f2-2* mutant by Henning Frerigmann.

Received June 8, 2018; accepted July 27, 2018; published August 3, 2018.

## LITERATURE CITED

- Ahuja I, Kissen R, Bones AM (2012) Phytoalexins in defense against pathogens. *Trends Plant Sci* 17: 73–90
- Arasimowicz-Jelonek M, Floryszak-Wieczorek J (2011) Understanding the fate of peroxy nitrite in plant cells: from physiology to pathophysiology. *Phytochemistry* 72: 681–688
- Bardou P, Mariette J, Escudé E, Djemiel C, Klopp C (2014) jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics* 15: 293
- Bayles CJ, Ghemawat MS, Aist JR (1990) Inhibition by 2-deoxy-D-glucose of callose formation, papilla deposition, and resistance to powdery mildew in an ml-o barley mutant. *Physiol Mol Plant Pathol* 36: 63–72
- Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubek J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323: 101–106
- Beevers L, Hageman RH (1969) Nitrate reduction in higher plants. *Annu Rev Plant Physiol* 20: 495–522
- Berardini TZ, Reiser L, Li D, Mezheritsky Y, Muller R, Strait E, Huala E (2015) The *Arabidopsis* Information Resource: making and mining the “gold standard” annotated reference plant genome. *Genes* 53: 474–485
- Bigeard J, Colcombet J, Hirt H (2015) Signaling mechanisms in pattern-triggered immunity (PTI). *Mol Plant* 8: 521–539
- Blanco F, Salinas P, Cecchini NM, Jordana X, Van Hummelen P, Alvarez ME, Holuigue L (2009) Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Mol Biol* 70: 79–102
- Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol* 60: 379–406
- Böttcher C, Westphal L, Schmotz C, Prade E, Scheel D, Glawischnig E (2009) The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*. *Plant Cell* 21: 1830–1845
- Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng SH, Sheen J (2010) Differential innate immune signalling via Ca<sup>2+</sup> sensor protein kinases. *Nature* 464: 418–422
- Browse J (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annu Rev Plant Biol* 60: 183–205
- Bruce TJ, Matthes MC, Chamberlain K, Woodcock CM, Mohib A, Webster B, Smart LE, Birkett M, Pickett J, Napier J (2008) cis-Jasmone induces *Arabidopsis* genes that affect the chemical ecology of multitrophic interactions with aphids and their parasitoids. *Proc Natl Acad Sci USA* 105: 4553–4558
- Caarls L, Pieterse CMJ, Van Wees SCM (2015) How salicylic acid takes transcriptional control over jasmonic acid signaling. *Front Plant Sci* 6: 170
- Caarls L, Elberse J, Awwanah M, Ludwig NR, de Vries M, Zeilmaker T, Van Wees SCM, Schuurink RC, Van den Ackerveken G (2017) *Arabidopsis* JASMONATE-INDUCED OXYGENASES down-regulate plant immunity by hydroxylation and inactivation of the hormone jasmonic acid. *Proc Natl Acad Sci USA* 114: 6388–6393
- Castillo M-C, Lozano-Juste J, González-Guzmán M, Rodríguez L, Rodríguez PL, León J (2015) Inactivation of PYR/PYL/RCAR ABA receptors by tyrosine nitration may enable rapid inhibition of ABA signaling by nitric oxide in plants. *Sci Signal* 8: ra89
- Chaerle L, Van Der Straeten D (2000) Imaging techniques and the early detection of plant stress. *Trends Plant Sci* 5: 495–501
- Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* 323: 95–101
- Corpas FJ, Barroso JB (2013) Nitro-oxidative stress vs oxidative or nitrosative stress in higher plants. *New Phytol* 199: 633–635
- Couto D, Zipfel C (2016) Regulation of pattern recognition receptor signalling in plants. *Nat Rev Immunol* 16: 537–552
- Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, (1994) A central role of salicylic acid in plant disease resistance. *Science* 266: 1247–1250
- Ellinger D, Voigt CA (2014) Callose biosynthesis in *Arabidopsis* with a focus on pathogen response: what we have learned within the last decade. *Ann Bot* 114: 1349–1358
- Ellinger D, Naumann M, Falter C, Zwikowics C, Jamrow T, Manisseri C, Somerville SC, Voigt CA (2013) Elevated early callose deposition results in complete penetration resistance to powdery mildew in *Arabidopsis*. *Plant Physiol* 161: 1433–1444
- Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J* 35: 193–205
- Ferrari S, Galletti R, Denoux C, De Lorenzo G, Ausubel FM, Dewdney J (2007) Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiol* 144: 367–379
- Frerigmann H, Glawischnig E, Gigolashvili T (2015) The role of MYB34, MYB51 and MYB122 in the regulation of camalexin biosynthesis in *Arabidopsis thaliana*. *Front Plant Sci* 6: 654
- Frerigmann H, Pislewska-Bednarek M, Sánchez-Vallet A, Molina A, Glawischnig E, Gigolashvili T, Bednarek P (2016) Regulation of pathogen-triggered tryptophan metabolism in *Arabidopsis thaliana* by MYB transcription factors and indole glucosinolate conversion products. *Mol Plant* 9: 682–695
- García-Andrade J, Ramírez V, Flors V, Vera P (2011) *Arabidopsis* ocp3 mutant reveals a mechanism linking ABA and JA to pathogen-induced callose deposition. *Plant J* 67: 783–794
- Gaupels F, Kuruthukulangarakoola GT, Durner J (2011) Upstream and downstream signals of nitric oxide in pathogen defence. *Curr Opin Plant Biol* 14: 707–714
- Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy: analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* 20: 307–315
- Georgii E, Jin M, Zhao J, Kanawati B, Schmitt-Kopplin P, Albert A, Winkler JB, Schäffner AR (2017) Relationships between drought, heat and air humidity responses revealed by transcriptome-metabolome co-analysis. *BMC Plant Biol* 17: 120
- Ghirardo A, Koch K, Taipale R, Zimmer I, Schnitzler JP, Rinne J (2010) Determination of de novo and pool emissions of terpenes from four common boreal/alpine trees by 13C<sub>2</sub> labelling and PTR-MS analysis. *Plant Cell Environ* 33: 781–792
- Ghirardo A, Gutknecht J, Zimmer I, Brüggemann N, Schnitzler JP (2011) Biogenic volatile organic compound and respiratory CO<sub>2</sub> emissions after 13C-labeling: online tracing of C translocation dynamics in poplar plants. *PLoS ONE* 6: e17393
- Ghirardo A, Heller W, Fladung M, Schnitzler JP, Schroeder H (2012) Function of defensive volatiles in pedunculate oak (*Quercus robur*) is tricked by the moth *Tortrix viridana*. *Plant Cell Environ* 35: 2192–2207
- Ghirardo A, Wright LP, Bi Z, Rosenkranz M, Pulido P, Rodríguez-Concepción M, Niinemets Ü, Brüggemann N, Gershenzon J, Schnitzler JP (2014) Metabolic flux analysis of plastidic isoprenoid biosynthesis in poplar leaves emitting and nonemitting isoprene. *Plant Physiol* 165: 37–51
- Ghirardo A, Xie J, Zheng X, Wang Y, Grote R, Block K, Wildt J, Mentel T, Kändler-Scharr A, Hallquist M, (2016) Urban stress-induced biogenic VOC emissions and SOA-forming potentials in Beijing. *Atmos Chem Phys* 16: 2901–2920
- Glawischnig E (2007) Camalexin. *Phytochemistry* 68: 401–406

- Glawischnig E, Hansen BG, Olsen CE, Halkier BA (2004) Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. *Proc Natl Acad Sci USA* 101: 8245–8250
- Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol* 43: 205–227
- Groß E, Durner J, Gaupels F (2013) Nitric oxide, antioxidants and prooxidants in plant defence responses. *Front Plant Sci* 4: 419
- Heil M, Land WG (2014) Danger signals: damaged-self recognition across the tree of life. *Front Plant Sci* 5: 578
- Heitz T, Widemann E, Lugin R, Miesch L, Ullmann P, Désaubry L, Holder E, Grausem B, Kandel S, Miesch M, (2012) Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone jasmonoyl-isoleucine for catabolic turnover. *J Biol Chem* 287: 6296–6306
- Heitz T, Smirnova E, Widemann E, Aubert Y, Pinot F, Ménard R (2016) The rise and fall of jasmonate biological activities. In Y Nakamura, Y Li-Beisson, eds, *Lipids in Plant and Algae Development*. Springer, pp 405–426
- Himejima M, Hobson KR, Otsuka T, Wood DL, Kubo I (1992) Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa*: a defense mechanism against microbial invasion. *J Chem Ecol* 18: 1809–1818
- Huber DPW, Philippe RN, Madilao LL, Sturrock RN, Bohlmann J (2005) Changes in anatomy and terpene chemistry in roots of Douglas-fir seedlings following treatment with methyl jasmonate. *Tree Physiol* 25: 1075–1083
- Hull AK, Vij R, Celenza JL (2000) Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *Proc Natl Acad Sci USA* 97: 2379–2384
- Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* 15: 2503–2513
- Joudoi T, Shichiri Y, Kamizono N, Akaike T, Sawa T, Yoshitake J, Yamada N, Iwai S (2013) Nitrated cyclic GMP modulates guard cell signaling in Arabidopsis. *Plant Cell* 25: 558–571
- Kasten D, Mithöfer A, Georgii E, Lang H, Durner J, Gaupels F (2016) Nitrite is the driver, phytohormones are modulators while NO and H<sub>2</sub>O<sub>2</sub> act as promoters of NO<sub>2</sub>-induced cell death. *J Exp Bot* 67: 6337–6349
- Kasten D, Durner J, Gaupels F (2017) Gas alert: the NO<sub>2</sub> pitfall during NO fumigation of plants. *Front Plant Sci* 8: 85
- Kitaoka N, Matsubara T, Sato M, Takahashi K, Wakuta S, Kawaide H, Matsui H, Nabeta K, Matsuura H (2011) Arabidopsis CYP94B3 encodes jasmonyl-L-isoleucine 12-hydroxylase, a key enzyme in the oxidative catabolism of jasmonate. *Plant Cell Physiol* 52: 1757–1765
- Klepper L (1979) Nitric oxide (NO) and nitrogen dioxide (NO<sub>2</sub>) emissions from herbicide-treated soybean plants. *Atmos Environ* 13: 537–542
- Klepper L (1990) Comparison between NO<sub>x</sub> evolution mechanisms of wild-type and nr<sub>1</sub> mutant soybean leaves. *Plant Physiol* 93: 26–32
- Kliebenstein DJ, Rowe HC, Denby KJ (2005) Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. *Plant J* 44: 25–36
- Köhle H, Jeblick W, Poten F, Blaschek W, Kauss H (1985) Chitosan-elicited callose synthesis in soybean cells as a Ca-dependent process. *Plant Physiol* 77: 544–551
- Kolbert Z, Feigl G, Bordé Á, Molnár Á, Erdei L (2017) Protein tyrosine nitration in plants: present knowledge, computational prediction and future perspectives. *Plant Physiol Biochem* 113: 56–63
- Kolesnikov N, Hastings E, Keays M, Melnichuk O, Tang YA, Williams E, Dylag M, Kurbatova N, Brandizi M, Burdett T, (2015) ArrayExpress update: simplifying data submissions. *Nucleic Acids Res* 43: D1113–D1116
- Koo AJK, Cooke TE, Howe GA (2011) Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. *Proc Natl Acad Sci USA* 108: 9298–9303
- Lewis LA, Polanski K, de Torres-Zabala M, Jayaraman S, Bowden L, Moore J, Penfold CA, Jenkins DJ, Hill C, Baxter L, (2015) Transcriptional dynamics driving MAMP-triggered immunity and pathogen effector-mediated immunosuppression in Arabidopsis leaves following infection with *Pseudomonas syringae* pv *tomato* DC3000. *Plant Cell* 27: 3038–3064
- Lichtenthaler H, Miehe J (1997) Fluorescence imaging as a diagnostic tool for plant stress. *Trends Plant Sci* 2: 6–10
- Liu X, Hou F, Li G, Sang N (2015) Effects of nitrogen dioxide and its acid mist on reactive oxygen species production and antioxidant enzyme activity in Arabidopsis plants. *J Environ Sci (China)* 34: 93–99
- Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: a multifaceted plant defense response. *Mol Plant Microbe Interact* 24: 183–193
- Maassen A, Hennig J (2011) Effect of *Medicago sativa* Mhb1 gene expression on defense response of Arabidopsis thaliana plants. *Acta Biochim Pol* 58: 427–432
- Martin DM, Fäldt J, Bohlmann J (2004) Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. *Plant Physiol* 135: 1908–1927
- Mata-Pérez C, Begara-Morales JC, Chaki M, Sánchez-Calvo B, Valderrama R, Padilla MN, Corpas FJ, Barroso JB (2016a) Protein tyrosine nitration during development and abiotic stress response in plants. *Front Plant Sci* 7: 1699
- Mata-Pérez C, Sánchez-Calvo B, Padilla MN, Begara-Morales JC, Luque F, Melguizo M, Jiménez-Ruiz J, Fierro-Risco J, Peñas-Sanjuán A, Valderrama R, (2016b) Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in Arabidopsis. *Plant Physiol* 170: 686–701
- Mengiste T (2012) Plant immunity to necrotrophs. *Annu Rev Phytopathol* 50: 267–294
- Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, Thomas PD (2017) PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res* 45: D183–D189
- Miersch O, Neumerkel J, Dippe M, Stenzel I, Wasternack C (2008) Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. *New Phytol* 177: 114–127
- Mishina TE, Zeier J (2007) Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant J* 50: 500–513
- Müller TM, Böttcher C, Morbitzer R, Götz CC, Lehmann J, Lahaye T, Glawischnig E (2015) TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASE-mediated generation and metabolic analysis of camalexin-deficient *cyp71a12 cyp71a13* double knockout lines. *Plant Physiol* 168: 849–858
- Mur LAJ, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova GV, Hall MA, Harren FJM, Hebelstrup KH, Gupta KJ (2013) Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* 5: pls052
- Nafis M, Goregaoker S, Botanga CJ, Glawischnig E, Olsen CE, Halkier BA, Glazebrook J (2007) Arabidopsis cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. *Plant Cell* 19: 2039–2052
- Nawrath C, Métraux JP (1999) Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11: 1393–1404
- Niinemets U (2010) Mild versus severe stress and BVOCs: thresholds, priming and consequences. *Trends Plant Sci* 15: 145–153
- Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301: 969–972
- Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. *Plant J* 31: 1–12
- Patkar RN, Benke PI, Qu Z, Chen YY, Yang F, Swarup S, Naqvi NI (2015) A fungal monooxygenase-derived jasmonate attenuates host innate immunity. *Nat Chem Biol* 11: 733–740
- Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. *Annu Rev Cell Dev Biol* 28: 489–521
- Pryor WA, Houk KN, Foote CS, Fukuto JM, Ignarro LJ, Squadrito GL, Davies KJ (2006) Free radical biology and medicine: it's a gas, man! *Am J Physiol Regul Integr Comp Physiol* 291: R491–R511
- Radi R (2013) Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects. *Acc Chem Res* 46: 550–559
- Ramonell K, Berrocal-Lobo M, Koh S, Wan J, Edwards H, Stacey G, Somerville S (2005) Loss-of-function mutations in chitin responsive genes show increased susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum*. *Plant Physiol* 138: 1027–1036

- Rauhut T, Luberaack B, Seitz HU, Glawischnig E (2009) Inducible expression of a Nep1-like protein serves as a model trigger system of camalexin biosynthesis. *Phytochemistry* 70: 185–189
- R Core Team (2014) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna
- Riedlmeier M, Ghirardo A, Wenig M, Knappe C, Koch K, Georgii E, Dey S, Parker JE, Schnitzler JP, Vlot C (2017) Monoterpenes support systemic acquired resistance within and between plants. *Plant Cell* 29: 1440–1459
- Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43: e47
- Robert-Seilaniantz A, Grant M, Jones JDG (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu Rev Phytopathol* 49: 317–343
- Roden LC, Ingle RA (2009) Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant-pathogen interactions. *Plant Cell* 21: 2546–2552
- Rogers EE, Glazebrook J, Ausubel FM (1996) Mode of action of the Arabidopsis thaliana phytoalexin camalexin and its role in Arabidopsis-pathogen interactions. *Mol Plant Microbe Interact* 9: 748–757
- Sakamoto A, Sakurao SH, Fukunaga K, Matsubara T, Ueda-Hashimoto M, Tsukamoto S, Takahashi M, Morikawa H (2004) Three distinct Arabidopsis hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration. *FEBS Lett* 572: 27–32
- Sakihama Y, Tamaki R, Shimoji H, Ichiba T, Fukushi Y, Tahara S, Yamasaki H (2003) Enzymatic nitration of phytophenolics: evidence for peroxynitrite-independent nitration of plant secondary metabolites. *FEBS Lett* 553: 377–380
- Schopfer FJ, Cipollina C, Freeman BA (2011) Formation and signaling actions of electrophilic fatty acids. *Chem Rev* 111: 5997–6021
- Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatos A, Halkier BA, Glawischnig E (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol* 141: 1248–1254
- Shibata H, Kono Y, Yamshita S, Sawa Y, Ochiai H, Tanaka K (1995) Degradation of chlorophyll by nitrogen dioxide generated from the peroxidase reaction. *Biochim Biophys Acta* 1230: 45–50
- Shimazaki KI, Yu SW, Sakaki T, Tanaka K (1992) Differences between spinach and kidney bean plants in terms of sensitivity to fumigation with NO<sub>2</sub>. *Plant Cell Physiol* 33: 267
- Smirnova E, Marquis V, Poirier L, Aubert Y, Zumsteg J, Ménard R, Miesch L, Heitz T (2017) Jasmonic Acid Oxidase 2 hydroxylates jasmonic acid and represses basal defense and resistance responses against Botrytis cinerea infection. *Mol Plant* 10: 1159–1173
- Smith CA O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G (2005) METLIN: a metabolite mass spectral database. *Therapeutic Drug Monitoring* 27: 747–751
- Smyth GK (2005) Limma: linear models for microarray data. In R Gentleman, VJ Carey, W Huber, RA Irizarry, S Dudoit, eds, *Bioinformatics and Computational Biology Solutions Using R and Bioconductor: Statistics for Biology and Health*. Springer, New York, pp 397–420
- Sparks JP (2009) Ecological ramifications of the direct foliar uptake of nitrogen. *Oecologia* 159: 1–13
- Srivastava HS, Ormrod DP, Hale BA (1994) Responses of greening bean seedling leaves to nitrogen dioxide and nutrient nitrate supply. *Environ Pollut* 86: 37–42
- Stintzi A, Browse J (2000) The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. *Proc Natl Acad Sci USA* 97: 10625–10630
- Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proc Natl Acad Sci USA* 98: 12837–12842
- Suhre K, Schmitt-Kopplin P (2008) MassTRIX: mass translator into pathways. *Nucleic Acids Res* 36: W481–W484
- Supek F, Bošnjak M, Škunca N, Šmuc T (2011) REVIGO summarizes and visualizes long lists of Gene Ontology terms. *PLoS ONE* 6: e21800
- Takahashi M, Furuhashi T, Ishikawa N, Horiguchi G, Sakamoto A, Tsukaya H, Morikawa H (2014) Nitrogen dioxide regulates organ growth by controlling cell proliferation and enlargement in Arabidopsis. *New Phytol* 201: 1304–1315
- Tholl D, Lee S (2011) Terpene specialized metabolism in Arabidopsis thaliana. *The Arabidopsis Book* 9: e0143
- Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolucci N, Ambs S, (2008) The chemical biology of nitric oxide: implications in cellular signaling. *Free Radic Biol Med* 45: 18–31
- Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci USA* 95: 15107–15111
- Vadassery J, Reichelt M, Hause B, Gershenzon J, Boland W, Mithöfer A (2012) CML42-mediated calcium signaling coordinates responses to Spodoptera herbivory and abiotic stresses in Arabidopsis. *Plant Physiol* 159: 1159–1175
- Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol* 47: 177–206
- von Saint Paul V, Zhang W, Kanawati B, Geist B, Faus-Kessler T, Schmitt-Kopplin P, Schäffner AR (2011) The Arabidopsis glucosyltransferase UGT76B1 conjugates isoleucic acid and modulates plant defense and senescence. *Plant Cell* 23: 4124–4145
- Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in *Annals of Botany*. *Ann Bot* 111: 1021–1058
- Weigl E, Ghirardo A, Schnitzler JP, Pritsch K (2016) Sesquiterpene emissions from Alternaria alternata and Fusarium oxysporum: effects of age, nutrient availability, and co-cultivation. *Sci Rep* 6: 22152
- Wellburn AR (1990) Tansley Review No. 24. Why are atmospheric oxides of nitrogen usually phytotoxic and not alternative fertilizers? *New Phytol* 115: 395–429
- Widemann E, Miesch L, Lugan R, Holder E, Heinrich C, Aubert Y, Miesch M, Pinot F, Heitz T (2013) The amidohydrolases IAR3 and ILL6 contribute to jasmonoyl-isoleucine hormone turnover and generate 12-hydroxyjasmonic acid upon wounding in Arabidopsis leaves. *J Biol Chem* 288: 31701–31714
- Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414: 562–565
- Xu Q, Zhou B, Ma C, Xu X, Xu J, Jiang Y, Liu C, Li G, Herbert SJ, Hao L (2010) Salicylic acid-altering Arabidopsis mutants response to NO<sub>2</sub> exposure. *Bull Environ Contam Toxicol* 84: 106–111
- Yoneyama T, Sasakawa H (1979) Transformation of atmospheric NO<sub>2</sub> absorbed in spinach leaves. *Plant Cell Physiol* 20: 263–266
- Zeevaert AJ (1976) Some effects of fumigating plants for short periods with NO<sub>2</sub>. *Environ Pollut* 11: 97–108
- Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428: 764–767