Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes

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Lipopolysaccharides (LPS) are cell-surface components of Gram-negative bacteria and are microbe-/pathogen-associated molecular patterns in animal pathosystems. As for plants, the molecular mechanisms of signal transduction in response to LPS are not known. Here, we show that *Arabidopsis thaliana* reacts to LPS with a rapid burst of NO, a hallmark of innate immunity in animals. Fifteen LPS preparations (among them *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Erwinia carotovora*) as well as lipoteichoic acid from Gram-positive *Staphylococcus aureus* were found to trigger NO production in suspension-cultured *Arabidopsis* cells as well as in leaves. NO was detected by confocal laser-scanning microscopy in conjunction with the fluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate, by electron paramagnetic resonance, and by a NO synthase (NOS) assay. The source of NO was addressed by using T-DNA insertion lines. Interestingly, LPS did not activate the pathogen-inducible *varP* NOS, but *AtNOS1*, a distinct NOS previously associated with hormonal signaling in plants. A prominent feature of LPS treatment was activation of defense genes, which proved to be mediated by NO. Northern analyses and transcription profiling by using DNA microarrays revealed induction of defense-associated genes both locally and systemically. Finally, AtNOS1 mutants showed dramatic susceptibility to the pathogen *Pseudomonas syringae* pv. tomato DC3000. In sum, perception of LPS and induction of NOS contribute toward the activation of plant defense responses.

The past few years have seen dramatic changes in our understanding of the molecular principles of disease resistance. A growing body of evidence indicates that some principles involved in innate immunity in mammalian and insect systems are strikingly similar to the molecular mechanisms underlying plant disease-resistance responses (1). It has been proposed, therefore, that innate immunity might be an evolutionarily ancient system of host defense (2).

In many cases, the plant response is initiated by a "gene-for-gene" interaction that involves a dominant *R* gene in the plant and a corresponding avirulence (*avr*) gene in the pathogen (3, 4). However, in addition to the *R* gene-mediated and highly specific mechanisms, plants have acquired the ability to recognize basal and more general elicitors of plant defense. It is a key function of innate immunity in animals and plants to recognize invariant pathogen-associated molecular patterns (PAMPs) that are characteristic of microbial organisms but that are not found in potential hosts (5, 6). PAMPs include peptidoglycans, lipoteichoic acid (LTA) of Gram-positive bacteria, and lipopolysaccharides (LPS) of Gram-negative bacteria. In insects and vertebrates, perception of PAMPs frequently is mediated by Toll-like receptors (TLRs) containing extracellular leucine-rich repeats (7). As recently demonstrated for flagellin perception, there is remarkable conservation in the recognition of PAMPs by plants, insects, and mammals (7–9).

LPS are a key component of the outer membrane of Gram-negative bacteria and serve in many experimental systems as a prototypic model PAMP (10). LPS have been shown to activate the synthesis of antimicrobial peptides in *Drosophila*, as well as the production of immunoregulatory and cytotoxic molecules in humans (11). One of the most important hallmarks of innate immunity activation by LPS is the induction of cellular mediators and antimicrobial defense mechanisms such as the production of NO (12), a molecule whose importance in plant defense is just emerging (13).

In contrast to the well documented effects of LPS on mammalian cells, much remains to be elucidated about the effect of LPS on plants. LPS from nonpathogenic plant growth-promoting rhizobacteria can stimulate plants to develop induced systemic resistance. In the systemic protection of carnation against *Fusarium* wilt by *Pseudomonas fluorescens* WCS417, LPS induced resistance just as live bacteria (14). LPS extracted from an endophytic strain of *Burkholderia cepacia* had a protective effect on the *Nicotiana tabacum–Phytophthora nicotianae* interaction (15). In radish, bacterial mutants lacking the O-antigen (OA) did induce systemic resistance (14).

Currently, strong efforts are being made to elucidate the molecular mechanisms of LPS in the stimulation of plant defense. LPS from various sources could trigger defense responses in pepper, without the synthesis of the resistance-related salicylic acid and without the triggering of an oxidative burst (16). In contrast, LPS from the phytopathogen *Xanthomonas campestris* pv. *campestris* could induce an oxidative burst in tobacco cells (17), and LPS isolated from *B. cepacia* were found to trigger a rapid influx of Ca^{2+} into the cytoplasm (18).

We have examined whether one of the most prominent features of animal innate immunity, LPS-mediated NO production, is apparent in plants. In the present work, we show that LPS from animal and plant pathogens induce NO synthase (AtNOS1) and activate an array of defense genes in *Arabidopsis thaliana*. NO proved to play an important role in defense-gene induction as well as in basal resistance.

Materials and Methods

Cell Culture and Plant Material. *A. thaliana* suspension cells were grown in the dark in medium modified after Murashige and Skoog (19). *A. thaliana* eco. type Col were grown as described in ref. 20.

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Abbreviations: DA, diacetate; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; LPS, lipopolysaccharides; LTA, lipoteichoic acid; NOS, NO synthase; iNOS, pathogen-inducible NOS; NR, nitrate reductase; PAMP, pathogen-associated molecular patterns; PR, pathogenesis related; TLR, Toll-like receptor.

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LPS. LPS were dissolved at 1 mg/ml in water containing 2.5 mM $MgCl_2$ plus 1 mM $CaCl_2$ and shaken for 3 h on a rotary shaker (300 rpm). Lipid A and LTA were prepared in water (1 mg/ml). The working concentration for all experiments was 100 μg/ml. If not mentioned otherwise, experiments were performed with LPS from an endophytic strain of *B. cepacia* (ASP B 2D) or for control with buffer A containing 1.0 mM $CaCl_2$ and 2.5 mM $MgCl_2$ (pH 7.6) (18).

Microscopy. Arabidopsis *cells.* For confocal laser-scanning microscopy, 100 μl of cell suspension was placed on a coverslip bottom dish and treated with 100 μg/ml LPS (buffer A for control) and 5 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). Settings and laser of the Zeiss Axiovert 100M inverted microscope were as described in ref. 20.
Arabidopsis *leaves.* Epidermal abaxial peels were treated with LPS or buffer A and with DAF-FM DA (5 μM). Images were obtained with a Canon G2 camera. Autofluorescence of chloroplasts was captured with a 585-nm long-pass filter.

Electron Paramagnetic Resonance (EPR) Imaging of NO. For EPR analysis of NO, 500 μl of cells were harvested 10 min after LPS or buffer A treatment and then were incubated in 0.6 ml of buffered solution (50 mM Hepes/1 mM DTT/1 mM $MgCl_2$, pH 7.6) at 37°C for 2 min. After sample preparation, the supernatant was added to 300 μl of freshly made Fe_2/diethyldithiocarbamate solution (21) and analyzed with a Bruker ESP300 X-band spectrometer (Billerica, MA) (20).

Fluorimetric Quantification of NO. To monitor the NO accumulation in LPS-treated *Arabidopsis* cells or leaf peels, the DAF-FM DA fluorescence was measured with a GENios plate reader (Tecan Group, Maennedorf, Switzerland) with FITC excitation and emission filters. Fluorescence intensity was measured every minute for 30 min. The plate was rocked before measuring for 20 sec.

Determination of NOS Activity. One gram of frozen leaves was pounded together with NOS extraction buffer in liquid nitrogen by using a mortal and pestle as described in ref. 22. The resulting protein solution was used to measure NOS activity with the NOS assay kit from Sigma (22, 23).

Northern Analysis. Northern analyses followed standard protocols (digoxygenin method; Roche Diagnostics). Probes were amplified in full from the EST clones corresponding to At2g14610 (PR1), At3g57260 (PR2), At3g12500 (PR3), At3g04720 (PR4), and At1g75040 (PR5).

Microarray Analyses. Microarray analyses were performed as described in refs. 19 and 24 with some modifications. Briefly, amino-modified PCR products were arrayed onto silylated microscope slides (CEL Associates, Houston) by using a DNA array robot (GMR, Cambridge, U.K.).

Probes were made by using an indirect aminoallyl labeling method with Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia) and purified according to standard protocols (19, 24). The arrays were scanned by using an Axon GenePix 4000 scanner (Axon Instruments, Union City, CA) and the GENEPIX PRO 4.1 and ACUITY (Axon Instruments) software packages.

Bacterial Growth Assay. The bacterial strain used in this study was *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Growth of bacteria and spray inoculations were performed as described in ref. 9. In brief, overnight *Pst* DC3000 cultures were collected, washed, and resuspended in sterile water. Plants were sprayed with a bacterial suspension containing 5×10^8 colony-forming units per milliliter bacteria. Extracts from leaf discs (from three different leaves) were prepared as described and analyzed in a colony-forming units test (9).

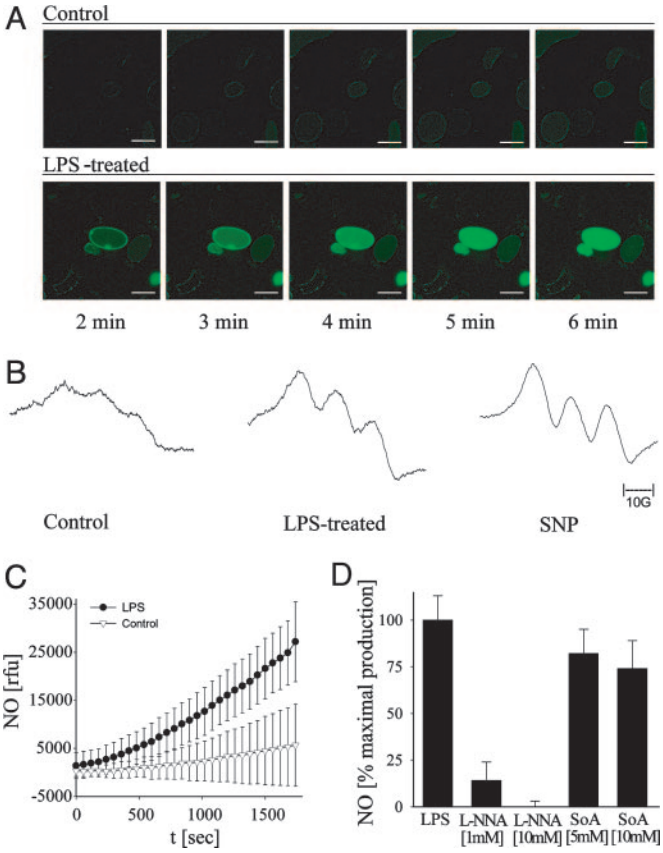

Fig. 1. LPS induce a NO burst in *Arabidopsis* suspension cells. (*A*) Time course of the LPS-induced NO burst as detected by confocal laser-scanning microscopy. *Arabidopsis* cells were loaded with 5 μM DAF-FM DA and treated with buffer (*Upper*) or LPS (*B. cepacia*; 100 μg/ml) (*Lower*). Green fluorescence is indicative for NO. (Scale bars, 25 μm.) (*B*) LPS-induced increases of NO in *Arabidopsis* cells as detected by EPR. NO was detected by EPR by using the spin trap Fe_2 plus diethyldithiocarbamate. Shown are an extract obtained from untreated *Arabidopsis* cells, an extract from cells 10 min after LPS treatment, and a NO control (5 μM sodium nitroprusside in Hepes). The signals were recorded at identical EPR settings. (*C*) Time course of NO burst after LPS treatment. NO production was estimated by measuring fluorescence intensity with a microplate reader. The values (relative units) represent a mean of 25 independent experiments. (*D*) Effects of NOS and NR inhibitors on LPS-induced NO burst. *Arabidopsis* cells were treated with LPS and analyzed for NO by using 1 μM DAF-FM DA. In the case of inhibitor studies, cells were pretreated for 10 min with L-NNA or SoA before addition of LPS. Values represent a mean of five independent experiments.

Results

LPS Induce a Strong and Rapid NO Burst in *A. thaliana* Cells. LPS are strong inducers of mammalian innate immunity including NO production, and activation of pathogen-inducible NOS (iNOS) by LPS is the most applied readout to analyze innate immune responses (11). Because NO seems to be a key player regulating plant defense response (25), we examined the potential of LPS to induce a NO burst in *Arabidopsis* suspension cells. Real-time imaging of NO is best performed with DAF-FM DA in combination with confocal laser-scanning microscopy (26, 27).

Fig. 1*A* shows real-time imaging of NO production in *Arabidopsis* cells after loading with 5 μM DAF-FM DA and subsequent LPS treatment (100 μg/ml). Effective LPS concentrations were between 10 and 200 μg/ml, concentrations that are routinely applied by others (17, 18). LPS treatment resulted in a

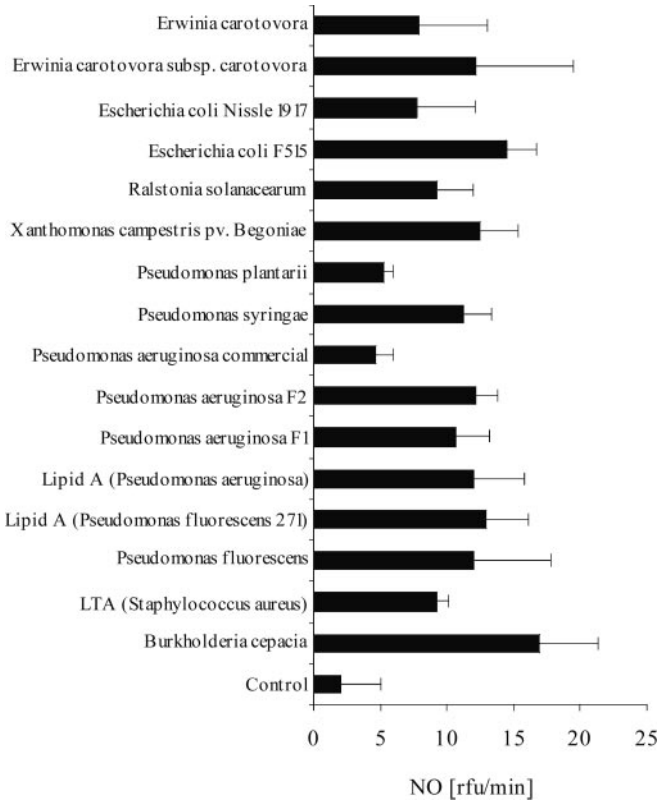

Fig. 2. Comparison of LPS-induced NO burst by diverse LPS preparations, Lipid A, and LTA. Cells were treated with the same concentration (100 μg/μl) of LPS, Lipid A, or LTA and/or 1 μM DAF-FM diacetate as described (Fig. 1 *C* and *D*). NO production was determined with a microplate reader (Fig. 1*C*). Values are expressed as NO production per minute and represent a mean of 10 independent experiments.

rapid burst of green fluorescence, indicative of NO production (*Lower*). In our hands, the effective minimum concentration of *B. cepacia* LPS was 20 μg/ml, and *Ralstonia* LPS showed activity at 50 μg/ml. In animals, TLR4-mediated signal transduction in macrophages is sensitive to LPS in the picograms-per-milliliter range (28). Conversely, the activation of leukocytes, resulting in the oxidative burst, required LPS in the low micrograms-per-milliliter range (29). To characterize also the less-active preparations shown by Fig. 2, we used 100 μg/ml as the standard concentration.

To specifically detect NO, the use of more than one technique is highly recommended (20). Probably the most specific NO detection is EPR imaging with Fe^2 plus diethyldithiocarbamate as spin trap (20, 21). The presented data clearly demonstrate strong NO production 10 min after LPS treatment in *Arabidopsis* cells (Fig. 1*B*). To quantitate NO in LPS-treated *Arabidopsis* cells, a spectrofluorometric assay was developed, by using DAF-FM DA and a multiwell-plate reader. Here, we demonstrate the time course of LPS-induced NO after treatment with *B. cepacia* LPS (Fig. 1*C*). For an estimation of NO production, see Fig. 2.

In plants, NO can be produced by NOS-like enzymes or by nitrate reductase (NR) (22, 30, 31). To find out which NO-source becomes activated by LPS, we first resorted to a pharmacological approach. LPS-induced NO production in *Arabidopsis* cells was reduced dramatically by the general NOS inhibitor N_ω-nitro-L-arginine (L-NNA) (Fig. 1*D*). However, the LPS-induced NO burst was insensitive to sodium azide (SoA), a potent inhibitor of NR, indicating that NR is not involved in NO synthesis (Fig. 1*D*).

Many LPS preparations (also commercial ones) contain other bacterial components such as peptidoglycans that can stimulate animal cells independently of LPS (7). We used as many as 18 different LPS batches (shown are 15) from an array of plant- or animal-associated bacteria and prepared by several different laboratories. Here, we show that LPS from *Pseudomonas aeruginosa, P. fluorescens, Erwinia carotovora, Escherichia coli*, *B. cepacia*, and others induce immediate production of NO in *Arabidopsis* cells (Fig. 2). *P. aeruginosa* and *B. cepacia* are opportunistic pathogens in cystic fibrosis patients. Other *B. cepacia* isolates, like the one shown, have been used as biocontrol agents in agriculture. The strong response of *Arabidopsis* cells toward lipid A suggests that this component may be at least partially responsible for LPS perception by plants. In addition to LPS, *Arabidopsis* cells responded strongly to LTA from *Staphylococcus aureus* (Fig. 2). If calibrated against DAF-T (the fluorescent adduct of NO and DAF-FM) real-time NO production within the cells can be estimated. Here, the control produced 0.06 nmol of NO per g fresh weight per min, and the highest induction was found after stimulation with *B. cepacia* LPS (0.49 nmol per g fresh weight per min).

LPS Induces *A. thaliana* NOS (AtNOS1). To distinguish between the two reported plant NOS enzymes (varP, a glycine decarboxylase subunit variant, and AtNOS1, a plant homologue of snail NOS) (22, 30) and to verify the data on the NO burst in *Arabidopsis* suspension cells, we analyzed the action of LPS in epidermal cells of *Arabidopsis* leaves. Epidermal (abaxial) peels were loaded with DAF-FM DA and analyzed with fluorescence microscopy as described in refs. 20 and 27. LPS-induced NO production became apparent within a few minutes (Fig. 3*A e* and *f*). The NO scavenger 2-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) (100 μM) suppressed the elicited bursts of fluorescence (Fig. 3*Ad*).

The spectrofluorometric assay to detect NO accumulation (Fig. 2) was used to assay peels of wild-type, a varP-iNOS mutant (Salk T-DNA insertion line no. 110091), and AtNOS1 mutant plants (22). After stimulation with LPS, NO production in the varP-iNOS insertion line wild-type leaves was approximately as high as in wild-type plants, indicating that variant P is not involved in LPS-triggered NO synthesis (Fig. 3*B*). In contrast, in the AtNOS1 mutant the LPS-induced NO was reduced by ≈80%, suggesting that it is the AtNOS1 enzyme that is generating the LPS-stimulated NO. To analyze the NO burst in more detail, leaf extracts were assayed for NOS activity by using a conventional citrulline/arginine assay (22). Here, basal NOS activity was 3.6 pmol per mg·min. Immediately after LPS treatment, NOS activity could not be reduced by the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA). In contrast, the significantly increased NOS activity 20 min after LPS administration was clearly repressed (4-fold) by L-NMMA (Fig. 3*C*). These results confirm that AtNOS1 is involved in LPS-induced NO production.

Gene Induction by LPS. Host defense becomes apparent not only by triggering production of reactive oxygen and NO, but also in induction of defense genes (9). Almost no data are available for gene induction by LPS. However, LPS pretreatment potentiated the expression of several genes involved in defense upon subsequent bacterial infection (16). This finding, together with our results on NO production after LPS treatment, prompted us to analyze for alterations in plant gene expression after LPS stimulation.

We studied gene expression dynamics in LPS-treated *Arabidopsis* plants by using a (biased) custom-designed cDNA microarray that included ≈700 defense-related genes encoding pathogenesis-related (PR) proteins or proteins induced by pathogens and abiotic stresses (19). Array hybridizations were

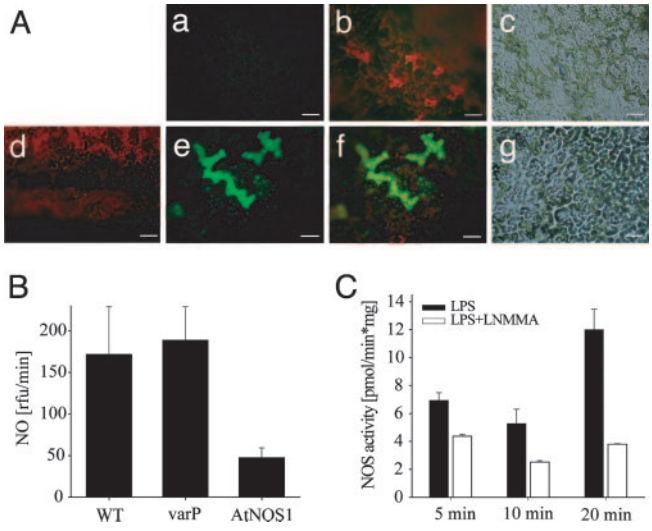

Fig. 3. LPS elicited NO and NOS activity in *Arabidopsis* plants. (*A*) Fluorescence microscopy of LPS-induced increases in intracellular DAF-FM DA signals in epidermal cells from *A. thaliana*. The lower epidermis of *Arabidopsis* leaves was loaded with 1 μM DAF-FM DA in absence (*Upper*) or presence (*B. cepacia*; 100 μg/ml) (*Lower*) of LPS. The images were obtained 10 min after LPS treatment under bright field (*c* and *g*) and under fluorescence light (green light filter, 505–530 nm) (*a*, *b*, and *d–f*). Chlorophyll fluorescence was captured with a long-pass filter (585 nm) (*b*, *d*, and *f*). *d* shows an LPS-treated leaf coinfiltrated with the NO scavenger PTIO (1 mM). (Scale bars, 100 μm.) (*B*) LPS-induced NO in epidermal cells of *Arabidopsis* wild-type (WT), variantP-iNOS (varP), and AtNOS1 mutant plants. The NO burst was determined with a microplate reader during the first 60 min of treatment. The relative values represent a mean of four independent experiments. (*C*) NOS activity in wild-type *Arabidopsis* leaf extracts after LPS treatment. LPS-treated and control leaves were harvested at different time points, and an extract of leaf tissue was prepared. The NOS activity was determined with the NOS assay kit. Values represent a mean of four independent experiments.

based on two independent biological repeats, with two technical replicates each (hybridization) plus a red-green dye-swap. Only signals >2-fold above local background level were considered, and values with coefficient of variation values >10 were omitted. These very rigorous criteria imply that our procedure ignores such genes with relatively low basal expression ratios.

The results shown in Fig. 4 demonstrate that LPS induces an array of defense or stress-associated genes including glutathione S-transferases, cytochrome P450, and many genes encoding PR proteins (for a complete set of data see Table 1, which is published as supporting information on the PNAS web site). Interestingly, whereas the local response is stronger, several of the LPS-induced genes were activated in systemic leaves, too. Most importantly, gene expression was abolished almost completely in *AtNOS1* mutant plants. This result suggests a functional link between LPS-induced NO production and gene induction. To verify the induction of defense genes by LPS, we performed Northern blot analysis for several PR genes. Again, gene expression became apparent both in local (treated) and, albeit to a much lesser extent, in systemic tissues (Fig. 5). We want to emphasize again, however, that our application-based array is biased toward stress and defense genes. To get a clear picture of transcriptional changes after LPS treatment, genome-covering arrays should be used.

NOS Is Involved in Bacterial Disease Resistance. Because NO is involved in disease resistance in plants (32), we tested whether plants lacking NOS are more susceptible to pathogenic bacteria. We therefore infected *A. thaliana* plants by spraying *P. syringae* pv. *tomato* DC3000 bacteria onto leaf surfaces. Under these

Name	acc#	WT local 4 hr	WT local 8 hr	WT local 48 hr	WT sytemic 24 hr	WT sytemic 48 hr	*AtNOS1* local 24 hr	*AtNOS1* local 48 hr	cells 0.5 hr	cells 1 hr	cells 2 hr	cells 8 hr	cells 24 hr
ABC transporter family protein	At1g59870	0.59	1.68	2.15	0.45	1.31	0.83	1.05	2.14	2.62	1.73	2.26	2.32
ABC transporter homolog PnATH - like	At5g60790	2.17	0.92	0.93	1.09	0.80	-	-	1.46	1.05	1.34	1.86	2.81
Atpm24.1 glutathione S transferase	At4g02520	0.19	5.52	2.59	0.40	1.61	0.71	0.73	2.44	1.92	3.07	2.51	1.76
blue copper binding protein	At5g20230	0.45	2.37	2.27	0.74	1.38	0.78	1.08	1.74	2.22	1.43	0.67	0.78
cytochrome P450 71B15, putative	At3g26830	0.19	14.57	4.20	0.27	1.70	-	-	2.76	1.75	4.25	2.78	2.31
cytochrome P450 family protein	At2g24180	0.55	2.08	1.42	0.85	1.40	0.89	1.03	1.16	0.59	1.24	1.14	1.70
cytochrome P450 family protein	At2g45560	2.21	0.75	0.57	1.15	0.89	0.68	-	0.74	-	0.91	1.31	1.08
cytochrome P450 family protein	At4g12320	1.89	0.60	0.57	1.15	1.03	1.34	-	-	-	0.41	0.59	0.22
cytochrome P450 family protein	At4g37370	0.37	5.28	2.20	0.48	1.57	-	-	0.79	1.00	1.21	0.88	0.99
fasciclin-like arabinogalactan-protein 7	T88134	0.67	2.49	1.98	0.72	1.22	0.98	-	1.40	1.38	2.06	0.90	1.14
flavanone 3-hydroxylase (FH3);	At3g51240	0.48	1.72	1.95	0.72	1.29	0.81	1.11	1.10	0.53	1.42	1.58	1.09
germin-like protein (GLP4)	At1g09560	0.41	1.58	2.07	0.93	1.22	0.73	1.58	0.85	-	1.09	1.05	1.43
glutathione peroxidase 1	At2g25080	2.04	2.52	0.61	1.01	0.91	1.18	1.02	0.74	-	0.86	0.98	1.24
glutathione reductase	At3g24170	0.44	2.18	1.55	0.76	1.30	0.83	1.12	1.38	0.95	1.21	1.55	1.86
glutathione S-transferase	At1g02920	0.49	3.49	3.20	0.21	1.87	0.64	1.11	1.92	1.88	2.67	0.88	1.75
glutathione S-transferase (GST14)	At5g62480	0.19	4.91	2.48	0.39	1.63	-	0.81	-	-	-	-	-
gluthatione s-transferase	At1g02930	0.65	2.72	2.95	0.25	1.42	0.76	1.19	1.75	1.74	2.41	3.18	1.42
HIN1 family protein	At2g35980	0.44	1.81	1.50	1.02	1.18	0.97	-	2.60	3.00	1.47	1.12	1.66
pathogenesis-related protein 1 (PR-1)	At2g14610	0.10	10.86	3.63	0.12	6.10	0.71	1.09	0.83	0.28	0.90	1.01	1.06
pathogenesis-related protein 2 (PR-2)	At3g57260	0.25	4.86	2.18	0.26	2.32	0.75	1.43	0.42	-	0.57	1.23	0.44
pathogenesis-related protein 3 (PR-3)	At3g12500	0.42	2.45	2.03	0.90	1.57	0.88	1.12	1.63	1.41	0.90	1.16	7.07
pathogenesis-related protein 4 (PR-4)	At3g04720	0.62	2.59	2.48	0.41	1.52	0.78	0.94	1.70	1.78	1.92	1.04	1.83
pathogenesis-related protein 5 (PR-5)	At1g75040	0.12	6.47	3.43	0.27	2.47	0.73	1.57	0.38	-	0.74	1.19	0.54
peroxidase; prxCb	At3g49120	0.41	2.46	2.30	0.71	1.63	0.62	0.94	1.56	1.62	1.40	1.23	2.00
RuBisCO activase	At2g39730	2.33	0.66	0.79	0.92	0.68	-	1.16	1.33	1.12	1.03	1.64	1.54
Triose phosphate isomerase	U02949	0.36	3.13	2.00	0.59	1.47	-	-	-	-	-	-	-
violaxanthin de-epoxidase precursor	TC109750	2.03	0.98	0.78	0.91	0.82	1.12	-	0.91	0.86	0.96	0.93	1.04

Fig. 4. DNA microarray analyses of transcriptional changes in *A. thaliana* plants (WT and *AtNOS1*) and suspension cells in response to LPS treatment. At the indicated time points after LPS treatment, mRNA from local and systemic leaf tissue or cells was hybridized to the cDNA array. A complete data set is presented in Table 1. Here, we present genes that respond to LPS in both test-systems (cells and leaves). White boxes, activation ≤1.5-fold; yellow, genes with >1.5- to <2.0-fold activation; light orange, 2.0- to <2.5-fold activation; orange, 2.5- to <3.0-fold activation; red, activation ≥3.0-fold. Greenish colors indicate repression. Gray numbers indicate weak signals <2-fold higher than surrounding background. The genes are arranged in alphabetical order.

conditions, *AtNOS1* plants showed a faster and much more severe development of disease symptoms than wild-type plants. These stronger symptoms correlated with higher numbers of bacteria in *AtNOS1* leaves (Fig. 6). The water-treated control did not show any symptoms (data not shown). Thus, AtNOS1 is involved in bacterial disease resistance in *A. thaliana*.

Discussion

Specific *R*-mediated innate immunity is only one aspect of plant resistance against pathogens. PAMPs that trigger innate immune responses in various vertebrate and invertebrate organisms have long been known to act as general elicitors of defense responses in a multitude of plant species, too (33). In animals, one of the most important hallmarks of innate immunity is the LPS-mediated induction of NO production (11, 34), a molecule whose importance in plant growth and defense is just emerging (13).

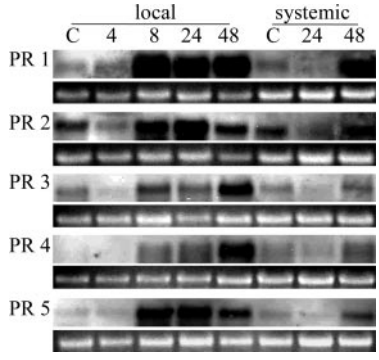

Fig. 5. Induction of local and systemic PR gene expression in *Arabidopsis* leaves by LPS. *Arabidopsis* leaves were treated with LPS (100 μg/ml) and collected at the times indicated for RNA preparation (4–48 h). Northern blots were probed with cDNAs for PR1, PR2, PR3, PR4, and PR5. Shown is the region between 1.8 and 1.0 kb. Ethidium bromide staining shows equal loading.

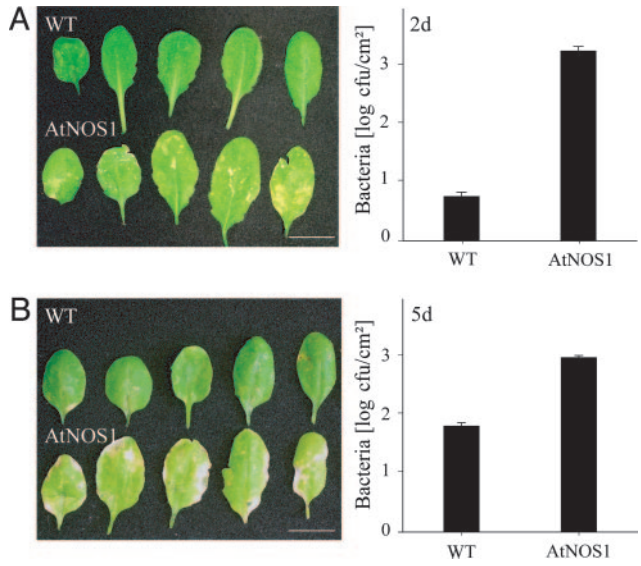

Fig. 6. An AtNOS1 mutant shows enhanced disease susceptibility against *Pst* DC3000. Wild-type (WT) and *AtNOS1* mutant plants were sprayed with *Pst* DC3000 bacteria or with water and photographed 2 (*A*) and 5 (*B*) days later, respectively. (*Left*) Symptoms after 2 and 5 days in a series of leaves. (*Right*) Bar graphs indicate the number of *Pst* DC3000 bacteria extracted from wild-type and *AtNOS1* mutant plants 2 and 5 days after infection, respectively.

NO and ROS play a major regulatory and/or executive role in plant defense responses and cell-death events associated with microbial pathogen attack (23, 35, 36). Here, we demonstrate NO production by the use of the NO-specific fluorophore DAF-FM DA in conjunction with confocal laser-scanning microscopy, by a highly specific EPR method using Fe_2 plus diethyldithiocarbamate as a spin trap (Fig. 1) and with a conventional NOS assay (Fig. 3*C*). The induction of NO seems to be a very early LPS response, similar to an elicitor-induced NO burst in tobacco, mechanical stress of various gymnosperms, or wounding (20, 27, 37).

Next, we investigated the source of LPS-induced NO. In animals, NO is generated primarily by NOS, a group of evolutionarily conserved isoenzymes that convert L-arginine to L-citrulline and NO (38). In plants, two unrelated NOS-like enzymes have been identified: a pathogen-inducible NOS from *Arabidopsis* and tobacco (iNOS; ref. 30) and a hormone-activated NOS from *Arabidopsis* (AtNOS1; ref. 22). iNOS is a variant of the P protein of the glycine decarboxylase complex. It displays typical NOS activity and requires the same cofactors as its mammalian counterparts. iNOS was shown to produce NO in *Arabidopsis* plants that were resisting infection by turnip crinkle virus and in tobacco plants treated with tobacco mosaic virus (23). Furthermore, iNOS is a key enzyme for the maintenance of basal resistance to *P. syringae* in tomato (13). The association of iNOS with pathogen responses suggested this enzyme to be responsible for a LPS-induced NO burst. However, T-DNA insertion lines of iNOS turned out to be unaffected in LPS-induced NO production (Fig. 3*B*). A similar outcome was observed when we silenced iNOS (data not shown). This result was not unexpected. The slow (transcriptional) induction of iNOS (23, 30) does not correlate with the almost immediate NO burst after contact with LPS (Figs. 1–3).

In addition to iNOS, Crawford and coworkers (22) have cloned a NOS on the basis of its sequence similarity to a protein implicated in NO synthesis in the snail *Helix pomatia*. AtNOS1 does not share sequence identity with either mammalian NOS or the plant iNOS and, surprisingly, displays a flavin-, heme-, and tetrahydrobiopterin-independent NOS activity. AtNOS1 has been implicated in NO production in response to hormonal signals including abscisic acid and seems to be constitutively expressed. Strikingly, AtNOS1 appears to be the initial source for the LPS-mediated NO burst in *Arabidopsis* leaves (Fig. 3*B*). However, the LPS-induced NOS activity in *Arabidopsis* (≈12 pmol per min·mg) was definitely much lower than the strong iNOS activity (up to 75 pmol per min·mg) found in tomato or tobacco elicited with *Pseudomonas* or tobacco mosaic virus (32). NR, another enzyme capable of producing NO in plants (39), does not seem to be involved in NO generation in our experimental system (Fig. 1*D*).

What might be the molecular mechanisms underlying perception of LPS in plants? LPS is comprised of three distinct regions: lipid A, the oligosaccharide core, and commonly a long-chain polysaccharide O antigen that causes a smooth phenotype. Lipid A is the most conserved part of LPS. It is connected to the core part, which links it to the highly polymorphic O repeating units. In our hands, ≈15 LPS preparations and LTA from Gram-positive *S. aureus* were found to trigger rapid NO production in suspension-cultured *Arabidopsis* cells and in leaves, respectively. Lipid A was as effective as most LPS preparations (Fig. 3). Thus, lipid A may serve as the active part of LPS, as reported for animal–microbe interactions (11). However, interpretations of differences in LPS-mediated responses should be made with caution, because any readout (such as NO) might be related to the LPS conformation. Biological activities of LPS are determined by the shape of their lipid A portion, and it is still unclear whether monomeric LPS molecules are able to activate cells or whether only larger aggregates or even an intact bacterial surface are active (18, 40, 41).

In all probability, the first step in the signal perception and transduction of the LPS-induced defense responses is the interaction of LPS with a plant cell wall- or plasma membrane-bound receptor or binding protein. LPS have been reported to bind to the mesophyll cell wall of tobacco cells and to induce ultrastructural changes such as vesiculation (42). In animals, specific cellular recognition of LPS/lipid A is binding of LPS by CD14, and transmission of the signal by TLR4 (7). What might the LPS receptor in plants look like? The genome of *Arabidopsis* contains numerous putative receptors featuring toll-interleukin domains, and several plant genes show strong homology to mammalian NOD factors that also are involved in signal transmission after LPS contact (4). The receptor-like kinase FLS2 that binds flagellin shares homology with the TLR family, and TLR5 is responsible for flagellin perception in mammals (8, 43). However, there is no sequence with (convincing) homology to TLR4. Furthermore, CD 14/TLR4-mediated perception of LPS operates in the picograms- or nanograms-per-milliliter range (28), whereas in most plants defense responses require higher amounts of LPS (Fig. 1–3 and refs. 15–18). Conversely, animals possess additional, low-affinity systems to detect LPS. Heat shock proteins 70 and 90, chemokine receptor 4, and growth differentiation factor 5 are the main mediators of activation by bacterial LPS (44). Other LPS receptors with affinity in the micrograms-per-milliliter range are L-selectins, which mediate production of oxygen free radicals (29). In our opinion, the putative LPS receptor in plants may be of such a low-affinity type.

The effects of Gram-negative bacterial LPS on mammalian and insect cells have been well documented. LPS have been shown to activate the synthesis of antimicrobial peptides in *Drosophila*, as well as the production of immunoregulatory and cytotoxic molecules in humans (5, 45). As for plants, evidence is emerging implicating bacterial LPS in enhancement of the plant's response to subsequent pathogen attack by pretreatment with LPS. Although treatment of leaves with LPS from a number of bacteria did not induce the synthesis of defense-related secondary conjugates, it primed its induction upon subsequent

bacterial inoculation (16). LPS pretreatment also potentiated the expression PR genes upon subsequent bacterial inoculation (46).

Currently, we can only speculate how the activation of innate immune responses in plants as a consequence of PAMP recognition works together with the more specific recognition by means of *avr* factors/*R* genes. In a first step, we studied gene expression in LPS-treated *Arabidopsis* plants with a custom-designed, stress-response-biased cDNA microarray. Fig. 4 shows that LPS induced an array of defense- or stress-associated genes, including glutathione S-transferases, cytochrome P450, and many genes encoding PR proteins, both locally and systemically. Interestingly, PR-protein accumulation was independent of cell death (data not shown), reminiscent of the action of flg22 peptide (8, 9). Flagellin also acted as an elicitor in whole *Arabidopsis* plants, inducing an oxidative burst and leading to the induction of defense-related genes such as *PR1*, *PR5*, *PAL1*, and *GST1*, but just like LPS never induced a hypersensitive response type of necrosis. Most interestingly, (defense) gene expression was abolished almost completely when *AtNOS1* mutant plants were treated with LPS. This result suggests a functional link between LPS-induced NO production and gene induction. As a matter of fact, many of the LPS-responsive genes have been found to be NO inducible (19). Note that these data do not imply that gene induction by LPS is always dependent on NO. Unlike a genome-wide array, our application-based array (Table 1) is biased toward redox-sensitive genes and genes associated with defense.

Innate immunity becomes apparent as basal resistance against pathogens. In a reverse genetics approach, it was demonstrated recently that defective flagellin perception leads to enhanced susceptibility of *Arabidopsis* to *Pst* DC3000 (9). Because NO is an important component of innate immunity and induced by LPS, we asked for its role in plant disease resistance. Here, we show that plants lacking NOS are more susceptible to pathogenic bacteria. *AtNOS1* plants showed a faster and much more severe development of disease symptoms than wild-type plants (Fig. 6). Recently, it was demonstrated that suppression of pathogen-inducible NO synthase activity in tomato increases susceptibility to Pst DC3000 as well as Pst DC3000/AvrPto (32). Thus, NO production appears to be important for basal resistance in Arabidopsis and tomato as well as for *R*-gene-dependent resistance in tomato.

Taken together, perception of LPS and generation of NO appear to be part of an important signaling and response system in plant–pathogen interactions involved in broad-spectrum defense mechanisms. The demonstration of the biological relevance of putative PAMPs and the identification of their corresponding receptors represent an exciting goal for the future.

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