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## Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*

Received: 14 August 2003 / Accepted: 12 November 2003 / Published online: 10 January 2004  
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**Abstract** Nitric oxide (NO) has been associated with plant defense responses during microbial attack, and with induction and/or regulation of programmed cell death. Here, we addressed whether NO participates in wound responses in *Arabidopsis thaliana* (L.) Heynh.. Real-time imaging by confocal laser-scanning microscopy in conjunction with the NO-selective fluorescence indicator 4,5-diaminofluorescein diacetate (DAF-2 DA) uncovered a strong NO burst after wounding or after treatment with JA. The NO burst was triggered within minutes, reminiscent of the oxidative burst during hypersensitive responses. Furthermore, we were able to detect NO in plants (here induced by wounding) by means of electron paramagnetic resonance measurements using diethyldithiocarbamate as a spin trap. When plants were treated with NO, Northern analyses revealed that NO strongly induces key enzymes of jasmonic acid (JA) biosynthesis such as allene oxide synthase (AOS) and lipoxygenase (LOX2). On the other hand, wound-induced AOS gene expression was independent of NO. Furthermore, JA-responsive genes such as defensin (*PDF1.2*) were not induced, and NO induction of JA-biosynthesis enzymes did not result in elevated levels of JA. However, treatment with NO resulted in accumulation of salicylic acid (SA). In transgenic NahG plants (impaired in SA accumulation and/or

signaling), NO did induce JA production and expression of JA-responsive genes. Altogether, the presented data demonstrate that wounding in *Arabidopsis* induces a fast accumulation of NO, and that NO may be involved in JA-associated defense responses and adjustments.

**Keywords** *Arabidopsis* · Jasmonic acid · Nitric oxide · Salicylic acid · Wounding

**Abbreviations** AOS Allene oxide synthase · *cPTIO* Carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl · DAF-2 DA 4,5-Diaminofluorescein diacetate · DETC Diethyldithiocarbamate · EPR Electron paramagnetic resonance · *iNOS* Inducible nitric oxide synthase · JA Jasmonic acid · JIP Jasmonic acid-induced protein · LOX2 Lipoxygenase 2 · NO Nitric oxide · OPR3 12-Oxophytodienoate reductase · PDF1.2 Plant defensin · ROS Reactive oxygen species · SA Salicylic acid · SNP Sodium nitroprusside

### Introduction

Due to the diversity of its physiological functions and general ubiquity, nitric oxide (NO) has attracted a great deal of attention. NO is now recognized to be an intra- and intercellular mediator of signal transduction pathways controlling smooth muscle tone, neurotransmission, cell proliferation, apoptosis, and host responses to infection (Torreilles 2001; Wendehenne et al. 2001). In mammals, the generation of NO by inducible nitric oxide synthase (iNOS) plays an important role in inflammation, host defense responses and tissue repair (Nathan and Shiloh 2000).

Recent studies have revealed that many of the biological functions of NO have an evolutionarily ancient origin. The chemistry of NO in a biological environment is characterized by its reaction with O<sub>2</sub>, superoxide (O<sub>2</sub><sup>-</sup>) and transition metals including those found in

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metalloproteins of the heme and FeS classes (Stamler et al. 1992). Several well-known targets of NO in animals, e.g. aconitase and MAP kinases, are also modulated by NO in plants. In plants, NO is associated with nitrate assimilation (Kaiser et al. 2002; Stohr and Ullrich 2002), and NO regulates early events of plant resistance responses. In tobacco, *Arabidopsis* and soybean, NO triggered expression of several defense related genes, and pathogen infection or treatment with elicitors resulted in enhanced NO production (Delledonne et al. 1998; Durner et al. 1998; Foissner et al. 2000). Very recently, it was demonstrated that the pathogen-induced, NO-synthesizing enzyme in tobacco and *Arabidopsis* is a variant form of the P-protein of glycine decarboxylase (GDC; Chandok et al. 2003).

In many cases, NO collaborates with reactive oxygen species (ROS) (McDowell and Dangl 2000). Several very recent publications all demonstrate the participation of NO and its activated derivative peroxynitrite (ONOO<sup>-</sup>) in plant apoptosis, gene regulation and defense responses against pathogens (Clark et al. 2000; Huang et al. 2002b; Wendehenne et al. 2001). It is noteworthy that the interactions of NO with reactive oxygen can be cytotoxic (promoting cell death) or protective (Beligni et al. 2002; Orozco-Cárdenas and Ryan 2002).

The role of NO in plants is by far not limited to host responses to microbial attack. In addition to its role in defense, a mounting body of evidence suggests that NO is a novel effector of plant growth and development. For example, NO was shown to be involved in photomorphogenesis, mitochondrial activity, leaf expansion, root growth, stomatal closure, senescence and phytoalexin production (Leshem et al. 1998; Neill et al. 2002; Schaller and Weiler 1997; Wendehenne et al. 2001). First investigations into NO functions suggested that plants use NO as a signaling molecule via pathways remarkably similar to those found in mammals. Two downstream signaling molecules, cyclic GMP (cGMP) and cyclic ADP ribose (cADPR), appear to mediate plant defense gene activation. In some cases defense gene induction depended on salicylic acid (SA; e.g. PR 1 induction in tobacco). Interestingly, while there are many parallels between NO's action in plants and animals, some interesting differences such as the involvement of cGMP in plant apoptosis have been reported (Clarke et al. 2000). Furthermore, NO seems to be linked to the plant hormonal network, namely abscisic acid (ABA) and cytokinin (Desikan et al. 2002; Tun et al. 2001).

Very recent data point to a possible role of NO as modulator in responses to wounding and/or mechanical stress such as hypergravity (e.g. Garcês et al. 2001; Pedroso et al. 2000). In tomato, NO inhibited wound-inducible H<sub>2</sub>O<sub>2</sub> generation and proteinase inhibitor gene expression downstream of jasmonic acid (JA) synthesis, and it has been shown that NO has a role in down-regulating the expression of wound-inducible defense genes during pathogenesis (Orozco-Cárdenas and Ryan 2002). Here we elucidated whether NO is part of the wound response in *Arabidopsis*. Real-time imaging by confocal laser-

scanning microscopy in conjunction with an NO-selective fluorophore uncovered a strong NO burst after wounding. Furthermore, we were able to confirm fluorescence-based data by a highly specific electron paramagnetic resonance (EPR) method using diethyldithiocarbamate (DETC) as a spin trap. This finding prompted us to evaluate a putative role of NO in wound signaling.

## Materials and methods

### Plant material

*Arabidopsis* plants [*Arabidopsis thaliana* (L.) Heynh., ecotype Columbia; Lehle Seeds, Round Rock, USA] were grown at 22°C in growth chambers programmed for a 14-h light and 10-h dark cycle. Five- to six-week-old plants were used for experimentation.

### Treatment of *Arabidopsis* plants (NO and wounding)

The experimental setups to study the effect of NO on whole plants consisted of controlled-environment cabinets as well as complete instrumentation to adjust and control gaseous NO through an electrochemical sensor. *Arabidopsis* plants were treated with NO concentrations of 1,250 µl l<sup>-1</sup> for 10 min. At this NO concentration, the plants did not show any symptoms. After this treatment, the plants were maintained in growth chambers until sample collection.

For wounding treatments, we crushed all rosette leaves several times across the apical lamina with forceps, which effectively wounded approximately 40% of the leaf area.

### Chemicals

4,5-Diaminofluorescein diacetate (DAF-2 DA) and carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (cPTIO) were purchased from Alexis Corp..

### Microscopy

To analyze NO production by fluorescence microscopy, epidermal cell layers from the abaxial surface of leaves were peeled with forceps and placed on a slide containing 30 µl of loading buffer (10 mM Tris-KCl, pH 7.2) with DAF-2 DA at a final concentration of 10 µM [added from a 10 mM stock in dimethyl sulfoxide (DMSO)]. Peels from control preparations did not show NO or ROS production. For cPTIO treatment, the epidermal peels were rinsed in 100 µM cPTIO for 1 min, before being transferred to the slide containing DAF-2 DA. After overlaying the glass cover, the slides were placed under the microscope(s) and the images were taken within 10 min.

For confocal laser-scanning microscopy a Zeiss Axiovert 100 M inverted microscope equipped with a confocal laser scanner (LSM 510; Zeiss, Oberkochen, Germany) was used in this study and sections were excited with the 488 line of an argon laser. Dye emissions were recorded using a 505- to 530-nm band pass filter; autofluorescence of chloroplasts was captured with a 585-nm long pass filter. Microscope, laser and photomultiplier settings were held constant during the course of an experiment in order to obtain comparable data. Images were processed and analyzed using the Zeiss LSM 510 software.

### NO detection by EPR

For EPR analysis of NO, entire rosette leaves were cut after wounding and frozen at once in liquid nitrogen. About 0.6 g frozen

leaves was crushed with a mortar and pestle under liquid nitrogen and incubated in 1.2 ml of buffered solution (50 mM Hepes, 1 mM DTT, 1 mM MgCl<sub>2</sub>, pH 7.6) for 2 min. The mixture was centrifuged at 13,200 g for 2 min. The supernatant was added to 300 µl of freshly made [Fe(II)(DETC)<sub>2</sub>] solution (2 M Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 3.3 mM DETC, 3.3 mM FeSO<sub>4</sub>, 33 mg ml<sup>-1</sup> BSA; Tsuchiya et al. 1996), incubated for 2 min at room temperature and frozen again in liquid nitrogen. EPR measurements were performed on a Bruker ESP300 X-band spectrometer under the following conditions: room temperature; microwave power, 20 mW; modulation amplitude, 3 G; scan rate, approx. 2.5 G S<sup>-1</sup>; time constant, 164 ms.

#### Northern analysis

RNA from *Arabidopsis* leaves was extracted using the TRIZOL reagent according to the suppliers instructions (BRL). Northern analysis followed standard protocols. The *Arabidopsis* sequences used as probes for hybridization (identical to those used for the array) were amplified in full from the expressed sequence tag (EST) clones corresponding to At5g42650 (allene oxide synthase: AOS), At3g45140 (lipoxygenase: LOX2), At2g06050 (12-oxophytodienoate reductase: OPR3), At5g44420 (plant defensin: PDF1.2) and At3g16420 (jasmonic acid-induced protein: JIP). Labelling was with digoxigenin as recommended by the manufacturer (Boehringer, Mannheim). Northern blots were done at least in triplicate. Typical expression profiles are shown.

#### Determination of SA and JA

Free SA was extracted and quantified basically according to Meuwly and Metraux (1993). SA was detected by using a Shimadzu RF 535 fluorescence detector at excitation and emission wavelengths of 305 and 407 nm, respectively.

For jasmonate determinations, plant tissue was shock-frozen with liquid nitrogen and processed as described by Mueller and Brodschelm (1994). For quantification, 100 ng of 9,10-dihydro-JA was added to the frozen cells prior to work-up. Jasmonates were analyzed as pentafluorobenzyl (PFB) ester derivatives by gas chromatography (GC)-mass spectrometry (SSQ quadrupole instrument; Finnigan, San Jose, CA, USA) operated in the negative ion chemical ionization mode using isobutane as reactant gas. [Molecular anions-PFB]-ions of JA-PFB ( $m/z=209$ ) and dihydro-JA-PFB ( $m/z=211$ ) were monitored and JA levels were calculated from the GC peak areas of the selected ions.

## Results

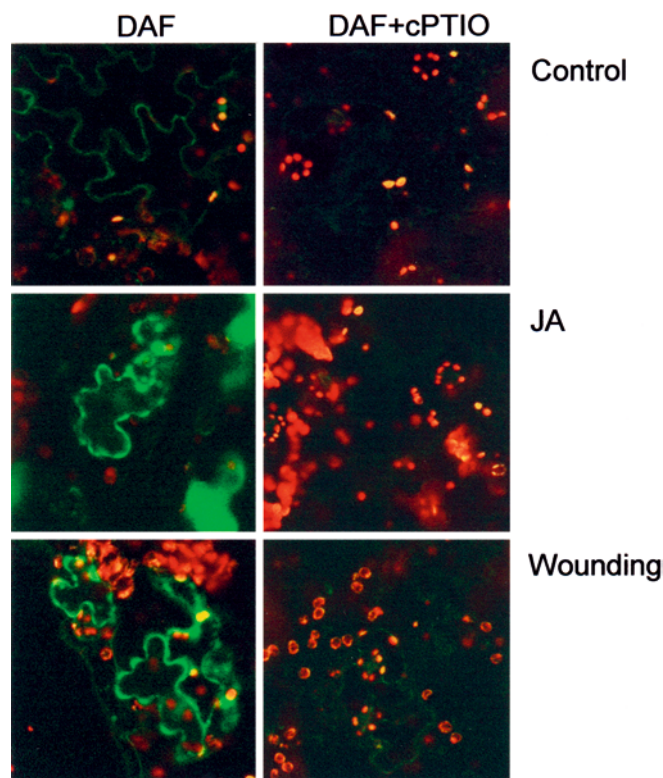
### Wounding induces NO

The induction of NO and ROS by pathogens seems to regulate plant defense responses and/or cell death (Delledonne et al. 1998, 2001; Durner et al. 1998). NO seems to feed into the well-described SA-dependent signaling system (Durner et al. 1998). We were interested in whether NO plays a role in wound responses, which, in part, are mediated through generation of other downstream redox messengers such as H<sub>2</sub>O<sub>2</sub> (Orozco-Cárdenas and Ryan 1999; Orozco-Cárdenas et al. 2001).

Real-time imaging of NO is best done by diamino-fluoresceins (DAFs) in conjunction with confocal laser-scanning microscopy. DAFs do not react with any ROS, and lower the detection limit for NO to 5 nM. The diacetate derivative (DAF-2 DA) is used to load living

cells, where it is hydrolyzed by cytosolic esterases to release DAF-2, which in the presence of NO is converted to the fluorescent triazole derivative (Kojima et al. 1998). DAF has been used by us and others to visualize NO production in *Kalanchoe* (Pedroso et al. 2000), mung bean (Lum et al. 2002) and tobacco (Foissner et al. 2000). The ability of DAFs to specifically detect NO in biological systems has been confirmed (e.g. Suzuki et al. 2002).

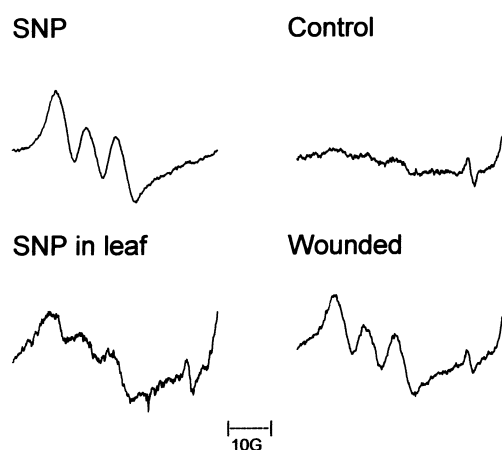
Figure 1 shows real-time imaging of NO production in epidermal *Arabidopsis* cells either wounded or treated with JA. Epidermal (abaxial) peels were loaded with DAF-2 DA and analyzed by confocal laser-scanning microscopy. We have focused on the NO burst in epidermal cells since the peels (but not entire leaves or leaf sections) could effectively be loaded with DAF-2 DA; cell types other than epidermal cells, however, might also react to wounding. Wounding resulted in a rapid burst of fluorescence, indicative of NO production. Fluorescence became visible in the cytosol and along the plasma



**Fig. 1** Confocal laser-scanning microscopy of wound-induced increases in intracellular DAF-2 DA fluorescence in epidermal cells from *Arabidopsis thaliana* (Col). Plants were wounded as described or sprayed with JA (100 µM). Epidermal peels were loaded with DAF-2 DA in the absence (left column) or the presence (right column) of the NO scavenger cPTIO (100 µM), washed, and examined under the microscope. The upper row shows DAF-loaded, but otherwise untreated controls. The strong green fluorescence along the plasma membranes and/or cell walls in response to wounding or JA is indicative of NO production, and could be suppressed by addition of cPTIO. Note the strong red chlorophyll fluorescence within the chloroplasts. Pictures were taken 10 min after wounding

membrane or cell wall. However, since DAF-2 DA is a single-wavelength probe, no adjustments can be made for differential accumulation of the probe in the cell. While there is no evidence for differential loading, DAF-2 DA or other fluorescein derivatives might preferentially accumulate in specific cellular compartments. The basal fluorescence shown in Fig. 1 represents basal NO. Scanning by using the laser (488 nm excitation) alone did not result in a measurable increase in fluorescence (data not shown). While DAF-2 DA is reported to be highly specific for NO (Kojima et al. 1998), we used a membrane-permeable NO scavenger as a control. Carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (cPTIO) is highly specific for NO scavenging, does not react with any ROS and has been used to block NO production as well as NO-dependent cell death and defense gene activation in tobacco, soybean, *Arabidopsis* and barley (Delledonne et al. 1998; Durner et al. 1998; Foissner et al. 2000). 100  $\mu$ M cPTIO completely suppressed both the basal fluorescence level as well as the elicited bursts of fluorescence. The inhibitor of animal nitric oxide synthase (NOS), N<sup>G</sup>-monomethyl-arginine monoacetate (L-NMMA; 1 mM) reduced the burst of DAF-2 DA fluorescence only slightly (data not shown). The wound response started to decay between 30 min and 1 h.

A highly specific method for NO detection in plants and animals is EPR with ferrous and mononitrosyl dithiocarbamate [ $\text{Fe}_2^+$  (DETC)<sub>2</sub>] or other dithiocarbamate derivatives for spin trapping. To overcome the low solubility of  $\text{Fe}_2^+$  (DETC)<sub>2</sub>, and the rapid oxidation of the  $\text{NOFe}_2^+$  (DETC)<sub>2</sub> complex (Pieper et al. 2003),  $\text{Na}_2\text{S}_2\text{O}_4$  was used as a strong reductant to increase the sensitivity and stability of the EPR spectrum of the complex (Tsuchiya et al. 1996). By this modified method, we were able to demonstrate NO production from wounded *Arabidopsis* leaves (Fig. 2). It should be noted,



**Fig. 2** Wound-induced increases of NO in *Arabidopsis* as detected by EPR. Plants were wounded as described, and NO was detected by EPR using the spin trap  $\text{Fe}_2^+$  (DETC)<sub>2</sub>. Shown are an NO control (5  $\mu$ M SNP in HEPES), an extract obtained from an unwounded leaf, an extract of a plant containing 5  $\mu$ M SNP and an extract from a leaf 20 min after wounding. The signals were recorded at identical EPR settings

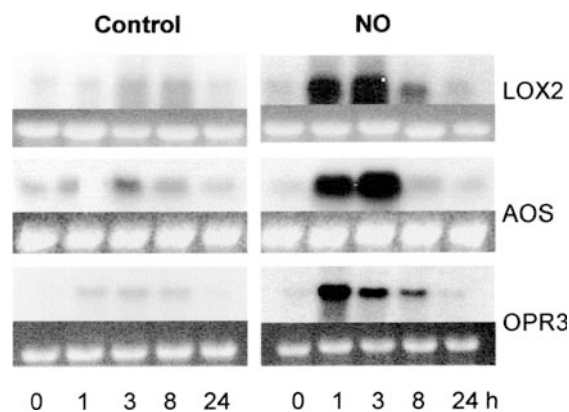
that the EPR experiments were carried out with bulk tissue (in contrast to microscopy experiments that were carried out with epidermal peels only). Strikingly, the wound-induced EPR signal was even higher than a signal obtained by injection into the leaf of 5  $\mu$ M sodium nitroprusside (SNP), an NO donor frequently used by others (e.g. Delledonne et al. 2001; Orozco-Cárdenas and Ryan 2002). Low concentrations of SNP (50  $\mu$ M) injected into the leaves initiated AOS-induction (data not shown), but this experiment does not distinguish between a general wounding response and a specific NO-mediated gene induction.

### NO induces LOX2, AOS and OPR3

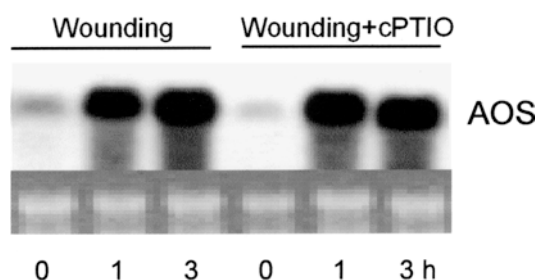
To unravel the interaction and/or participation of NO with known signaling pathways, we studied gene expression dynamics in NO-treated *A. thaliana* (Col-0) plants and suspension cells by using a cDNA microarray that included about 330 defense-related genes (Huang et al. 2002b, and data not shown). Plants were treated with gaseous NO as described. Note that the use of gaseous NO is the only method of NO delivery without any carrier involved (Bredt 2003; Shimizu et al. 2003). Physiological effects of gaseous NO on plants have been addressed earlier and are summarized in a comprehensive review (Wellburn and Wellburn 1997). We adjusted the NO concentration to a level where no effect on plant health or growth could be observed (Huang et al. 2002a). Among the most prominent genes induced at least 2.5-fold by NO were those for the key enzymes of the octadecanoid pathway, AOS, LOX2 and OPR3. To allow for an effective increase in pathway output capacity yielding JA, LOX2, AOS and OPR3 are often induced in a coordinated manner (Schaller 2001). Here we present transcript accumulation by Northern analysis. The probes constructed for Northern hybridization were full length. Total RNA was extracted at the indicated time-points and subjected to Northern blot hybridization. There was strong induction of LOX2, AOS and OPR3 by NO. Expression was transient and peaked between 1 and 3 h post treatment (Fig. 3).

### Wound induction of AOS is independent of NO

The induction of enzymes involved in JA biosynthesis, such as AOS, is considered to represent a key event of wound responses. Since wounding induces NO (Fig. 1, 2), and NO in turn leads to accumulation of AOS transcripts (Fig. 3), we asked whether NO plays a role in wound-induction of AOS. For this purpose we tried to suppress NO production *in planta* by infiltration of leaves with cPTIO, a cell-permeable NO scavenger. cPTIO did not influence AOS induction (Fig. 4). Thus, we conclude that during/after wounding, transcription of AOS is driven by additional factors other than NO.



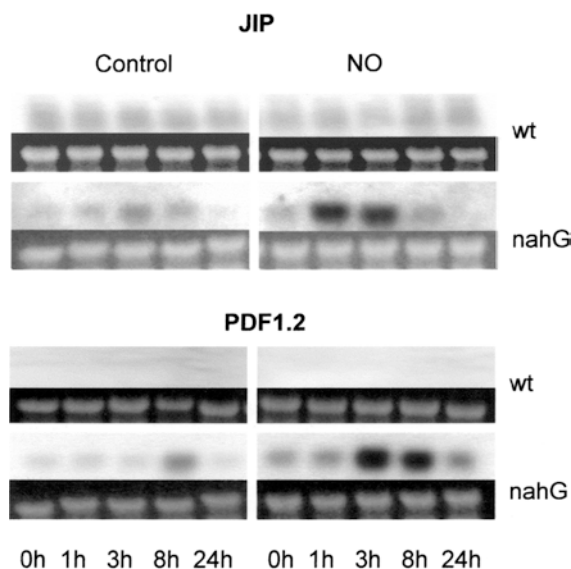
**Fig. 3** NO induces AOS, LOX2 and OPR3 gene expression in *Arabidopsis*. *Arabidopsis* plants were treated with NO as described. Leaf material was collected at the times indicated for RNA preparation (0–24 h). Northern blots were probed with cDNAs for AOS, LOX2, and OPR3, which were based on primers as described in Materials and Methods. Shown is the region between 1.8 and 1.0 kb. Ethidium bromide staining shows loading of the gels



**Fig. 4** Expression of AOS after wounding does not depend on NO. *Arabidopsis* plants (wild-type Col) were infiltrated with 10 mM potassium phosphate buffer with or without 100  $\mu$ M cPTIO, and subsequently wounded as described. Leaf material was collected at the times indicated for RNA preparation (0–23 h). Northern blots were probed with cDNAs for AOS as described. Ethidium bromide staining shows loading of the gels

NO induces PDF1.2 and JIP only in the absence of SA

Since NO-treatment of *Arabidopsis* was activating at least part of the JA biosynthetic machinery and early signaling genes, we were interested to see whether NO would also induce typical defense-related (and JA-inducible) genes. Surprisingly, there was an extremely weak or almost no accumulation of JA-dependent late defense genes in NO-treated plants. For example, NO did not induce any PDF1.2 or JIP accumulation (Fig. 5). As a control, and as previously reported by many others, JA-treatment led to strong PDF1.2 accumulation (data not shown). The JA signaling cascade in *Arabidopsis* as well as in tomato was shown previously to be linked with/inhibited by SA, indicating cross-talk between pathogen- and herbivore-inducible defense responses (Thomma et al. 1998). When we carried out corresponding experiments with SA-deficient NahG plants, PDF1.2 and JIP, which are insensitive to NO in the wild type, were rendered NO-responsive (Fig. 5). These data



**Fig. 5** NO induces PDF1.2 and JIP gene expression in NahG *Arabidopsis*, but not in the wild type (*wt*). *Arabidopsis* plants (wild-type Col) and transgenic NahG plants were treated with NO as described. Leaf material was collected at the times indicated for RNA preparation (0–24 h), and analyzed for PDF1.2 and JIP expression. Northern blots were probed with cDNAs that were based on primers as described in Materials and Methods. Ethidium bromide staining shows loading of the gels

suggest a role for SA in suppression of JA- or NO-responsive genes.

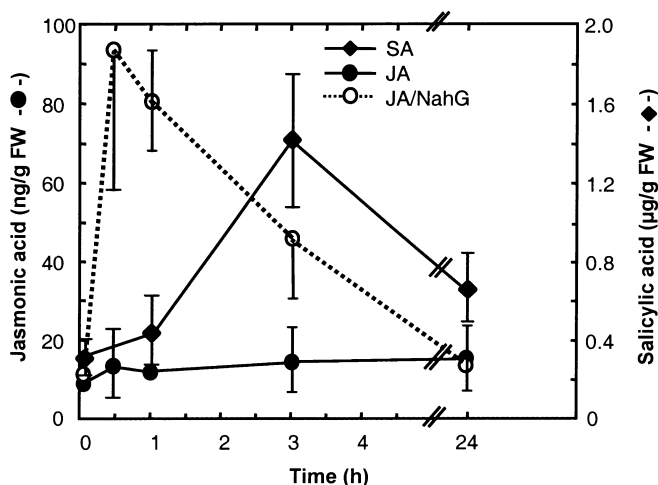
#### Influence of NO on SA and JA levels

A link between NO action in plants and SA has been made previously (Klessig et al. 2000). Treatment of tobacco leaves with NO induced a strong increase in the endogenous SA (Durner et al. 1998). Here, direct quantification of free SA in leaves of NO-treated *Arabidopsis* plants revealed that NO was inducing SA biosynthesis (Fig. 6). As expected after the results shown by Fig. 5, NO did not affect JA levels in *Arabidopsis*. However, in NahG there was substantial JA accumulation. These results suggest that AOS/LOX/OPR3 activation by NO may be antagonized by inhibition of JA biosynthesis by SA, as reported previously (Glazebrook 2001). Nevertheless, a wild-type plant does contain SA, and therefore we conclude that despite activation of LOX2, AOS and OPR3, NO is not able to induce accumulation of JA.

#### Discussion

Previously, it has been suggested that NO and ROS play a major regulatory and/or executive role in plant defense responses and cell death events associated with microbial pathogen attack (McDowell and Dangl 2000). NO acts synergistically with ROS to increase host cell death





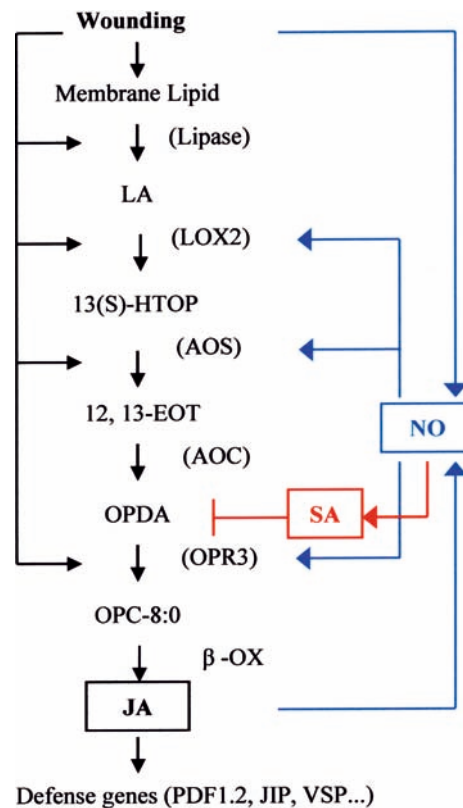
**Fig. 6** Effects of NO on SA and JA concentrations in *Arabidopsis* leaves. NO treatment and determination of the levels of free SA and JA were as described. JA was measured in wild-type and NahG plants (dotted lines). Data are means  $\pm$  SD of two (SA, JA/NahG) or four (JA) independent experiments. For the sake of clarity some SD bars are not shown

of soybean suspension cells, and inhibitors of NOS compromise the hypersensitive response (HR) in *A. thaliana* and tobacco (Delledonne et al. 2001). The interaction of ROS with NO during plant defense responses, including cell death induction, is currently a subject of intensive research.

ROS are also messengers in wound responses (for a recent review, see (Kessler and Baldwin 2002). In tomato,  $H_2O_2$  generated in response to wounding can be detected at wound sites and in distal leaf veins within 1 h after wounding (Orozco-Cárdenas and Ryan 1999). In tomato, NO inhibited wound-inducible  $H_2O_2$  generation and proteinase inhibitor gene expression downstream of JA, and it has been shown that NO has a role in down-regulating the expression of wound-inducible defense genes during pathogenesis (Orozco-Cárdenas and Ryan 2002). These findings prompted us to ask if NO has a role in wounding.

To specifically detect NO, the use of more than one technique is highly recommended. In addition to methods based on detection by fluorescence bioimagers (Foissner et al. 2000) we, and others, have used the traditional arginine-citrulline assay, the hemoglobin-based assay and the Greiss method (Delledonne et al. 1998; Orozco-Cárdenas and Ryan 2002; Chandok et al. 2003). Here, we demonstrate NO production by the use of the NO-specific fluorophore DAF-2 DA and by a highly specific EPR method.

First, the NO burst in epidermal cells of wounded *Arabidopsis* leaves was detected by the NO-sensitive fluorophore DAF-2 DA in conjunction with confocal laser-scanning microscopy (Fig. 1). The kinetic of NO production was similar to an elicitor-induced NO burst in tobacco or mechanical stress in various gymnosperms (Foissner et al. 2000; Pedroso et al. 2000). Thus, the induction of NO seems to be an early wounding



**Fig. 7** Induction of NO by wounding and effect of NO on the JA and SA signaling network in *Arabidopsis* leaves. The figure was drawn according to Schaller (2001), and Laudert and Weiler (1998). Only those regulatory circuits that are relevant for the present study are shown. For further information see the above-mentioned references. LA  $\alpha$ -Linolenic acid; AOC allene oxide cyclase; 13(S)-HPOT (9Z, 11E, 15Z, 13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 12, 13-EOT (9Z, 11E, 15Z, 13S, 12R-12, 13-epoxy-9,11,15-octadecatrienoic acid; OPDA 12-oxo-10,15(Z)-octadecatrienoic acid; OPC-8:0 3-oxo-2 (2'(Z)-pentenyl)-cyclopentane-1-octanoic acid

response. For a comparison,  $H_2O_2$  generated in response to wounding can be detected at wound sites and in distal leaf veins of tomato within 1 h after wounding, the response maximizes at about 4–6 h in both wounded and unwounded leaves, and then declines. The time course of wound-inducible  $H_2O_2$  in *A. thaliana* leaves was similar to that found in tomato (Orozco-Cárdenas and Ryan 1999, and data not shown). Since methyl jasmonate (MJ) as well as JA-inducing signals such as systemin and chitosan all induce the accumulation of  $H_2O_2$  in leaves (and possibly also NO), we asked whether JA treatment could trigger the NO burst. JA treatment resulted in strong NO production, suggesting a self-amplifying JA–NO loop (Fig. 7). Recently, a role for NO in the wound-inducible signaling pathway in tomato plants was demonstrated, in which NO could suppress the  $H_2O_2$ -induced activation of proteinase inhibitor genes (Orozco-Cárdenas and Ryan 2002). In these studies, wounding did not result in enhanced NO production. A possible reason for this discrepancy might be the very different detection limits of the applied NO assays

(Foissner et al. 2000). Furthermore, while microscopy-based assays cannot be used as a quantitative tool, they can detect isolated NO-producing tissues or even cells within tissues (see Fig. 1).

A recent method for NO detection, and probably the most specific one, is electron paramagnetic resonance (EPR) with ferrous and mononitrosyl dithiocarbamate [ $\text{Fe}_2 + (\text{DETC})_2$ ] for spin trapping (Pieper et al. 2003; Tsuchiya et al. 1996). Under the energy of microwave frequency and at adequate magnetic field strength, unpaired electrons of radicals are promoted to higher energy levels and relaxation to ground state produces characteristic spectra. The identity of the spin-trapped radicals, defined by EPR, is characterized by the multiplicity of the hyperfine splitting, and in the case of NO is a triplet feature (see Fig. 2). The amount of radical present is proportional to the magnitude of the signal. To overcome problems with the low solubility of  $\text{Fe}_2 + (\text{DETC})_2$  and rapid oxidation of the  $\text{NOFe}_2 + (\text{DETC})_2$  complex, we followed the suggestions of Tsuchiya and used albumin to solubilize  $\text{Fe}_2 + (\text{DETC})_2$ , and  $\text{Na}_2\text{S}_2\text{O}_4$  as a strong reductant to increase the sensitivity and stability of the EPR spectrum of the  $\text{NOFe}_2 + (\text{DETC})_2$  complex. The detection limit is less than  $10 \text{ pmol ml}^{-1}$  under these conditions (Tsuchiya et al. 1996). By this modified method, we succeeded in detecting NO production from wounded *Arabidopsis* leaves. The magnitude of the signal was between those obtained with  $5 \mu\text{M}$  sodium nitroprusside (SNP) in buffer or in plant tissue. This finding suggests some caution in the use of high doses of SNP, which is applied to plants in concentrations as high as  $10 \text{ mM}$  (Modolo et al. 2002). However, as shown by Fig. 1, NO might be produced by specific tissues and cells, and EPR data might not reflect local NO concentrations.

So far, the source of NO during wound responses remains unknown. There are at least two, and possibly more, enzymes responsible for NO synthesis in plants: nitrate reductase, which is associated with non-elicited NO generation (Desikan et al. 2002; Rockel et al. 2002); iNOS (a variant of the P-protein of glycine decarboxylase), which is responsible for the dramatic and sustained NO production in plants resisting pathogens (Chandok et al. 2003); and perhaps a constitutive NOS that is rapidly (and possibly transiently) activated by pathogens or pathogen-derived elicitors (Foissner et al. 2000). Another possible source of NO generation has been described for tobacco roots, where a plasma-membrane-bound enzyme catalyses the formation of NO from nitrite (Stöhr et al. 2001). We were not able to inhibit the wound-induced NO burst with injected inhibitors. However, considering the complex regulation of iNOS or nitrate reductase (e.g. Kaiser et al. 2002), such pharmacological approaches would provide very limited evidence. To identify the wound-responsive, NO-producing enzyme the generation of transgenic plants with impaired NO synthesis seems to be a promising strategy.

To elucidate whether wound-induced NO plays a role in downstream events, we studied NO-dependent gene

expression *A. thaliana* plants. To do so we used gaseous NO, which is the only method of NO delivery without any side-effects (Bredt 2003; Shimizu et al. 2003). The effects of gaseous NO on plants have been addressed earlier (Wellburn and Wellburn 1997). We adjusted the NO concentration to a level where no effect on plant health or growth could be observed (Huang et al. 2002a). Interestingly, NO was a powerful inducer of LOX2, AOS and OPR3, as shown here by Northern analyses (Fig. 3). AOS is of particular importance in biosynthesis of JA. The enzyme catalyzes the first reaction specific to the pathway, the dehydration of 13(*S*)-hydroperoxy-9(*Z*), 11(*E*),15(*Z*)-octadecatrienoic acid to 12,13(*S*)-epoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid. AOS transcript levels as well as AOS polypeptide levels rise after mechanical stimulation, elicitation or wounding (Schaller 2001). Thus, AOS is regarded as a central point of control of the octadecanoid biosynthetic pathway (Laudert and Weiler 1998). We next asked whether NO is a *necessary* component of wound signaling. However, induction of AOS during wounding did not depend on NO (Fig. 4). Similar results were obtained for LOX2 and OPR3 (data not shown). However, we have no proof that NO was completely removed (by cPTIO) in these experiments. cPTIO is considered a specific scavenger of NO and used as a control, but there is no definite proof that the substance enters the cell; the results should be interpreted cautiously. We conclude that although NO is a downstream event of wounding and although NO can induce AOS and LOX genes, NO seems to have a redundant role in wound-induction of these genes. In tomato, administration of the NO donor SNP (actually a nitrosonium anion donor) through stems did not influence early JA signaling genes (Orozco-Cárdenas and Ryan 2002).

Despite the strong activation by NO of the biosynthetic enzymes involved in jasmonate production, typical JA-inducible genes such as defensins or JIPs were not induced in wild-type *Arabidopsis* (Fig. 5). Interestingly, in tomato, NO donors even down-regulated JA-responsive defense genes if applied before wounding (Orozco-Cárdenas and Ryan 2002). Analyses of signaling molecules in NO-treated *Arabidopsis* provided a likely answer for this observation. Although AOS, LOX2 and OPR3 were strongly induced, NO treatment did not result in elevated JA levels (Fig. 6). Several reasons might account for this paradox. Recently, it has been suggested, that the output of the jasmonate pathway appears to be strictly limited by substrate availability. Overexpression as well as knock-outs of AOS do not alter the basal level of JA in unwounded plants (Laudert et al. 2000). In our hands, NO-treatment did not alter the expression of phospholipase (D), the key enzyme feeding lipids into the LOX2/AOS pathway (data not shown).

In addition to substrate limitation, JA biosynthesis seems to be under control of other factors such as SA. The relationship between the SA and the JA/ethylene defense response pathways is not well understood. JA

and SA are now regarded as global signals for defense gene activation (Reymond and Farmer 1998; O'Donnell et al. 2003). Some studies have demonstrated that these signals work synergistically to induce defense responses. However, other evidence suggests that these pathways function antagonistically (Farmer et al. 1998; Glazebrook 2001, and references therein). SA and JA have been shown to antagonize the activation of each other's defense responses, and SA can inhibit JA biosynthesis (Laudert and Weiler 1998; Van Loon et al. 1998; Glazebrook 2001). Since NO has been shown to induce SA biosynthesis in tobacco (Durner et al. 1998; Durner and Klessig 1999) we asked whether NO treatment was activating SA biosynthesis in *Arabidopsis*. Figure 6 demonstrates a substantial increase of SA in plants exposed to NO. Furthermore, NO treatment of NahG plants resulted in activation of PDF1.2 and JIP (Fig. 5), and most importantly, a strong increase in JA (Fig. 6). These results are in contrast to recent data on tomato, where NO was still effective in proteinase inhibitor suppression even in NahG plants and where NO did not elevate endogenous SA levels of wild-type plants (Orozco-Cárdenas and Ryan 2002). On the other hand, there are several reports that there are differences in principal between signals involved in wounding responses in *Arabidopsis* and those in the Solanaceae (León et al. 2001).

In conclusion, we have demonstrated that wounding induces NO, and provided evidence for cross-talk of NO with jasmonate signaling in *Arabidopsis*. However, while NO activates early JA signaling genes, it is apparently not (activating) key player in the wounding response in *Arabidopsis*, a situation similar to tomato where NO is even a negative regulator of wound signaling. Thus, AOS/LOX/OPR3 induction by NO might play a role in fine-tuning of wound responses or in a pathogenesis-related context such as induced systemic resistance (Orozco-Cárdenas and Ryan 2002; Van Loon et al. 1998).

**Acknowledgements** We thank E. Mattes and G. Fricke-Bode for excellent technical assistance, and Wolfgang Mayr for SA measurements. Furthermore, we thank Syngenta (San Diego, CA) for NahG lines (Col). This work was supported by Freistaat Bayern (BStMLU) and by grant DU 246/4-1 (Deutsche Forschungsgemeinschaft).

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