The Plant Journal (2000) **23**(6), 817–824

TECHNICAL ADVANCE

In vivo imaging of an elicitor-induced nitric oxide burst in tobacco

Ilse Foissner[1], David Wendehenne[2], Christian Langebartels[3] and Jörg Durner[3,*]
[1]*Institut für Pflanzenphysiologie, Universität Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria,*
[2]*Biochimie, Biologie Cellulaire et Moleculaire Ecologie des Interactions Plantes/Microorganismes, Unite Mixte INRA – Universite de Bourgogne, INRA, BV 1540, 17 rue Sully, 21034 Dijon, France, and*
[3]*Institute of Biochemical Plant Pathology, GSF – National Research Center for Environment and Health, D-85764 Oberschleissheim, Germany*

Received 25 April 2000; revised 15 June 2000; accepted 15 June 2000.
*For correspondence (fax +49 89 31873383; e-mail durner@gsf.de).

Summary

A growing body of evidence suggests that nitric oxide (NO), an important signalling and defence molecule in mammals, plays a key role in activating disease resistance in plants, acting as signalling molecule and possibly as direct anti-microbial agent. Recently, a novel fluorophore (diaminofluorescein diacetate, DAF-2 DA) has been developed which allows bio-imaging of NO *in vivo*. Here we use the cell-permeable DAF-2 DA, in conjunction with confocal laser scanning microscopy, for real-time imaging of NO in living plant cells. Epidermal tobacco cells treated with cryptogein, a fungal elicitor from *Phytophthora cryptogea,* respond to the elicitor with a strong increase of intracellular NO. NO-induced fluorescence was found in several cellular compartments, and could be inhibited by a NO scavenger and an inhibitor of nitric oxide synthase. The NO burst was triggered within minutes, reminiscent of the oxidative burst during hypersensitive response reactions. These results reveal additional similarities between plant and animal host responses to infection.

Keywords: nitric oxide, cryptogein, hypersensitive response, oxidative burst, fluorescence, confocal laser scanning microscopy.

Introduction

A key difference between resistant and susceptible plants is the timely recognition of the invading pathogen and the rapid and effective activation of host defences. The activated defence response is frequently manifested in part as the so-called hypersensitive response (HR), which is characterized by necrosis at the sites of infection (resembling animal programmed cell death) and restriction of pathogen growth and spread (Dangl *et al.*, 1996; Scheel, 1998). The HR is associated with the induction of defence-related genes which play important roles in containing pathogen growth either indirectly, by helping to reinforce the plant cell walls, or directly, by providing anti-microbial enzymes and phytoalexins.

Following infection, plants resistant to the invading pathogen mount a sustained increase of reactive oxygen species (ROS, oxidative burst). ROS such as the superoxide radical ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2) appear to play key roles in early and later stages of the plant response against pathogens (Bolwell, 1999; Scheel, 1998). A prominent feature is the induction of host cell death by ROS. In addition, ROS can serve as second messengers for the activation of defence gene expression (Alvarez *et al.*, 1998). The ability of ROS, and thus the cellular redox state, to activate plant defences may parallel the mechanism by which oxidative stress induces the genes associated with mammalian immune and inflammatory responses (Finkel, 1998).

The participation of ROS seems to be a necessary, but not sufficient, requirement not only for the induction of host cell death but also for pathogen killing (Dangl, 1998).

In animals, ROS often function together with nitric oxide (NO). For example, in the immune system, NO collaborates with ROS in macrophage execution of bacterial pathogens (Durner *et al.*, 1999; Mayer and Hemmens, 1997; Nathan, 1995). Due to the great diversity of its physiological functions and general ubiquity, NO has attracted a great deal of attention. NO, first identified as an endothelium-derived relaxation factor, is now recognized to be a general intra- and intercellular mediator of cell function. It is freely diffusible, and its biological effects are determined by its chemical reactivity towards oxygen, ROS and metals (Hippeli and Elstner, 1998; Stamler *et al.*, 1992). NO and its redox-activated forms are often regarded as the only ROS that can fulfil the requirements of a true inter- and intracellular signalling molecule.

Originally, research on the effects of NO in plants focused on atmospheric pollution by nitrogen oxides, NO and NO_2. Uptake of NO into foliage, as well as its subsequent metabolism and phytotoxicity, are well documented (Hufton *et al.*, 1996). Later it became clear that plants not only *respond* to atmospheric NO, but that they are also able to *produce* substantial amounts of NO (Wildt *et al.*, 1997). A mounting body of evidence suggests that NO is a novel effector of plant growth, development and defence. For example, NO was shown to be involved in photomorphogenesis, leaf expansion, root growth, senescence and phytoalexin production (Beligni and Lamattina, 2000; Leshem, 1996; Leshem *et al.*, 1998; Noritake *et al.*, 1996).

Recently, it was shown that NO plays a prominent role in plant defence against microbial pathogens. NO treatment of tobacco and soybean suspension cells triggered expression of several defence-related genes (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Infection of resistant, but not susceptible, tobacco with tobacco mosaic virus resulted in enhanced nitric oxide synthase (NOS) activity (Durner *et al.*, 1998). Similar results were obtained with soybean cells and *Arabidopsis*, treated either with a bacterial pathogen (*Pseudomonas syringae*) or an elicitor (Delledonne *et al.*, 1998). Strikingly, NOS inhibitors were able to compromise the resistance response in *Arabidopsis* and the induction of programmed cell death in pathogen-treated soybean suspension cells (Delledonne *et al.*, 1998). These experiments suggest that NO has a key role in the early events of plant resistance responses.

While several recent reports have confirmed NOS activity in plants (Barroso *et al.*, 1999; Ribeiro *et al.*, 1999), our understanding of NO production by plants is very limited (Durner and Klessig, 1999). NO emission from plants occurs under stress situations such as herbicide treatment or even under normal growing conditions, and it is believed that the ability to produce NO is common to many, if not all, plant species (Wildt *et al.*, 1997). Contrary to the common view, NO synthesis is not confined to NOS. In plants, NO is also a by-product of denitrification, nitrogen fixation or respiration. In many cases, NO production has been linked to the accumulation of NO_2^- in plant tissues (Klepper, 1991; Wildt *et al.*, 1997).

Many proposed physiological roles of NO are difficult to demonstrate directly. The extremely short half-life of NO, a gaseous free radical species, limits the study of its physiological effects *in vivo*. In this study, we used the newly developed NO sensitive fluorophore 4,5-diaminofluorescein diacetate (DAF-2 DA) to directly measure the NO production of tobacco cells by confocal laser scanning microscopy. This fluorophore allows real-time NO detection *in vivo* and has recently been used to detect NO accumulation in resting endothelial cells of mammals (Kojima *et al.*, 1998). Here, we describe the use of DAF-2 DA to monitor the NO accumulation in epidermal tobacco cells treated with cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea* which induces HR in tobacco. This analysis revealed that tobacco cells responding to the elicitor mount a fast NO burst.

Results and Discussion

Up to now, bio-imaging of NO has been reported by chemoluminescence and electron spin resonance (ESR). In addition, fluorescence detection of intracellular NO based on dichlorofluorescein (DCFH) was reported (Vowells *et al.*, 1995). However, while DCFH exhibits certain selectivity for H_2O_2, it is unable to distinguish between reactive oxygen species (ROS) and NO, and the detection limit of NO is 16 μM (Kojima *et al.*, 1998). Discrimination of which primary species is produced is then based on differential sensitivity to applied agents such as ROS- or NO-scavenging agents or enzymes. Using such a strategy, Allan and Fluhr (1997) were able to identify two distinct sources of cryptogein-elicited ROS in tobacco.

Recently, a new class of fluorescent probes highly specific for NO has been developed, that in conjunction with confocal laser microscopy can be used for real-time imaging of NO. Diaminofluoresceins (DAFs) do not react with any ROS (see Table 1), and lower the detection limit for NO to 5 nM. DAFs were used to detect NO production in cytokine-activated rat smooth muscle cells and even in resting endothelial cells (Kojima *et al.*, 1998). The diacetate derivative (DAF-2 DA) is used to load living cells. Subsequently, hydrolysis by cytosolic esterases releases DAF-2, which in the presence of NO is converted to the fluorescent triazole derivative, DAF-2T (Kojima *et al.*, 1998). Esterases able to modify diacetate fluorescein derivatives are also present in the cytosol of plant cells.

Here we report on the real-time imaging of NO production in epidermal tobacco cells treated with the fungal elicitor cryptogein (from *P. cryptogea*). In non-host tobacco cells, cryptogein induces rapid changes in ion

Table 1. *In vitro* fluorescence of DAF-2 DA in the presence of NO and ROS

	Relative fluorescence changes after 10 min (NOC-9 treatment set as 100)
No treatment	1
NOC-9 (1 μM)	100
NOC-9 + 100 μM PTIO	4
H_2O_2 (100 μM)	1
Xanthine/xanthine oxidase (10 U)	0

1 μM DAF-2 DA was dissolved in Tris-KCl, pH 7.2, and treated as indicated (final volume: 350 μl). NOC-9 is MAHMA NoNOate, an NO donor with a half-life of 3 min for NO release. Carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (PTIO) is a specific NO scavenger. H_2O_2 was added directly from stock. Xanthine oxidase converts xanthine to uric acid, thereby generating superoxide (O_2^-): 10 units produce 10 μM O_2^- per min. After incubation for 10 min, samples were analysed using a fluorescence spectrophotometer. Data represent the mean of two independent experiments.

fluxes (Ca^{2+} influx, Cl^- and K^+ efflux), phosphorylation events, changes in extracellular and cytosolic pH, transient and fast ROS production, ethylene and phytoalexin production and finally cell death (Bourque *et al.*, 1999; Lebrun-Garcia *et al.*, 1998; Pugin *et al.*, 1997). Recently, it was demonstrated that ROS act synergistically with NO to increase the host cell death of soybean suspension cells, and that inhibitors of NOS compromise the induction of cell death and defence gene expression in *Arabidopsis* and tobacco (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Huang and Knopp, 1998).

Epidermal (abaxial) sections from tobacco were loaded with DAF-2 DA and analysed with confocal laser scanning microscopy. Figure 1(a) shows a bright-field image of an epidermal tissue section. Figure 1(b) represents the same sample after loading with DAF-2 DA (10 μM) and after incubation in fresh loading buffer without the probe, but before stimulation with cryptogein. Figure 1(c) shows the cells 6 min after addition of 50 nM cryptogein, a concentration which promotes induction of cell death (Bonnet *et al.*, 1996). Cryptogein addition during image acquisition resulted in a rapid burst of fluorescence, indicative of massive NO production. Fluorescence became visible in the cytosol, along the plasma membrane, in chloroplasts and organelles probably representing peroxisomes (see Figures 2 and 3 for higher magnifications). The basal fluorescence shown in Figure 1(b) results from prolonged incubation of the loaded cells (50 min) and represents basal NO production (Wildt *et al.*, 1997). Scanning by using the laser alone (488 nm excitation) was ineffective in inducing a measurable increase of fluorescence (data not shown). While DAF-2 DA is reported to be highly specific for NO (Kojima *et al.*, 1998) (Table 1), we used a membrane permeable NO scavenger as a control. Carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (carboxy-PTIO) is highly specific for NO scavenging, does not react with any ROS (Barchowsky *et al.*, 1999) and has been used to block NO-dependent cell death and defence gene activation in tobacco and soybean (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Addition of 100 μM carboxy-PTIO completely suppressed both the basal level (Figure 1e) as well as the elicited burst of fluorescence (Figure 1f). Thus, DAF-2 DA specifically detects intracellular NO.

While carboxy-PTIO is a useful control to determine whether DAF-2 DA fluorescence is due to NO accumulation, it does not give any hints on the source of NO. In mammals, NO is produced by nitric oxide synthase (NOS), which occurs in several constitutive and inducible (iNOS) isoforms. NOS catalyses NO formation from O_2 and L-arginine (Poulos *et al.*, 1998). iNOS plays a central role in the animal immune system where it is induced at the transcriptional and post-translational level by endotoxins and cytokines (Mayer and Hemmens, 1997).

In plants, initial evidence for the presence of a mammalian-type NOS was first reported in 1996 (Cueto *et al.*, 1996; Leshem, 1996; Ninnemann and Maier, 1996). NO and NOS seem to play a prominent role in plant defence against pathogens. Tobacco, soybean cells and *Arabidopsis*, treated with tobacco mosaic virus, bacterial pathogens (*Ralstonia solanacearum* and *Pseudomonas syringae*) or elicitors responded with enhanced NOS activity (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Huang and Knopp, 1998).

In addition to applying highly specific NOS assays, most data on plant NOS are based on and/or supported by the use of well-characterized NOS inhibitors, mostly inactive substrate analogues. Several previous reports presented *in vitro* data on inhibition of plant NOS by NOS inhibitors (Cueto *et al.*, 1996; Delledonne *et al.*, 1998; Durner *et al.*, 1998; Leshem, 1996; Ninnemann and Maier, 1996). Figure 1(h,i) shows that the NOS inhibitor N^G-monomethyl-arginine monoacetate (L-NMMA; 1 mM) reduced both the basal level as well as the elicited burst of DAF-2 DA fluorescence by more than 50%. The inactive D-enantiomer of NMMA did not influence DAF-2 DA fluorescence (data not shown).

The partial inhibition of DAF-2 DA fluorescence by a NOS-inhibitor (Figure 1g–i), which is in contrast to the complete fluorescence suppression by the NO scavenger PTIO (Figure 1d–f), may be due to the high specificity of NOS inhibitors for any given NOS isoform. As a matter of fact, the individual forms of animal NOS as well as plant NOS activity differ in their degree of sensitivity to NOS inhibitors (Durner and Klessig, 1999; Ogden and Moore, 1995). Nevertheless, while our data are in line with previous reports on NOS-like activity in plants, they do

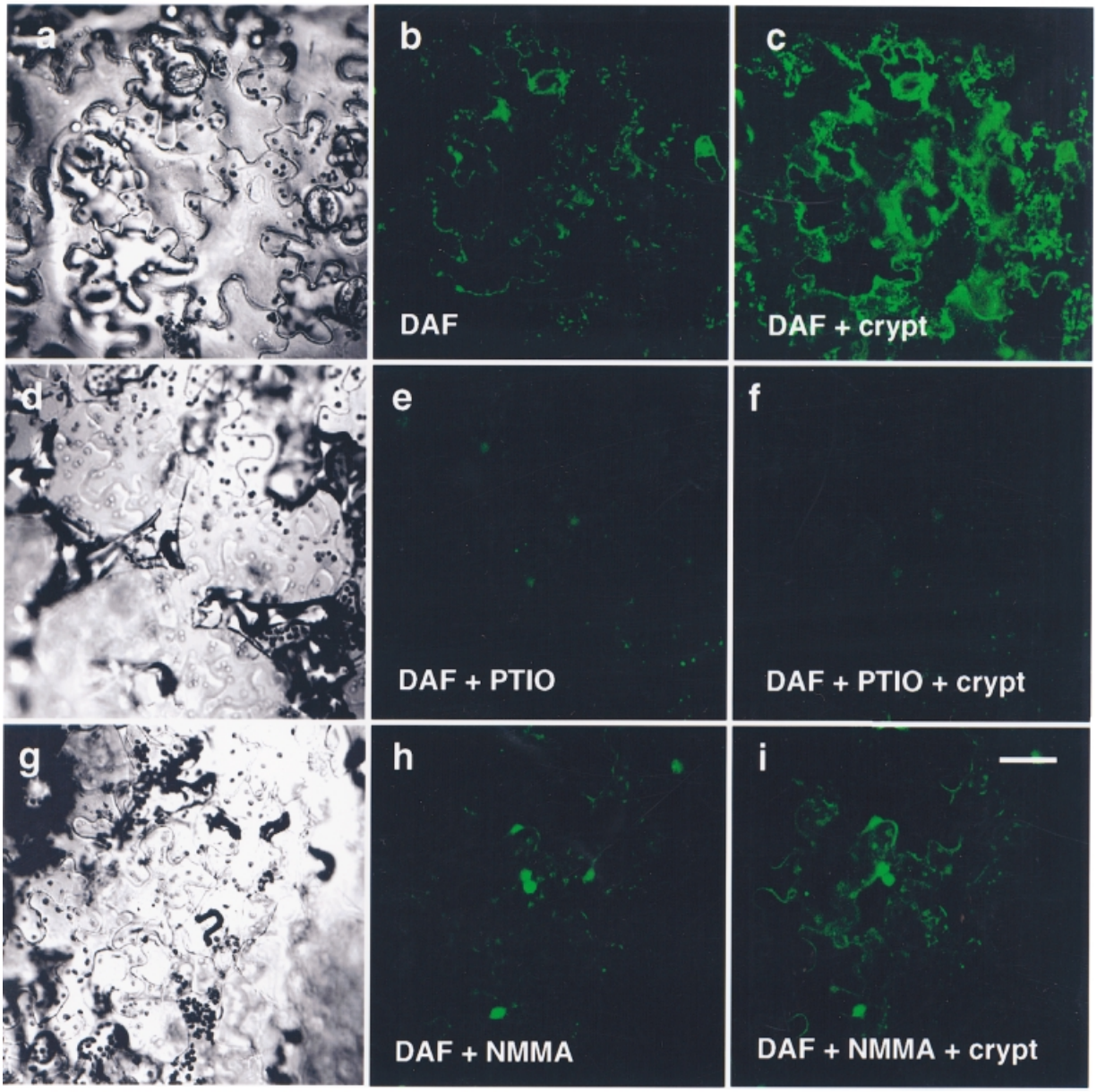

Figure 1. Confocal laser scanning microscopy of cryptogein-induced increases in intracellular DAF-2 DA fluorescence.
Epidermal cells were loaded with DAF-2 DA, washed, treated with cryptogein ± scavenger or inhibitor and examined by confocal laser scanning microscopy. (a) Bright-field image of epidermal cells. (b) Fluorescence of DAF-2 DA (505–530 nm) before stimulation of the cell shown in (a). (c) Fluorescence 6 min after treatment with 50 nM cryptogein. (d,e,f) No fluorescence is seen when DAF-2 DA-loaded cells are elicited by cryptogein in the presence of the NO scavenger carboxy-PTIO (100 μM). (g,h,i) Elicitation by cryptogein of DAF-2 DA-loaded cells in the presence of the NOS inhibitor L-NMMA (1 mM) results in reduced fluorescence. Bar = 50 μm.

not rule out alternative sources of NO. NO may be synthesized non-enzymatically from NO_2^- accumulating in chloroplasts and in the cytosol, or through an NO_2^--dependent side reaction of nitrate reductases, which can also catalyse formation of the highly toxic peroxynitrite (Cooney *et al.*, 1994; Klepper, 1991; Wildt *et al.*, 1997; Yamasaki and Sakihama, 2000). However, currently there is no evidence for a possible role of nitrate reductase as an NO source during early defence responses.

Imaging of DAF-2 DA fluorescence was also used to follow the time course of NO accumulation. Figure 2 shows a close-up of an epidermal cell responding to cryptogein. Figure 2(a,c) represents bright-field images (asterisks in Figure 2c indicate regions analysed for changes in pixel intensity as shown in Figure 2d). Figure 2(b) shows an image obtained with a long-pass filter (LP 585), revealing the strong chlorophyll fluorescence within the chloroplasts. After elicitation with cryptogein, the earliest

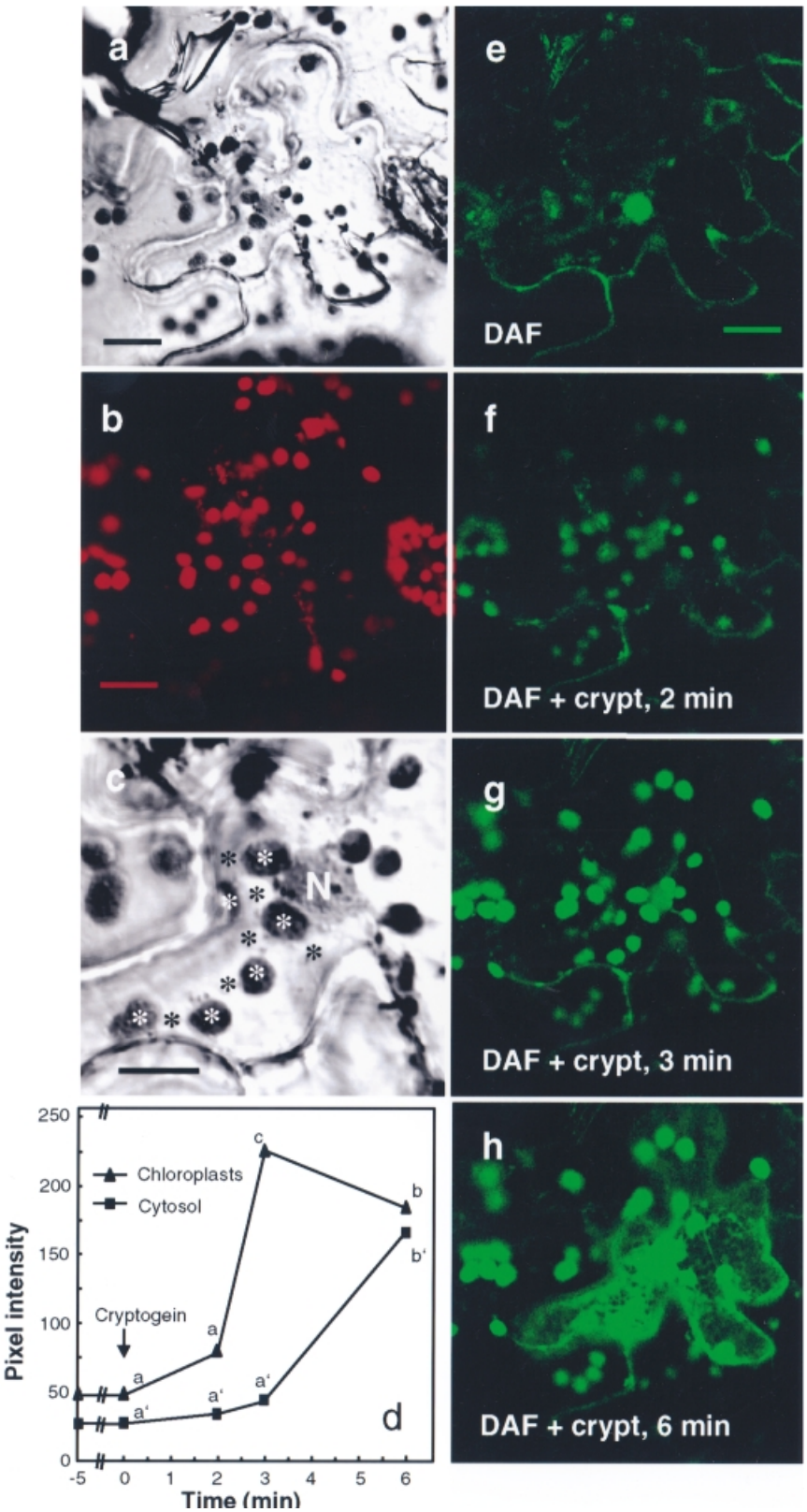

Figure 2. Time course of intracellular DAF-2 DA fluorescence after cryptogein stimulation.

Epidermal cells were loaded with DAF-2 DA, washed, treated with cryptogein and examined by confocal laser scanning microscopy. (a) Bright-field image of a single epidermal cell. (b) Image obtained with a long-pass filter (⩾585 nm) reveals chlorophyll fluorescence within the plastids. The image was taken before the addition of cryptogein. (c) Close-up of (a). Chloroplasts and cytosolic regions analysed for pixel intensities (see (d)) are marked with asterisks. (d) Time course of pixel intensities of selected regions in a DAF-2 DA-loaded epidermal cell treated with cryptogein. At time zero, 50 nM cryptogein was added (arrow). Each time point represents the mean pixel intensity in six chloroplasts and six adjacent cytoplasmic areas of the epidermal cell shown in (a). Means ($n = 6$) marked by the same letter are not significantly different ($P < 0.05$) according to Tukey's multiple range test. (e) DAF-2 DA fluorescence before addition of cryptogein. (f–h) Chloroplast autofluorescence and DAF-2 DA fluorescence 2, 3 and 6 min after stimulation with 50 nM cryptogein. Bars = 20 μm (a,b,e–h) and 15 μm (c).

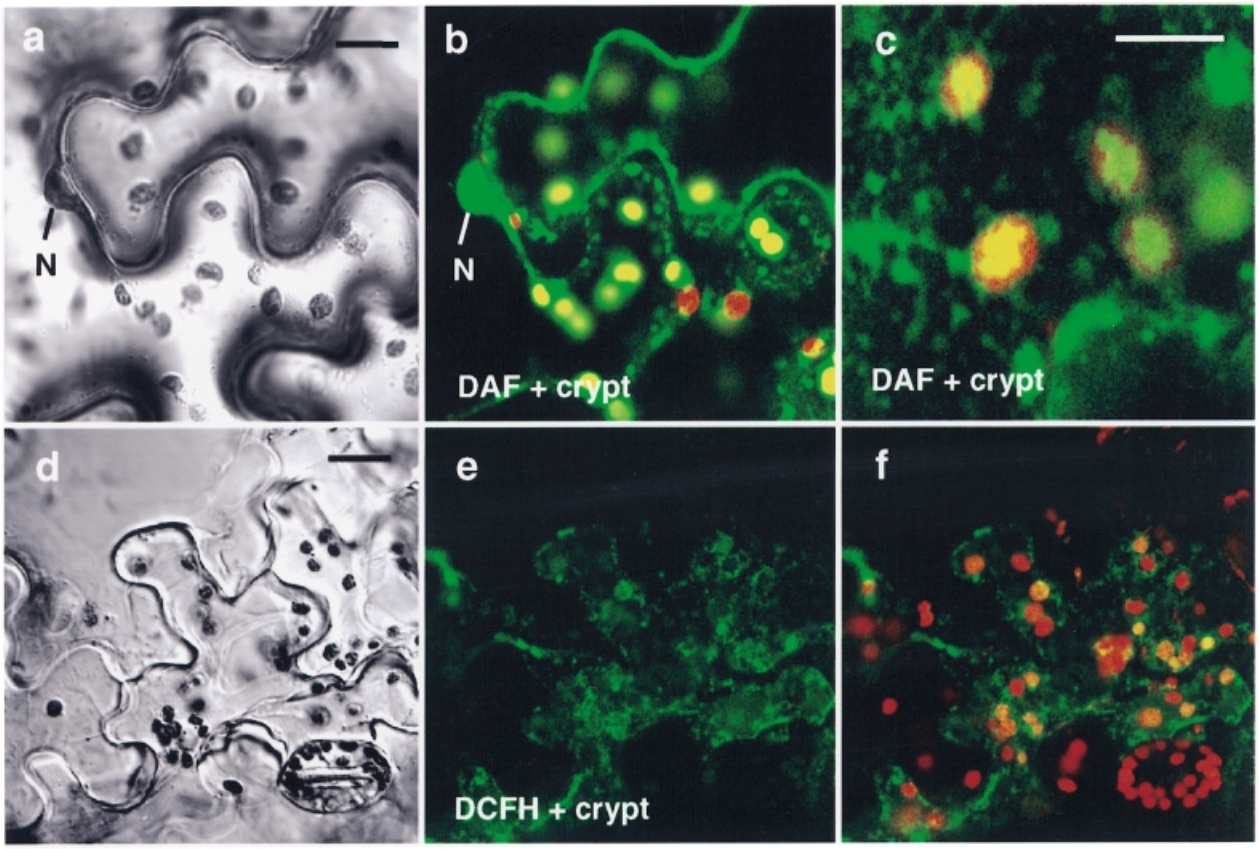

Figure 3. Confocal laser scanning microscopy of cryptogein-induced increases in intracellular DAF-2 DA and DCFH fluorescence.
(a) Bright-field image of two epidermal cells loaded with DAF-2 DA. (b) DAF-2 DA fluorescence of cells shown in (a) 10 min after stimulation with 50 nM cryptogein. Autofluorescence of chloroplasts and DAF-2DA fluorescence are superimposed. Most of the chloroplasts show strong DAF-2 DA fluorescence, which is also seen along the plasma membrane and in the nucleus (N). (c) Close-up of a DAF-2 DA-loaded cell 6 min after treatment with 50 nM cryptogein. DAF-2 DA fluorescence is localized to distinct regions within the chloroplasts as well as to other subcellular structures, which may represent peroxisomes. The image was superimposed with an image scanned with a long-pass filter to reveal chlorophyll autofluorescence. (d) Bright-field image of a single epidermal cell loaded with DCFH (ROS-indicating fluorophore). (e) DCFH fluorescence of the cell shown in (d) 12 min after stimulation with 50 nM cryptogein. (f) Same image as shown in (e), but superimposed with chlorophyll autofluorescence. In contrast to (c), chloroplasts show a very weak fluorophore signal. Bars = 10 μm (a), 15 μm (b,c) and 20 μm (d–f).

increases in NO were in the chloroplasts, where NO accumulation occurred within the first 3 min (Figure 2e–g). After 6 min, the cytosol and the cell periphery, probably the plasma membrane, showed strong DAF-2 DA fluorescence (Figure 2h). The time course of DAF-2DA fluorescence measured as pixel intensities of six selected chloroplasts and six cytosolic regions is shown in Figure 2(d). Similar results have been reported regarding production of ROS in epidermal peels of tobacco (Allan and Fluhr, 1997). While chloroplasts show an early increase in DAF-2 DA fluorescence, NO production and/or accumulation is also found in the nucleus and along the plasma membrane (Figure 3a,b), and in distinct cellular compartments in the vicinity of chloroplasts, most likely peroxisomes (Figure 3c). These data are consistent with both nitrite accumulation in the chloroplast as a possible NO source (Klepper, 1991) and with the localization of putative plant NOS, respectively. Most recently, electron microscopy immunogold-labelling with antibodies made against rabbit brain NOS or murine iNOS has confirmed the subcellular localization of an NOS-like protein in the matrix of peroxisomes, in chloroplasts and in the nucleus of tobacco, maize and pea (Barroso *et al.*, 1999; Huang and Knopp, 1998; Ribeiro *et al.*, 1999). However, data on intracellular localization of NO based on the use of dyes should be interpreted with caution. DAF-2 DA is a single-wavelength probe, and no adjustments can be made for differential accumulation of the probe within the cell. While there is no evidence for differential loading, DAF-2 DA or other fluorescein derivatives might preferentially accumulate in specific cellular compartments.

In animals, ROS collaborate with NO to induce apoptosis, to execute invading pathogens and to induce defence genes. While it is assumed that both NO and ROS act as both cellular signals and direct weapons, their biological effects may depend on multiple interactions (Mayer and Hemmens, 1997). Both NO and H_2O_2 are freely diffusible and membrane-permeable. In plants, NO was shown to act

synergistically with ROS to increase host cell death of soybean suspension cells (Delledonne *et al.*, 1998). Previously, it has been shown that cryptogein treatment of tobacco cells results in a fast ROS burst (Allan and Fluhr, 1997). In our study also, elicitation by cryptogein revealed strong DCFH fluorescence indicating the presence of ROS (Figure 3d,e). The overall time courses of ROS and NO production after cryptogein activation are almost identical, with earliest responses detectable after less than 1 min and full activation between 6 and 12 min (Figure 2) (Allan and Fluhr, 1997). The synchronized bursts suggest a concerted action of ROS and NO as described for animals (Mayer and Hemmens, 1997) and for plants (McDowell and Dangl, 2000; Van Camp *et al.*, 1998).

In summary, we have shown that the NO-sensitive fluorophore DAF-2 DA in conjunction with confocal laser scanning microscopy can be used to directly measure the NO burst of tobacco cells elicited by cryptogein. Real-time detection of NO *in vivo* might be an extremely valuable tool to study the involvement of NO in other plant–pathogen interactions as well as in other fields of plant biology such as plant photomorphogenesis, root–microbe interactions and nitrate sensing.

Experimental procedures

Plant material

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown at 22°C in growth chambers programmed for a 14 h light and 10 h dark cycle. Six- to eight-week-old plants were used for experimentation.

Chemicals

4,5-diaminofluorescein diacetate (DAF-2 DA), L-N^G-monomethyl-arginine monoacetate (L-NMMA), carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl (PTIO) and (*Z*)-1-*N*-methyl-*N*[6-(*N*-methyl-ammoniohexyl)amino])-diazen-1-ium-1,2-diolate (NOC-9, also known as MAHMA NoNOate) were purchased from Alexis Corp. Xanthine oxidase (from milk) and xanthine were from Sigma.

Cryptogein

Cryptogein was purified according to Bonnet *et al.* (1996).

Fluorescence spectrophotometry

To assess the specificity of DAF-2 DA, the NO probe was treated with various ROS or NO donors. Fluorescence spectroscopy was performed using a LS-3B/R 100A system (Perkin Elmer) in 0.5 ml fluorescence cells. After excitation at 488 nm, emission at 515 nm was recorded.

Confocal laser scanning microscopy

Fully expanded leaves were removed from greenhouse-grown tobacco (*N. tabacum* cv. Xanthi nc). Using a razor blade, epidermal sections were then produced from the abaxial surface of the leaves and placed in a Petri dish containing 5 ml of loading buffer (10 mM Tris/KCl, pH 7.2) and DAF-2 DA at a final concentration of 10 μM (added from a 10 mM stock in DMSO). If not otherwise indicated, epidermal sections were maintained in the dark for 10 min. The sections were then removed and transferred to a dish of fresh loading buffer (without probe) to wash off excess fluorophore. After 10–20 min, sections were affixed to the cover slip bottom of a chamber slide with silicon grease where they remained immersed in 250 μl of fresh loading buffer. Elicitor and inhibitors were directly added to the buffer at volumes < 10 μl.

A Zeiss Axiovert 100M inverted microscope equipped with a confocal laser scanner (Zeiss LSM 510, 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 505–530 nm band-pass filter, autofluorescence of chloroplasts was captured with a 585 nm long-pass filter. Microscope, laser and photo-multiplier settings were held constant during the course of an experiment in order to obtain comparable data. Images were processed and analysed using the Zeiss LSM 510 software. Plates were produced with Microsoft PowerPoint and printed on a Kodak dye sublimation printer.

Acknowledgements

We thank Wolfgang Pfeiffer (Salzburg) for helpful discussion, and Friedrich-Wilhelm Bentrup (Salzburg) and Heinrich Sandermann Jr (GSF) for encouraging cooperation. This work was supported by grant Du 246/4-1 (Deutsche Forschungsgemeinschaft, DFG). Additional support was given by the Österreichische Nationalbank, project number 6675, and by Freistaat Bayern (BStMLU).

References

Allan, A.C. and Fluhr, R. (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell*, **9**, 1559–1572.

Alvarez, M.E., Pennell, R.I., Meijer, P.-J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell*, **92**, 773–784.

Barchowsky, A., Klei, L.R., Dudek, E.J., Swartz, H.M. and P.E.J. (1999) Stimulation of reactive oxygen, but not reactive nitrogen species in vascular endothelial cells exposed to low levels of arsenite. *Free Rad. Biol. Med.* **27**, 1405–1412.

Barroso, J.B., Corpas, F.J., Carreras, A., Sandalio, L.M., Valderrama, R., Palma, J.M., Lupianez, J.A. and del Rio, L.A. (1999) Localization of nitric-oxide synthase in plant peroxisomes. *J. Biol. Chem.* **274**, 36729–36733.

Beligni, M.V. and Lamattina, L. (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta*, **210**, 215–221.

Bolwell, G.P. (1999) Role of active oxygen species and NO in plant defence responses. *Curr. Opin. Plant Biol.* **2**, 287–294.

Bonnet, P., Bourdon, E., Ponchet, M., Blein, J.-P. and Ricci, P. (1996) Acquired resistance triggered by elicitins in tobacco and other plants. *Eur. J. Plant Pathol.* **102**, 181–192.

Bourque, S., Binet, M.N., Ponchet, M., Pugin, A. and Lebrun-

Garcia, A. (1999) Characterization of the cryptogein binding sites on plant plasma membranes. *J. Biol. Chem.* **274**, 34699–34705.

Cooney, R.V., Harwood, P.J., Custer, L.J. and Franke, A.A. (1994) Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. *Environ. Health Perspect.* **102**, 460–462.

Cueto, M., Hernandéz-Perera, O., Martin, R., Bentura, M.L., Rodrigo, J., Lamas, S. and Golvano, M.P. (1996) Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. *FEBS Lett.* **398**, 159–164.

Dangl, J. (1998) Plants just say NO to pathogens. *Nature*, **394**, 525–526.

Dangl, J.L., Dietrich, R.A. and Richberg, M.H. (1996) Death don't have no mercy: cell death programs in plant–microbe interactions. *Plant Cell*, **8**, 1793–1807.

Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature*, **394**, 585–588.

Durner, J. and Klessig, D.F. (1999) Nitric oxide as a signal in plants. *Curr. Opin. Plant Biol.* **2**, 369–372.

Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP ribose. *Proc. Natl Acad. Sci. USA*, **95**, 10328–10333.

Durner, J., Gow, A.J., Stamler, J.S. and Glazebrook, J. (1999) Ancient origins of nitric oxide signaling in biological systems. *Proc. Natl Acad. Sci. USA*, **96**, 14206–14207.

Finkel, T. (1998) Oxygen radicals and signaling. *Curr. Opin. Cell Biol.* **10**, 248–253.

Hippeli, S. and Elstner, E.F. (1998) Transition metal ion-catalyzed oxygen activation during pathogenic processes. *FEBS Lett.* **443**, 1–7.

Huang, J.-S. and Knopp, J.A. (1998) Involvement of nitric oxide in *Ralstonia solanacearum*-induced hypersensitive reaction in tobacco. In *Bacterial Wilt Disease: Molecular and Ecological Aspects* (Prior, P., Elphinstone, J. and Allen, C, eds). Berlin: INRA and Springer Editions, pp. 218–224.

Hufton, C.A., Besford, R.T. and Wellburn, R.A. (1996) Effects of NO ($+NO_2$) pollution on growth, nitrate reductase activities and associated protein contents in glasshouse lettuce grown hydroponically in winter with CO_2 enrichment. *New Phytol.* **133**, 495–501.

Klepper, L. (1991) NOx evolution by soybean leaves treated with salicylic acid and selected derivatives. *Pesticide Biochem. Physiol.* **39**, 43–48.

Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y. and Nagano, T. (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* **70**, 2446–2453.

Lebrun-Garcia, A., Ouaked, F., Chiltz, A. and Pugin, A. (1998) Activation of MAPK homologues by elicitors in tobacco cells. *Plant J.* **15**, 773–781.

Leshem, Y.Y. (1996) Nitric oxide in biological systems. *Plant Growth Regul.* **18**, 155–159.

Leshem, Y.Y., Wills, R.B.H. and Ku, V.V.V. (1998) Evidence for the function of the free reduced gas – nitric oxide (NO·) – as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiol. Biochem.* **36**, 825–826.

McDowell, J.M. and Dangl, J.L. (2000) Signal transduction in the plant immune response. *Trends Biochem. Sci.* **25**, 79–82.

Mayer, B. and Hemmens, B. (1997) Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem. Sci.* **22**, 477–481.

Nathan, C. (1995) Natural resistance and nitric oxide. *Cell*, **82**, 873–876.

Ninnemann, H. and Maier, J. (1996) Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidation of *Neurospora crassa*. *Photochem. Photobiol.* **64**, 393–398.

Noritake, T., Kawakita, K. and Doke, N. (1996) Nitric oxide induces phytoalexin accumulation in potato tuber tissue. *Plant Cell Physiol.* **37**, 113–116.

Ogden, J.E. and Moore, P.K. (1995) Inhibition of nitric oxide synthase – potential for a novel class of therapeutic agent? *Trends Biotechnol.* **13**, 70–78.

Poulos, T.L., Raman, C.S. and Li, H. (1998) NO news is good news. *Structure*, **6**, 255–258.

Pugin, A., Frachisse, J.-M., Tavernier, E., Bligny, R., Gout, E., Douce, R. and Guern, J. (1997) Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. *Plant Cell*, **9**, 2077–2091.

Ribeiro, E.A., Cunha, F.Q., Tamashiro, W.M.S.C. and Martins, I.S. (1999) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS Lett.* **445**, 283–286.

Scheel, D. (1998) Resistance response physiology and signal transduction. *Curr. Opin. Plant. Biol.* **1**, 305–310.

Stamler, J.S., Singel, D.L. and Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science*, **258**, 1898–1902.

Van Camp, W., Van Montagu, M. and Inzé, D. (1998) H_2O_2 and NO: redox signals in disease resistance. *Trends Plant Sci.* **3**, 330–334.

Vowells, S.J., Sekhsaria, S., Malech, H.L., Shalit, M. and Fleisher, T.A. (1995) Flow cytometric analysis of the granulocyte respiratory burst: a comparison of fluorescent probes. *J. Immunol. Methods*, **178**, 89–97.

Wildt, J., Kley, D., Rockel, A., Rockel, P. and Segschneider, H.J. (1997) Emission of NO from higher plant species. *J. Geophys. Res.* **102**, 5919–5927.

Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* **468**, 89–92.