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RESEARCH PAPER

Salicylic acid activates nitric oxide synthesis in *Arabidopsis*

Michela Zottini[1,*], Alex Costa[1], Roberto De Michele[1], Maria Ruzzene[2], Francesco Carimi[3] and Fiorella Lo Schiavo[1]

[1] *Dipartimento di Biologia, Università Degli Studi di Padova, Via U. Bassi 58/B, I-35131 Padova, Italy*
[2] *Dipartimento di Chimica Biologica, Università Degli Studi di Padova, Viale G. Colombo 3, I-35121 Padova, Italy*
[3] *Istituto di Genetica Vegetale, Palermo (CNR), Corso Calatafimi 414, I-90128 Palermo, Italy*

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Abstract

The relationship between nitric oxide (NO) and salicylic acid (SA) was investigated in *Arabidopsis thaliana*. Here it is shown that SA is able to induce NO synthesis in a dose-dependent manner in *Arabidopsis*. NO production was detected by confocal microscopic analysis and spectrofluorometric assay in plant roots and cultured cells. To identify the metabolic pathways involved in SA-induced NO synthesis, genetic and pharmacological approaches were adopted. The analysis of the *nia1,nia2* mutant showed that nitrate reductase activity was not required for SA-induced NO production. Experiments performed in the presence of a nitric oxide synthase (NOS) inhibitor suggested the involvement of NOS-like enzyme activity in this metabolic pathway. Moreover, the production of NO by SA treatment of *Atnos1* mutant plants was strongly reduced compared with wild-type plants. Components of the SA signalling pathway giving rise to NO production were identified, and both calcium and casein kinase 2 (CK2) were demonstrated to be involved. Taken together, these results suggest that SA induces NO production at least in part through the activity of a NOS-like enzyme and that calcium and CK2 activity are essential components of the signalling cascade.

Key words: *Arabidopsis thaliana*, *Atnos1*, casein kinase 2, *nia1,nia2*, nitrate reductase, nitric oxide, salicylic acid signals.

Introduction

Salicylic acid (SA) is a phenolic compound affecting a number of physiological processes in plants, including thermogenesis, ethylene synthesis, and fruit ripening (Rhoads and McIntosh, 1992). It also has a role in plant responses to different abiotic stresses such as UV radiation and ozone exposure (Rao and Davis, 1999; Senaratna *et al*., 2000), in different developmental conditions or in response to various biological stimuli such as during nodulation (Stacey *et al*., 2006). The role of SA during pathogen attack is particularly relevant (Yang *et al*., 1997) both in promotion of a local response and in systemic acquired resistance (SAR) (Alvarez, 2000). In fact, upon infiltration of SA in *Arabidopsis* leaves, the same set of genes activated by pathogen infection is induced (Uknes *et al*., 1992). It has also been reported that, following inoculation of the leaves with a micro-organism capable of inducing SAR, SA accumulates at very high concentrations, not only in leaves and in the stem, but also in the roots where SA reaches concentrations higher than in tissues close to the infection site (Kubota and Nishia 2006).

Nitric oxide (NO) is a highly active gaseous molecule involved in diverse pathophysiological processes (Neill *et al*., 2003; Grün *et al*., 2006). A major advance in the understanding of NO functions in plants was the identification of different NO biosynthetic pathways (Lamotte *et al*., 2005) and, in fact, NO can be formed by cytoplasmic nitrate reductase (cNR), nitrite-NO reductase (NI-NOR), or by unidentified protein(s) displaying NOS (nitric oxide synthase) activity. cNR appears to operate under anaerobic conditions in the presence of high nitrite

* To whom correspondence should be addressed. E-mail: mzottini@bio.unipd.it
Abbreviations: CK2, casein kinase 2; cNR, cytoplasmic nitrate reductase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, diaminofluorescein-FM diacetate; DMSO, dimethylsulphoxide; HbO_2, oxyhaemoglobin; K252a, protein kinase inhibitor; l-NMMA, N^G-monomethyl-L-arginine; metHb, methaemoglobin; NI-NOR, nitrite-NO reductase; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; TBB, 4,5,6,7-tetrabromobenzotriazole.

concentrations (Yamasaki, 2000; Yamasaki and Sakihama, 2000), more often in roots than in leaves (Stöhr, 1999). In roots, NO can also be formed by NI-NOR, which uses nitrite as a substrate (Stöhr *et al*., 2001). Recently, an *Arabidopsis* mutant altered in NO production has been isolated *(Atnos1*) (Guo *et al*., 2003). It was initially considered to be altered in a NOS activity localized at the mitochondrial level (Guo and Crawford, 2005). More recently, however, it has been excluded that the AtNOS1 protein has NOS activity (Zemojtel *et al*., 2006), but this mutant is still useful for its phenotype, which shows reduced levels of NO (He *et al*., 2004; Zeidler *et al*., 2004; Bright *et al*., 2006).

In the pathogen-activated hypersensitive response, both NO and reactive oxygen species (ROS) act as signal molecules (Delledonne, 2005). ROS and NO are also involved in the regulation of SA biosynthesis (Durner *et al*., 1998). Several models suggest that redox signalling through NO and ROS is enhanced by SA in a self-amplifying process (Klessig *et al*., 2000). Nonetheless, the relationship between NO, SA, and ROS in the activation of defence genes and/or induction of host cell death is not clearly defined.

In this report, experimental evidence is presented that SA activates NO synthesis both in *Arabidopsis* roots and in cultured cells, and that this NO production proceeds at least in part through a NOS-dependent pathway.

Materials and methods

Materials

The NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) was from Alexis (Vinci, Italy). The casein kinase 2 (CK2)-specific inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) was used at a final concentration of 30 μM (Ruzzene *et al*., 2002). All other chemicals were from Sigma-Aldrich (Milan, Italy). Sodium salicylate was dissolved in water. Kinase inhibitors were dissolved in dimethylsulphoxide (DMSO) at the indicated concentrations, and the same amount of the solvent was used for control experiments.

Cell cultures and plant material

The *Arabidopsis* cell line was obtained as described in Carimi *et al.* (2005). The experiments on plant seedlings were performed on *Arabidopsis* wild type, *nia1,nia2* (Nottingham *Arabidopsis* Stock Centre), and *Atnos1* mutants ecotype Columbia-0. Seeds were surface-sterilized by immersion in 4% sodium hypochloride (v/v) and rinsed five times with distilled sterile water before transfer to MS (Murashige and Skoog, 1962) solidified medium (7 g l^{-1} agar type M). All experiments were performed using 5-d-old seedlings.

NO quantification

Fluorometric assay: The cell-permeable diacetate derivative diaminofluorescein-FM (DAF-FM DA; Alexis Biochemicals, Cod 620-071-M001) was used as a specific fluorescent probe for the detection of intracellular NO (Gould *et al*., 2003).

Five-day-old *Arabidopsis* seedlings were incubated for 15 min in loading buffer (5 mM MES-KOH, pH 5.7, 0.25 mM KCl, 1 mM $CaCl_2$) with or without NOS inhibitor (1 mM L-NMMA), protein kinase inhibitors (2 μM K252a, 30 μM TBB) or the NO scavenger cPTIO [0.5 mM, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide]. The seedlings were then incubated for 15 min in the same loading buffer containing 15 μM DAF-FM DA, followed by 20 min of washing in loading buffer in order to remove excess fluorescent probe. The seedlings were subsequently incubated in buffer with or without SA for different times in the presence or absence of various inhibitors. In the calcium-free experiments, calcium was removed from the buffers after DAF loading.

DAF-FM fluorescence was estimated by using confocal laser scanning microscopy (excitation 488 nm, emission 515–530 nm; Nikon PCM2000). The pixel intensities of fluorescence images, acquired using a confocal microscope, were determined by using ImageJ software (NIH, USA). Values were corrected for background.

The confocal images shown are representative of three different experiments in which at least five seedlings were analysed for each treatment (n=15).

Spectrophotometric assay: The NO concentration was measured by monitoring the conversion of oxyhaemoglobin (HbO_2) to methaemoglobin (metHb) in the medium of cultured cells, as described by Murphy and Noack (1994). For the NO assay, 1 ml of *Arabidopsis* cell suspension was incubated with 100 U ml^{-1} catalase (Sigma) and 100 U ml^{-1} superoxide dismutase (Sigma) for 5 min to remove ROS before the addition of HbO_2 (10 μM final concentration, haemoglobin-A_0 human, Sigma). Next, cells were separated by filtration through chromatography columns (Bio-Rad, 731-1550) and 0.8 ml of medium was collected for NO determination. NO was quantified by measuring spectrophotometrically (Perkin-Elmer lambda spectrophotometer) following the conversion of HbO_2 to metHb at 401 nm and 421 nm, using an extinction coefficient of 77 mM^{-1} cm^{-1} (A_{401} HbO_2–A_{421} metHb). The values reported have been corrected for the basal NO content of untreated cells.

For these experiments, a randomized complete block design was used with three replicates (individual Erlenmeyer flasks). Each experiment was repeated three times (n=9).

CK2 activity assay

Endogenous CK2 activity was measured on protein extracts obtained from 5-d-old *Arabidopsis* seedlings according to Zottini *et al.* (2002). A 2.5–5 μg aliquot of protein extracts was incubated for 10 min at 30 °C with the CK2-specific peptide RRRADDSDDDDD (0.5 mM) in the presence of a phosphorylation mixture consisting of 50 mM TRIS-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM ATP, [γ-^{33}P]ATP (specific activity 3000–6000 cpm $pmol^{-1}$), 100 mM NaCl, 10 mM NaF, and 1 mM okadaic acid. Samples were spotted onto phosphocellulose filters, washed, and counted by scintillation as described (Ruzzene *et al*., 2002). A blank value obtained from control experiments performed under the same conditions but in the presence of 4 μM TBB, thus preventing phosphorylation by CK2, was subtracted from each value. Protein concentration was determined by the method of Bradford (1976) and normalized by western blot with anti-ATPase β-subunit antibodies, loading 2.5 μg of protein. Each experiment was repeated three times. One hundred seedlings were used for each experiment and the enzymatic assay was repeated four times.

Results

SA induces NO production in a dose-dependent manner

In order to evaluate the ability of SA to induce a NO burst, NO production was analysed in *Arabidopsis* seedlings by

using the cell-permeable fluorescent probe DAF-FM DA in combination with confocal laser-scanning microscopy. Figure 1A shows real-time imaging of NO production in *Arabidopsis* seedlings preloaded with 15 μM DAF-FM DA and subsequently treated with SA (1 mM). SA at 1 mM is not toxic for *Arabidopsis* seedlings (data not shown).

SA treatment resulted in a strong increase of fluorescence, indicative of NO production. To ascertain that the fluorescence signal was due only to NO, control experiments were performed. First the effectiveness of cPTIO, a widely used NO scavenger, was tested. As seen in Fig. 1B, treatment with 0.5 mM cPTIO prevented fluorescence increase in roots of *Arabidopsis* seedlings incubated with 0.75 mM and 1 mM SA. Figure 1C shows a control experiment performed by treating seedlings with an inactive analogue of SA (1 mM hydroxybenzoate; Norman *et al*., 2004), and no variation in DAF-FM fluorescence was observed.

In Fig. 2A, the kinetics of NO accumulation in DAF-loaded seedlings treated with increasing concentrations of SA (0.5, 0.75, and 1 mM) are reported. A clear dose dependence of the NO accumulation was observed, with a maximum response reached within 2 h.

To detect NO, the use of more than one technique is highly recommended (Zeidler *et al*., 2004). For this reason, NO was also determined spectrophotometrically by measuring the conversion of HbO_2 to metHb in *Arabidopsis* cultured cells. This method is quantitative and provides the opportunity to determine the precise amount of NO produced by differentially treated samples. In Fig. 2B, the kinetics of the accumulation of NO in cultured cells treated with increasing concentrations of SA are shown. The latter data confirm the dose dependence of SA-induced NO production and correlate well with the data obtained in roots by using the fluorometric assay.

Sources of SA-induced NO synthesis

In order to identify the metabolic pathways involved in NO synthesis, a genetic approach was adopted. First the contribution of nitrate reductase (NR) activity was evaluated. In *Arabidopsis*, NR is encoded by two genes (*NIA1* and *NIA2*), and the double mutant *nia1,nia2* shows <1% of wild-type NR activity (Wilkinson and Crawford, 1993).

Low levels of fluorescence were observed in the untreated wild-type and *nia1,nia2* mutant (Fig. 3). The *nia1,nia2* mutant did not emit NO when incubated in the presence of 1 mM nitrite, as expected, while wild-type roots showed >3-fold increased levels of fluorescence under the same experimental conditions. Seedlings treated with 1 mM SA for 2 h showed a similar induction of NO production in both wild-type and *nia1,nia2* mutant roots (Fig. 2). These results indicate an NR-independent route for SA-induced NO synthesis.

To determine whether SA-induced NO synthesis proceeds via a NOS-like activity, the experiments were

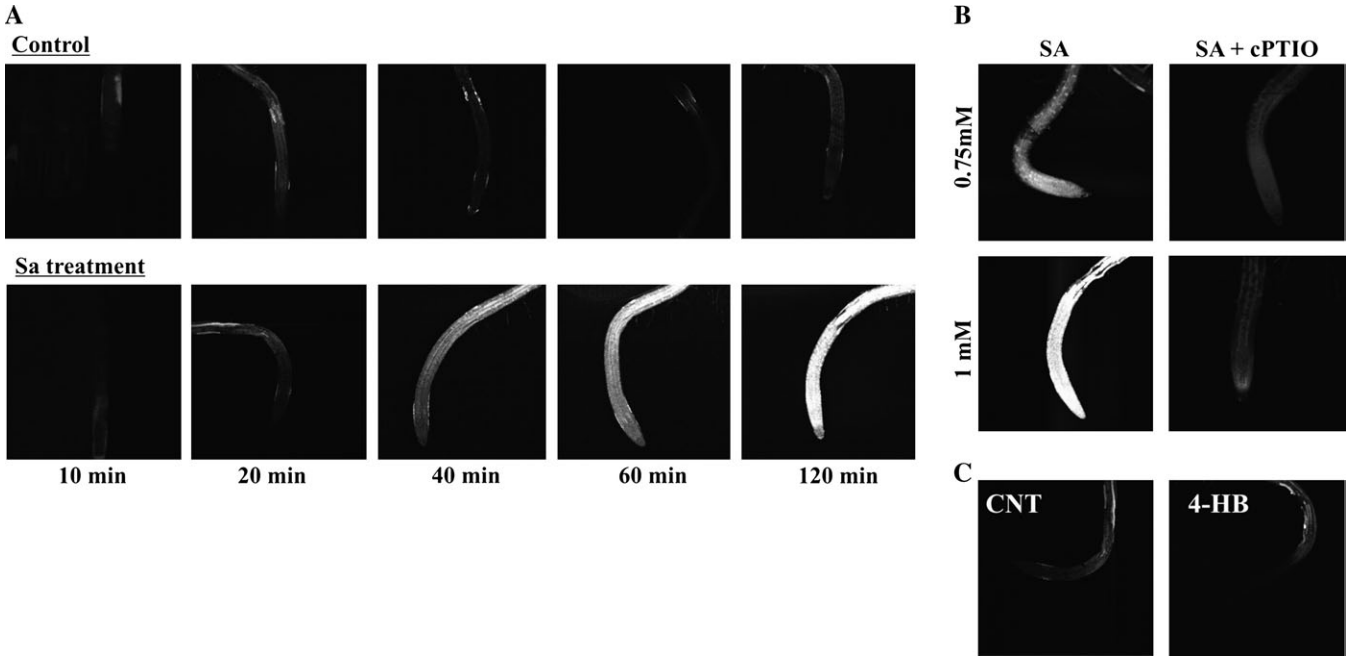

Fig. 1. SA stimulates NO production in *Arabidopsis*. (A) Time-course of SA-induced NO burst as detected by confocal laser-scanning microscopy. *Arabidopsis* 5-d-old seedlings were loaded with 15 μM DAF-FM DA and treated with water (upper) or 1 mM SA (lower). (B) Fluorescence imaging of DAF-FM-loaded seedlings treated for 2 h with SA with or without 0.5 mM cPTIO. (C) Fluorescence imaging of DAF-FM-loaded seedling treated for 2 h with water (CNT) or 1 mM hydroxybenzoate (4-HB).

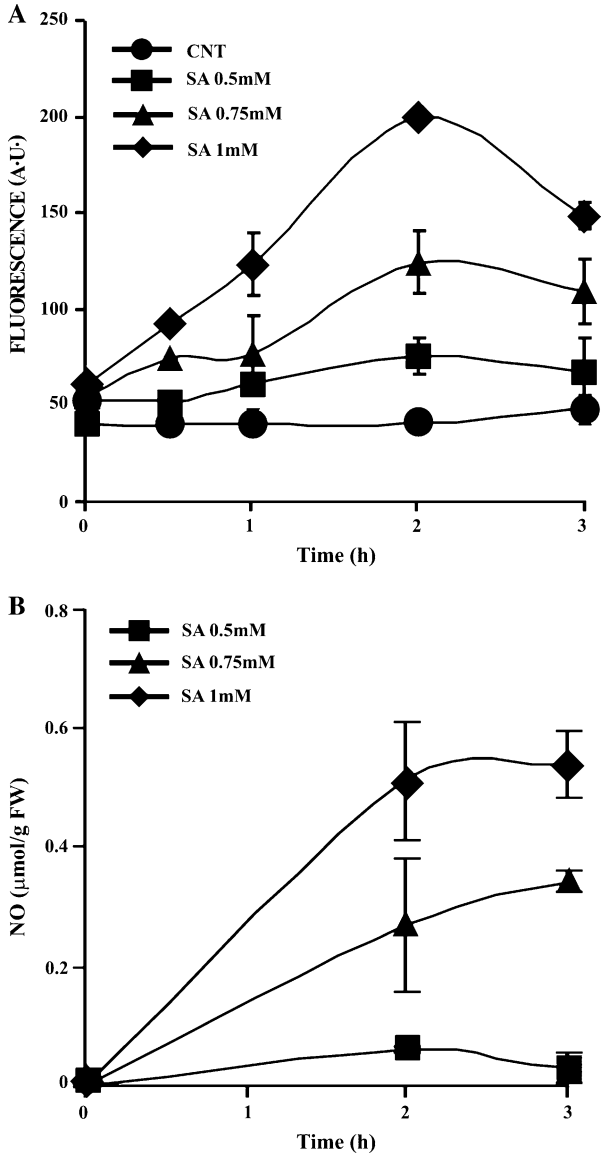

Fig. 2. (A) Time-course of SA-induced NO production in DAF-FM-loaded seedlings treated with different concentrations of SA. DAF-FM fluorescence is indicated as pixel intensity arbitrary units (A.U.). (B) Time-course of SA-induced NO production in cultured cells estimated spectrophotometrically following the conversion of oxyhaemoglobin to methaemoglobin. Values represent the mean ±SD.

repeated with SA after pretreatment of the seedlings with L-NMMA, an arginine-based NOS inhibitor widely used in plant systems (Zeidler *et al*., 2004). As shown in Fig. 3, pretreatment with 1 mM L-NMMA reduced NO production in both wild-type and mutant roots incubated with 1 mM SA. The results showed a decrease in the DAF-FM signal in both samples which, however, was not completely abolished; in fact, a similar fluorescence signal remained in wild-type and mutant roots. This suggests that, besides the involvement of NOS activity(ies), a minor NOS-independent pathway contributes to the total production of NO in roots.

Is AtNOS1 involved in SA-induced NO production?

An *Arabidopsis* mutant (*Atnos1*), impaired in NO production, has recently been characterized (Guo *et al*., 2003). To evaluate whether the *Atnos1* mutation would be involved in SA-induced NO production, experiments were performed on wild-type and *Atnos1* mutant seedlings.

In Fig. 4 it can be seen that treatment with SA (1 mM) for 2 h increased NO production in both wild-type and *Atnos1* mutant roots, although not to the same extent. In mutant roots treated with SA, the DAF-FM florescence signal was ~60% of that detected in wild-type roots. The experiments were next repeated in the presence of the NOS inhibitor L-NMMA (1 mM). The results showed a reduction of the DAF-FM signal in both samples which, however, was not completely abolished, while a similar fluorescence signal remained in wild-type and mutant roots. This result suggests that AtNOS1, despite not having a definite NOS activity by itself (Guo, 2006; Zemojtel *et al*., 2006), is however involved in a NOS-like activity.

Components of the signal transduction pathway induced by SA

To investigate signalling components involved in SA-increased NO synthesis, the role of calcium and protein phosphorylation was studied.

Since a role for calcium was detected in other NO-induced elicitor pathways (Lamotte *et al*., 2004), experiments were performed on *Arabidopsis* seedlings incubated in calcium-free medium. As reported in Fig. 5, the absence of calcium ions in the medium completely prevents SA-induced NO synthesis, demonstrating that calcium is strictly required in this signalling pathway. These data are confirmed by experiments performed in the presence of the calcium chelator EGTA (2.5 mM; data not shown).

Protein kinases have been indicated as critical components of SA signalling. To understand if a kinase activity could be involved in SA-induced NO production, the NO level was determined in *Arabidopsis* seedlings treated with different protein kinase inhibitors. As shown in Fig. 5, the general serine/threonine kinase inhibitor K252a did not prevent NO production upon SA treatment. It has been reported recently that a CK2 participates in SA-induced phosphorylation of specific proteins involved in SA signalling (Hidalgo *et al*., 2001). Thus the effect of TBB, a specific inhibitor of CK2 (Ruzzene *et al*., 2002), on SA-induced NO production was tested. As shown in Fig. 5, significant inhibition was observed in seedlings pre-exposed to 30 μM TBB. Control experiments with DMSO and SA have been performed showing that DMSO does not have any effect on the SA response (data not shown).

To understand better how this specific protein kinase is involved in SA signalling leading to NO production, a CK2 activity assay was performed. As shown in Fig. 6, CK2 activity is already present in untreated seedlings and

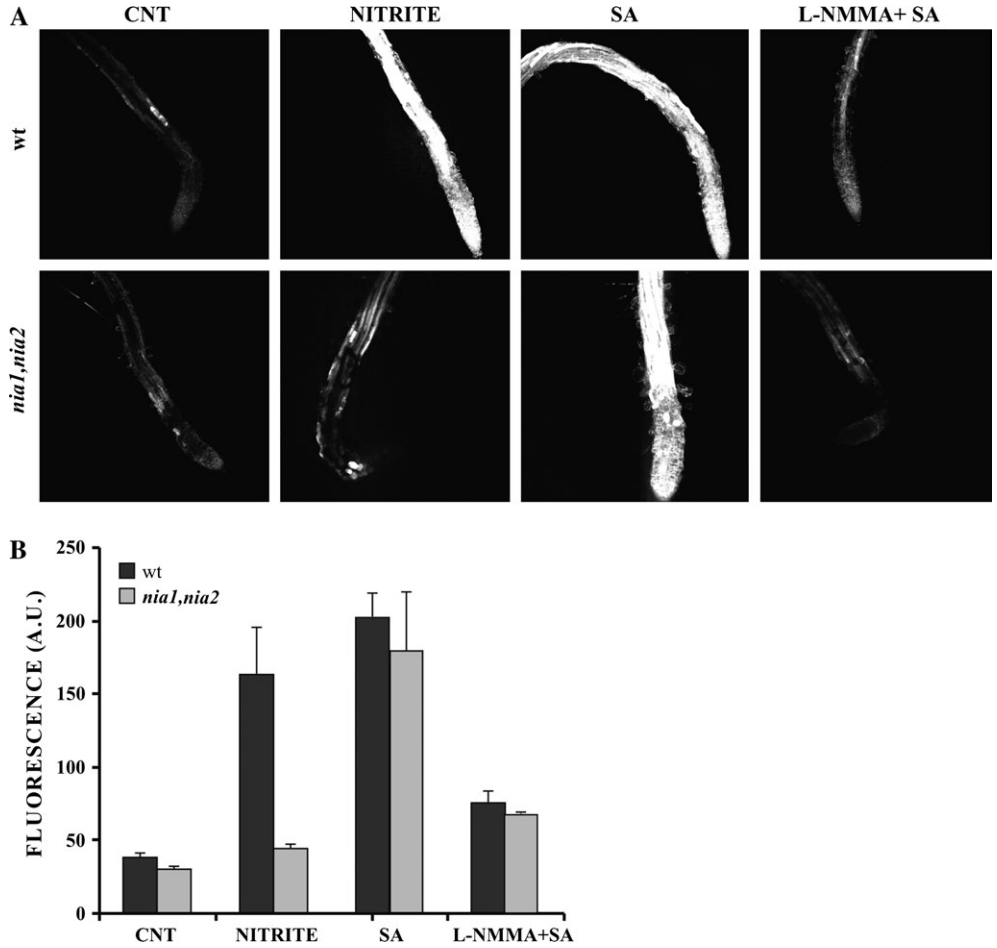

Fig. 3. SA-induced NO production in wild-type and *nia1,nia2* mutant seedlings. (A) NO production (shown as DAF-FM fluorescence) in 5-d-old seedlings after treatment for 2 h with 1 mM nitrite, 1 mM SA, and 1 mM L-NMMA+1 mM SA. (B) Pixel intensity of DAF-FM fluorescence of roots. Bars correspond to mean values ±SD.

is not affected by SA treatment. CK2 activity is strongly inhibited by 30 μM TBB, even in the presence of SA, while it is not affected by pretreatment with K252a. Moreover, the ineffectiveness of K252a was also assessed by an *in vitro* assay performed using a recombinant form of CK2. Using this assay, CK2 activity was not inhibited by K252a at concentrations up to 5 μM (data not shown).

Discussion

In this report, it is demonstrated that SA is able to trigger NO synthesis in *Arabidopsis* seedlings. Studying the kinetics of accumulation of NO, a clear response was observed that was dependent on the concentration of SA. To corroborate this result, two different techniques were adopted to detect NO specifically: a fluorometric assay that evaluates NO production at the cellular level in *Arabidopsis* roots and a spectrophotometric assay that quantifies NO released by cultured cells. There was good correlation between the two techniques and the two different biological systems (cells and seedlings). This point strengthens the validity of the results, suggesting a broader occurrence of SA-induced NO production not limited to specific tissues or organs.

To define the metabolic pathway(s) involved in this process, both genetic and pharmacological approaches were used. It is likely that plants have several enzyme systems to produce NO, even though the most intensively studied are based on NR and a putative NOS. In a previous report, Klepper (1991) showed that SA induces NO evolution from soybean leaves in the presence of photosynthesis-inhibiting herbicides, suggesting that NO production was due to a stimulating action of an NR activity coupled with an inefficient photosynthetic process. For this reason, a first attempt was made to define a role for NR activity in the induction of NO by a genetic approach using an NR-deficient *Arabidopsis* mutant (*nia1,nia2*). When wild-type and mutant seedlings were incubated in the presence of SA, NO synthesis was clearly induced in both lines, showing that NR activity was not involved in this process.

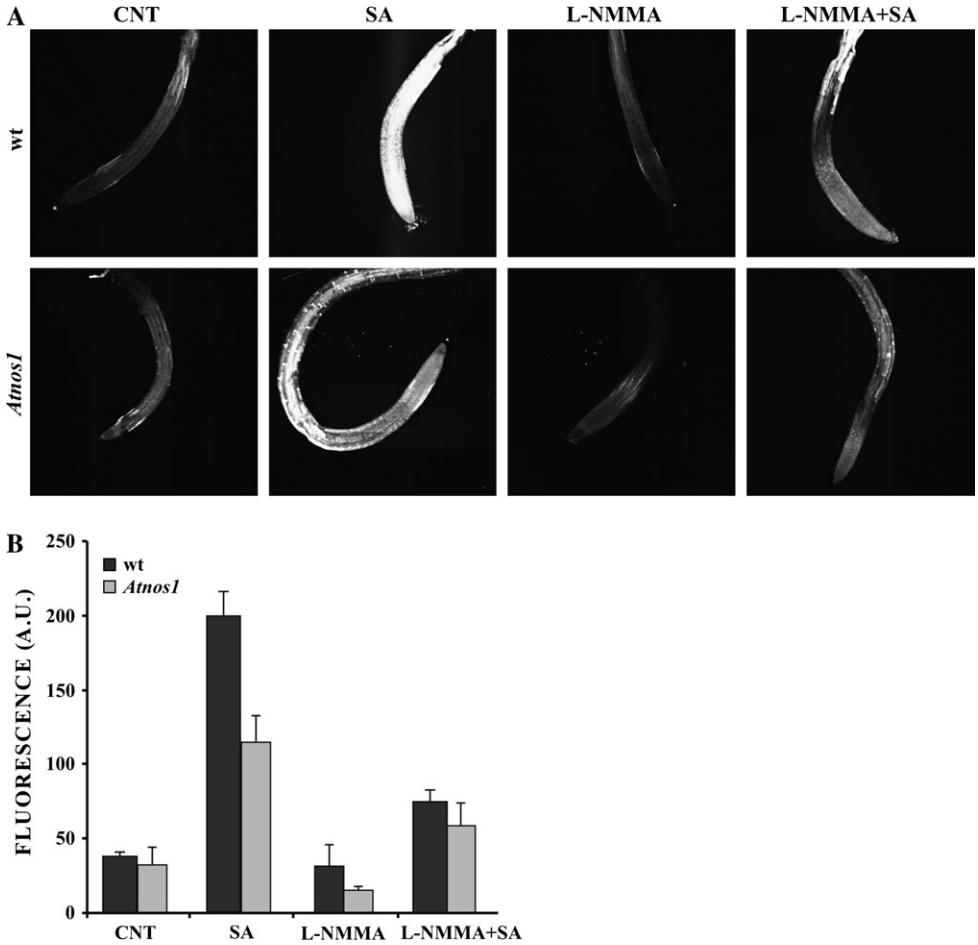

Fig. 4. SA-induced NO production in wild-type and *Atnos1* mutant seedlings. (A) NO production (shown as DAF-FM fluorescence) in 5-d-old seedlings after treatment for 2 h with 1 mM SA, 1 mM L-NMMA, or both. (B) Pixel intensity of DAF-FM fluorescence of roots. Bars correspond to mean values ±SD.

To evaluate the participation of NOS-type enzymatic activities in SA-induced NO production, wild-type and *nia1,nia2* seedlings were incubated with the NOS inhibitor L-NMMA before the addition of SA. This experiment defines an important role for NOS activities since NO production was strongly reduced by this pretreatment. SA-induced NO production was also detected in the *Atnos1* mutant, but not to the same extent as in the wild type, since the fluorescence signal in the mutant was ~60% of that detected in the wild type. These data suggest that AtNOS1 is involved in this process, even though not in an exclusive manner. Upon combined treatment (SA+L-NMMA) of *Atnos1* as well as of wild-type seedlings, SA-induced NO production was further reduced. These results suggest that SA-induced NO production involves AtNOS1 via a NOS-like activity. Even if the engaged molecular mechanism is still unknown, the data agree with those reported by other authors showing a strong impairment in NO production in the *Atnos1* mutant in response to different biological stimuli (i.e. abscisic acid, lipopolysaccharide, etc) (Guo *et al*., 2003; Zeidler *et al*., 2004; Bright *et al*., 2006).

Upon combined treatment (SA+L-NMMA), a similar fluorescence signal, higher than that of controls, was observed in the wild type and in *nia1,nia2* and *Atnos1* mutants. This residual fluorescence signal could be attributed to NO synthesized through a different route, for example, the recently discovered plasma membrane-bound NI-NOR. This activity has been shown to induce NO synthesis from nitrite, and it is known to be specifically expressed in plant roots (Stöhr *et al*., 2001).

In a previous report (Song and Goodman, 2001), it was shown that SAR induction was significantly attenuated when tobacco plants were co-injected with both SA and NOS inhibitors. The present data clearly demonstrate that NO is a downstream signal in the SA-induced response in plants and that this NO production proceeds mainly through a NOS-dependent pathway.

A pharmacological approach was adopted to investigate the signalling components involved in NO synthesis upon

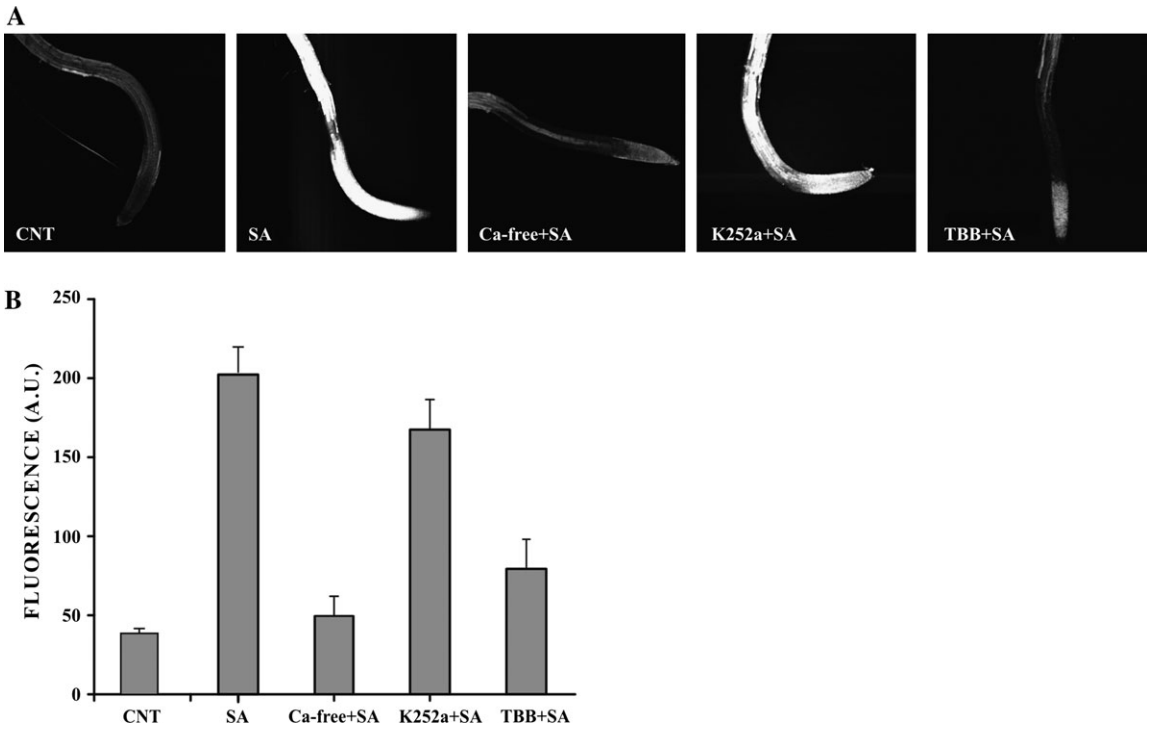

Fig. 5. NO synthesis is dependent on calcium and protein phosphorylation. (A) NO production (shown as DAF-FM fluorescence) in 5-d-old seedlings after treatment for 2 h with 1 mM SA, or in combination with 2 μM K252a or 30 μM TBB, or in a calcium-free medium. (B) Pixel intensity of DAF-FM fluorescence of roots. Bars correspond to mean values ±SD.

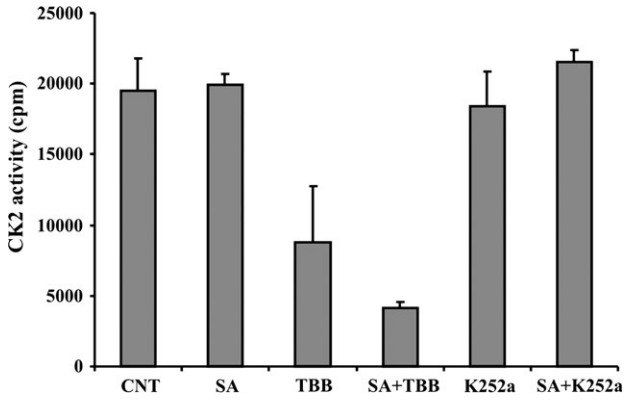

Fig. 6. Endogenous CK2 activity was measured towards a specific peptide (see Materials and methods), using 2.5–5 μg of protein extracts from *Arabidopsis* seedlings treated as indicated. Bars correspond to mean values ±SD.

SA treatment. Using protein kinase inhibitors, evidence was provided that phosphorylation events participate in the SA-induced signalling cascade leading to NO production. In particular, the results indicate that NO production is completely dependent on the activity of a specific CK2, since only the specific CK2 inhibitor (TBB) was effective on both NO production and CK2 activity. In contrast, the general serine/threonine kinase inhibitor (K252a), completely ineffective on NO production, was also unable to affect CK2 activity. The data are in agreement with previous results showing the involvement of CK2 in SA-induced activity (Kang and Klessig, 2005). In these experiments, CK2 activity was not increased by SA treatment, in contrast to what was observed in tobacco leaves by Kang and Klessig (2005). The finding that CK2 activity is already high in the seedlings, independently of SA treatment, is fully consistent with the current view of this kinase as a constitutively active enzyme, unaffected by external stimuli (Pinna, 2002). Moreover, the results are in agreement with the recent report by Salinas *et al.* (2006), where no changes were detected in the expression of *CK2* genes after SA treatment. Despite its unmodified activity, the involvement of CK2 in the SA response is clearly indicated by the present data, suggesting that some changes induced by SA treatment absolutely require CK2-dependent phosphorylation events to be effective: whenever CK2 activity is low, SA is not sufficient in itself to induce NO production. Further investigation will be required to clarify at which level and on which substrates CK2 exerts its fundamental effect.

It should also be considered that the SA-induced NO production is probably controlled by multiple mechanisms, as suggested by the experiments on calcium signalling. There is increasing evidence of the existence of cross-talk between NO and calcium signalling systems in plants (Lamotte *et al.*, 2004). In order to study the involvement of calcium in SA-induced NO synthesis, experiments were performed in calcium-free medium, which indicated that

this component is crucial to the SA signalling pathway. The role played by calcium in NO synthesis induced by SA may be that of a signal molecule and/or of an enzymatic cofactor.

In conclusion, the present results clearly demonstrate that at least part of SA-induced NO synthesis occurs through a NOS-dependent route; calcium signalling and protein phosphorylation, through CK2, are early and essential components of the SA-induced pathway mediating NO synthesis. The data that demonstrate the existence of an SA-dependent NO production in plants are of interest because they suggest a regulatory loop able to amplify the signal involving NO and SA. Moreover, by better defining the relationship between SA and NO, these results contribute to a more detailed understanding of the metabolic pathways in which these molecules are involved.

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