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Polyamines Induce Rapid Biosynthesis of Nitric Oxide (NO) in *Arabidopsis thaliana* Seedlings

Ni Ni Tun [1,3], Claudete Santa-Catarina [2,3], Tahmina Begum [1], Vanildo Silveira [2], Walter Handro [2], Eny Iochevet Segal Floh [2] and Günther F. E. Scherer [1,*]

[1] *Universität Hannover, Institut für Zierpflanzenbau, AG Molekulare Ertragsphysiologie, Herrenhäuser Str. 2, D-30419 Hannover, Germany*
[2] *Plant Cell Biology Laboratory, Department of Botany, IB-University of São Paulo, São Paulo, CP 11461, 05422-970, Brazil*

In this study, we examined the regulation by putrescine, spermidine and spermine of nitric oxide (NO) biosynthesis in *Arabidopsis thaliana* seedlings. Using a fluorimetric method employing the cell-impermeable NO-binding dye diaminorhodamine-4M (DAR-4M), we observed that the polyamines (PAs) spermidine and spermine greatly increased NO release in the seedlings, whereas arginine and putrescine had little or no effect. Spermine, the most active PA, stimulated NO release with no apparent lag phase. The response was quenched by addition of 2-aminoethyl-2-thiopseudourea (AET), an inhibitor of the animal nitric oxide synthase (NOS) and plant NO biosynthesis, and by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide (PTIO), an NO scavenger. By fluorescence microscopy, using the cell-permeable NO-binding dye diaminorhodamine-4M acetoxymethyl ester (DAR-4M AM), we observed that PAs induced NO biosynthesis in specific tissues in *Arabidopsis* seedlings. Spermine and spermidine increased NO biosynthesis in the elongation zone of the *Arabidopsis* root tip and in primary leaves, especially in the veins and trichomes, while in cotyledons little or no effect of PAs beyond the endogenous levels of NO-induced fluorescence was observed. We conclude that PAs induce NO biosynthesis in plants.

Keywords: Arginine — Nitric oxide — Polyamines — Putrescine — Spermidine — Spermine.

Abbreviations: AET, 2-aminoethyl-2-thiopseudourea; DAR 4M, diaminorhodamine-4M; DAR 4M AM, diaminorhodamine-4M acetoxymethyl ester; iNOS, inducible nitric oxide synthase; MAP, mitogen-acitvated protein; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PA, polyamine; PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide.

Introduction

Polyamines (PAs), spermidine, spermine and their diamine obligate precursor putrescine, are ubiquitous aliphatic amines that occur in all plant cells. The biosynthesis of spermidine and spermine from arginine and ornithine as precursors and putrescine as common intermediate was reviewed recently (Bouchereau et al. 1999, Sebela et al. 2001). Alterations in PA contents and in the enzymes of their biosynthesis have been related to controlling environmental stresses such as cold stress, salt and drought stress, and heat stress (Bouchereau et al. 1999). Under potassium deficiency, PAs accumulate (Richards and Coleman 1952). More recently, it was observed that spermine plays a role as a mediator in defence signalling against pathogens (Takahashi et al. 2003, Takahashi et al. 2004), accumulates during defence (Mo and Pua 2002), and that PAs enhance resistance against viruses (Yamakawa et al. 1998).

Evidence gathered in recent years also supports their role as regulators of cell proliferation and differentiation in plants, and in various physiological processes including rhizogenesis, development of flowers and fruits, zygotic and somatic embryogenesis, dormancy and senescence (Feirer et al. 1984, Galston et al. 1995, Bouchereau et al. 1999, Kakkar et al. 2000).

PAs and cytokinins have some overlapping physiological functions (Cohen et al. 1979, Greenland and Lewis 1984, Galston et al. 1995, Laxalt et al. 1997). Due to our common interest in PA effects on somatic embryogenesis (Santa-Catarina et al. 2003, Santa-Catarina et al. 2004, Silveira et al. 2004) and cytokinin-induced nitric oxide (NO) biosynthesis (Scherer and Holk 2000, Tun et al. 2001, Scherer 2004), we were led to investigate a role for PA in the regulation of NO biosynthesis. We observed a reduction of growth in an embryogenic *Ocotea* culture treated with 0.5, 1.0 and 2.0 mM spermidine and spermine added to the culture medium. However, the addition of the same concentrations of putrescine to the growth medium did not interfere with the growth of the embryogenic cultures (C. Santa-Catarina and E. I. S. Floh, unpublished data). The second initial observation was that NO biosynthesis was rapidly induced by cytokinins in plant cell cultures of *Arabidopsis*, parsley and tobacco (Tun et al. 2001). As cytokinins also have a role in regeneration, these observations led us to investigate whether PAs might also induce NO biosynthesis.

NO is a highly diffusible gaseous free radical that plays a key role as an intra- and intercellular messenger to induce various processes in plants, including the expression of defence-

[3] These authors contributed equally to this work.
[*] Corresponding author: E-mail, scherer@zier.uni-hannover.de; Fax, +49-511-762-3608.

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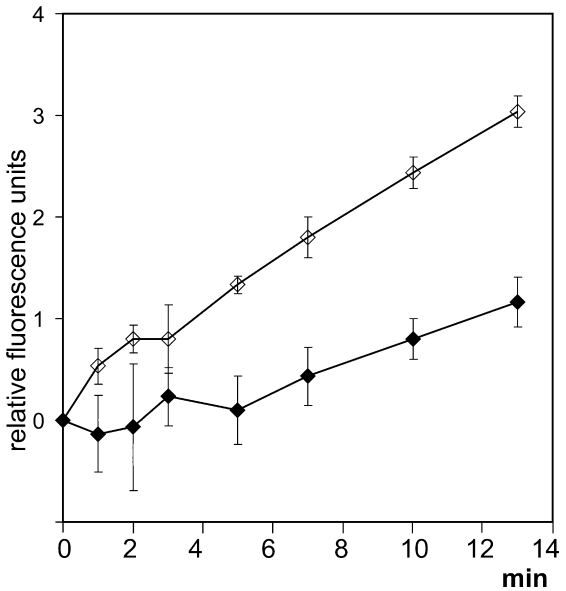

Fig. 1 Rapid induction by spermine of NO release from *Arabidopsis* seedlings. Endogenous release (no addition), filled diamonds; spermine (1 mM), open diamonds ($n = 3$; SD).

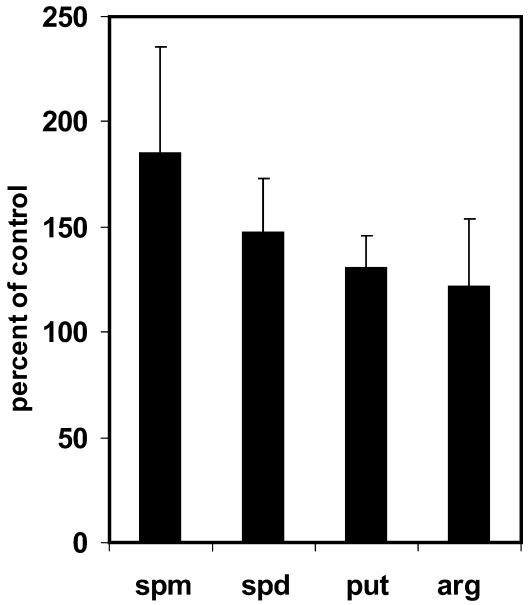

Fig. 2 Relative efficiency of different polyamines and arginine for NO release from *Arabidopsis* seedlings. Spm, spermine ($n = 15$); spd, spermidine ($n = 10$); put, putrescine ($n = 7$); arg, arginine ($n = 8$) (SD). Tests with no additions were done for each individual experiment and set as 100%. All other values were then calculated relative to that. Values were determined after 4 h incubation time, and the DAR-4M concentration was 2.5 μM.

related genes and programmed cell death, stomatal closure, seed germination and root development (Wendehenne et al. 2001, Neill et al. 2003, Lamotte et al. 2004). In plants, NO can be synthesized from nitrite by nitrate reductase (NR) and can also be formed by the oxidation of arginine by enzyme(s) homologous to snail nitric oxide synthase (NOS) (Guo et al. 2003, Neill et al. 2003). Xanthine oxidase and non-enzymatic sources of NO were described (Neill et al. 2003), but the magnitude of their contribution is unclear and the physiological conditions for non-enzymatic synthesis seem to be limited to specialized situations (Bethke et al. 2004). Here, we present data showing that addition of PA to Arabidopsis seedlings induced rapid synthesis of NO. This may provide a new angle for research into the overlapping functions of PAs and cytokinin.

Results

NO release

With respect to our previous study on tobacco and other cells in cytokinin research, we modified our previously described method (Tun et al. 2001). Instead of DAF-4M (4,5-diaminofluoresceine acetoxymethylester) we used the cell-impermeable dye DAR-4M (diaminorhodamine-4M) (Kojima et al. 2000, Kojima et al. 2001). This dye also reacts with NO but in a pH-independent manner at pH > 4 so that it can be used in the usually slightly acidic plant growth media.

When *Arabidopsis* seedlings were used to measure the response to PAs, a very rapid increase of NO release by addition of 1 mM spermine to the medium above endogenous levels was observed with no apparent lag phase (Fig. 1).

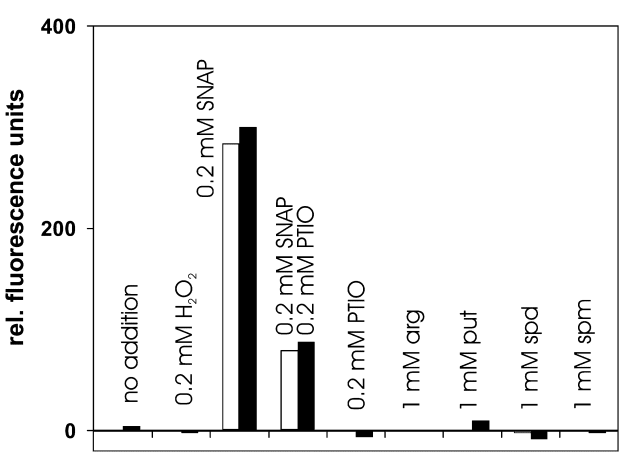

Fig. 3 Effects of chemicals on the fluorescence of 2.5 μM DAR-4M. Open columns (when exceeding the baseline), $t = 0$ h; closed columns, $t = 4$ h.

When spermine, spermidine, putrescine and arginine were compared, on average, only the PAs stimulated NO release in a statistically significant manner. This was not the case for arginine [122 ± 25% ($n = 8$; SD)] even though in individual tests stimulation by arginine reached up to 48%. Spermine was consistently the most active PA (Fig. 2).

The non-biological amines Tris and imidazole were inactive (data not shown). PAs are also known to be oxidized by PA

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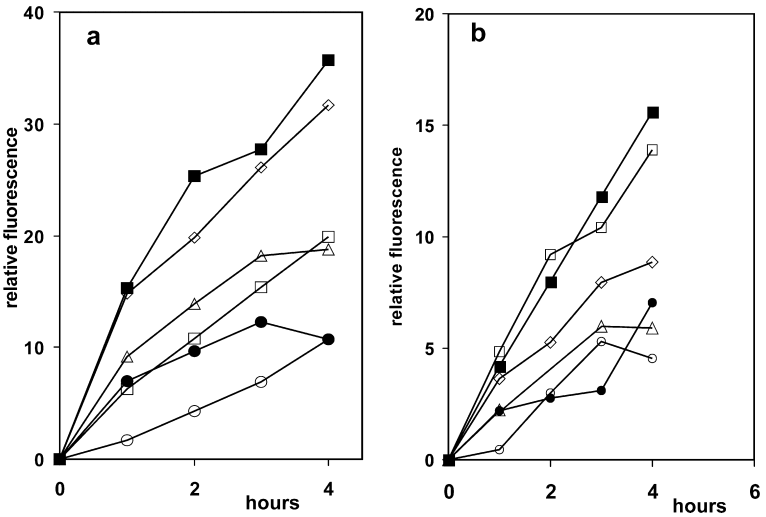

Fig. 4 Induction by spermine and inhibition by AET and PTIO of NO released from *Arabidopsis* seedlings. (a) Control (no addition), filled circles; 0.2 mM AET, open circles; 0.5 mM spermine, open triangles; 1 mM spermine, open diamonds; 2 mM spermine, filled squares; 1 mM spermine and 0.2 mM AET, open squares. The DAR-4M concentration was 2.5 μM. (b) Spermine effects and quenching by increasing concentrations of PTIO. Control (no addition), closed circles; 1 mM PTIO, open circles; 1 mM spermine, filled squares; 1 mM spermine and 0.1 mM PTIO, open squares; 1 mM spermine and 0.5 mM PTIO, open diamonds; 1 mM spermine and 0.1 mM PTIO, open triangles. The DAR-4M concentration was 1 μM.

oxidases whereby H_2O_2 originates (Binda et al. 2002). H_2O_2 might also influence DAR-4M fluorescence but failed to do so in a chemical test with 1 mM H_2O_2 (Fig. 3). Similarly, PAs and arginine did not influence DAR-4M fluorescence in a purely chemical way. Incubations with plant material showed that the inhibition by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1–1-oxy-3-oxide (PTIO) together with 2.5 μM DAR-4M often was only partial (not shown). Indeed, even the fluorescence increase induced by the NO donor SNAP was only partially quenched (by about 70%) by an equal concentration of PTIO so that competition of the regularly used concentrations of 2.5 μM DAR-4M and 0.2 mM PTIO for NO might explain the sometimes incomplete inhibition of NO-induced DAR or DAF fluorescence in experiments with plants. Interestingly, nitrite applied to seedlings induced an approximately 10-fold stronger fluorescence increase of DAR-4M than any PA, so that caution is advised in interpreting nitrite effects visualized with these fluorescent dyes (data not shown). Also, when 0.1 mM nitrite plus 2.5 μM DAR-4M was tested in the cuvette, a strong fluorescence increase was observed even without any plant (data not shown).

2-Aminoethyl-2-thiopseudourea (AET), an inhibitor of cytokinin-induced NO release (Tun et al. 2001), inhibited both endogenous and spermine-induced NO release (Fig. 4a). The NO-specific scavenger PTIO also decreased PA-induced NO fluorescence in assays with *Arabidopsis* seedlings at the decreased concentration of 1 μM DAR-4M (Fig. 4b), proving that the PA-induced fluorescence change was due to NO release. Similar inhibition by PTIO was obtained when spermidine and putrescine were tested (data not shown).

NO fluorescence microscopy

Measuring the NO-induced fluorescence increase using DAR-4M in the medium has the advantage of high sensitivity but it cannot show the tissue distribution of this response. Therefore, to analyse NO-induced fluorescence further, we used fluorescence microscopy analysis of *Arabidopsis* seedlings with the cell-permeable indicator diaminorhodamine-4M acetoxymethyl ester (DAR-4M AM) (Fig. 5). Again, the NO-induced fluorescence increase of this compound is independent both of the pH above 4 in the binding reaction and of the fluorescence emission (Kojima et al. 2000, Kojima et al. 2001). Since we used a cut-off filter for Chl fluorescence emission, the increase in fluorescence is not due to interference from Chl, as controls without DAR-4M AM showed. Another potential interfering compound, H_2O_2, originating from PA conversion by PA oxidases, did not increase the fluorescence in a chemical test (Fig. 3). We conclude that NO, by covalently binding to DAR-4M AM, was the chemical that induced the increase of fluorescence as compared with controls without DAR-4M AM. We observed clear differences in the increase of NO-induced

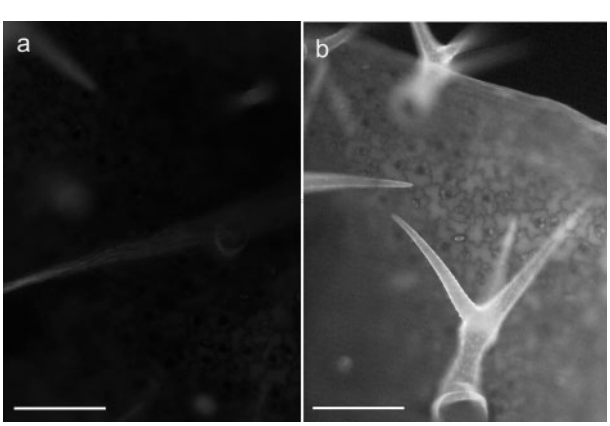

Fig. 5 Spermidine-induced NO-dependent fluorescence in *Arabidopsis* primary leaf. (a) In the leaf treated for 18 h by 2.5 μM DAR-4M, only epidermal cells and stomata are visible. A vein crosses through the centre of the micrograph and trichomes are visible as shadows. (b) The leaf was treated with 1 mM spermine and 2.5 μM DAR-4M for 18 h. Fluorescence in epidermal cells, trichomes and veins was strongly enhanced. Bar = 500 μm

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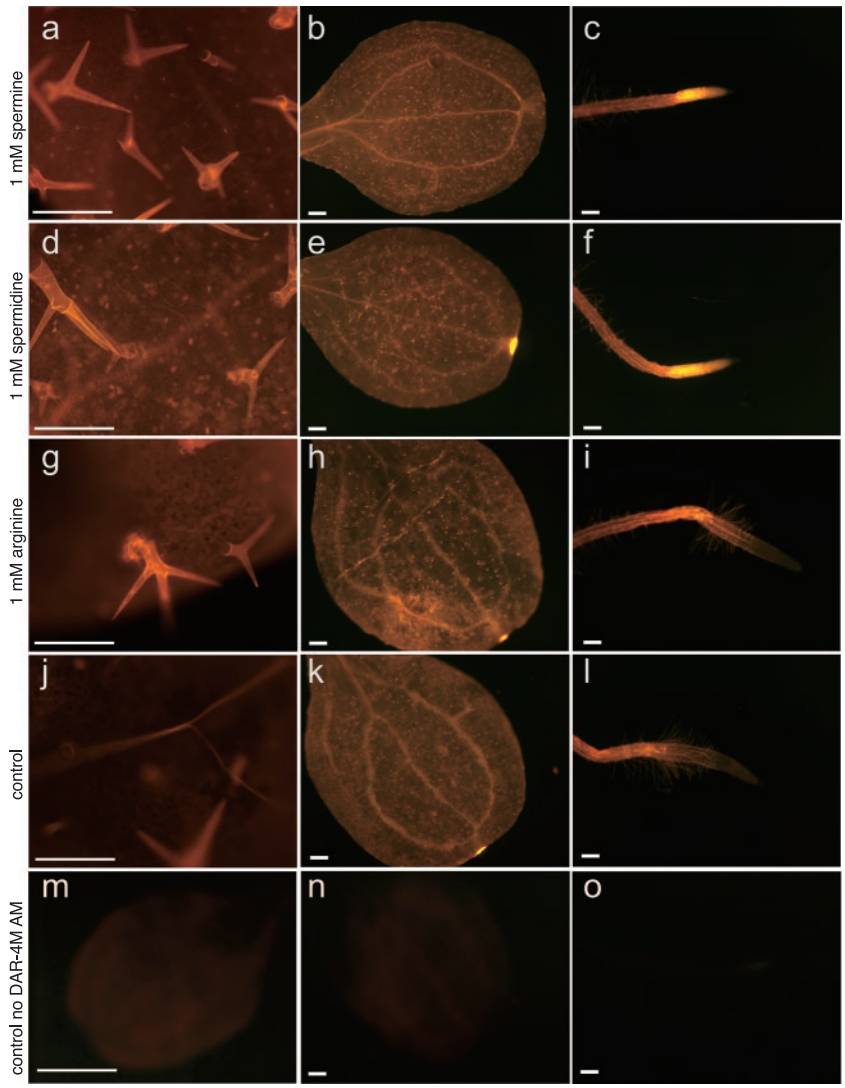

Fig. 6 NO-induced fluorescence as observed by fluorescence microscopy in *Arabidopsis* seedlings treated with arginine and polyamines for 18 h. Comparable micrographs were obtained with the same setting. (a) Leaf segment, 1 mM spermine, (b) cotyledon, 1 mM spermine, (c) root, 1 mM spermine, (d) leaf segment, 1 mM spermidine, (e) cotyledon, 1 mM spermidine, (f) root, 1 mM spermidine, (g) leaf segment, 1 mM arginine, (h) cotyledon, 1 mM arginine, (i) root, 1 mM arginine, (j) leaf segment, no addition, (k) cotyledon, no addition, (l) root, no addition, (m) leaf without DAR-4M AM and PA, (n) cotyledon without DAR-4M AM and PA, (o) root without DAR-4M AM and PA. White bars correspond to 200 μM.

fluorescence in primary leaves treated with spermidine (Fig. 5). Trichomes light up most dramatically within the cells and at their cuticles, revealing a characteristic sculpture. Guard cells remained rather dark but the cuticles around the opening cleft became bright by PA treatment and veins shone through the epidermis tissue. Hence certain lipophilic structures such as cuticles may attract the dye. Otherwise, the dye seems to be concentrated in the vacuoles as one would expect from a cationic dye.

When different tissues and several PAs were analysed, the PAs spermidine and spermine induced higher NO-dependent fluorescence in the *Arabidopsis* root tip in the elongation zone as compared with control or arginine treatment. In addition, an increase of NO-induced fluorescence was observed in primary leaves that followed the quantitative trends shown in Fig. 1 and 2, i.e. spermine was the most active. In the cotyledons, no increase in NO-induced fluorescence was found or only a weak increase (Fig. 6). PTIO quenched the PA-induced fluorescence increase in the leaves (Fig. 7) and roots (data not shown). These data suggest a tissue specificity of PA-induced NO biosynthesis in *Arabidopsis*, according to the treatments, and possibly indicate different enzymatic activities and localizations of these activities.

Taken together, the tissue distribution of a cationic dye and tissue-specific NO release will obviously influence each other, and the dye, once modified by NO, may even redistribute to an unknown extent. However, as guard cells remained relatively dark next to epidermal cells, tissue specificity must be assumed. Generally, it is assumed that NO cannot diffuse a longer distance than 500 μm in a plant before it is bound (Yamasaki 2005) so that the observed PA-induced tissue pattern is at least a good approximation of the true NO biosynthetic activity pattern.

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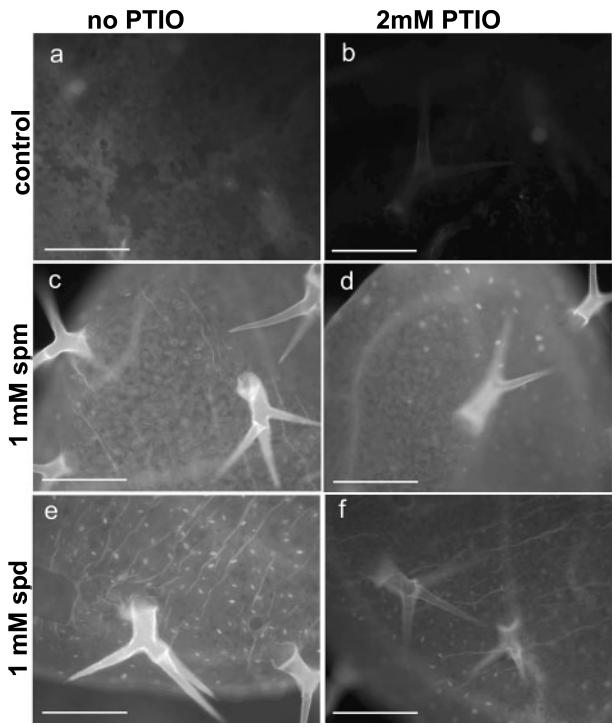

Fig. 7 PA-induced fluorescence and quenching by PTIO as observed by fluorescence microscopy in *Arabidopsis* seedlings. Seedlings were treated with 1 mM PAs with and without 2 mM PTIO and incubated in buffered 1 μM DAR-4M AM for 18 h. Micrographs were taken at the same magnification and with the same microscope settings. Treatments were: (a) no addition, (b) 2 mM PTIO, (c) 1 mM spermine, (d) 1 mM spermine + 2 mM PTIO, (e) 1 mM spermidine, (f) 1 mM spermidine + 2 mM PTIO. White bars correspond to 200 μm.

Discussion

What is the regulatory step in NO biosynthesis induced by added polyamines?

The observation that addition of PAs to seedlings and cell cultures causes rapid release of NO (Fig. 1) is new, to the best of our knowledge. Besides the presentation of the empirical evidence, two questions must be asked: what could be the biosynthetic enzyme(s) for PA-induced NO and how does it relate to known functions or experiments with PAs?

Polyamine transport

Linked to these questions is the conjecture that the observed quantitative differences between the different PAs could be caused by differences in uptake or transport of the compounds added rather than by the different effects or properties of the compounds inside cells. PA transporters were molecularly identified only recently in yeast (Tachihara et al. 2005, Uemura et al. 2005a, Uemura et al. 2005b), and previous work indicated both passive and active uptake (Kerschbaum et al. 2003). PAs were added to plants in many experiments and, in general, the observed quantitative differences were similar to those observed here, generally increasing from putrescine to spermidine and spermine (reviewed by Galston et al. 1995, Bouchereau et al. 1999, Kakkar et al. 2000). In most publications, arginine was not compared with the other PAs but, as it is the precursor to PAs, it was included in our experiments (Bouchereau et al. 1999). Amino acid transporters have been molecularly identified in plants (Wipf et al. 2002), but PA transporters have not been identified yet, so that the potential quantitative contribution of transport activity in any quantitative considerations on PA effects cannot, presently, be accurately estimated. However, the short-term kinetics (Fig. 1) with spermine showed that the effect on NO synthesis was directly apparent and fast, at least in this example. In the 4 h quantification by fluorometry (Fig. 4), no lag phases were apparent, which could have been due to a delay in uptake. In microscopy, the experiment duration was 18 h and the observed effects of different compounds were similar to the effects in the shorter fluorometric quantification experiments. This does not argue for an influence of transport on the experimental outcome, but this also cannot be completely ruled out. Hence, we rather assume that the observed differential effects of these PAs and of arginine can be interpreted as being realistic as in previous reports on fluorometric measurements of NO in plants (Zeidler et al. 2004).

Regulation of NO synthesis-catalyzing enzymes

Several potential enzymatic sources of PA-stimulated NO release or accumulation in cells must be discussed. Non-enzymatic conversion of PAs to NO is unknown, and the rapid regulation of known non-enzymatic sources by PAs seems unlikely (Neill et al. 2003, Yamasaki 2005). Known enzymatic sources of NO in plants are the plant NOS (Guo et al. 2003) and NR (Harper 1981). The NO-generating activity of added compounds increased from arginine, to putrescine, spermidine and spermine. With the methods used here, arginine did not have a statistically significant effect on NO biosynthesis [122 ± 25% (n = 8; SD)]. Since arginine is a precursor for NO biosynthesis by the plant NOS (Guo et al. 2003), this could suggest that the quantitative contribution of the known plant NOS should have played a minor role under our experimental conditions because this plant NOS could be activated strongly by elicitor without exogenous arginine (Guo et al. 2003, Zeidler et al. 2004). Arginine in its role as biosynthetic precursor for PAs (Bouchereau et al. 1999), possibly generating PAs from arginine during the experiment, also seemed to be a minor contributor to NO biosynthesis here. Because the enzymatic source of NO remains unclear, an explanation for the tissue distribution, at present, could only be speculative.

NR as another NO-releasing enzyme could be present, which could generate NO from nitrite (Harper 1981). It remains unclear whether or not PAs can regulate NR or the plant NOS directly or by a PA-mediated process.

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Yet another possibility could be that regulatory effects of PAs on NO-producing enzymes could be exerted by a PA receptor, which is not the NO-synthesizing enzyme itself, leading to regulation of the known or unknown enzymes that produce NO, i.e. NR, plant NOS, or others. As PAs exert their biological effects in the millimolar range (Kakkar et al. 2000), such receptors would have an unusually low affinity for their ligands. PA receptors, however, are unknown, and their role remains entirely speculative.

The last possibility or speculation could be the conversion of PA by as yet unknown enzymes or by PA oxidases to generate NO. PA oxidases are not known to generate NO in animal systems, but some plant PA oxidases possess an enzymatic mechanism different from that of the otherwise homologous animal enzymes (Sebela et al. 2001, Binda et al. 2002) so that this remains a viable speculation. A first indication for this possibility is the observation by Allan and Fluhr (1997) that PA oxidase using the substrate putrescine could be inhibited by L-NAME, also an inhibitor of the plant NOS (Delledonne et al. 1998, Durner et al. 1998, Zeidler et al. 2004). Clearly, the very rapid induction of NO release by spermine without an apparent lag phase (Fig. 1) argues in favour of the presence of an enzyme which directly converts PA to NO and other products.

Polyamine-induced NO biosynthesis: a potential link of PA physiology to other physiological processes

Investigations on the function of PAs in plants generated a multitude of articles and findings. Here, we add the finding that PA addition to seedlings generates NO. When one compares this finding with other processes or signals in plants generating NO, could NO be a common theme of or a link to these other physiological responses? Three topics at least seem to have PAs as mediators and NO biosynthesis in common: pathogen defence, abiotic stresses and senescence.

Polyamines, pathogen defence and NO

A prominent process employing NO as a mediator is pathogen defence (Delledonne et al. 1998, Durner et al. 1998, Wendehenne et al. 2001, Delledonne et al. 2001, Lamotte et al. 2004, Zeidler et al. 2004) and it can be generated within a few minutes upon signal application (Foissner et al. 2000, Zeidler et al. 2004). On the other hand, an increase in PA synthesis in response to various pathogens was observed earlier (Mo and Pua 2002), and resistance to virus infection was enhanced by PA inasmuch as virus-induced lesions were smaller and several pathogenesis-related genes were transcriptionally activated (Yamakawa et al. 1998). Spermine, but not putrescine or spermidine, stimulated after 6 h the activity of two important mitogen-activated protein kinases involved in plant defence, WIPK and SIPK, and defence gene expression (Takahashi et al. 2003, Takahashi et al. 2004). Reactive oxygen species and Ca^{2+} influx were upstream of PA action on MAP kinases. The far more rapid time course of PA-induced NO biosynthesis observed in our experiments indicates that NO should be upstream of the activation of defence MAP kinases, if it is involved in MAP kinase activation at all. Stimulation of expression of defence genes by NO was shown earlier (Huang et al. 2002, Polverari et al. 2003) so PA-induced NO as an element of both PA-mediated defence reactions and defence gene induction is not a contradiction. Interestingly, spermine inhibited the animal inducible nitric oxide synthase (iNOS) activity in infections with *Helicobacter pylori* by inhibiting iNOS translation (Bussiere et al. 2005). Since the animal iNOS is missing in *Arabidopsis*, the relationship of this finding to plant systems remains unclear.

Polyamines and abiotic stress—and NO?

Several forms of abiotic stress lead to enhanced PA biosynthesis and can be ameliorated by PAs (review by Bouchereau et al. 1999). PA increases were found in rice, sorghum, maize and tomato under salt stress and osmotic stress (Flores and Galston 1984, Krishnamurthy and Bhagnat 1984, Prakash and Prathapsenan 1988, Erdei et al. 1996, Willidiano et al. 1996, Santa-Cruz et al. 1997). Remarkably, the osmotic stress- and, partially, the salt stress-mediating hormone ABA also uses NO as a second messenger (reviewed by Neill et al. 2003). The accumulation of PAs under potassium deficiency has been known for some time (Richards and Coleman 1952, Watson and Malmberg 1996, Geny et al. 1997). Recently, it was found that NO has a function in iron deficiency and stress (Murgia et al. 2002). Hypoxia led to PA accumulation in rice, wheat and zucchini (Reggiani and Bertani 1989, Reggiani et al. 1989, Reggiani et al. 1990, Racz et al. 1996), and NO accumulation (Dordas et al. 2003), so that NO generated during hypoxia might, in part, originate from this accumulating PA. However, rapid release of NO by hypoxia was also mediated by NO release from nitrite (Rockel et al. 2002). Again, NO may be a link between PA-mediated stress responses and other stress mediators using NO as an intermediate. In conclusion, several biotic and abiotic stresses may use NO as a mediator where PAs are also involved.

Hormones, embryogenesis, and PAs and NO

Our findings on PA-stimulated NO release may have a bearing on the anti-senescence effects of PAs (Shi et al. 1982, Lester 2000, Bregoli et al. 2002) since NO counteracts the senescence-enhancing effects of ethylene (Leshem et al. 1998). Also, cytokinins have an anti-senescence effect (Hajouj et al. 2000, Li et al. 2000) and induced rapid release of NO (Tun et al. 2001). The initiation of this study was embryogenesis (Moura-Costa et al. 1993, Viana and Mantell 1999, Santa-Catarina et al. 2003, Santa-Catarina et al. 2004, Silveira et al. 2004) and PAs and their potential link to NO biosynthesis. PAs were described as mediators of embryogenesis (Galston et al. 1995, Shoeb et al. 2001, Bertoldi et al. 2004), and PA-induced NO could be involved in this function. We found that embryogenic cells of *Ocotea catharinensis* responded to PAs by synthesizing more NO and that the round and embryogenic

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cells of *Araucaria angustifolia* were accumulating more NO than the suspensor-like elongated cells (V. Silveira, N. N. Tun, C. Santa-Catarina, Floh, I. E. S. Handro and G. F. E. Scherer, unpublished data), suggesting that embryogenic cells might have a distinct NO physiology. Our findings should have bearings on future research on PAs in embryogenesis.

Materials and Methods

Plant material

Arabidopsis seeds (Columbia ecotype) were surface-sterilized, maintained for 3 d at 4°C for vernalization, and grown in half-strength MS (Murashige and Skoog 1962) medium at 22°C on a rotatory shaker (60 rpm), under a 16 h photoperiod and photon flux of 20–23 μmol m^{-2} s^{-1}. Seedlings of 7–8 d of age were used in the experiments.

NO release quantification

NO release to the medium was determined by binding to the cell-impermeable DAR-4M in a fluorometric assay (Kojima et al. 2000, Kojima et al. 2001). Twenty *Arabidopsis* seedlings (equivalent to 88.9 ± 7.7 mg FW) were used for each sample. When, with 12 identical samples of lots of 20 seedlings, the average units were set as 100%, an SD of ± 9.2% was obtained. When—in the same experiment using 20 seedlings per 12 samples—units were based on fresh weight directly, the average was 100 ± 13.8% or, when based on protein extracted by 0.5 M KOH and measured by a Bradford (1976) assay, 100 ± 14.0% was obtained. Hence, variance of data was lowest within one experiment when based on seedling number. In order to show inhibition of NO-induced fluorescence by the NO scavenger PTIO, only 1 μM DAR-4M was used in the assays. *Arabidopsis* seedlings were incubated in the light, on a rotatory shaker (60 rpm). Usually, after 2–4 h incubation or appropriate intervals, the supernatant was taken and the relative fluorescence was measured by excitation at 560 nm and emission at 575 nm in an LS-5 Luminescence spectrometer (Perkin-Elmer, Überlingen, Germany). In chemical experiments with 2.5 μM DAR-4M, chemicals were dissolved in water, except for the PAs which were dissolved in 30 mM MES-KOH pH 5.8, and blanks were subtracted. The cuvette volume was 2 ml. All experiments were repeated at least 3–5 times with similar results, and the data presented are of a single representative experiment or the average.

NO observation by microscopy

NO was visualized under a fluorescence microscope by binding to the cell-permeable derivative DAR-4M AM. For microscopy, seedlings were loaded with 1 μM dye for 4 h, then washed twice with water, incubated with PAs or arginine (1 mM) with and without 1 mM PTIO in 30 mM MES-KOH pH 5.8 buffer for 18 h and washed again twice with water prior to observation. Incubation was in the light at 22.5°C on a rotatory shaker (60 rpm) and then samples were kept at 4°C prior to microscopy. Plates were prepared and observed under a fluorescence Axioskop2 Mot Plus microscope (Zeiss), Filter set no. 20 from Zeiss (excitation, BP 546/12; beam splitter, FT 560; emission, BP 575–640). Digital photos which are compared were taken at exactly the same camera settings of a digital camera and were not processed further.

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