FEBS Letters 582 (2008) 874–880

Does NO play a role in cytokinin signal transduction?

Georgy A. Romanov[a,b,*], Sergey N. Lomin[a], Natalia Yu. Rakova[a], Alexander Heyl[b], Thomas Schmülling[b]

[a] *Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya 35, 127276 Moscow, Russia*
[b] *Freie Universität Berlin, Institute of Biology, Applied Genetics, Albrecht-Thaer-Weg 6, D-14195 Berlin, Germany*

Received 12 January 2008; revised 6 February 2008; accepted 8 February 2008

Available online 20 February 2008

Edited by Julian Schroeder

Abstract **We tested the hypothesis that nitric oxide (NO) plays an important role in cytokinin signaling. Inhibitors of NO-synthase (NOS), L-NMMA and L-NAME, inhibited the expression of the *GUS* gene controlled by the cytokinin-responsive *ARR5* promoter. However, the inactive analogues D-NMMA and D-NAME had a similar inhibitory activity. NO donors alone did not induce GUS activity and the NO scavenger cPTIO did not prevent the induction of the *ARR5* promoter by cytokinin. Northern blot analysis of the P_{ARR5}::*GUS* transgene and the host *ARR5* gene revealed that cytokinin-induced transcript accumulation was not altered by NMMA-treatment, indicating that NMMA acts post-transcriptionally. Together the data show that NO has no direct role in eliciting the primary cytokinin response in plants.**

Keywords: Cytokinin; Nitric oxide; Signal transduction; *Arabidopsis*; *Amaranthus*

1. Introduction

Nitric oxide (NO) is a unique ubiquitous signaling molecule in animals and plants [1]. The molecular mechanisms of NO action are well studied in animals but have only come under the scrutiny of plant science in recent years. Plants produce NO through different pathways: enzymatically by nitrate reductase, peroxidases and/or xanthine oxidoreductases and putative nitric oxide synthases (NOS); and non-enzymatically via, for example, nitrite reduction by ascorbic acid at low pH or by carotenoids in light [1–5]. At low concentrations NO stimulates seed germination, leaf expansion, root growth and stomatal closure. NO retards senescence and fruit maturation and participates in stress/pathogen resistance [2–6]. It was suggested that NO can tightly interact with the plant hormonal system and may serve as a second messenger [7]. Indeed, it has been demonstrated that NO mediates ABA-induced stomatal closure, and interferes with ethylene during the maturation and senescence of plant tissues [4,7]. The auxin effects on lateral root formation [8,9] and root gravitropic bending [10] were shown to be NO-dependent. Several lines of evidence point to an involvement of NO in jasmonic acid, salicylic acid and polyamine signaling [3,11,12]. Other data suggested that NO is also involved in cytokinin signaling [13,14]. One study [13] used *Amaranthus* seedlings, which respond to cytokinin by the rapid accumulation of the red pigment betacyanin, as a test system. It was shown that inhibitors of animal NOS suppressed the cytokinin action and that treatment of seedlings with NO (as a gas or by applying chemical NO donors) led to a limited enhancement of the betacyanin level. A second study [14] reported a rapid increase of NO production in tobacco, parsley and *Arabidopsis* cell cultures after treatment with cytokinin. This effect was specific to cytokinin and not reproduced by other phytohormones or by adenine. On the basis of these results the authors suggested that NO may participate as a second messenger in cytokinin signal transduction. A recent study [15] showing that cytokinin-induced apoptosis is mediated via a NO burst corroborated the link between cytokinins and NO biosynthesis in plant cells.

However, although suggestive, the above-mentioned studies did not provide definitive proof of a role of NO in cytokinin signaling. The concentrations of NOS inhibitors used to block cytokinin action in *Amaranthus* were rather high [13] and did not exclude non-specific action. Furthermore, induction of NO biosynthesis by cytokinin was shown to occur only at high hormone concentrations causing cell damage [15]. In order to obtain more conclusive evidence we have performed a study using P_{ARR5}::*GUS* transgenic *Arabidopsis* plants which harbor the *GUS* reporter gene under the transcriptional control of the cytokinin-responsive *ARR5* promoter [16,17]. This experimental system allows us to monitor the primary cytokinin response at the mRNA level very shortly after cytokinin treatment and to quantify the response by measuring the GUS enzymatic activity. Additional experiments were performed using *Amaranthus* seedlings where rapid transcriptional gene activation is needed as well to induce the cytokinin response [18]. Our study did not yield results that support the participation of NO in cytokinin signaling, rather it excluded NO participation, at least during the early signaling events leading to the activation of a primary response gene in *Arabidopsis*.

2. Materials and methods

2.1. Plant material

Transgenic P_{ARR5}::GUS *Arabidopsis* plants (*Arabidopsis thaliana* L., ecotype Wassilewskija), expressing the *GUS* reporter gene under the control of a cytokinin-dependent *ARR5* promoter were described earlier [16]. Transgenic P_{FER}::*GUS Arabidopsis* plants (ecotype Col-0),

*Corresponding author. Address: Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya 35, 127276 Moscow, Russia. Fax: +7 495 977 80 18.
E-mail address: gar@ippras.ru (G.A. Romanov).

0014-5793/$34.00
doi:10.1016/j.febslet.2008.02.016

expressing the *GUS* reporter gene under the control of an iron and NO-sensitive *AtFER1* promoter [19,20] were kindly provided by Dr. F. Cellier (Montpellier, France). Seeds were surface sterilized and soaked for 3 days at 4 °C in sterilized distilled water. Then seeds were placed in a growth chamber at 24 °C, 16 h light/8 h dark cycles. Seedlings grown for 3–4 days were used for experiments. A set of experiments was also made with etiolated *Amaranthus* seedlings (*Amaranthus caudatum* L.). Seeds of *Amaranthus* were germinated in the dark at 24 °C for 3 days on filter paper moistened with distilled water and then used for the assay.

2.2. Chemicals

NOS inhibitors (L-NMMA monoacetate salt, L-NAME hydrochloride and their non-active analogs D-NMMA and D-NAME) and NO donors (NOR3, SNAP and SNP) were from Calbiochem (Schwalbach, Germany); 6-benzyladenine (BA) was from Sigma (Deisenhofen, Germany). NOS inhibitors and NO scavenger carboxy-PTIO (cPTIO, Calbiochem) were dissolved in sterile bidistilled water in concentrations of 0.1–0.2 M and used freshly or stored at −20 °C before use. NO donors were dissolved in DMSO (50–100 mM) and stored at −20 °C. Immediately before use these compounds were diluted with water to the desired concentrations. The fluorescent probes DAF-2 and DAF-2DA (Calbiochem) were dissolved in DMSO at 5 mM and stored at −20 °C before use.

2.3. Plant assay systems

The *Arabidopsis* and *Amaranthus* assay systems were described in detail earlier [17,18]; descriptions are included as Supplementary data. Compounds to be tested were added in H_2O to the desired final concentration 10 min before BA (5 μM). The fluorescence of the reaction product 4-methylumbelliferone (MU) was measured on DyNa Quant 2000 fluorometer (Amersham, England). In graphics, 100% corresponds to 600–1000 nmol MU mg protein^{-1} h^{-1}. Control measurements have shown that the direct addition of compounds under examination to MU solution did not change its fluorescence. Amaranthin was measured spectrophotometrically at 540 nm. In graphics, 100% corresponds to OD_{540} 0.12–0.16.

2.4. NO fluorescence assays

The NO production by NO donors was monitored in our assay using the fluorescent indicator DAF-2 [21]. DAF-2 fluorescence was measured by a Hitachi 850 fluorometer (Japan), with excitation wavelength set at 495 nm and emission wavelength set at 515 nm. NO production was shown to be dose-dependent both in plain solution and in the presence of *Arabidopsis* seedlings. cPTIO (0.5 mM) removed NO effectively from the assay solution (Fig. 1S).

Endogenous NO was imaged using the cell-permeable probe DAF-2DA and fluorescence microscopy. Seedlings were incubated for 40 min at 24 °C in the dark in the presence of selected chemicals, then 5 μM DAF-2DA was added followed by another 30 min incubation. Subsequently seedlings were washed briefly in water and roots were viewed immediately under a fluorescence Axioscop 2 Plus microscope (Zeiss), filter set number 9 (excitation: BP 450–490 nm, emission: LP 515 nm). The images shown are representative examples of four independent experiments and were not further processed.

2.5. RNA blot analysis

3-Day-old *Arabidopsis* seedlings were placed in Petri dishes with 10 ml of distilled water and incubated with BA (5 μM) for 35 min at 24 °C. Compounds to be tested were added 10 min before BA. After incubation, seedlings were immediately frozen in liquid nitrogen and kept at −80 °C. Total RNA was isolated from about 2×10^3 seedlings by means of RNeasy kit (Qiagen, Germany). Twenty five micrograms of RNA was separated in a denaturing 1.5% agarose–formaldehyde gel, transferred to nylon membranes (Amersham Hybond N) and hybridized with radioactive-labelled DNA probe. The probes were full length ^{32}P-labelled *GUS* DNA and *ARR5* DNA. Hybridization was performed in phosphate buffer with 7% SDS at 68 °C. The lowest stringency wash was done in 0.2 × SSC, 0.1% SDS at 65 °C. For radioautography blots were exposed with BioMax MS films (Kodak) at −70 °C for 1–3 days. Quantification of signals was done on an integrative densitometer CD-50 (Desaga, USA). As a control for loading, the blot was rehybridized with an *Actin 2* probe.

2.6. Statistics

Each value on graphics represents an average of 2–3 independent determinations. Mean values, standard deviations and significance of experimental differences were calculated using a *t*-test statistical program. According to the program, difference is assessed as significant if $P < 0.05$; marginally significant if $0.05 < P < 0.1$, and not significant if $P > 0.1$.

3. Results

It was reported earlier [13] that inhibitors of NOS activity (L-NMMA, L-NAME, L-NNA, L-NIO and M-ITU) suppressed the cytokinin action in *Amaranthus* seedlings. In initial experiments we confirmed this result as one of these compounds, L-NMMA, a strong competitive NOS inhibitor,

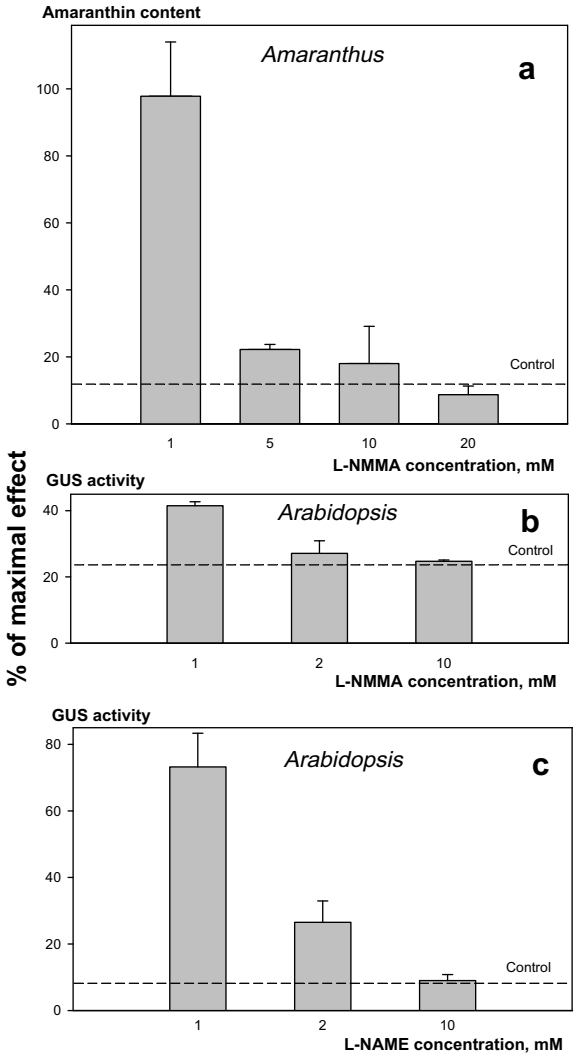

Fig. 1. L-NMMA and L-NAME inhibition of cytokinin-dependent reactions in *Amaranthus* and P_{ARR5}::*GUS Arabidopsis*. *Amaranthus* (a) or *Arabidopsis* (b) seedlings were incubated with BA (5 μM) in the presence of indicated concentrations of L-NMMA during 8 h or 5 h, respectively; (c) *Arabidopsis* seedlings incubated with BA (5 μM) and L-NAME during 5 h. Data from seedlings treated with BA alone were set as 100% (positive control). For solvent controls (dash line), seedlings were incubated in sterilized distilled water without addition of any chemical.

suppressed cytokinin-dependent betacyanin accumulation in *Amaranthus* seedlings. Fig. 1a shows that 5–10 mM L-NMMA inhibited the cytokinin-induced pigment accumulation up to 90–95% and 20 mM fully blocked the response ($P < 0.05$).

In the *Arabidopsis* reporter gene assay the effectiveness of L-NMMA was even higher than in *Amararanthus* (Fig. 1b): 2 mM L-NMMA inhibited up to 90% of the cytokinin-induced GUS activity. Another strong NOS inhibitor, L-NAME, inhibited cytokinin-induced GUS activity in a similar concentration range. A decrease of about 75% corresponded to 2 mM NAME and 10 mM totally blocked the response ($P < 0.05$) (Fig. 1c). The dose–response curve of NOS inhibition seems to be rather steep as 1 mM concentration was much less effective as compared to 2 mM (Fig. 1) and concentrations lower than 1 mM were not effective at all (not shown).

In addition to L-NMMA and L-NAME we used their D-isomers (D-NMMA and D-NAME) which do not markedly affect NOS activity [22]. Experiments have shown (Fig. 2) that D-isomers were able to inhibit cytokinin action with similar effectiveness (in the case of D-NMMA, Fig. 2a and b) or even more effectively (in the case of D-NAME, Fig. 2c) than the corresponding L-isomers. Thus the expected functional difference between the active L-form and its inactive D-analog was not revealed in the *Arabidopsis* assay system, indicating the possibility of a non-specific mode of action.

To obtain more direct evidence of an eventual role for NO in cytokinin signaling, we treated seedlings with NO and measured the response of the cytokinin-sensitive reporter gene.

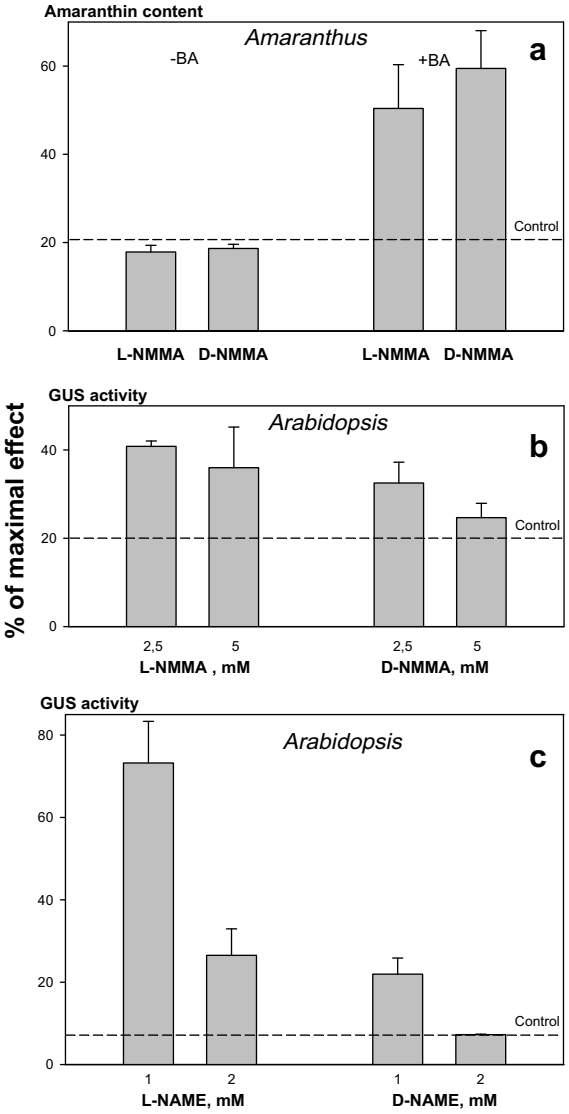

Fig. 2. Comparison of the inhibitory activities of L- and D-NMMA and L- and D-NAME on cytokinin-dependent processes in *Amaranthus* and P_{ARR5}::*GUS Arabidopsis*. *Amaranthus* (a) or *Arabidopsis* (b) seedlings were incubated with BA (5 μM) in the presence of 5 mM L-NMMA or D-NMMA during 8 h or 5 h, respectively; (c) *Arabidopsis* seedlings incubated with BA (5 μM) and L-NAME or D-NAME during 5 h. Data from seedlings treated with BA alone were set as 100% (positive control). For solvent controls (dash line), seedlings were incubated in sterilized distilled water without addition of any chemical.

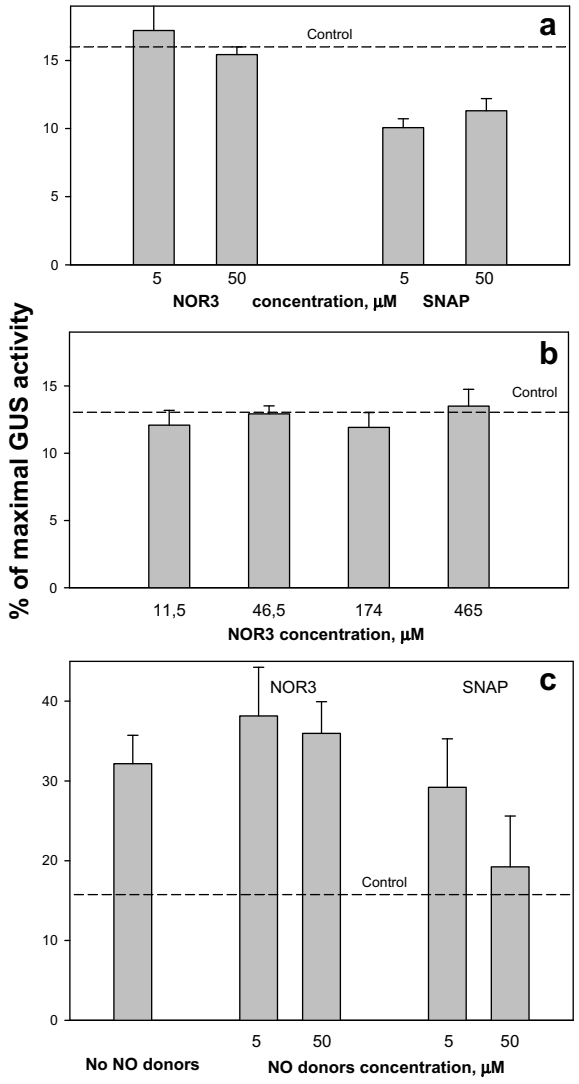

Fig. 3. GUS activity after treatment of P_{ARR5}::*GUS Arabidopsis* with NO donors. Seedlings were incubated with two concentrations of NOR3 or SNAP (a) or various concentrations of NOR3 (b) during 5 h; (c) treatment with NO donors in combination with BA and L-NMMA. L-NMMA alone or together with NO donors was added 10 min before BA. The column on the left shows the effect of L-NMMA (2.5 mM) on seedlings treated with BA (5 μM) in the absence of NO donors. Data from seedlings treated with BA alone were set as 100% (positive control). For solvent controls (dash line), seedlings were incubated in sterilized distilled water without addition of any chemical.

Arabidopsis seedlings were incubated in the presence of the NO donors NOR3 or SNAP. NOR3 is a cell-permeable compound that releases NO spontaneously in a rate-controlled manner ($t_{1/2}$ = 40 min) generating biologically inactive by-products. SNAP acts similarly ($t_{1/2}$ = 10.3 h) and, according to Scherer and Holk [13], evoked a weak cytokinin-like effect in *Amaranthus* seedlings. Control tests showed extensive nitric oxide production by NO donors in our assay system (Fig. 1S and data not shown). In our experiments with transgenic *Arabidopsis* neither SNAP nor NOR3 (the latter was tested in a wide concentration range, i.e. 0.01–0.5 mM), caused a cytokinin-like effect, namely the enhancement of GUS activity (Fig. 3a and b). SNAP produced a moderate inhibitory effect which was statistically only marginally significant ($P \approx 0.06$). These results argue against a role for NO as a direct messenger of the cytokinin signal. However, they do not exclude the possibility that NO is required directly or indirectly for cytokinin activity. For example, NO might have a role in a parallel transduction pathway, which could be indispensable for cytokinin signaling. This possibility was tested experimentally by adding NO donors to *Arabidopsis* plants treated with BA and L-NMMA. If two parallel signaling pathways exist, BA would activate both of them, L-NMMA would block the NO-dependent one and NO would overcome this inhibition. However experiments showed that NO did not alleviate the L-NMMA inhibition of cytokinin-induced GUS activity (Fig. 3c).

SNP, another NO donor producing charged NO^+ molecules, was also ineffective in activating the *ARR5* promoter (Fig. 4). In contrast, the same concentration (1 mM) of SNP activated the NO-responsive *Arabidopsis AtFER1* promoter ($P < 0.05$) (Fig. 4, inset), in accordance with earlier reports [19]. This activation could be significantly reduced by cPTIO ($P < 0.05$), a frequently used NO scavenger. However, when used on P_{ARR5}::*GUS Arabidopsis*, cPTIO (0.5–2 mM) was unable to reduce the cytokinin-dependent increase in GUS activity (Fig. 4). These results show that (i) an NO-responsive transgene is activated under our experimental condition and (ii) this NO-dependent gene activation – but not a cytokinin-dependent gene activation – can be inhibited by a NO scavenger.

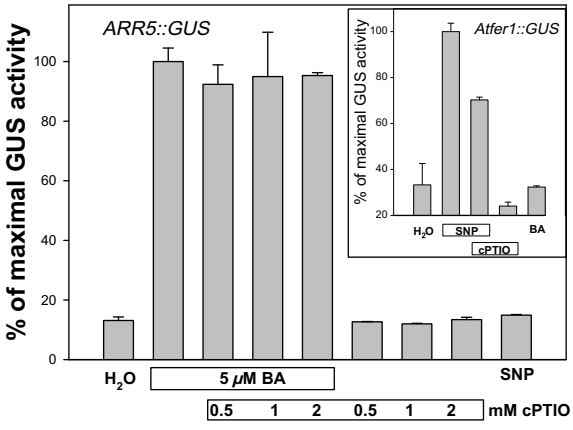

Fig. 4. GUS activity after treatment of transgenic *Arabidopsis* with BA, SNP, cPTIO and their combinations. The main graphic shows the influence of different compounds on GUS activity in P_{ARR5}::*GUS* transgenic *Arabidopsis*. The inset shows the influence of the same compounds on P_{FER}::*GUS* transgenic *Arabidopsis*. Concentrations used: SNP, 1 mM; BA, 5 μM; cPTIO (inset), 1 mM. GUS activity in seedlings treated with BA alone (main graphic) or SNP alone (inset) was taken as 100%.

DAF-2DA, a cell-permeable NO probe, showed the presence of endogenous NO in *Arabidopsis* root tissue (Fig. 2Sa and b). The positive effect of cytokinin on NO production was rather ambiguous if any (Fig. 2Sc and d). NO scavenger cPTIO markedly decreased the fluorescence intensity, supporting the specificity of imaging toward NO (Fig. 2Se and f). In contrast, L-NMMA (10 mM) did not cause a marked change of fluorescence intensity in the presence or absence of cytokinin (Fig. 2Sg and data not shown), indicating that this compound did not provoke a rapid decrease in NO concentration.

Next we explored whether L-NMMA inhibits cytokinin signaling at an early stage (before gene activation) or acts post-transcriptionally. To this end we analyzed the steady state levels of *GUS* and *ARR5* transcripts 35 min after cytokinin treatment of *Arabidopsis* seedlings. Northern blots were normalized by *Actin 2*, which does not respond to cytokinin [17]. Results demonstrated that with no cytokinin treatment *GUS* gene expression was very low, the radioactive signal being hardly detectable (Fig. 5a). Thirty-five minutes after cytokinin treatment an approximately 30-fold increase of the *GUS* transcript was detected. Simultaneous treatment with 0.5% 1-butanol, a known inhibitor of the early cytokinin response [17], reduced the accumulation of *GUS* transcripts to about one third (Fig. 5a), confirming the adequacy of the assay system. In contrast to 1-butanol, 5 mM L-NMMA, which strongly inhibited the cytokinin-induced increase in GUS activity (see Fig. 1), had no influence on the cytokinin-induced accumulation of *GUS* transcripts (Fig. 5a).

A similar result was obtained when the transcript level of the endogenous *ARR5* gene was tested. Cytokinin rapidly induced *ARR5* transcript accumulation, and this was not inhibited by 5 mM L-NMMA (Fig. 5b). Cytokinin-dependent transcript accumulation of both genes in the presence of L-NMMA clearly indicates that this inhibitor acts post-transcriptionally.

We then extended the analysis to our second experimental system, *Amaranthus*. To determine the relevant period of L-NMMA action in *Amaranthus* we performed a kinetic analysis of inhibitor activity. This approach allows the determination of the effective time interval for different inhibitors or activators over the entire period of cytokinin action [18]. Using this type of analysis we have shown earlier [18] that actinomycin D, a strong inhibitor of transcription, has its half-maximal effect ($\tau_{1/2}$) around 1.7–1.8 h after cytokinin application, the $\tau_{1/2}$ for cycloheximide, a strong inhibitor of translation, corresponds to 3.8–3.9 h and the $\tau_{1/2}$ for the enzymatic inhibitor of betacyanin synthesis 1,10-phenanthroline to 5.3–5.4 h. By comparing $\tau_{1/2}$ of the compound under study with $\tau_{1/2}$ of these known inhibitors one can judge the nature of the process affected by this compound.

In our study BA was added simultaneously to all test plants incubated in the assay solution whereas NMMA was added to different samples at different time points over the whole 8-h incubation period. Fig. 6 shows the level of amaranthin accumulation in relation to the timing of NMMA addition versus BA. The resulting inhibition curve for NMMA fell between the inhibition curves for a transcriptional inhibitor (actinomycin D) and a translational inhibitor (cycloheximide), with a half-maximal effect ($\tau_{1/2}$) for NMMA at about 2.6–2.7 h. This indicated that in the *Amaranthus* assay system as well L-NMMA acted later than at the onset of transcriptional induction. In other words, after the stage of cytokinin signal transduction leading to gene activation.

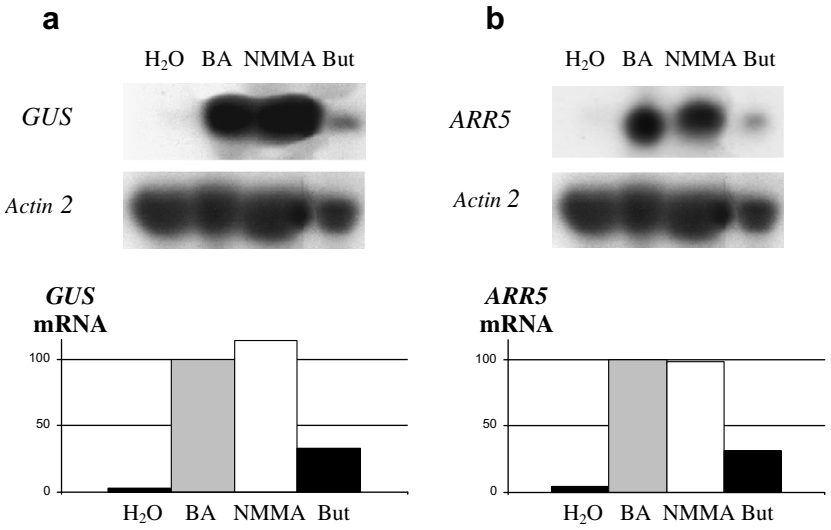

Fig. 5. *GUS* and *ARR5* transcript accumulation in cytokinin- and inhibitor-treated P_{ARR5}::*GUS Arabidopsis*. The steady state levels of *GUS* (a) and *ARR5* (b) mRNA as determined by Northern blot analysis and normalized by *Actin 2* mRNA. The result of the quantification of the Northern blots is shown below. H_2O – incubation in aqueous solution; BA – incubation in the presence of BA (5 μM BA, 35 min) alone; NMMA – incubation in the presence of BA and 5 mM L-NMMA; But – incubation in the presence of BA and 0.5% 1-butanol.

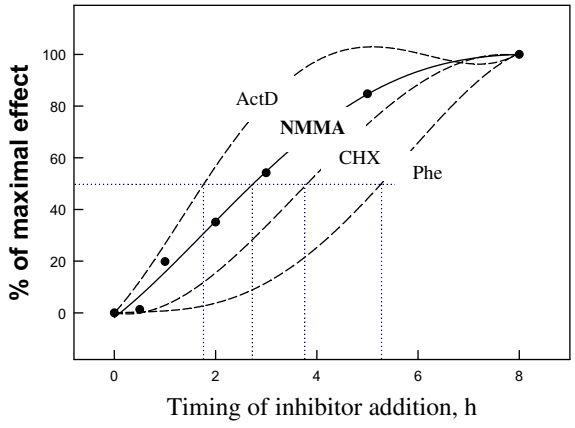

Fig. 6. Kinetic analysis of L-NMMA inhibitory action in *Amaranthus* seedlings. Seedlings were induced with BA (5 μM) and then L-NMMA (5 mM) was added to different samples after different time intervals as indicated on the abscissa. The total incubation period with BA was 8 h. The maximum pigment accumulation at the end of assay was set 100% (the ordinate). Dashed lines show analogous curves [12] for inhibition of pigment accumulation by actinomycin D (Act D), cycloheximide (CHX) and 1,10-phenanthroline (Phe). The time of the half-maximal effects is indicated by vertical dotted lines.

4. Discussion

In recent years a breakthrough in the study of the molecular mechanism of cytokinin action took place: membrane-located cytokinin receptors (sensor histidine kinases), primary response genes and essential components of the signal transduction pathway were discovered (for reviews, see: [23–26]). The main pathway of cytokinin signal transduction is now assumed to be a His-Asp phosphorelay leading from a sensor histidine kinase through phosphotransmitter proteins to intranuclear transcription factors. However, some important aspects of cytokinin signal transduction, as well as the mode of its interaction with other known signaling systems are still unclear and remain under intensive study.

The suggestion that NO might have a role in cytokinin signaling was an attractive possibility [13,14]. NO has, similar to cytokinin, pleiotropic activities in plants and some consequences of NO treatment resemble the consequences of cytokinin treatment. Both compounds stimulate leaf expansion, seed germination, delay senescence and promote de-etiolation [1–6]. However, the detailed analysis of the physiological action of NO on some plant models also shows differences from the mode of cytokinin action. For example, NO stimulates the formation and growth of lateral roots [8], whereas cytokinin has been shown to be a negative regulator of these processes [27,28]. The notion that cytokinins induce NO production [13,14] was not supported by recent observations: highly effective (but not damaging) cytokinin concentration (4 μM BA) did not induce NO release from cultured *Arabidopsis* cells [15] and even an inverse correlation was found between NO production and cytokinin levels during leaf development [29].

In our study we have used two potent inhibitors, L-NMMA and L-NAME, which block the activity of putative plant NOSs [1–4]. These compounds were shown to inhibit the cytokinin response in *Amaranthus* and *Arabidopsis*, though at rather high concentrations [13, this study]. For example, strong inhibition of NO production in pea guard cells required only 25 μM L-NAME [30]. Moreover, not only active L-isomers blocked the expression of cytokinin primary response genes (Fig. 1), but their usually inactive analogs D-NMMA and D-NAME acted similarly (Fig. 2). The effect of the inhibitors was not alleviated by NO donors (Fig. 3). In addition, different NO donors (NOR3, SNAP, SNP) were not able to mimic the cytokinin-dependent activation of a primary response gene (Figs. 3 and 4), and the NO scavenger cPTIO did not inhibit the cytokinin action (Fig. 4). Most importantly, the presence of effective concentrations of inhibitor (L-NMMA) did not prevent the primary transcriptional response to cytokinin in *Arabidopsis*, indicating the inhibition of posttranscriptional processes (Fig. 5). A posttranscriptional action of L-NMMA was also shown in the *Amaranthus* assay system (Fig. 6) by a

kinetic analysis. Together these results argue against the participation of NO in cytokinin signal transduction in *Arabidopsis* and *Amaranthus* seedlings, at least in early signaling events under the imposed conditions.

The fact that NOS inhibitors were able to block different early cytokinin responses (GUS enzyme accumulation; betacyanin accumulation) in different plant species (*Arabidopsis*, *Amaranthus*) raises the possibility that their inhibitory activities in mM concentrations used in former [13] and present studies are non-specific. Indeed, L-NMMA also inhibited an auxin-dependent increase of reporter gene activity in P_{DR5}::*GUS* transgenic *Arabidopsis* [31]. High concentrations of NOS inhibitors may block a common process important for the maturation, translocation or translation of newly formed transcripts. Thus one important part of the experimental basis to classify NO as second messenger in cytokinin signaling [13,14] seems to be doubtful. NOS inhibitors must be used with caution in plants as they apparently have non-specific inhibitory activity.

Evidence arguing against a role of NO in cytokinin signaling comes also from the recent analysis of the global transcriptional response of *Arabidopsis* to NO and cytokinin by the Affymetrix ATH1 GeneChip [32,33]. The almost complete lack of overlap between NO- and cytokinin-regulated genes found in these experiments precludes the tight interaction of these hormonal compounds in signal transduction to their response genes, at least in *Arabidopsis*.

However, it cannot be excluded that in some particular cases cytokinin action is indeed associated with NO. For example, some cytokinin late response genes may require also NO for their regulation. The rapid NO release by cells treated with cytokinin [14] might result from the cytokinin-dependent activation of nitrate reductase which represents one of the major NO producers in plants, including *Arabidopsis* [4,7]. Nitrate reductase is rapidly activated by cytokinin at the transcriptional and, possibly, posttranscriptional level [34,35]. However, the possibility that NO produced by any source serves as second messenger in cytokinin signaling is not supported by our NO donor and NO scavenger experiments, at least not for the induction of the *Arabidopsis ARR5* gene. The observed weak cytokinin-like effect of NO-donors on pigment biosynthesis in *Amaranthus* [13] could be a consequence of the direct interaction of NO with the amaranthin biosynthesis enzyme(s), which most likely contain(s) iron as a cofactor [1,36]. Taken together, the direct involvement of NO in cytokinin signal transduction appears to be unlikely, at least at early signaling stages. However, other possibilities for a regulatory relationship between cytokinin and NO start to emerge and deserve further molecular and physiological investigations.

Acknowledgement: This work was supported by grants of the Deutsche Forschungsgemeinschaft, the Russian Foundation of Basic Research (NN 04-04-49120/07-04-00331) and INTAS N 01-0602.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.016.

References

[1] Wendehenne, D., Pugin, A., Klessig, D.F. and Durner, J. (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells. Trends Plant Sci. 6, 177–183.
[2] Belligni, M.V. and Lamattina, L. (2001) Nitric oxide in plants: the history is just beginning. Plant Cell Env. 24, 267–278.
[3] Wendehenne, D., Durner, J. and Klessig, D.F. (2004) Nitric oxide: a new player in plant signalling and defence responses. Curr. Op. Plant Biol. 7, 449–455.
[4] Desikan, R., Cheung, M.K., Bright, J., Henson, D., Hancock, J.T. and Neill, S.J. (2004) ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J. Exp. Bot. 55, 205–212.
[5] Zemojtel, T., Fröhlich, T., Palmieri, M.C., Kolanczyk, M., Mikula, I., Wyrwicz, L.S., Wanker, E.E., Mundlos, S., Vingron, M., Martasek, P. and Durner, J. (2006) Plant nitric oxide synthase: a never-ending story? Trends Plant Sci. 11, 524–525.
[6] Romero-Puertas, M.C., Perazzolli, M., Zago, E.D. and Delledonne, M. (2004) Nitric oxide signalling functions in plant–pathogen interactions. Cell Microbiol. 6, 795–803.
[7] Lamattina, L., Garcia-Mata, C., Graziano, M. and Pagnussat, G. (2003) Nitric oxide: the versatility of an extensive signal molecule. Annu. Rev. Plant Biol. 54, 109–136.
[8] Correa-Aragunde, N., Graziano, M. and Lamattina, L. (2004) Nitric oxide plays a central role in determining lateral root development in tomato. Planta 218, 900–905.
[9] Correa-Aragunde, N., Graziano, M., Chevalier, C. and Lamattina, L. (2006) Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. J. Exp. Bot. 57, 581–588.
[10] Hu, X., Neill, S.J., Tang, Z. and Cai, W. (2005) Nitric oxide mediates gravitropic bending in soybean roots. Plant Physiol. 137, 663–670.
[11] Tun, N.N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Floh, E. and Scherer, G.F. (2006) Polyamines induce biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant Cell Physiol. 47, 346–354.
[12] Zottini, M., Costa, A., De Michele, R., Ruzzene, M., Carimi, F. and Lo Schiavo, L. (2007) Salicylic acid activates nitric oxide synthesis in *Arabidopsis*. J. Exp. Bot. 58, 1397–1405.
[13] Scherer, G.F.E. and Holk, A. (2000) NO donors mimic and NO inhibitors inhibit cytokinin action in betalaine accumulation in *Amaranthus caudatus*. Plant Growth Regul. 32, 345–350.
[14] Tun, N.N., Holk, A. and Scherer, G.F.E. (2001) Rapid increase of NO release in plant cell cultures induced by cytokinin. FEBS Lett. 509, 174–176.
[15] Carimi, F., Zottini, M., Costa, A., Cattelan, I., De Michele, R., Terzi, M. and Lo Schiavo, F. (2005) NO signalling in cytokinin-induced programmed cell death. Plant Cell Env. 28, 1171–1178.
[16] D'Agostino, I.B., Deruère, J. and Kieber, J.J. (2000) Characterization of the response of the *Arabidopsis* response regulator gene family to cytokinin. Plant Physiol. 124, 1706–1717.
[17] Romanov, G.A., Kieber, J.J. and Schmülling, T. (2002) A rapid cytokinin response assay in *Arabidopsis* indicates a role for phospholipase D in cytokinin signalling. FEBS Lett. 515, 39–43.
[18] Romanov, G.A., Getman, I.A. and Schmülling, T. (2000) Investigation of early cytokinin effects in a rapid *Amaranthus* seedlings test. Plant Growth Regul. 32, 337–344.
[19] Murgia, I., Delledonne, M. and Soave, C. (2002) Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. Plant J. 30, 521–528.
[20] Tarantino, D., Petit, J.-M., Lobreaux, S., Briat, J.-F., Soave, C. and Murgia, I. (2003) Differential involvement of the IDRS *cis*-element in the developmental and environmental regulation of the *Atfer1* ferritin gene from *Arabidopsis*. Planta 217, 709–716.
[21] 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.
[22] Chung, B.H. and Chang, K.C. (1994) Photo-induced adequate nitric oxide (PIANO)-mediated relaxation in isolated rabbit corpus cavernosum. Gen. Pharmacol. 25, 893–898.
[23] Hutchison, C.E. and Kieber, J.J. (2002) Cytokinin signaling in *Arabidopsis*. Plant Cell (Suppl.), S47–S59.

[24] Romanov, G.A. (2002) The phytohormone receptors. Russian J. Plant Physiol. 49, 552–560.
[25] Heyl, A. and Schmülling, T. (2003) Cytokinin signal perception and transduction. Curr. Op. Plant Biol. 6, 480–488.
[26] Kakimoto, T. (2003) Perception and signal transduction of cytokinins. Annu. Rev. Plant Biol. 54, 605–627.
[27] Werner, T., Motyka, V., Strnad, M. and Schmülling, T. (2001) Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA 98, 10487–10492.
[28] Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H. and Schmülling, T. (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 2532–2550.
[29] Wilhelmova, N., Fuksova, H., Srbova, M., Mikova, D., Mytinova, Z., Prochazkova, D., Vytasek, R. and Wilhelm, J. (2006) The effect of plant cytokinin hormones on the production of ethylene, nitric oxide, and protein nitrotyrosine in ageing tobacco leaves. Biofactors 27, 203–211.
[30] Neill, S.J., Desikan, R., Clarke, A. and Hankock, J.T. (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. Plant Physiol. 128, 13–16.
[31] Rakova, N.Yu. (2005) Some peculiarities of the regulation of primary response gene expression by cytokinin. Doctoral thesis, Moscow (in Russian).
[32] Parani, M., Rudrabhatla, S., Myers, R., Weirich, H., Smith, B., Leaman, D.W. and Goldman, S.L. (2004) Microarray analysis of nitric oxide responsive transcripts in *Arabidopsis*. Plant Biotech. J. 2, 359–366.
[33] Brenner, W., Romanov, G.A., Köllmer, I., Bürkle, L. and Schmülling, T. (2005) Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J. 44, 314–333.
[34] Kamínek, M., Šolcová, B., Trčková, M., Motyka, V., Daskalova, S. and Elliott, M.C. (2003) Exploitation of mechanisms regulating cytokinin levels to improve cereals in: Phytohormones in Plant Biotechnology and Agriculture (Macháčková, I. and Romanov, G.A., Eds.), pp. 109–128, Kluwer Acad. Publ., Dordrecht/Boston/London.
[35] Lillo, C., Meyer, C., Lea, U.S., Provan, F. and Oltedal, S. (2004) Mechanism and importance of post-translational regulation of nitrate reductase. J. Exp. Bot. 55, 1275–1282.
[36] Christinet, L., Burdet, F.X., Zaiko, M., Hinz, U. and Zryd, J.-P. (2004) Characterization and functional identification of a novel plant 4,5-extradiol dioxygenase involved in betalain pigment biosynthesis in *Portulaca grandiflora*. Plant Physiol. 134, 265–274.