Journal of Plant Physiology 167 (2010) 77–80

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Short communication

Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of *Arabidopsis thaliana* L. roots

Zsuzsanna Kolbert [a,*], Leandro Ortega [b], László Erdei [a]

[a] Department of Plant Biology, Faculty of Science and Informatics, University of Szeged, 6701 Szeged POB 654, Hungary
[b] Instituto de Fitopatología y Fisiología Vegetal (IFFIVE), INTA, Camino a 60 Cuadras Km 5,5; X5020ICA Córdoba, Argentina

ARTICLE INFO

Article history:
Received 6 August 2009
Received in revised form
31 August 2009
Accepted 31 August 2009

Keywords:
Arabidopsis thaliana
Nia1
Nia2
Nitric oxide
Osmotic stress

ABSTRACT

Nitric oxide (NO) is undoubtedly a potential signal molecule in diverse developmental processes and stress responses. Despite our extensive knowledge about the role of NO in physiological and stress responses, the source of this gaseous molecule is still unresolved. The aim of this study was to investigate the potential role of nitrate reductase (NR) as the source of NO accumulation in the root system of wild-type and NR-deficient *nia1*, *nia2* mutant *Arabidopsis* plants under osmotic stress conditions induced by a polyethylene glycol (PEG 6000) treatment. Reduction of primary root (PR) length was detected as the effect of osmotic stress in wild-type and NR-deficient plants. We found that osmotic stress-induced lateral root (LR) initiation in wild-type, but not in NR-mutant plants. High levels of NO formation occurred in roots of Col-1 plants as the effect of PEG treatment. The mammalian nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) had no effect on LR initiation or NO generation, while tungstate, an NR inhibitor, inhibited the later phase of osmotic stress-induced NO accumulation and slightly decreased the LR development. In *nia1*, *nia2* roots, the PEG treatment induced the first phase of NO production, but later NO production was inhibited. We conclude that the first phase of PEG-induced NO generation is not dependent on NOS-like or NR activity. It is also suggested that the activity of NR in roots is required for the later phase of osmotic stress-induced NO formation.

Introduction

Nitric oxide (NO) is known to be an important signal molecule in diverse abiotic stress responses such as drought and osmotic stress and also during normal developmental processes. Correa-Aragunde et al. (2004) provided the first evidence for the involvement of NO in auxin-induced lateral root development of tomato. It was later observed that NO can modulate the expression of certain cell cycle regulatory genes induced by auxin (Correa-Aragunde et al., 2006).

Application of NO donor in wheat plants led to stomatal closure and resulted in enhanced tolerance against drought, which is coupled with the accumulation of LEA (late embryogenesis abundant) proteins (García-Mata and Lamattina, 2001). In osmotic stress-treated wheat seedlings, treatment with NO resulted in a decrease in water loss and accumulation of abscisic acid (ABA), and these effects were reversible by NO scavengers (Xing et al., 2004). Lamotte et al. (2006) found that the protein kinase NtOSAK (*Nicotiana tabacum* osmotic stress-activated protein kinase) is activated by NO donor molecules. It is clear that, under stress conditions, nitric oxide is a key component of protective signaling pathways at low concentrations only, as high concentrations can induce cell damage (del Rio et al., 2004). It is therefore important for plants to regulate the production of nitric oxide under stress conditions. The generation of nitric oxide in plants is quite complex compared to animal systems because there are two major pathways: L-arginine and nitrate pathways. A plant nitric oxide synthase (NOS) similar to that in animals has not yet been identified (Crawford et al., 2006; Zemojtel et al., 2006), and recently, Moreau et al. (2008) demonstrated that the previously identified putative plant NOS, AtNOA1 (*Arabidopsis thaliana* nitric oxide synthase associated 1) is not able to bind and oxidize arginine, so it has no direct role in NO biosynthesis. Despite this uncertainty, experiments using different inhibitors of the animal NOS enzyme (L-NMMA, L-NAME) still provide evidence supporting the role of the L-arginine pathway in NO production under salt stress in *Arabidopsis* (Zhao et al., 2007) or during cell division in alfalfa suspension cultures (Ötvös et al., 2005). In roots, NO production from nitrate by the activity of nitrate reductase (NR; Yamasaki and Sakihama, 2000) and nitrite:NO reductase (Ni:NOR; Stöhr et al., 2001) has significant importance. The involvement of NR in NO synthesis has also been observed during ABA-induced stomatal closure (Desikan et al., 2002) and during

Abbreviations: DAF-2DA, 4,5-diaminofluorescein diacetate; L-NMMA, N^G–monomethyl-L-arginine; LR, Lateral root; MES, 2-(N-morpholino) ethanesulfonic acid; NO, Nitric oxide; NOS, Nitric oxide synthase; NR, Nitrate reductase; PEG, Polyethylene glycol; PR, Primary root

* Corresponding author. Tel.: +36 62 544 307; fax: +36 62 544 307.
E-mail address: kolzsu@bio.u-szeged.hu (Zs. Kolbert).

0176-1617/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2009.08.013

indole-3-butyric acid (IBA)-induced lateral root initiation (Kolbert et al., 2008a). In addition, NO can be produced from nitrite at low pH by a non-enzymatic process, which was observed in the barley aleuron layer (Bethke et al., 2004).

Osmotic or drought stress is a major stress factor that negatively influences productivity in plants. Roots are especially important in acclimation to stresses, and in our previous work, we detected elevated NO levels in roots of different osmotic stress-treated plant species. Two phases of NO generation (the transient "stress-NO" was followed by a slower second phase) were determined in roots of pea (Kolbert et al., 2008b), wheat, and *Arabidopsis* (Kolbert et al., 2008c) under osmotic stress. To our knowledge, there are no data in the literature describing the mechanisms of NO production under osmotic stress conditions, so our aim was to identify the possible enzymatic source of osmotic stress-induced NO in lateral root primordia of *Arabidopsis thaliana* plants using biochemical and genetic methods.

Materials and methods

Plant material and growth conditions

Three-week-old wild-type (Col-1) and *nia1*, *nia2* mutant *Arabidopsis thaliana* L. were used for our experiments. The seeds were surface sterilized with 5% sodium hypochlorite (v/v) for 20 min and rinsed 5 times with distilled sterile water before transferring to half strength MS (Murashige and Skoog, 1962) medium (7 g/L agar). To check the seed purity of *nia1*, *nia2* mutants, 5 mM $KClO_3$ was also added to the medium. Three-week-old plants were transferred to Petri dishes containing modified Hoagland solution and were treated for 5 days. Plants were grown under controlled conditions in greenhouse at a photo flux density of 300 $\mu mol\, m^{-2} s^{-1}$ (12/12 h day/night period) at a relative humidity of 55–60%, and a temperature of $25 \pm 2\,^{\circ}C$.

Treatments

Osmotic stress was administrated by dissolving polyethylene glycol 6000 (PEG, Sigma-Aldrich, St. Louis, MO) into the nutrient solution at a 400 mOsm osmotic concentration as measured by a digital automatic osmometer (Micro GMS, Hungary). This concentration is equivalent to 19% (w/v), or −0.976 MPa ψ_s, according to calculations of Wyn Jones and Gorham (1983), respectively. The animal NOS inhibitor N^G–monomethyl-L-arginine (L-NMMA, Sigma-Aldrich, St. Louis, MO) and the NR enzyme inhibitor sodium tungstate (Reanal, Hungary) were both applied in 1 mM concentrations.

Detection of NO

Visualization of NO was performed using the highly sensitive *in situ* and *in vivo* method of Kojima et al. (1998) applied for root tissues (Kolbert et al., 2008a–c). *Arabidopsis* root segments were dyed with 10 μM DAF-2DA (in MES/KCl buffer, 10^{-3} M, pH 6.15) for 20 min at $25 \pm 2\,^{\circ}C$ in the dark. Samples were washed 4 times within 20 min with MES buffer and were monitored under a Zeiss Axiowert 200M-type microscope (Carl Zeiss, Germany) equipped with filter set 10 (exc.: 450–490 nm, em.:515–565 nm) and a high resolution digital camera (Axiocam HR). The fluorescence intensity was determined within the area of circles with 20 μm radii using Axiovision Rel. 4.6 software. The radii of circles were not modified during the experiments. Each digital image was recorded with the same camera settings and was not processed further. At least 10 samples were measured in each treatment. The lengths of primary roots were measured manually using a scale, and lateral roots were counted under the microscope using 5 × magnifications, and were expressed as LR frequency (number/mm root).

Results and discussion

Osmotic stress decreases primary root length, induces lateral root initiation and results in high levels of NO production in Arabidopsis roots

The 400 mOsm PEG added to nutrient solution significantly reduced the length of PRs (Fig. 1A) and resulted in a 2-fold increase in LR frequency (Fig. 1B) of wild-type *Arabidopsis* plants compared to controls. This effect of osmotic stress on LR development was also found in pea plants (Kolbert et al., 2008b). In contrast, Deak and Malamy (2005) demonstrated that the formation of lateral roots from LR primordia is repressed as water availability is reduced. It was also observed that the number of lateral roots decreased following PEG treatment; however,

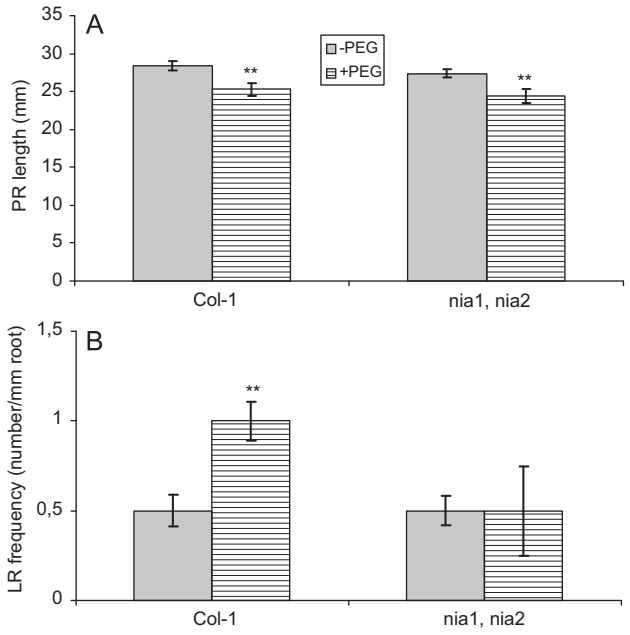

Fig. 1. Primary root length (A) and lateral root frequency (B) in control (grey bars) and 400 mOsm PEG-treated (stripped bars) wild-type (Col-1) and *nia1*, *nia2* *Arabidopsis* plants. Vertical bars are standard errors (n=9, $^{**}P \leq 0.01$).

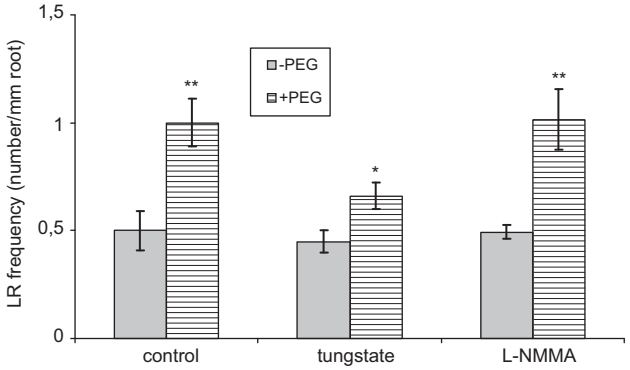

Fig. 2. Lateral root frequency of wild-type plants. Grey bars: control, stripped bars: 400 mOsm PEG treatment. Vertical bars are standard errors (n=9, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$).

osmotic stress had no effect on the LR initiation process (van der Weele et al., 2000). These opposing results may be partially explained by the use of a different growth system (there nutrient agar medium with PEG 8000). Osmotic stress did not alter LR number per mm root in the NR double mutant plants, but inhibited the elongation of PR in a manner similar to that in wild-type plants (Fig. 1AB). This indicates the involvement of NR enzyme activity in PEG-induced LR development processes. Monitoring of NO-associated fluorescence under osmotic stress revealed high levels of NO production (Kolbert et al., 2008c). The kinetics of early NO transients may depend on the source of NO. Very early NO accumulation (within 30 min) was observed in response to Fe^{2+}-treatment in *Arabidopsis* (Arnaud et al., 2006) and in the case of Cu^{2+} treatments in *Pisum sativum* and *Brassica juncea* (2 h after metal treatments) (Bartha et al., 2005). Tossi et al. (2009) discovered a fast apocynin-induced NO production in maize leaves. A slower early NO burst was observed in a fungal elicitor induced process that had a maximum value around 5 h after treatment (Xu et al., 2006), or at 24 h after the treatment of wheat plants with stripe rust (Guo et al., 2004). These stress-induced early NO generations may have a significant role in acclimation processes.

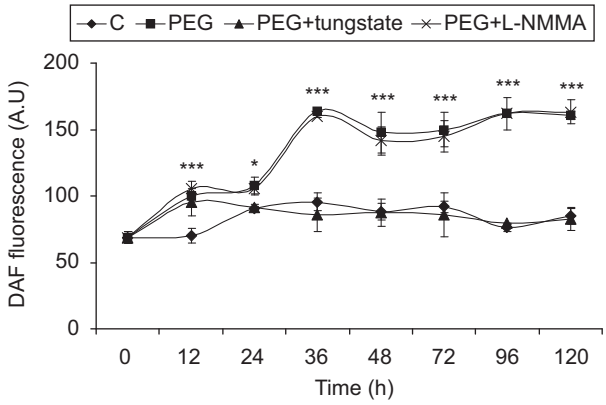

Fig. 3. Relative NO fluorescence in wild-type *Arabidopsis* roots under control (◆), 400 mOsm PEG (■), 400 mOsm PEG+L-NMMA (×) and 400 mOsm PEG+tungstate (▲) treatments. Vertical bars are standard errors ($n=9$, $^{*}P \leq 0.05$, $^{***}P \leq 0.001$).

Tungstate decreases osmotic stress-induced LR development and second phase NO, but not "stress NO" production

To biochemically examine the possible enzymatic source of osmotic stress-induced phases of NO production, plants were

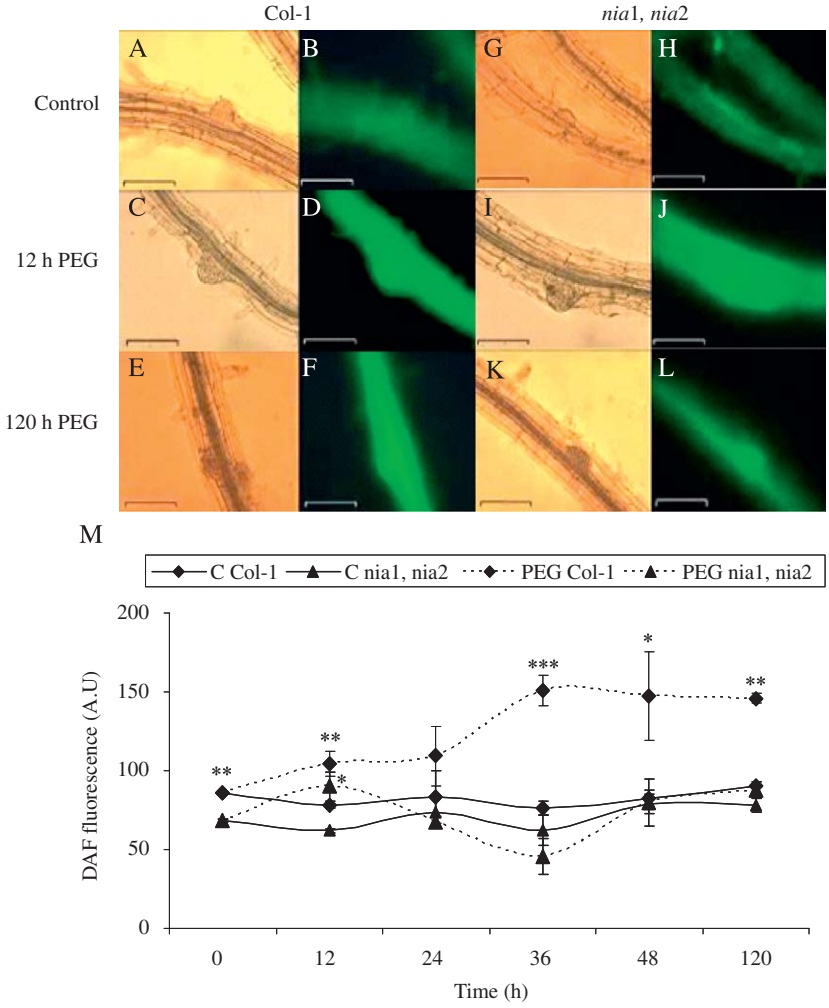

Fig. 4. Light- and fluorescent microscopic visualization of non-treated (Col-1: AB; *nia1*, *nia2*: GH), 12 h PEG-treated (Col-1: CD; *nia1*, *nia2*: IJ) and 120 h PEG-treated (Col-1: EF; *nia1*, *nia2*: KL) *Arabidopsis thaliana* roots. Samples were prepared and processed as described in the Materials and methods section. Images are representatives of 9 replicates in each treatment. Bars = 100 μm. (M) Relative fluorescence values of control and 400 mOsm PEG-treated *A. thaliana* roots. Vertical bars are standard errors ($n=9$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$).

treated either with 1 mM sodium tungstate ($NaWO_3$), an inhibitor of NR enzyme, or with the inhibitor of mammalian NOS, L-NMMA (1 mM). Tungstate slightly decreased the LR number of control and osmotic stress-treated plants, while L-NMMA had no effect on LR development under control or under osmotic conditions (Fig. 2). In PEG-treated roots, the early phase of NO accumulation was observed 12 h after the osmotic treatment. L-NMMA did not alter the osmotic stress-induced NO levels in this early phase, which suggests that L-NMMA sensitive pathway has no role in NO production in roots under osmotic stress. In PEG-treated roots, the presence of tungstate had no effect on the transient NO production; however, the later phase of NO accumulation was completely inhibited (Fig. 3). This indicates that the early NO generation does not involve either NR or NOS-like activity, while the accumulation of later NO is mediated by an NR-associated pathway.

PEG-induced "stress NO" is generated in nia1, nia2 roots

For further investigation of the source of PEG-induced NO, time-dependent experiments were carried out in wild-type and *nia1*, *nia2* mutant plants. Throughout the experiment NR-mutant roots showed lower NO-associated fluorescence compared to wild-type roots, which indicates a role for NR in NO production in *Arabidopsis* roots. Under osmotic stress Col-1 roots accumulated NO in the previously observed manner, while in *nia1*, *nia2* roots, only the transient NO appeared. This shows that early NO accumulation does not require NR activity. In PEG-treated NR-deficient roots, NO levels remained below or at the control level after the 24th h of the treatment (Fig. 4). This result provides genetic evidence for the role of NR in the osmotic stress-induced later phase of NO generation in *Arabidopsis* roots. Under osmotic stress, significant levels of abscisic acid (ABA) generate in roots (Ribaut and Pilet, 1991). Since it was shown that ABA is able to induce NO synthesis via a NR-associated process in guard cells (Desikan et al., 2002), we can speculate that similar events may occur in *Arabidopsis* roots under osmotic stress.

Based on these results, we conclude that osmotic stress-induced transient NO is generated without involving NR or NOS-like activities. We suggest that osmotic stress-induced early NO generates via non-enzymatic processes or via liberation from mobile storages (e.g. S-nitroso-gluthatione). In contrast, the later phase of PEG-induced NO accumulation involves NR activity in roots of *Arabidopsis thaliana*.

Acknowledgements

NR double mutant *nia1*, *nia2* plants were kindly provided by Prof. Dr. F.E. Scherer (Universität Hannover, Germany). This work was supported by the Hungarian Scientific Research Fund Grant no. OTKA T048436. This study was carried out during a one-month stay by Leandro Ortega at the Department of Plant Biology, University of Szeged, in the framework of the Hungarian–Argentine research project no. OMFB-00008/2008 TéT ARG-15/2006, sponsored by NKTH, the National Office of Research and Technology of Hungary and MinCyT, Ministerio de Ciencia, Tecnología e Innovación Productiva of Argentina. We thank Barnabás Wodala for critical reading of the manuscript.

References

Arnaud N, Murgia I, Boucherez J, Briat J-F, Cellier F, Gaymard F. J Biol Chem 2006;281:23579–88.
Bartha B, Kolbert Zs, Erdei L. Acta Biol Szeged 2005;49(1–2):9–12.
Bethke PC, Badger MR, Jones RL. Plant Cell 2004;16:332–41.
Correa-Aragunde N, Graziano M, Lamattina L. Planta 2004;218:900–5.
Correa-Aragunde N, Graziano M, Chevalier Ch, Lamattina L. J Exp Bot 2006;57:581–588.
Crawford NM, Galli M, Tischner R, Heimer YM, Okamoto M, Mack A. Trends Plant Sci 2006;11:526.
Deak KI, Malamy J. Plant J 2005;43:17–28.
Desikan R, Griffiths R, Hancock J, Neill S. Proc Natl Acad Sci 2002;99:16314–8.
García-Mata C, Lamattina L. Plant Physiol 2001;126:1196–204.
Guo P, Cao Y, Li Z, Zhao B. Plant Cell Environ 2004;27:473–7.
Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, et al. Anal Chem 1998;70:2446–53.
Kolbert Zs, Bartha B, Erdei L. J Plant Physiol 2008a;165:967–75.
Kolbert Zs, Bartha B, Erdei L. Physiol Plant 2008b;133:406–16.
Kolbert Zs, Sahin N, Erdei L. Acta Biol Szeged 2008c;52:63–5.
Lamotte O, Courtois C, Dobrowolska G, Besson A, Pugin A, Wendehenne D. Free Rad Biol Med 2006;40:1369–76.
Moreau M, Lee GI, Wang Y, Crane BR, Klessig GF. J Biol Chem 2008;283:32957–67.
Murashige T, Skoog F. Physiol Plant 1962;15:473–97.
Ötvös K, Pasternak TP, Miskolczi P, Domoki M, Dorgjotov D, Szűcs A, et al. Plant J 2005;43:849–60.
Ribaut JM, Pilet PE. Physiol Plant 1991;81(2):156–62.
del Rio LA, Corpas FJ, Barroso JB. Phytochemistry 2004;65:783–92.
Stöhr C, Strube F, Marx G, Ullrich WR, Rockel P. Planta 2001;212:835–41.
Tossi V, Cassia R, Lamattina L. J Plant Physiol 2009; doi:10.1016/j.jplph.2009.02.001.
van der Weele CM, Spollen WG, Sharp RE, Baskin TI. J Exp Bot 2000;51:1555–62.
Wyn Jones RG, Gorham J, editors. Encyclopedia of plant physiology. New series, Vol. 12C. Berlin: Springer-Verlag; 1983. p. 35–58.
Xing H, Tan L, An L, Zhao Z, Wang S, Zhang C. Plant Growth Regul 2004;42:61–8.
Xu M, Dong J, Zhu M. Sci China Ser C: Life Sci 2006;49:379–89.
Yamasaki H, Sakihama Y. FEBS Lett 2000;468:89–92.
Zemojtel T, Fröhlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, et al. Trends Plant Sci 2006;11:524–5.
Zhao MG, Tian QY, Zhang WH. Plant Physiol 2007;144:206–17.