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ARTICLE

Early nitric oxide (NO) responses to osmotic stress in pea, *Arabidopsis* and wheat

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ABSTRACT Nitric oxide (NO) is a novel diffusible, lipophylic gas, which acts in diverse physiological processes in plants. The 4,5-diaminofluorescein diacetate (DAF-2DA)- based *in vivo* and *in situ* fluorescence method of NO detection gives an excellent opportunity to investigate the transient NO generations in plant tissues. During our work with the help of this method time-dependent kinetics of NO formation were investigated in osmotic stress treated- *Pisum sativum* L., *Triticum aestivum* L. and *Arabidopsis thaliana* L. roots. Osmotic stress was provoked by addition polyethylene glycol (PEG 6000) to the nutrient solution. In the case of pea plants an early phase of NO generation was distinguishable, which reached a maximum point at 24th hours after PEG treatment. This transient NO accumulation was followed by a slower but more significant NO formation. In *Arabidopsis* roots NO was formed already in the first 12 hours, the highest NO level was detected only 36 hours after treatment. Interestingly, in PEG-treated wheat roots the first NO peak appeared already after 1 hour treatment, which slightly moderated in the following 12 hours. In the cases of all the investigated plant species two phased NO generation was observed. The significant transient NO bursts were followed by slower NO accumulations. These early NO formations could have an important role in acclimation of plants to osmotic stress.
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KEY WORDS

nitric oxide
osmotic stress
Pisum sativum
Triticum aestivum
Arabidopsis thaliana

Nitric oxide, a novel, bioactive signal molecule became special attention in the last decade, since it acts in several physiological processes. This multifaced molecule has three forms in animals and plants: nitrosonium cation (NO^+), nitroxyl anion (NO^-) and nitric oxide radical ($NO^·$). The presence of NO was determined in different plant species during different stress conditions, however the source of it is not well understood (Kolbert et al. 2008a and references therein). In roots, the most likely candidate of NO production is nitrate reductase (NR) enzyme (Deshikan et al. 2002). Recently, nitrit:nitric oxide reductase (NiNOR) was described as another possible NO-producing enzyme in roots (Stöhr et al. 2001). The two major non-enzymatic sources of NO are the reduction of nitrite to NO at pH 3-6 (Stöhr and Stremlau 2006) and the light-mediated conversion of nitrogen dioxide (NO_2) to nitric oxide (Cooney et al. 1994). In the international literature an extended debate evolved on the existence of mammalian nitric oxide sythase-like (NOS-like) enzymes in plants (Kolbert et al. 2008a and references therein). Notwithstanding of this uncertainty, the research on NO is going on intensively and it proved to be involved in root development under stress conditions induced by heavy metals, salt, drought or different pathogens (Bartha et al. 2005; Kolbert et al. 2005; Xu et al. 2006; Kolbert et al. 2008b).

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In our study we hypothesize that NO plays a role during osmotic stress conditions. We observed two-phase kinetics of PEG-induced NO formation in which a rapid, early accumulation is followed by a long-lasting constant production.

Materials and Methods

Plant material and growth conditions

Pea (*Pisum sativum* L.) and wheat (*Triticum aestivum* L. cv. Öthalom) plants were grown in modified Hoagland nutrient solution under controlled conditions in greenhouse at photo flux density of 240 µmol m^{-2} s^{-1} (12/12 h day/night period) at relative humidity of 55-60%, and 25 ± 2°C temperature.

Arabidopsis thaliana plants (wild-type, Col-1) were grown on solid Murashige-Skoog (MS) medium (Murashige and Skoog 1962) for 3 weeks, then they were transferred to modified Hoagland solution where they were treated. Osmotic stress was induced by addition polyethylene glycol (PEG 6000) to the nutrient solution at 0, 50, 100, 200, 400 mOsm concentrations.

Detection of NO

For visualization of NO the *in situ* and *in vivo* method of Kojima et al. (1998) and Pedroso et al. (2000) was applied. 1.5-2 cm long root segments were dyed with 10 µM 4,5-diaminofluorescein-diacetate (DAF-2DA) dissolved in MES/KCl buf-

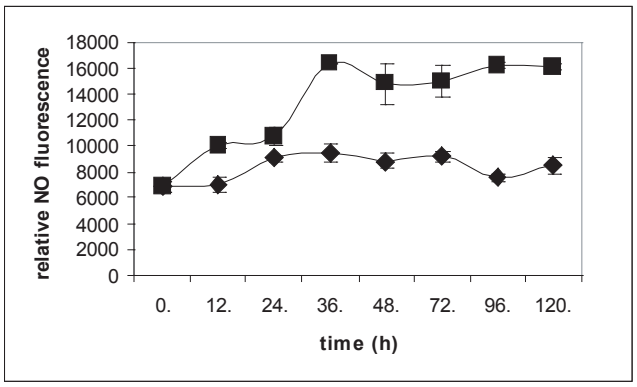

Figure 1. Time dependent NO generation in control (♦) and PEG treated (■)- *Arabidopsis* roots. Vertical bars are standard errors

fer for 20 minutes at 25 ± 2°C in darkness. After dyeing the samples were washed 4 times within 20 minutes with MES buffer (10^{-3} M, pH 6.15) and were prepared on microscopic slides. To detect fluorescence intensity Zeiss Axiowert 200M-type fluorescent microscope (Carl Zeiss, Germany) connected with a high resolution digital camera (Axiocam HR) was used. Measurement of the fluorescence intensity was done with the help of Axiovision Rel. 4.6 software. The detection of NO fluorescence was performed at 0, 24, 48, 72, 96 and 120 h after treatment in the case of pea and *Arabidopsis* plants. In wheat roots sampling times were: 0, 1, 2, 3, 4, 5, 6, 12, 18, 24th hours of treatments.

Results

Early NO appearance in osmotic stress treated-pea roots

In the case of pea roots, as the effect of osmotic treatment a two phase NO generation could be observed. A four-fold NO accumulation appeared in the first 12 hours of 400 mOsm PEG treatment. This transient NO formation showed a maximum point at 24th hours of treatments. During the following 96 hours a slower but more significant NO accumulation occurred in osmotic stressed-roots, while in control NO level remained low. The effect of osmotic stress on NO formation proved to be concentration dependent, since 400 mOsm PEG resulted in the highest NO fluorescence.

Rapid NO burst in PEG-treated *Arabidopsis thaliana* roots

Similarly to pea plants, the applied concentrations of PEG induced NO generation in *Arabidopsis* roots. In the first 12 hours 1.4 fold NO fluorescence enhancement was found, which intensified in the further 24 hours. In these plants the highest value of NO fluorescence was measured in the 36th hour of PEG treatment. In the 48th and 72nd hours a slight decrease of NO level was revealing and after this, increasing of NO content is followed till the 120th hour of osmotic treatment (Fig. 1).

Osmotic stress induced- transient NO accumulation in wheat roots

In the experiments with wheat plants we followed NO levels till 24th hour of PEG treatment. The concentration dependent effect of PEG predominated in the case of wheat plants as well, since 50 mOsm PEG resulted in 4-times higher NO content, while 400 mOsm PEG caused 5-fold increase in NO fluorescence compared to control. NO significantly accumulated already after the first hour, than decreased to a constant lower level in the following 5 hours. The highest NO peak in the first hour was caused by 400 mOsm PEG treatment. Between the 6th and 12th hours NO fluorescence stagnated at a high level compared to control. After this the amount of NO in roots increased slower but with a higher intensity.

Discussion

Nowadays events of NO function and signaling become known due to the active research. NO proved to be the key molecule in several stress response pathways (Gould et al. 2003; Wilson et al. 2008). Interestingly, during the most stress conditions transient NO generations were found. For instance, very rapid NO burst, developed in 30 min, was observed in response to Fe^{2+}-treatment in *Arabidopsis* (Arnaud et al. 2006), or in the case Cu^{2+} treatments of *Pisum sativum* and *Brassica juncea*, when the NO peak was the highest after 2 h after heavy metal treatment (Bartha et al. 2005). A slower early NO burst was determined in a fungal elicitor induced process which had a maximum point around 5 h after treatment, preceding salicylic acid response (Xu et al. 2005), or at 24 h after the treatment of wheat with stripe rust (Guo et al. 2004). According to these results we detected similar, rapid NO generations in pea, wheat and *Arabidopsis* roots under osmotic stress. In all the three plant species, two phased NO curve was determined, since the early burst was followed by a significant slower increase of NO fluorescence. The source and the role of the NO transient are certainly different from those of the following NO phase, which starts when the transient one already has decayed. This fast NO production can have an important role in acclimation processes to osmotic stress.

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