Osmotic stress- and indole-3-butyric acid-induced NO generation are partially distinct processes in root growth and development in *Pisum sativum*

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In this work, the effects of osmotic stress and exogenous auxin (indole-3-butyric acid, IBA) on root morphology and nitric oxide (NO) generation in roots were compared in pea plants. Five-day-old plants were treated with 0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} or 10^{-9} M IBA or with PEG 6000 at concentrations that determined 0, 50, 100, 200 or 400 mOsm in the medium, during 5 days. NO generation was examined by in situ and in vivo fluorescence method. Increasing concentrations of PEG as well as IBA resulted in shortening of primary root (PR), enhancement of lateral root (LR) number and significant increase of NO generation. Time-dependent investigations revealed that in the case of IBA treatments, the LR number increased in parallel with an intensified NO generation, while elongation of PR was not followed by changes in NO levels. Under osmotic stress, the time curve of NO development was distinct compared with that of IBA-treated roots, because significantly, the appearance of lateral initials was preceded by a transient burst of NO. This early phase of NO generation under osmotic stress was clearly distinguishable from that which accompanied LR initiation. It is concluded that osmotic stress and the presence of exogenous auxin resulted in partly similar root architecture but different time courses of NO synthesis. We suppose that the early phase of NO generation may fulfill a role in the osmotic stress-induced signalization process leading to the modification of root morphology.

Introduction

Development of root architecture greatly depends on both environmental and endogenous factors. Water and nutrient availability (Malamy and Ryan 2001, Zhang et al. 1999) as well as interplay between plant hormones like auxin, cytokinins and signaling substances, e.g. nitric oxide (NO), determine the growth of PR and initiation of LR (Correa-Aragunde et al. 2004, Kolbert et al. 2005, 2008, Malamy and Benfey 1997). Lateral roots (LR) are formed from specially positioned cells in the root pericycle postembryonically, which circumstance pro-

vides broad phenotypic plasticity during further growth and development. The frequency of initiation of LRs is, in part, determined by the auxin concentration: exogenous application of auxin increases the number of LR. The level of auxin, however, is the function of its basipetal polar transport to the shoot base followed by the acropetal movement to the root apex, and again, basipetal movement toward the shoot–root junction (Casimiro et al. 2001, Swarup et al. 2007) as well as its metabolism. Recently, it was shown that indole-3-butyric acid (IBA), a naturally occurring auxin besides the major IAA, is

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Abbreviations – cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid potassium salt; DAF-2DA, 4,5-diaminofluorescein diacetate; IBA, indole-3-butyric acid; LR, lateral root; NO, nitric oxide; NO^+, nitrosonium cation; NO^-, nitroxyl anion; NO·, nitric oxide radical; NPA, naphtylphtalamic acid; PR, primary root.

involved in root formation especially in case of adventitious rooting from stem tissue (Ludwig-Müller et al. 2005). Interestingly, the activity of IBA synthetase is enhanced by osmotic stress administered either as drought, sorbitol or NaCl as well as by ABA, jasmonic acid and salicylic acid, hormones that are involved in stress responses (Ludwig-Müller et al. 1995). The *rib1* (resistant to IBA) mutant of *Arabidopsis* is characterized by short primary root (PR) and increased number of LRs as well as variable responses to environmental stimuli; in this way in its responses, the mutant is separable from the IAA responsive phenotypes, suggesting IBA as a true endogenous auxin in *Arabidopsis* (Poupart and Waddell 2000). This was confirmed by the fact that IAA and IBA use different auxin transport protein complexes, distribute differently in the plant and the transport of IBA is resistant to the IAA transport inhibitor naphtylphtalamic acid (NPA) (Rashotte et al. 2003). Whether or not IBA is acting directly or serves as substrate for its conversion to IAA (Chhun et al. 2004, Kaldorf and Ludwig-Müller 2000, Zolman et al. 2000), this interrelationship between endogenous and environmental factors offers a genuine model for the study of the mechanism of root architecture development as influenced by external factors.

The molecular, biochemical and physiological events participating between signal transduction and root initiation and development, are, however, less understood. In the last years NO has emerged as a multifunctional bioactive molecule in plant signal transduction pathways participating in a broad spectrum of physiological and developmental processes via direct or indirect processes or through cross talking with the classical plant hormones (review; Lamattina et al. 2003). Root formation is not exception from the targets of NO action (Correa-Aragunde et al. 2004, 2006, Kolbert et al. 2005).

NO is a free radical diffusible, lipophylic gas which has three forms in both animals and plants: NO^+ (nitrosonium cation), NO^- (nitroxyl anion) and $NO^{\cdot}$ (nitric oxide radical). Because of its lipophylic characteristics, it can diffuse through membranes. NO has major role in PR growth and LR formation because it acts as a signal molecule in auxin-induced signal transduction. Auxin induces NO production in an unknown way, which activates guanylate cyclase enzyme and cGMP is produced (Pagnussat et al. 2003), which is in the route toward activation of cell division and differentiation.

Our previous experiments show that NO is an active signal component in different stress responses like drought and heavy metal load. Interestingly, the appearance of NO, as the function of time and spatial localization in roots, suggests different mechanisms

involved in NO production and in the role of signal transduction (Bartha et al. 2005, Kolbert et al. 2005).

Recently, it was suggested that stress-induced morphogenetic responses are controlled by auxin distribution in the plant (Potters et al. 2007). The question arose if there are similarities or differences in growth responses of a root system under external auxin load and osmotic stress, with focus on the role of NO in the phenotypic acclimation processes. In this work, the production of NO is investigated in the processes of IBA-induced LR initiation and PR elongation as compared with those under osmotic stress conditions. Using IBA and PEG as osmotic agent in broad concentration range during the time period of stress response as well as NO scavenger agent, different roles of NO could be distinguished under these conditions.

Materials and methods

Plant material and growth conditions

Pea (*Pisum sativum* L. cv. Rajnai törpe = Petit Provençal) seeds were surface sterilized with 5% sodium hypochlorite for 10 min, rinsed and imbibed for 2 h in running water. Germination took place between moisture filter papers in Petri dishes at 26°C for 2 days. Germs with 2-cm-long radicles were then placed into modified Hoagland solution (nine seedlings per 400 ml growth vessels) and were grown 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 for 5 days. The modified Hoagland solution contained the following chemicals: 2 mM Ca(NO$_3$)$_2$, 1 mM MgSO$_4$, 0.5 mM KCl, 0.5 mM KH$_2$PO$_4$ and 0.5 mM Na$_2$HPO$_4$. The micronutrient concentrations used for preparation of the nutrient solutions were: 1 μM MnSO$_4$, 5 μM ZnSO$_4$, 0.1 μM (NH$_4$)$_6$ MO$_7$O$_{24}$, 10 μM H$_3$BO$_4$, 0.1 μM AlCl$_3$, and 20 μM Fe-EDTA. Five-day-old plants were treated for another 5 days. Sampling was taken daily or as stated. The PR length was recorded in mm using a scale. The LR number was counted and expressed as initials per root.

Chemicals

NO was detected with the help of a fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma-Aldrich, St Louis, MO) dissolved in MES buffer (MES and KCl, pH 6.15). MES and KCl was obtained from Reanal (Budapest, Hungary). For inhibiting the polar transport of auxin, NPA (Sigma-Aldrich) was used. For scavenging NO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid potassium salt (cPTIO; Sigma-Aldrich), and as exogenous auxin, IBA (Fluka, Seelze, Germany) were used.

Treatments

Osmotic stress was administrated by dissolving PEG 6000 (Sigma) into the nutrient solution at 50, 100, 200 and 400 mOsm osmotic concentration as measured by a digital automatic osmometer (Micro GMS, Hungary). These concentrations are equivalent to 6.98, 9.97, 14.3 and 19% (w/v), or -0.122, -0.244, -0.488 and -0.976 MPa ψ_s, as calculated according to Wyn Jones and Gorham (1983), respectively. For auxin treatment, $10^{-3} - 10^{-9}$ M indole-3-butyric-acid (IBA) concentrations were applied. As for verification of NO detection, the NO scavenger cPTIO was used in 200 μM concentration. As polar auxin transport inhibitor, NPA was applied at 10 μM concentration.

Detection of NO

Visualization of NO was performed as the highly sensitive in situ and in vivo method of Kojima et al. (1998) as applied for plant tissues (Pedroso et al. 2000). Root segments of 1.5–2 cm length and cut cross-sections were dyed with 10 μM DAF-2DA dissolved in MES/KCl buffer for 20 min at $25 \pm 2°C$ in darkness. Cross-sections were cut with a specific plant microtome (NIHONIKA MTH-1, Tokyo, Japan). After dyeing the samples were washed $4\times$ within 20 min with MES buffer (1 mM, pH 6.15) and were prepared on microscopic slides.

To detect fluorescence intensity Zeiss Axiowert 200M-type fluorescent microscope (Carl Zeiss, Jena, Germany) connected with a high resolution digital camera (Axiocam HR) was used. FLUAR $5\times/0.25$ NA and FLUAR $10\times/0.50$ NA objective lenses were used to investigate the samples.

Digital photographs were taken from the samples with a high-resolution digital camera (Axiocam HR, HQ CCD camera; Carl Zeiss). To measure the fluorescence intensity, AXIOVISION REL. 4.5 software was applied using a filter set 10. The fluorescent values were relative to the area of the sample analyzed. The same camera settings were recorded for each digital image. The detection of NO fluorescence was performed at 0, 24, 48, 72, 96 and 120 h after treatment of roots, and in some cases also at 12 and 36 h (noted at those sites). In each treatment, at least three samples were measured. Three independent series of plants as biological repetitions were grown (n = 9). The selected fluorescent images are representatives of similar results from the three repetitions.

Statistics

The data presented as mean $\pm$ SE were obtained from at least three independently grown series of plants. Significant differences were determined using the Student's t-test applying SIGMASTAT 3.11 software. Significant differences among means (n = 9) are indicated by one ($P \leq 0.05$), two ($P \leq 0.01$), or three ($P \leq 0.001$) asterisk(s).

Results

The effects of osmotic stress and IBA treatment on root morphology and NO production are similar but not identical

Increasing osmolarities of PEG in the growth solution enhanced the number of LR while decreased the lengths of PR of pea plants (Fig. 1A). The concomitant NO
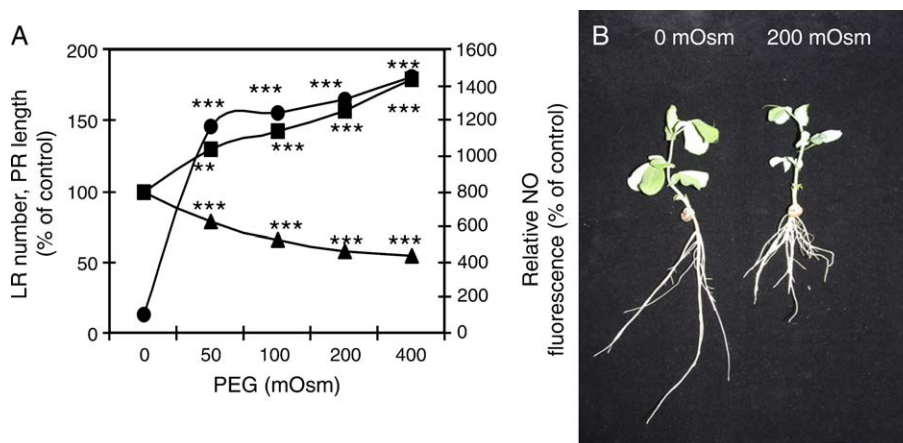

Fig. 1. (A) Effects of PEG-induced osmotic stress on the growth of PR (▲), development of LR (■) and NO formation in the roots (●) of *Pisum sativum* L. Data are expressed as % of control as follow: for PR, 100% = 154 ± 15 mm plant^{-1}, for LR, 100% = 14 ± 2 pieces plant^{-1} and for NO, 100% = 906 ± 250 pixel intensity. Each point is the mean ± SE of nine replicates per treatment (n = 9) and statistical differences are indicated by *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$. B) Morphological changes after PEG-treatment as compared with control plants.

fluorescence curve (Fig. 1A), after an intensive initial burst in response to the lowest osmotic value applied, followed the increase of numbers of LR initials in a parallel way. The phenotypic acclimation resulted in a strongly modified morphology of the plant, especially root architecture (Fig. 1B). This phenomenon is comparable with that which appeared after auxin treatments (Fig. 2). In agreement with earlier observations, we found that low IBA concentrations (10^{-9}–10^{-8} M) resulted in longer PR and fewer LR compared with those of control plants, while higher IBA concentrations (10^{-7}–10^{-3} M) caused shorter PR and enhanced LR number/root values. Concerning NO production, low (control) level NO fluorescence was detected in the cases of low auxin treatments; however, in roots treated with 10^{-6} and 10^{-5} M IBA, the intensity of NO fluorescence was two to three times higher than in the control plants. In the higher concentration range of IBA, the elongation of PRs was inhibited (Fig. 2B). The morphological alterations are shown in Fig. 2B. NO fluorescence for the low- and high-concentration region is represented by the photographs taken from cross sections of roots grown at 0, 50 and 400 mOsm PEG, and 0, 10^{-8} and 10^{-5} M IBA (Fig. 3A–C and D–F, respectively).

A very simple but informative experiment was carried out to investigate the relationship between exogenous auxin treatment and NO generation within pea roots. Three groups of 5-day-old plants were used: (1) plants treated for 10 days with 10^{-8} or 10^{-5} M IBA; (2) plants treated with 10^{-8} M IBA for 5 days and then transferred into solution containing 10^{-5} M IBA and (3) plants treated with 10^{-5} M IBA for 5 days then transferred into a solution containing 10^{-8} M IBA. Samples were taken at the 5th and the 10th day of treatments. The short laterals that appeared after 5-day-long 10^{-5} M IBA treatment were elongated during the following 5 days (Fig. 4f); however, in the case of plants transferred to the lower auxin concentration (10^{-8} M IBA), the elongation of laterals were more intensive (Fig. 4h). Importantly, along this developmental transient, the intensive NO fluorescence, which was earlier generated in the presence of 10^{-5} M IBA after 5 days, strongly decreased when the plants were transferred into 10^{-8} M IBA (Fig. 4H), while only slight decrease was detected in the roots staying in 10^{-5} M IBA for 10 days (Fig. 4F). Plants, with roots elongated in 10^{-8} M IBA during the first 5 days, developed short laterals after transferring them into higher IBA concentration and showed increased NO fluorescence (Fig. 4g,G). These results provide evidence for possible relationship between auxin level and NO generation of pea roots.

In our experiments, the application of the scavenger cPTIO, in agreement with Correa-Aragunde et al. (2004), clearly demonstrated that NO plays a role in LR formation because it completely inhibited LR formation both alone or together with 10^{-5} M IBA, but had no effect on PR elongation (data not shown).

Under osmotic treatments, root development was similar to that of IBA-treated plants as shown in Figs 1 and 2, respectively. The question arose if under osmotic
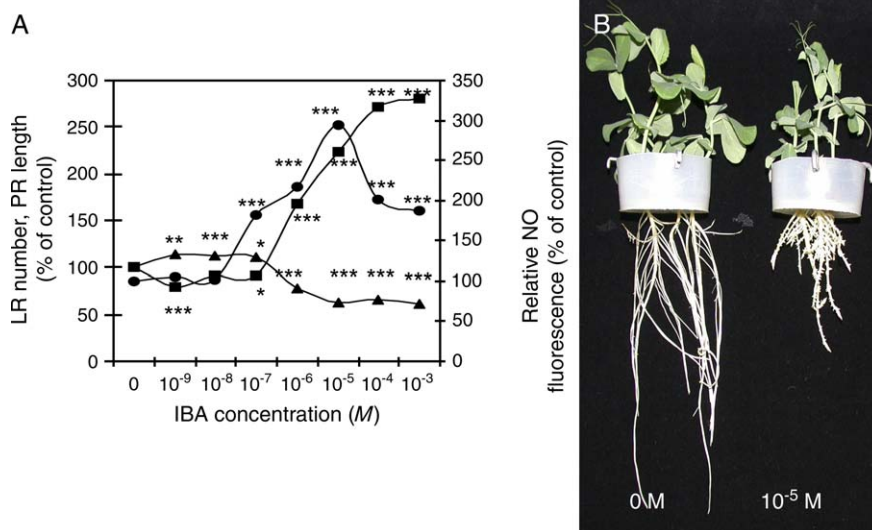

Fig. 2. (A) Effects of IBA concentrations on the growth of PR (▲), development of LR (■) and NO formation in the roots (●) of *Pisum sativum* L. Data are expressed as % of control as follow: for PR, 100% = 144 ± 24 mm plant^{-1}, for LR, 100% = 25 ± 3 pieces plant^{-1} and for NO, 100% = 1120 ± 126 pixel intensity. Each point is the mean ± SE of nine replicates per treatment (n = 9) and statistical differences are indicated by *$P \leq$ 0.05, **$P \leq$ 0.01 and ***$P \leq$ 0.001. (B) Morphological changes after 10^{-5} M IBA treatment as compared with control plants.
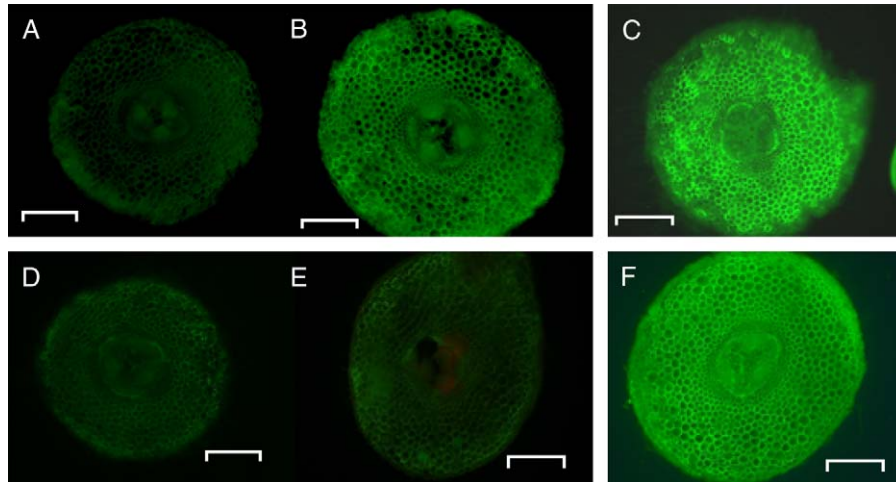
Fig. 3. Fluorescence visualization of NO production in cross sections of pea PRs in the control (0 mOsm PEG, A; 0 M IBA, D), 50 mOsm PEG (B), 400 mOsm PEG (C), 10^{-8} M IBA- (E) and 10^{-5} M IBA- (F) treated samples. Representative images for the concerned concentrations in the NO curve are shown in Fig. 2a. The selected images represent the mean values of nine replicates per treatment. Root samples were prepared and investigated as described in Materials and methods. Bars = 500 μm.

stress acropetally transported auxin was responsible for the intensified formation of LR and NO synthesis. Using the auxin polar transport inhibitor, NPA, the number of LR initials and the NO level were decreased, while the growth of PR practically was not affected (Fig. 5A–C). The relative low inhibition of LR development by NPA can be explained by the fact that NPA is not an inhibitor of transport of IBA (Rashotte et al. 2003) but it indicates that at least for a low extent, IAA can also be responsible for LR formation. The inhibition of NO generation was more significant under osmotic conditions, which could be because of the different time curves and levels of NO generation during the two treatments.

Osmotic stress- and IBA-induced NO generations have different time curves: 'the stress-NO'

In time-dependent investigations, the length of PR, number of LR and fluorescence of NO were measured at the 0, 24, 48, 72, 96 and 120th h of treatments. These experiments were carried out in all PEG and IBA concentrations, but for simplicity we chose two representative concentrations, namely 50 and 400 mOsm PEG, and 10^{-8} and 10^{-5} M IBA. For the NO production under osmotic stress, NO determination was carried out additionally also at 12 and 36 h.

As the function of time, constant linear growth of PR was observed in control roots and only the slopes not the linearity of the curves were changed by osmotic or IBA treatments (Fig. 6A, D). The length of PR was decreased by both concentrations of PEG (Fig. 6A). However, elongation was significantly enhanced by 10^{-8} M IBA,

while treatment with 10^{-5} M IBA resulted in immediate cease of PR elongation (Fig. 6D).

Initiation of LR development was observed after 48 h of osmotic treatment, showing proportionally increasing number of LR with increasing osmotic concentration (Fig. 6B). Concerning IBA treatments, enhancement of LR number was equal in time in the case of control and 10^{-8} M IBA-treated pea plants. Actually, the latter group of plants had much fewer LRs/root than the control. Until the 48th h of treatment, IBA at 10^{-5} M exhibited no significant effect on the visible appearance of LR number, but after this lag phase the number of LRs increased significantly and the most intensive enhancement was observed between the 48th and 72th h of treatment (Fig. 6E). In this respect, the effects of osmotic stress and high concentration of external auxin when compared with the time courses in Fig. 6B, E, were apparently identical.

There is, however, significant difference in the time courses of NO production under osmotic stress and IBA treatments. Under osmotic stress, a fast increase in NO level appeared reaching plateau between 24 and 36 h followed by steady increase afterwards (Fig. 6C).

Control and 10^{-8} M IBA-treated roots showed similar low levels of NO fluorescence intensity which increased slightly in time. In the case of 10^{-5} M IBA, visible fluorescence increase could be detected only after the 48th h of treatment (Fig. 6F). Comparison of time courses of NO production in Fig. 6C, F strongly suggest the occurrence of a transient NO burst that precedes the slow and steady auxin-coupled NO generation.
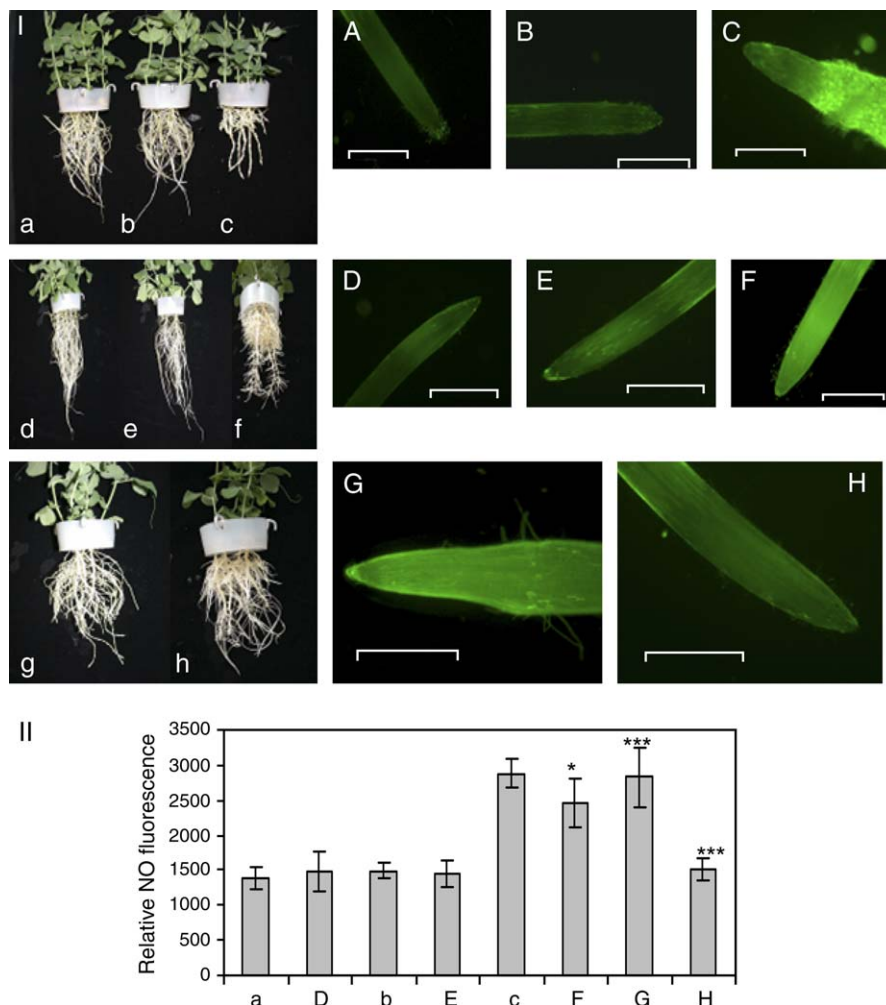
Fig. 4. 'I', Root morphology (a–h) and NO fluorescence (A–H) of pea plants after 5 days treatment (control: a,A; 10^{-8} M IBA: b,B; 10^{-5} M IBA: c,C) and after 10 days treatment (control: d,D; 10^{-8} M IBA: e,E; 10^{-5} M IBA: f,F). Pictures g and G represent plants transferred from 10^{-5} M IBA into the solution containing 10^{-8} M IBA, while h and H show plants transferred from 10^{-8} M IBA into 10^{-5} M IBA. Relative fluorescence values of NO production for A–H are shown in 'II'. Root samples were prepared and investigated as described in Materials and methods. Bars = 1 mm. The selected images are representative of nine photographs per treatments, and the data in 'II' are the mean $\pm$ SE (n = 9). Statistical differences are indicated by *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

To clearly separate the two types of NO, the rate of NO production is represented as the function of time of treatments (Fig. 7). The differential curves clearly distinguish between the osmotic stress-induced transient and the emergence of NO generation after 48 h of treatments.

To answer the question, whether the early NO burst has a role in PEG-induced LR development, we suppressed the PEG-induced transient NO burst with cPTIO in the first 36 h. These samples showed decreased LR number compared with control and 400 mOsm PEG-treated roots in the 120th h of treatment (Fig. 8). This strongly suggests that NO burst is needed to PEG-induced LR development.

Discussion

The development of root system architecture, besides intrinsic, mainly hormonal factors and cell cycle regulators (Malamy and Benfey 1997), is greatly influenced by environmental factors, most significantly water stress and nutrient availability (Lynch 1995, Malamy 2005, Malamy and Ryan 2001). In the build-up of the architecture, the number and frequency of LR initiations and root elongation are the main processes determined by 'intrinsic' and 'response' pathways (Malamy 2005). In this study, we investigated the effects of an environmental factor (osmotic stress) and a typically internal factor, auxin, on the elongation of PRs and initiation of secondary (lateral)
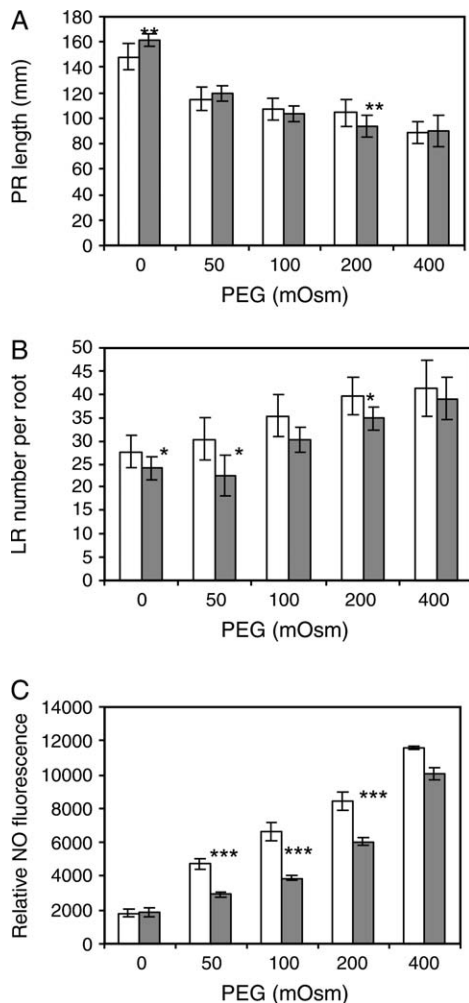
Fig. 5. Effects of PEG concentrations on PR length (A), LR number (B) and NO fluorescence (C) of pea roots. White columns represent treatments without NPA, grey bars represent PEG treatments together with 10 µ*M* NPA treatment. Each point is the mean ± SE of nine replicates per treatment (n = 9) and statistical differences are indicated by *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

roots. Although previous studies gave account that NO mediates the auxin response of LR formation (Correa-Aragunde et al. 2004, 2006, Pagnussat et al. 2002), further details and differences were found for the role of NO in root growth and development under the conditions of water stress and externally added auxin load.

The effects of osmotic stress and IBA treatment on root morphology and NO production are similar but not identical

Elongation of roots under water deficit is usually strongly hindered; while mild osmotic stress can stimulate growth (van der Weele et al. 2000). Recently, Pasternak et al.

(2005) suggested that key events in plant growth responses under environmental stress are interactions between the oxidative stress and auxin that result in altered auxin transport and hence, modified growth and development. In our experiments, the similarity between responses of PR elongation and LR initiation to increasing water stress and external auxin concentrations support this idea (Figs 1 and 2). In both cases, the reduction of PR length reached approximately 50% of the control, and the increase of numbers of LRs were 180% under osmotic stress and about 280% at the highest IBA concentration. These figures resulted in similar root architecture with slight differences in the length of LR (Figs 1B and 2B). Concerning the increase of LRs under osmotic stress in pea, it has to be mentioned that contrasting result was obtained for *Arabidopsis*, where the number of LRs decreased significantly and continuously from −0.49 through −0.92 to −1.21 MPa (van der Weele et al. 2000). The difference may origin from the different species and, in part, from the growing system (there, nutrient-agar medium with PEG 8000).

Despite the similarities in characteristics of root growth and development under osmotic stress and auxin load, there is a significant difference in the intensity of the accompanying NO generation: while the maximal value is three-fold in the roots with the high auxin load (at 10^{-5} *M* IBA), osmotic stress response resulted in 14-fold increase of NO level at 400 mOsm (−0.98 MPa) PEG 6000 concentration. Interestingly, the intensive increase of NO production started with and accompanied the LR initiation process in both cases.

The effects of IBA on PR growth and LR development, in connection with NO production, allowed to distinguish clearly between the two processes. It is well known that auxin at low concentration promotes PR elongation, while above a threshold concentration it induces LR initiation (Casimiro et al. 2001, Hinchee and Rost 1986, Torrey 1950). Still, in our actual experimental design, we applied a wide concentration range (10^{-9}–10^{-3} *M*) of exogenous auxin, IBA. Although IBA is mostly used to investigate adventitious rooting, we choose this auxin because of its higher stability than IAA and because it is found naturally in pea roots (Schneider et al. 1985). This wide concentration range of exogenous IBA treatment revealed the two-fold effect on root growth and development characteristics and NO fluorescence as well: low concentrations of IBA (10^{-9}–10^{-8} *M*) was favorable to PR elongation and this process was not followed by NO generation, while higher IBA concentrations (10^{-7}–10^{-3} *M*) were responsible for LR emergence and NO generation. The enhancement of LR number was continual but fluorescence of NO showed a maximum value at 10^{-5} *M* IBA (Fig. 2). Fig. 3 illustrates the difference in localization and intensities of NO generation under
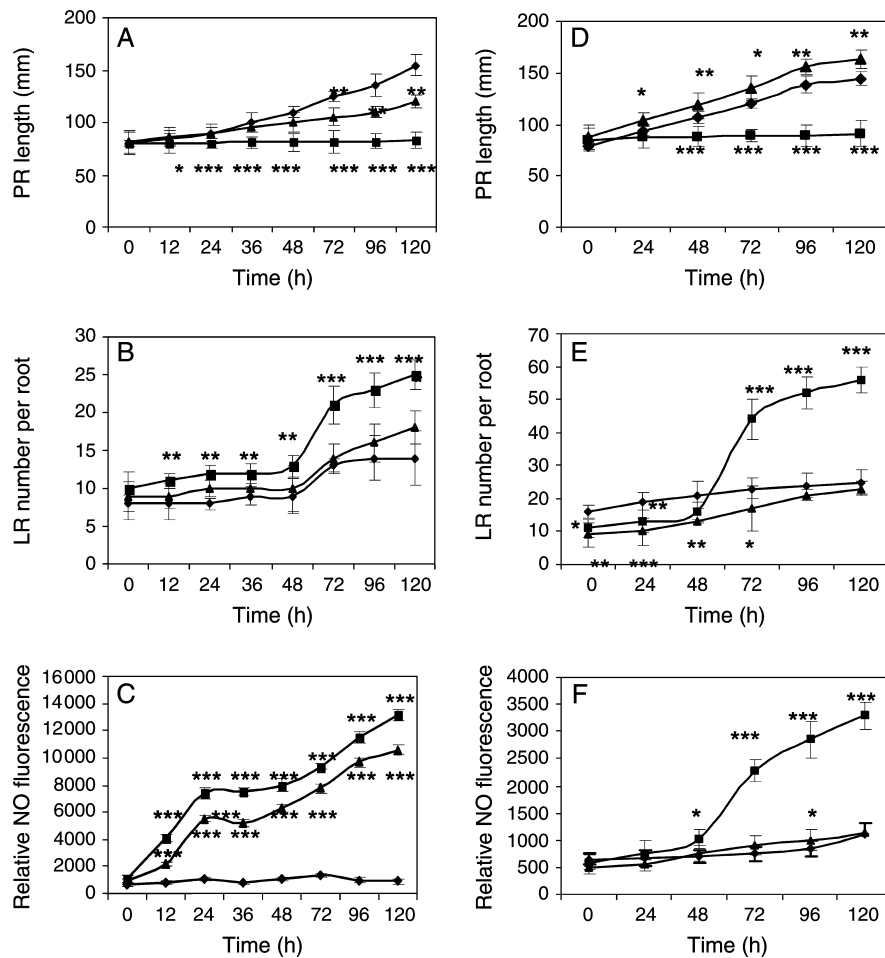
Fig. 6. Time dependence of PR growth (A), LR development (B) and NO formation (C) in control plants (♦), 50 mOsm PEG-treated plants (▲), 400 mOsm PEG-treated plants (■). Time dependence of PR growth (D), LR development (E) and NO formation (F) in control plants (♦), 10^{-8} *M* IBA-treated plants (▲), 10^{-5} *M* IBA-treated plants (■). The data represent the mean values of nine replicates per treatment and the vertical bars are standard errors and statistical differences are indicated by *$P \le 0.05$, **$P \le 0.01$ and ***$P \le 0.001$.

osmotic and auxin treatments. That the intensive NO signal is strongly coupled to initiation and emergence of LRs is proved its decay after transferring the plants to the medium of low IBA concentration that induced the elongation of the short primordial (Fig. 4, I and II). The first step of LR initiation is the auxin-mediated cell cycle activation (Himanen et al. 2002), whose process, as demonstrated in alfalfa cell cultures, is promoted by NO (Ötvös et al. 2005). NO fluorescence during LR formation was also observed in tomato roots, as illustrated by Correa-Aragunde et al. (2004, 2006).

Osmotic stress- and IBA-induced NO generations have different time curves: the 'stress-NO'

Investigating the time-dependent properties of rooting processes and NO generation, we observed that the effect

of 10^{-5} *M* IBA on LR number and NO fluorescence appeared after 48 h treatment. The similar temporal formation of auxin- induced NO synthesis and LR initiation suggests a functional relation between these processes (Fig. 6E, F). This supposition was confirmed by the result that NO fluorescence is strongly linked to auxin level in roots (Fig. 4).

During osmotic stress, the time dependence of NO development showed significant difference as compared with that of IBA-treated roots because the appearance of lateral initials was preceded by a transient burst of NO (Fig. 6). This early phase of NO generation under osmotic stress, culminating at 24 h, was clearly distinguishable from that which accompanied LR initiation under both osmotic and IBA treatments. The source and the role of this NO transient are certainly different from those of the constant and steady NO phase, which start after 48 h of
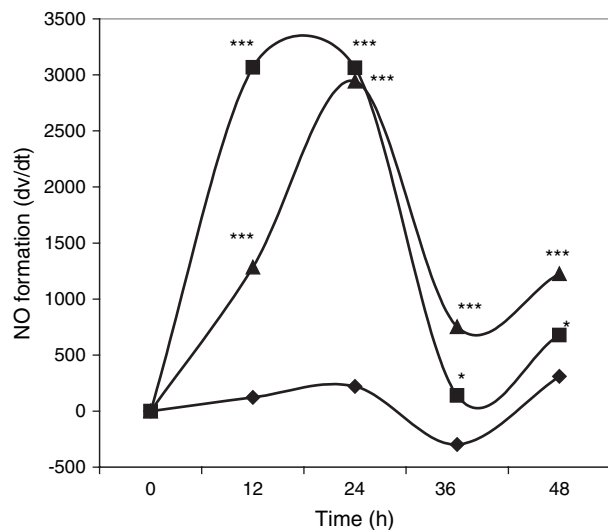
Fig. 7. Differential time curves of transient NO formation in control (♦), 50 mOsm PEG-treated (▲) and 400 mOsm PEG- treated (■) pea roots and statistical differences are indicated by *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

the treatments, when the transient one already has decayed. The kinetics of early NO transients seem to depend on the source of NO. For instance, very early NO burst, developed in 30 min, was observed in response to Fe^{2+} treatment in Arabidopsis, preceding *ATFer1D* gene expression (Arnaud et al. 2006), or in the case Cu^{2+} treatments of *Pisum sativum* and *Brassica juncea*, when the NO peak was obtained after 2 h after subjecting the plants to the metal ions (Bartha et al. 2005). Both these transition metal ions, in the Fenton reaction with H_2O_2, produce hydroxyl radicals leading to the formation other reactive oxygen species (ROS). Oxidative stress may involve NO as well playing a role in drought responses in the ABA pathway in wheat root (Zhao et al. 2001). A slower early NO burst was obtained in a fungal elicitor-induced process that had a maximal value around 5 h after treatment, preceding salicylic acid response (Xu et al. 2006), or at 24 h after the treatment of wheat plants with stripe rust (Guo et al. 2004). When the osmotic stress-induced NO burst was eliminated by the NO-scavenger cPTIO, less LRs were formed than in control and osmotic-stressed roots, indicating that the early NO burst ('stress-NO') is necessary for the osmotic stress-induced LR formation.

These findings refer to the different sources and the localization of NO in the signal transduction pathway, i.e. it seems that NO is signaling in the early steps in stress responses, preceding the specification of the different pathways.

Abiotic stressors, thus osmotic effects cause oxidative stress. Plant growth responses under these conditions are modified by altered auxin transport (Pasternak et al. 2005),
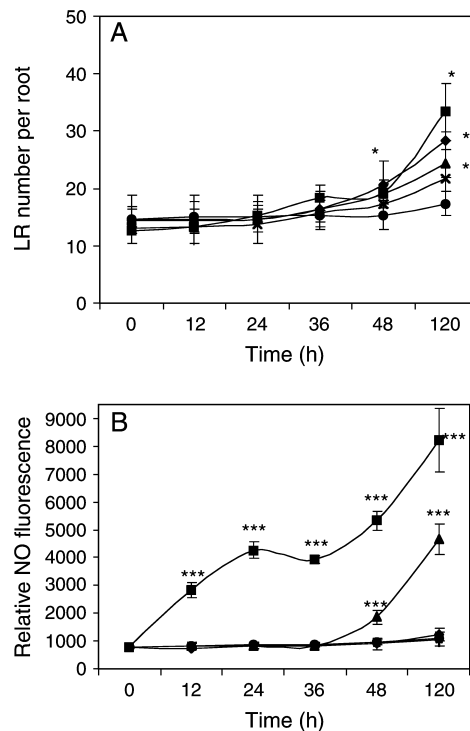

Fig. 8. Time curves of LR development (A) and NO generation (B) of control (♦), 400 mOsm PEG- (■), cPTIO + PEG- (✖), cPTIO- (●) treated pea plants and those of in plants transferred after the 36th hour from cPTIO + PEG into a solution containing 400 mOsm PEG (▲). The data represent the mean values of nine replicates per treatment and the vertical bars are standard errors. Statistical differences are indicated by *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

hence the similarity, though not identical, of osmotic stress-induced and IBA-induced root structures (Figs 1B and 2B). It was suggested that a stress-induced morphogenetic response, in general, is controlled by auxin distribution in the plant and ROS are part of the signalization between the stress and the morphogenetic response (Potters et al. 2007). In our experiments, the early appearance of the NO transient can be enrolled into the signal transduction pathway towards the auxin-induced changes in root morphology, this process has significance in the phenotypic adaptation under drought conditions.

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