Planta (2004) 218: 900–905
DOI 10.1007/s00425-003-1172-7

ORIGINAL ARTICLE

Natalia Correa-Aragunde · Magdalena Graziano
Lorenzo Lamattina

Nitric oxide plays a central role in determining lateral root development in tomato

Received: 28 August 2003 / Accepted: 15 November 2003 / Published online: 10 January 2004

Abstract Nitric oxide (NO) is a bioactive molecule that functions in numerous physiological processes in plants, most of them involving cross-talk with traditional phytohormones. Auxin is the main hormone that regulates root system architecture. In this communication we report that NO promotes lateral root (LR) development, an auxin-dependent process. Application of the NO donor sodium nitroprusside (SNP) to tomato (*Lycopersicon esculentum* Mill.) seedlings induced LR emergence and elongation in a dose-dependent manner, while primary root (PR) growth was diminished. The effect is specific for NO since the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) blocked the action of SNP. Depletion of endogenous NO with CPTIO resulted in the complete abolition of LR emergence and a 40% increase in PR length, confirming a physiological role for NO in the regulation of root system growth and development. Detection of endogenous NO by the specific probe 4,5-diaminofluorescein diacetate (DAF-2 DA) revealed that the NO signal was specifically located in LR primordia during all stages of their development. In another set of experiments, SNP was able to promote LR development in auxin-depleted seedlings treated with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA). Moreover, it was found that LR formation induced by the synthetic auxin 1-naphthylacetic acid (NAA) was prevented by CPTIO in a dose-dependent manner. All together, these results suggest a novel role for NO in the regulation of LR development, probably operating in the auxin signaling transduction pathway.

Keywords Nitric oxide · Auxin · *Lycopersicon* · Lateral root · Primary root

N. Correa-Aragunde · M. Graziano · L. Lamattina (✉)
Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CC 1245, 7600 Mar del Plata, Argentina
E-mail: lolama@mdp.edu.ar
Fax: +54-223-4753150

Abbreviations *CPTIO* 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide · *DAF-2 DA* 4,5-Diaminofluorescein diacetate · *LR* Lateral root · *NAA* 1-Naphthylacetic acid · *NO* Nitric oxide · *NPA* N-1-Naphthylphthalamic acid · *PR* Primary root · *SNP* Sodium nitroprusside

Introduction

Root systems perform the essential tasks of providing water, nutrients and physical support to plants. The length of the primary root (PR) and the density of lateral roots (LRs) determine the architecture of the root system, and this, in turn, plays a major role in determining whether a plant will succeed in a particular environment (Malamy and Benfey 1997). The number of LRs is not predetermined in plant development; therefore, each plant integrates information from its environment into the "decisions" it makes about root formation (Malamy and Ryan 2001). Nutrients are one of the major environmental signals that affect LR development. In soils or media with patchy nutrient distribution, LRs preferentially proliferate in the nutrient-rich zone (Robinson 1994; Zhang and Forde 1999). LR development is also under hormonal control. Many lines of experimental evidence strongly support a role for auxin during this process. Application of exogenous auxin results in increased initiation of LRs (Blakely et al. 1988) and several auxin-resistant mutants of *Arabidopsis thaliana* have a reduced number of LRs (Casimiro et al. 2003). Moreover, roots deprived of endogenous auxin by growing them in the presence of the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) failed to initiate LR primordia (Casimiro et al. 2001).

LRs originate in the root pericycle, in which individual quiescent cells are stimulated to dedifferentiate and proliferate to form the LR primordium. Cells in the LR primordium differentiate and elongate, causing

the LR to emerge through the PR epidermis (Dubrovsky et al. 2001). LR primordium development can be divided into two phases: an early phase which lasts until the LR primordia are three to five cell layers in size, and a later phase where the primordium develops a functional meristem and emerges from the main root. This distinction is based upon the results obtained from in vitro-cultured roots (Laskowski et al. 1995), which indicated that LR primordia acquire the capacity to synthesize their own auxin during the later phase. However, the signals and mechanisms triggered by auxin that initiate and direct this organogenesis have only recently been considered from a molecular standpoint (Bhalerao et al. 2002; Himanen et al. 2002; Xie et al. 2002).

Nitric oxide (NO) is a free radical involved in numerous and diverse cellular pathways in mammals (Torreilles 2001). In recent years, there has been much research about the presence of NO and its physiological roles in the plant kingdom. Thus, evidence has been obtained for the involvement of NO in growth and developmental processes, as well as in defense responses (for reviews, see: Durner and Klessig 1999; Wojtaszek 2000; Beligni and Lamattina 2001; Lamattina et al. 2003). Recently, Pagnussat et al. (2002) demonstrated that NO is required for the molecular events involved in auxin-induced adventitious root development in *Cucumis sativus*. NO donors induced adventitious root initiation in cucumber explants and an accumulation of endogenous NO was detected in explants after IAA treatment (Pagnussat et al. 2002). Since the involvement of NO in an auxin-signaling pathway opens a wide field of research for every known auxin effect, in this report we have evaluated the participation of NO in the auxin-induced pathway that directs LR development.

Materials and methods

Plant material and growth conditions

Tomato (*Lycopersicon esculentum* Mill. cv Ace 55) seeds were surface-sterilized in 5% sodium hypochlorite for 10 min, rinsed extensively and imbibed in water for 3 days. Seedlings with radicles 2–3 mm long were transferred to Petri dishes (60 mm diameter) containing filter paper soaked with 4 ml of the various treatments. Seedlings were grown in a chamber at 25 ± 1°C and a 14 h:10 h (L:D) photoperiod. After 5 days of treatment, the number of LRs per seedling and the length of the PR were quantified. LR number only included those roots that were > 1 mm in length.

Detection of endogenous NO

NO was monitored by incubating roots from 3-day-old seedlings with 15 μM of the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) in 20 mM Hepes–NaOH (pH 7.5). Thereafter, the roots were washed three times for 15 min with fresh buffer and examined by epi-fluorescence (excitation 490 nm; emission 525 nm) and bright-field microscopy in an Eclipse E 200 microscope (Nikon, Tokyo). For the experiment in Fig. 3, cortical cell layers were sloughed off leaving only the pericycle surrounding the vascular cylinder.

Chemicals

Sodium nitroprusside (SNP) and 1-naphthylacetic acid (NAA) were from Sigma (St. Louis, MO, USA), N-1-naphthylphthalamic acid (NPA) from Chemical Services (West Chester, PA, USA), 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) from Molecular Probes (Eugene, OR, USA) and DAF-2 DA from Calbiochem (San Diego, CA, USA).

Results

The NO donor SNP increased LR number and decreased PR growth in a dose-dependent manner in tomato seedlings. Figure 1a shows the number of emerged LRs after 5 days of treatment. Application of the permeable auxin NAA resulted in a similar response to that obtained with SNP (Fig. 1b). The promotion of LR development was maximal at 200 μM SNP and at 500 nM NAA. Concentrations between 50–200 μM SNP had previously been demonstrated to be functional in a number of biological systems (Beligni and Lamattina 2000; García-Mata and Lamattina 2002). Previous observations indicated that millimolar amounts of SNP in aqueous solution release nanomolar amounts of NO at 25°C (Ferrer and Ros Barcelo 1999; Beligni 2001). Anatomical studies demonstrated that: (i) at the highest SNP concentration tested (500 μM) many primordia failed to emerge (not shown), consequently diminishing the number of LRs (Fig. 1a); and (ii) LR primordia displayed similar morphology in control, NAA- and SNP-treated seedlings for all stages of development (not shown). SNP treatment also stimulated the elongation of LRs in a dose-dependent manner. Average LR lengths were (mean ± SE): 5.5 ± 0.6 mm in H_2O, 6.7 ± 1.1 mm in 50 μM SNP, 7.2 ± 1.0 mm in 100 μM SNP, 5.0 ± 0.3 mm in 200 μM SNP and 4.9 ± 0.4 mm in 300 μM SNP. We also observed that PR length decreases proportionally with increasing NAA and SNP concentrations (Fig. 1c,d). When the whole root system was analyzed, total root length (the sum of lengths of the PR and LRs) was unmodified until 200 μM SNP (Fig. 1e), indicating that higher branching and elongation of LRs compensated for a shorter PR in SNP-treated seedlings.

Promotion of LR development and inhibition of PR growth by the SNP treatment were NO-specific responses since the NO scavenger CPTIO abolished the effect of the NO donor (Fig. 2a,b). To strengthen the hypothesis that NO is involved in LR development, we analyzed the physiological role of endogenous NO. When applied alone, 1 mM CPTIO completely inhibited LR development (Fig. 2a,c). When anatomical studies were performed, no LR primordia were detected in roots of CPTIO-treated seedlings (not shown). This remarkable result suggests that LR promotion is highly dependent on NO, with NO probably being required in the early stages of primordium development. The inhibitory effect of CPTIO is reversible since LR emergence was triggered in seedlings that were shifted to H_2O

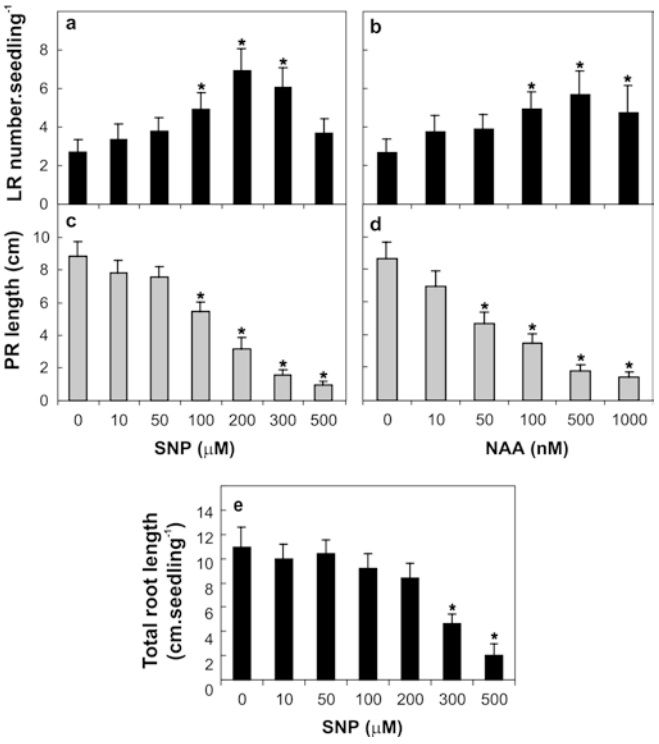

Fig. 1a–e Effect of NO and NAA on lateral root (LR) number and primary root (PR) length. Tomato (*Lycopersicon esculentum*) seedlings were treated with different concentrations of the NO donor SNP (**a,c**) or the auxin NAA (**b,d**). The number of LRs per seedling (**a,b**) and the length of the PRs (**c,d**) were analyzed. The values are means and SE of 5 independent experiments ($n = 10$). *Stars* indicate a significant difference with respect to the control (without SNP or NAA) at $P < 0.05$ (*t*-test). **e** The length of the PR and all LRs (> 1 mm) per seedling was measured for different SNP concentrations and the values were considered as a measure of total root length. The mean and SE were calculated from three independent experiments ($n = 10$). *Stars* indicate a significant difference with respect to the control (0 μM SNP) at $P < 0.05$ (*t*-test)

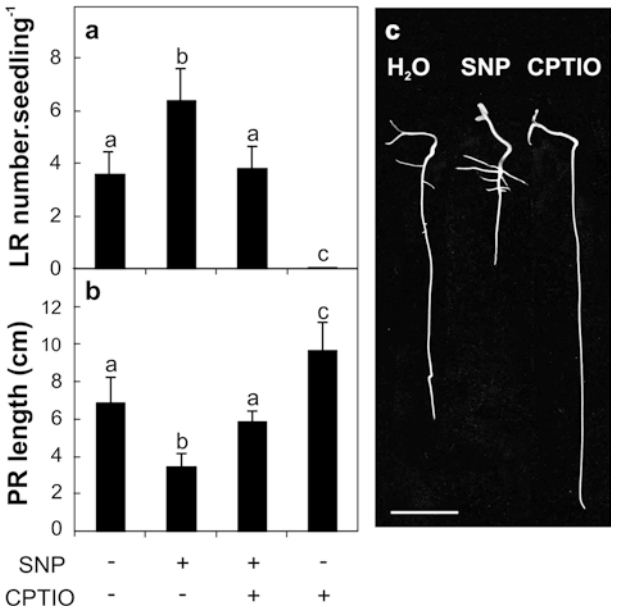

Fig. 2a–c Effect of endogenous NO depletion on LR number and PR length. **a,b** LR number (**a**) and PR length (**b**) were measured in tomato seedlings treated with H_2O, 200 μM SNP, 200 μM SNP plus 1 mM CPTIO or 1 mM CPTIO. Means and SE were calculated from three independent experiments ($n = 5$). *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test). **c** Representative photographs of 5-day-old seedlings treated with H_2O, SNP or CPTIO at the concentrations indicated above. Bar = 2 cm

after 5 days of treatment with CPTIO (not shown). Additionally, CPTIO not only inhibited LR formation, but also increased PR length by 40% compared with the control (H_2O) treatment (Fig. 2b,c). Figure 2c shows the effect of exogenous NO application and endogenous NO depletion on tomato root architecture after 5 days of treatment.

The early sequence of LR development in tomato is similar to that of *Arabidopsis* (Laskowski et al. 1995). The formation of an LR in tomato begins with transverse divisions in xylem-radius pericycle cells. These divisions are followed by radial expansion and subsequent periclinal division. Daughter cells continue to divide symmetrically and asymmetrically giving rise to a primordium. In order to further assess the role of endogenous NO during primordia formation, we analyzed the presence of NO in roots of control seedlings by using the fluorescent probe DAF-2 DA. Living cells incorporate DAF-2 DA and subsequently, it is hydrolyzed by cytosolic esterases to release DAF-2, which reacts with NO to produce the fluorescent triazole derivative DAF-2T (Kojima et al. 1998). When roots were loaded with DAF-2 DA, specific green fluorescence was clearly associated to primordia (Fig. 3e,f). Examining the progression of LR formation during its early stages, it was detected that NO also accumulates in those pericycle cells that exhibit transverse divisions and radial expansion (Fig. 3c,d); the primordium that will give place to an LR originates from these cells. Green fluorescence was not detected in undivided pericycle cells (Fig. 3a,b). These results strongly support an involvement of NO during the first stages of primordium development.

In order to study a possible link between NO and auxins in the regulation of LR development, the requirement of endogenous auxin for the NO response was assessed. The inhibitor of polar auxin transport NPA generates reduced levels of auxin in the root (Reed et al. 1998). Treatment of tomato seedlings with 1 μM NPA resulted in an almost complete abolition of LR emergence (Fig. 4). Application of exogenous NAA restored LR formation in NPA-treated tomato seedlings (Fig. 4), as was also reported for *Arabidopsis* (Casimiro et al. 2001). Hence, NPA-treated roots remain competent to respond to auxin and initiate LR development. NO was also able to rescue NPA-treated seedlings from the inhibition of LR formation (Fig. 4), indicating that NO can trigger LR development in seedlings containing suboptimal auxin levels in roots.

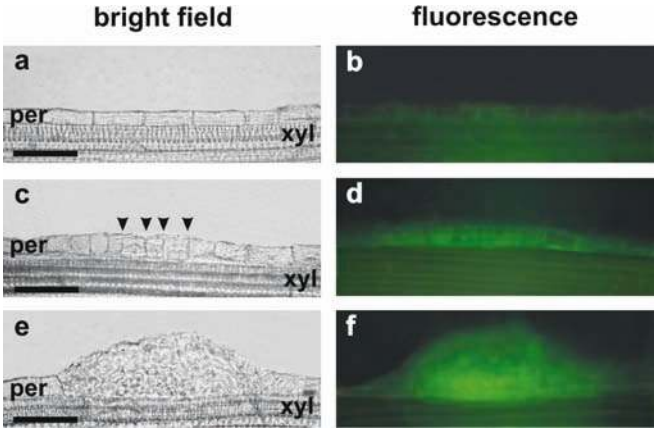

Fig. 3a–f NO accumulation during the development of LR primordia. Tomato seedlings were grown in water for 3 days. Roots were loaded with DAF-2 DA and observed by bright-field (**a,c,e**) and epi-fluorescence (**b,d,f**) microscopy. The outer cell layers of roots were sloughed off. **a,b** Longitudinal section in which LR primordia are not apparent. **c,d** Section showing radial expansion and transverse divisions (*arrowheads*) in pericycle cells. **e,f** Section showing an LR primordium with several cell layers. *per* Pericycle, *xyl* xylem. Bars = 0.1 mm

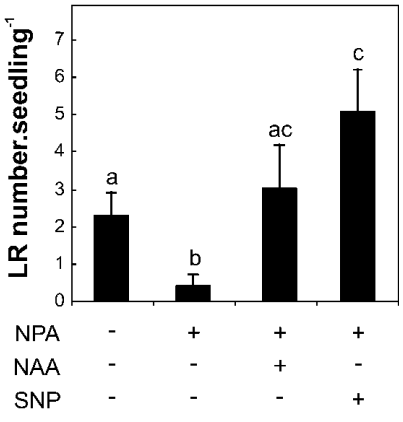

Fig. 4 NO restores LR formation in NPA-treated tomato seedlings. LR number was measured in seedlings treated as indicated. Concentrations used were 1 μM NPA, 100 nM NAA and 200 μM SNP. Data are means and SE obtained from three independent experiments ($n = 10$). *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test)

To further assess the cross-talk between NO and auxin during LR formation, we analyzed the requirement for NO in the NAA response. When NO was scavenged with CPTIO, the NAA-induced LR development was prevented in a dose-dependent manner (Fig. 5a). This result suggests that endogenous NO is involved in LR formation promoted by NAA. To analyze NO accumulation during NAA-induced LR formation we loaded seedlings with the specific NO probe DAF-2 DA. Our observations revealed that LR primordia displayed localized green fluorescence independently of the treatment (Fig. 5b). Thereby, the number of primordia correlates with NO-specific fluorescence detected by the probe, and thereafter, with NO levels within a root. Since exogenous NAA increases the number of LR primordia, NAA-treated seedlings consequently had higher levels of NO than control ones. Treatment with 1 μM NAA generates a large number of primordia, most of them failing to emerge (Figs. 5b, 1b). Figure 5b also shows that treatment with NAA plus CPTIO drastically shuts down primordia number and fluorescence at 72 h of treatment (Fig. 5b).

Discussion

The plasticity to modulate the root architecture is one way to overcome the inability of plants to move towards water or nutrients stores. In this report, we present evidence on NO participation in two processes that define the root architecture: LR development and PR growth. In tomato, NO can modify the root system structure without altering the root area by increasing branching and, thus, horizontal exploration of soil. Many nutrients can also affect the morphology of the root system. It was reported that NO_3^- treatment stimulates LR development (Granato and Rapper 1989; Zhang and Forde 1999). However, since enzymatic and non-enzymatic reduction of NO_3^- can lead to the production of NO in roots (Stöhr and Ullrich 2002), one interesting question is whether or not NO could be the signal that announces the presence of NO_3^- in soil and mediates its effect on LR formation.

The formation of LR primordia from pericycle cells through asymmetric divisions represents a general proliferative pattern (Dubrovsky et al. 2001; Casimiro et al. 2003). In *Arabidopsis*, primordia development begins with two pericycle cells undergoing almost simultaneous polarized transverse divisions. Daughter cells divide symmetrically and asymmetrically, creating groups with approximately 10 short cells that are similar in length, giving rise to the primordium (Casimiro et al. 2003). In this work we have shown that in tomato, an intense production of NO accompanied primordia development from its very first stages. Moreover, depletion of endogenous NO resulted in a complete absence of LR primordia. These results indicate that NO production plays an essential role during the formation of LR primordia and emergence of LRs from the parent root.

The involvement of NO in plant hormone responses has been well reported. NO mediates cytokinin-induced betalaine accumulation (Scherer and Holk 2000) and ABA-induced stomatal closure (García-Mata and Lamattina 2002; Neill et al. 2002). Furthermore, Pagnussat et al. (2002) provided the first evidence about NO and auxin cross-talk during adventitious root formation in cucumber. We are now reporting new evidence of NO participation in another auxin response. Our results show that: (i) NO is able to promote LR initiation in roots treated with the auxin transport inhibitor NPA, and (ii) the NAA promotion of LR development can be

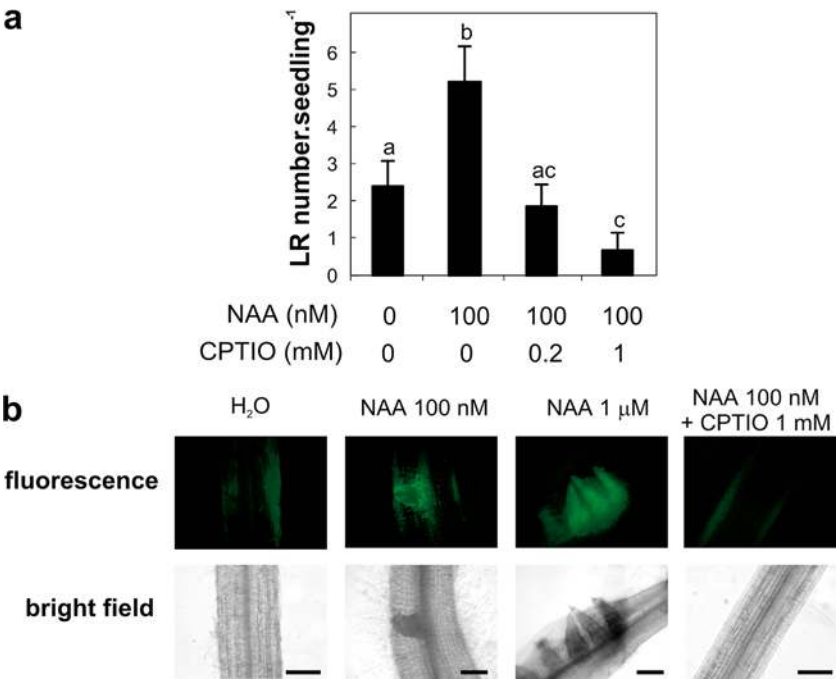

Fig. 5a,b LR development in NAA-treated tomato seedlings is NO dependent. **a** LR number was measured in seedlings treated as indicated for 5 days. Data are means and SE obtained from three independent experiments ($n = 10$). *Different letters* indicate a significant difference at $P < 0.05$ (Tukey test). **b** Longitudinal sections, taken at 5 mm from the hypocotyl–root junction, showing the green fluorescence corresponding to an NO-specific reaction with the probe DAF-2 DA. Seedlings were treated with H_2O, 0.1 or 1 μM NAA, or 0.1 μM NAA plus 1 mM CPTIO for 3 days, loaded with DAF-2 DA and observed by epi-fluorescence and bright-field microscopy. Bars = 0.5 mm

prevented by scavenging NO. Thus, this evidence supports the possibility that auxin and NO might be on a linear signaling pathway in the process of LR formation in tomato.

Genetic and physiological evidence suggest that auxin is required at several stages to facilitate LR formation. Young LR primordia are unable to continue to divide and emerge when they are excised from the parent root, probably due to the blockage on auxin supply. However, LR primordia became an autonomous meristem in advanced stages, indicating that they contain the cell types that act as an auxin source to trigger the outgrowth of LRs (Laskowski et al. 1995). The requirement for auxin during the early and late stages of LR primordium development can be correlated with the accumulation of NO detected by the probe DAF-2 DA (Figs. 3, 5b), supporting the idea that NO is involved in the auxin signaling pathway.

More than one hypothesis could be postulated to explain through which target molecules NO exerts its action to promote LR development. For example, it can be hypothesized that (i) NO produces an antioxidant environment (Beligni and Lamattina 2002), which might protect auxins from oxidation, and/or (ii) NO regulates cell-cycle genes or enzyme activities involved in auxin signal transduction. The ongoing analysis of the NO involvement in the regulation of cyclins, cyclin-dependent kinases and auxin-induced genes during LR development will surely contribute to our understanding of the molecular mechanisms that regulates root morphogenesis. Overall, the data presented here indicate that NO is a major participant that must be included in the intricate succession of events that conclude in LR emergence and modulation of the root system architecture.

Acknowledgments We thank Dr. Gabriela Pagnussat for critical reading and helpful discussions of the manuscript. This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICTs 1-6496/99 and 1-9767/00 to L.L.), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 0898/98 to L.L.), Fundación Antorchas and institutional grants from Universidad Nacional de Mar del Plata (UNMdP), Argentina. L.L. is a career research scientist and M.G. a postgraduate fellow from CONICET, Argentina. N.C.-A. is a student fellow from UNMdP, Argentina. N. C.-A. and M.G. contributed equally to this work.

References

Beligni MV (2001) Functions of nitric oxide in plants: antioxidant effect and participation in light responses. PhD thesis, Mar del Plata University, Argentina

Beligni MV, Lamattina L (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta 210:215–221

Beligni MV, Lamattina L (2001) Nitric oxide in plants: The history is just beginning. Plant Cell Environ 24:267–278

Beligni MV, Lamattina L (2002) Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. Plant Cell Environ 25:737–748

Bhalerao RP, Eklof J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. Plant J 29:325–332

Blakely LM, Blakely RM, Colowit PM, Elliot DS (1988) Experimental studies on lateral root formation in radish seedlings roots. Analysis of the dose-response to exogenous auxin. Plant Physiol 87:414–419

Casimiro I, Marchant A, Bhalerao R, Beeckman T, Dhooge S, Swarup R, Graham NS, Inzé D, Sandberg G, Casero PJ, Bennett MJ (2001) Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell 13:843–852

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting *Arabidopsis* lateral root development. Trends Plant Sci 8:165–171

Dubrovsky JG, Rost TL, Colon-Carmona A, Doerner P (2001) Early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana*. Planta 214:30–36

Durner J, Kessig DF (1999) Nitric oxide as a signal in plants. Curr Opin Plant Biol 2:369–374

Ferrer MA, Ros Barcelo A (1999) Differential effects of NO on peroxidase and H_2O_2 production by the xylem of *Zinnia elegans*. Plant Cell Environ 22:891–897

García-Mata C, Lamattina L (2002) Nitric oxide and abscisic acid cross talk in guard cells. Plant Physiol 128:790–792

Granato TC, Raper CD (1989) Proliferation of maize (*Zea mays* L.) roots in response to localized supply of nitrate. J Exp Bot 40:263–275

Himanen K, Boucheron E, Vanneste S, Almeida Engler J, Inzé D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14:2339–2351

Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal Chem 70:2446–2453

Lamattina L, García-Mata C, Graziano M, Pagnussat G (2003) Nitric oxide: The versatility of an extensive signal molecule. Annu Rev Plant Biol 54:109–136

Laskowski MJ, Williams ME, Nusbaum HC, Sussex IM (1995) Formation of lateral root meristems is a two-stage process. Development 121:3303–3310

Malamy JE, Benfey PN (1997) Down and out in *Arabidopsis*: the formation of lateral roots. Trends Plant Sci 2:390–396

Malamy JE, Ryan KS (2001) Environmental regulation of lateral root initiation in *Arabidopsis*. Plant Physiol 127:899–909

Neill SJ, Desikan R, Clarke A, Hancock JT (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. Plant Physiol 128:13–16

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L (2002) Nitric oxide is required for root organogenesis. Plant Physiol 129:954–956

Reed RC, Brady SR, Muday GK (1998) Inhibition of auxin movement from the shoot into the root inhibits lateral root development in *Arabidopsis*. Plant Physiol 118:1369–1378

Robinson D (1994) The responses of plants to non-uniform supplies of nutrients. New Phytol 127:635–674

Scherer GFE, Holk A (2000) NO donors mimic and NO inhibitors inhibit cytokinin action in betalain accumulation in *Amaranthus caudatus*. Plant Growth Regul 32:345–350

Stöhr C, Ullrich WJ (2002) Generation and possible roles of NO in plant roots and their apoplastic space. J Exp Bot 53:2293–2303

Torreilles J (2001) Nitric oxide: one of the more conserved and widespread signaling molecules. Front Biosci 6:1161–1172

Wojtaszek P (2000) Nitric oxide in plants: to NO or not to NO. Phytochemistry 54:1–4

Xie Q, Guo HS, Dallman G, Fang S, Weissman AM, Chua NH (2002) SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature 419:167–170

Zhang H, Forde BG (1999) Dual pathways for regulation of root branching by nitrate. Proc Natl Acad Sci USA 96:6529–6534