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RESEARCH PAPER

Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato

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Abstract

Nitric oxide (NO) is a bioactive molecule involved in diverse physiological functions in plants. It has previously been reported that the NO donor sodium nitroprusside (SNP) applied to germinated tomato seeds was able to induce lateral root (LR) formation in the same way that auxin treatment does. In this paper, it is shown that NO modulates the expression of cell cycle regulatory genes in tomato pericycle cells and leads, in turn, to induced LR formation. The addition of the NO scavenger CPTIO at different time points during auxin-mediated LR development indicates that NO is required for LR primordia formation and not for LR emergence. The SNP-mediated LR promotion could be prevented by the cell cycle inhibitor olomoucine, suggesting that NO is involved in cell cycle regulation. A system was developed in which the formation of LRs was synchronized. It was based on the control of NO availability in roots by treatment with the NO scavenger CPTIO. The expression of the cell cycle regulatory genes encoding CYCA2;1, CYCA3;1, CYCD3;1, CDKA1, and the Kip-Related Protein KRP2 was studied using RT-PCR analysis in roots with synchronized and non-synchronized LR formation. NO mediates the induction of the *CYCD3;*1 gene and the repression of the CDK inhibitor *KRP2* gene at the beginning of LR primordia formation. In addition, auxin-dependent cell cycle gene regulation was dependent on NO.

Key words: Auxin, cell cycle, cyclin, Cyclin Dependent Kinase, lateral root, nitric oxide.

Introduction

Development throughout the life cycle of a plant relies on the activity of meristems laid down during embryogenesis and the continuous production of new meristems. This ongoing development is generally considered to be an adaptation to the sedentary lifestyle of plants since they cannot move to more suitable environments (Leyser and Fitter, 1998). The discovery of the signal molecules that initiate and direct organogenesis and developmental patterning in plants remains a major goal for plant biologists. Lateral root (LR) formation is a good model to study the molecular mechanisms involved in plant organogenesis.

LRs are mainly responsible for providing water and nutrients that the plant requires from the soil. LR placement is not determined but is strongly influenced by the physiological state of the plant and the prevailing environmental conditions. Thus, the initiation of LRs is a fascinating developmental process, since it involves the post-embryonic production of an entire organ from a small number of differentiated cells in response to intrinsic and environmental cues (Malamy, 2005). LR formation occurs in the pericycle cells, in which individual quiescent cells are stimulated to dedifferentiate and proliferate to form an LR primordium. Finally, cells in the LR primordium differentiate and elongate causing the LR to emerge through the epidermis. LR emergence appears to be due to expansion of existing cells rather than cell division (Malamy and Benfey, 1997).

Strong evidence supports a central role for the plant hormone auxin in LR initiation. Auxin transported from the

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Abbreviations: CDK, Cyclin Dependent Kinase; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide; LR, lateral root; NAA, 1-naphthylacetic acid; NO, nitric oxide; NPA, naphthylphthalamic acid; SNP, sodium nitroprusside; KRP, Kip-Related Protein.

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shoot is necessary for LR formation in seedlings (Reed *et al.*, 1998; Casimiro *et al.*, 2001; Bhalerao *et al.*, 2002). In addition, the number of LRs is impaired in mutants that have altered auxin metabolism (Boerjan *et al.*, 1995; Celenza *et al.*, 1995; King *et al.*, 1995), transport (Marchant *et al.*, 2002), or signalling (Hobbie and Estelle, 1995). It has been extensively reported that auxin promotes LR initiation through the expression of cell cycle regulatory genes like cyclins and Cyclin Dependent Kinases (CDK) in the pericycle cells (reviewed in Casimiro *et al.*, 2003; Malamy, 2005). In higher eukaryotes, the association of CYCD and CDKA produces an active protein kinase that phosphorylates the retinoblastoma (Rb) protein at the G_1-to-S phase transition. This phosphorylation results in an inactivation of Rb and the subsequent release of E2F transcription factor, which is responsible for the transcription of the S-phase genes (Nakagami *et al.*, 2002). Conversely, Kip-Related Proteins (KRPs), which are specific inhibitors of CDK activity (De Veylder *et al.*, 2001), prevent the G_1-to-S phase transition (Jasinski *et al.*, 2002; Schnittger *et al.*, 2003). In the next checkpoint, namely at the G_2-to-M transition, B-type CDKs and cyclins A and B are involved (Reichheld *et al.*, 1996; Segers *et al.*, 1996; Joubès *et al.*, 2000*a*; Weingartner *et al.*, 2003). Although many auxin targets during root growth and development were identified, like CDKA;1 and mitotic cyclins (Hemerly *et al.*, 1993; Ferreira *et al.*, 1994; Doerner *et al.*, 1996; Himanen *et al.*, 2002) the intermediate molecules involved in the auxin signal transduction pathway remains poorly understood.

Nitric oxide (NO) is a diffusible second messenger whose physiological functions were first described in mammals and then in the plant kingdom, even though its presence was first reported in plants (Klepper, 1979). Novel and diverse roles are being attributed to this molecule in plants ranging from pathogen defence and growth and developmental processes to stress tolerance (reviewed in Lamattina *et al.*, 2003; Neill *et al.*, 2003). Recently, it was demonstrated that NO is involved in the auxin signalling cascade during root growth and development including adventitious root development (Pagnussat *et al.*, 2002) and LR formation (Correa-Aragunde *et al.*, 2004). NO was also shown to participate in the induction of LRs mediated by the plant-growth-promoting rhizobacterium *Azospirillum* (Creus *et al.*, 2005). In this report, the analysis of NO-regulated mechanisms leading to LR promotion is expanded. Evidence is presented that supports the NO modulation of cell cycle regulatory genes involved in G_1-to-S phase transition in tomato roots during LR initiation.

Materials and methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* Mill. cv. Ace 55) seeds were surface-sterilized in 5% sodium hypochlorite for 10 min, rinsed extensively, and imbibed in water for 3 d. Seedlings with radicles 2–3 mm long were transferred to Petri dishes containing a filter paper soaked with different compounds and grown in a chamber at 25±1 °C and at 14/10 h (light/dark) photoperiod. LR primordia were observed after 3 d of treatment by root squash preparations and quantified by bright-field microscopy. LR number was quantified after 5 d of treatment.

Chemicals

Sodium nitroprusside (SNP), 1-naphthylacetic acid (NAA) and olomoucine were from Sigma (St Louis, MO, USA), *N*-1-naphthylphthalamic acid (NPA) from Chemical Services (West Chester, PA, USA) and 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (CPTIO) from Molecular Probes (Eugene, OR, USA). Concentrations used were 200 μM SNP, 0.1 and 10 μM NAA, 5 μM NPA, 50 μM olomoucine, and 1 mM CPTIO.

Semi-quantitative RT-PCR analysis

Endogenous transcript levels of a set of cell cycle regulatory genes were analysed by semi-quantitative RT-PCR. The root apical meristems of the seedlings were cut off and the shoots were removed by cutting below the root–shoot junction in order to obtain RNA samples of only lateral root-inducible segments. Total RNA was extracted with Trizol reagent (Invitrogen, Gaithersburg, MD) and treated with DNAse I (Promega, Madison, WI). One μg of total RNA was used for reverse transcription with an oligo dT primer and M-MLV reverse transcriptase (Promega) in a reaction volume of 20 μl. PCR reactions were performed using 2 μl of a 5-fold dilution of the cDNA, 10 pmol of each oligonucleotide primer and 1 U of *Taq* polymerase (Invitrogen) in a 20 μl reaction volume. To verify the exponential phase of PCR amplification, a different number of amplification cycles ranging from 20 to 35 was tested for each gene. The oligonucleotide primers of *CYCA2;1* (accession number AJ243452), *CYCA3;1* (accession number AJ243453), *CYCD3;1* (accession number AJ245415), and *CDKA1* (accession number Y17225) were as described in Joubès *et al.* (2000*b*). *KRP2* (accession number AJ441250) oligonucleotide primers used were: forward CCCTCACTGCCCTCTGCTTCTG and reverse CAATTTCAT-CAGCCCCACCAGC (amplifying a 450 bp fragment) and actin (accession number BT012695) forward AAGAGCTATGAGCTCC-CAGATGG and reverse TTAATCTTCATGCTGCTAGGAGC (amplifying a 272 bp fragment). After 30 PCR cycles for the cell cycle genes and 25 cycles for actin with a primer annealing temperature of 50 °C, 10 μl samples of the PCR reaction products were loaded on a 1% (w/v) agarose gel, transferred to Nylon membranes and hybridized with *CYCA2;1*, *CYCA3;1*, *CYCD3;1*, *CDKA1*, *KRP2*, and actin ^{32}P-radiolabelled cDNA probes. Several expositions of the autoradiographs were done to obtain correct signals for densitometric analysis when indicated. Photographs of the autogradiographs are representative of at least three independent experiments.

Results

Nitric oxide is involved in the formation of lateral root primordia in tomato

It has previously been reported that NO increases LR number in tomato and that there is a high NO concentration within the growing LR primordium (Correa-Aragunde *et al.*, 2004). Since growth is determined by both cell division and elongation, NO may be involved either in inducing dedifferentiation and division in pericycle cells or in increasing the elongation rate of cells in LR primordia. To clarify the mechanism through which NO induces

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LR number in tomato, the formation of LR primordia was first analysed in roots treated with the NO donor sodium nitroprusside (SNP) for 3 d. SNP applied to germinated tomato seeds was able to mimic the effect of the synthetic auxin 1-naphthylacetic acid (NAA) in inducing LR primordia formation (Fig. 1A). This result correlates with the number of LRs observed after 5 d of treatment (Correa-Aragunde *et al.*, 2004). Microscopical analysis showed that NO- and NAA-induced LR primordia presented a similar anatomic structure (Fig. 1B). The effect achieved by SNP was prevented by the specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) indicating that the induction of LR primordia is due to an NO release from SNP (Fig. 1A). CPTIO applied alone drastically reduced the number of LR primordia compared with the control treatment (Fig. 1A). Microscopical analysis shows that CPTIO might block primordia formation at a very early stage of development since arrested primordia could not be detected. In NAA-treated seedlings, primordia formation was also inhibited by CPTIO suggesting that endogenous NO is involved in the LR primordia formation triggered by NAA (Fig. 1A). Himanen *et al*. (2002) developed a synchronized LR-inducible system in *Arabidopsis*, which consists of a treatment with the auxin transport inhibitor naphthylphthalamic acid (NPA). The treatment with NPA prevents the endogenous auxin-mediated induction of LR formation. Then, the shift to a medium containing auxin triggers a synchronized activation of pericycle cells. The inhibitory effect of CPTIO on auxin-induced LR formation led to the hypothesis that the control of the endogenous NO availability may synchronize the initiation of LRs. Thus, tomato seeds were incubated in the presence of CPTIO for 2 d to prevent LR primordia formation and subsequently incubated in the presence of NAA or SNP to induce pericycle activation. LR primordia formation was triggered when the seedlings were shifted to SNP or NAA after 2 d of CPTIO treatment (Fig. 1A).

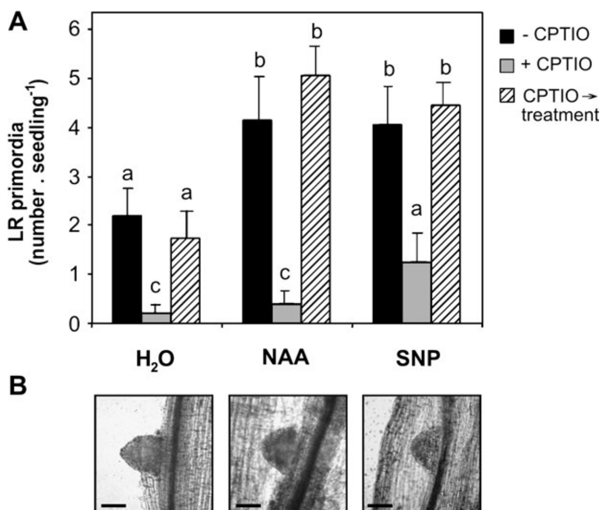

Fig. 1. Nitric oxide-mediated induction of lateral root (LR) primordia. (A) Germinated tomato seeds were incubated with water (control), 0.1 μM of the auxin 1-naphthylacetic acid (NAA) or 200 μM of the NO donor sodium nitroprusside (SNP) in the absence (black bars) or the presence (grey bars) of 1 mM of the NO scavenger CPTIO for 3 d. In an another set of experiments, seedlings incubated in 1 mM CPTIO for 2 d were shifted to water, 0.1 μM NAA or 200 μM SNP for another 3 d (shaded bars). LR primordia were observed by root squash preparations and quantified by bright-field microscopy. Means and SE were calculated from three independent experiments (n=5). Different letters indicate a significant difference at $P<0.05$ (t-test). (B) Photographs showing the LR primordia morphology in seedlings incubated in water, SNP or NAA. Bar: 200 μM.

Nitric oxide is required in the early stages of lateral root primordia formation

In order to assess the NO requirement during the formation and/or elongation of the auxin-mediated LR development further, tomato seedlings were incubated for 5 d in the presence of NAA, and CPTIO was added after 0, 1, 2, 3, or 4 d of the NAA treatment. Figure 2A shows that CPTIO when added at 0, 1 or 2 d of the NAA treatment produced a significant reduction (up to 5-fold) of LR formation. By contrast, only a slight effect could be observed in response to the addition of CPTIO after 3 or 4 d of the NAA treatment. Thus, the events in which NO might participate occur within the first 2 d of NAA treatment. These findings might suggest that NO is required for cell cycle progression and establishment of LR primordia in the pericycle and that NO would not be necessary for the elongation and emergence of LRs. According to this, the addition of the cell cycle inhibitor olomoucine to SNP-treated seedlings at different time points resulted in a similar response (Fig. 2B). However, a limited response was observed with olomoucine. Olomoucine has been reported to induce cell cycle arrest at G_1 phase by inhibiting CDKA activity in *Petunia* and *Arabidopsis* cell suspensions (Glab *et al.*, 1994). Similar results were also obtained with the cell cycle inhibitors apigenin and roscovitine (data not shown).

Nitric oxide modulates the expression of cell cycle regulatory genes

Based on the results described above, the influence of NO on the expression of cell cycle regulatory genes was analysed. Semi-quantitative RT-PCR analysis was carried out on RNA extracted from tomato roots treated with SNP, NAA, or NAA plus CPTIO for 1, 2, and 3 d. These time points were chosen because LR primordia were observed from the third day of the NAA or SNP treatments (Fig. 1A, B). Figure 3A shows that NAA induced the expression of the *CYCA2*;1, *CYCA3*;1, *CYCD3*;1, and *CDKA1* genes after 2 d of treatment. SNP induced the expression of *CYCD3*;1 and *CDKA1* and to a lesser extent *CYCA2*;1 after 2 d of treatment. The effect of SNP is sustained in time, inducing the expression of *CYCA2;*1, *CYCD3;*1 and *CDKA* up to 3 d. Interestingly, the NAA-induced expression of cell cycle regulatory genes was prevented or delayed when NO was

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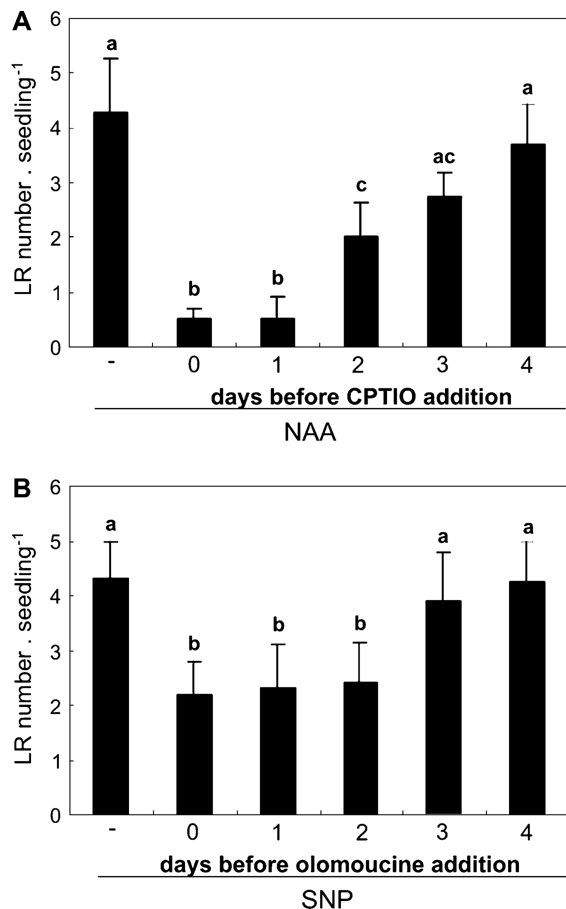

Fig. 2. Nitric oxide is required during the early events that trigger LR formation. (A) Germinated tomato seeds were incubated in 0.1 μM NAA alone or in the presence of 1 mM of the NO scavenger CPTIO after 0, 1, 2, 3, or 4 d of NAA treatment. LR number was quantified after 5 d of treatment. (B) Germinated tomato seeds were incubated in 200 μM SNP alone or in the presence of 50 μM of the cell cycle inhibitor olomoucine after 0, 1, 2, 3, or 4 d of SNP treatment. Lateral root (LR) number was quantified after 5 d of treatment. Means and SE were calculated from three independent experiments (n=5). Different letters indicate a significant difference at P <0.05 (t-test).

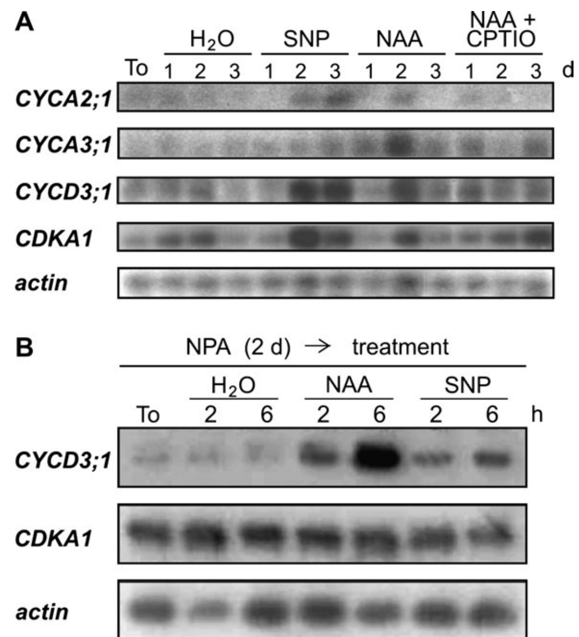

Fig. 3. Expression analysis of cell cycle regulatory genes by RT-PCR. Total RNA was extracted and used for reverse transcription with an oligo dT primer and M-MLV reverse transcriptase. The cDNA was used for semi-quantitative PCR. The PCR reaction products were loaded on an agarose gel, transferred to Nylon membranes and hybridized with specific ^{32}P-radiolabelled cDNA probes. (A) Germinated tomato seeds were incubated in water for 12 h (To) and then shifted to water or 200 μM SNP or 0.1 μM NAA or 0.1 μM NAA plus 1 mM CPTIO for 1, 2, and 3 d. (B) Germinated tomato seeds were incubated for 2 d in 5 μM NPA (To) and then shifted to water or 10 μM NAA or 200 μM SNP for 2 h and 6 h.

scavenged with CPTIO. In addition, the SNP plus CPTIO treatment prevented the induction of *CYCD3;*1 (data not shown), indicating that the effect is due to the NO molecule released by the donor. These findings gave preliminary evidence and suggested that endogenous NO could modulate the expression of cell cycle regulatory genes and that it is also required for the NAA-induced effect on these genes. In addition, the effect produced by SNP was more evident for the genes that encode proteins involved in the regulation of the G_1-to-S transition phase.

The lack of synchrony in the initial events leading to LR formation makes it difficult to attribute a more precise role to NO in cell cycle regulation. Therefore, the NPA-dependent synchronization of the pericycle cells developed by Himanen *et al*. (2002) in *Arabidopsis* was applied to tomato seedlings. The treatment with NPA induces cell cycle arrest in the G_1 phase (Himanen *et al*., 2002). It has been proposed that D-type cyclins play a prominent role in the G_1-to-S transition and hence have a major role in the commitment to the mitotic cell cycle. In the present study, the expression of *CYCD3*;1 was chosen for analysis since it was previously reported that the cyclin D3 overexpression in plants resulted in Rb inactivation and a strong increase of E2F levels (Nakagami *et al*., 2002) and because SNP treatment strongly induces *CYCD3;*1 (Fig. 3A). Semi-quantitative RT-PCR analysis was used in this system to study the effect of NO on the expression of cell cycle regulatory genes during the activation of the synchronized pericycle. The analysis was performed at short periods of activation time (2 h and 6 h) with NAA and SNP because the cell cycle progression was arrested with NPA for the first 2 d of treatment. Figure 3B shows that *CYCD3*;1 was induced by NAA and SNP after 2 h of treatment. The induction by SNP was lower than that obtained with NAA. Nevertheless, this result indicates that NO is able to induce *CYCD3*;1 independently of endogenous auxin. The induction of *CYCD3*;1 by NAA was higher than that observed in Fig. 3A as a result of the use of a higher NAA concentration (10 μM) as reported by Himanen *et al*. (2002) in this system. In contrast with the non-synchronized system, Fig. 3B shows that the level of the *CDKA1* transcript was constitutive, indicating that the

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synchronization of pericycle cells by NPA in tomato behaved similarly to that reported in *Arabidopsis* (Himanen *et al*., 2002).

According to the results presented in Fig. 1A showing that the effect of the NO scavenger CPTIO is reversible, the expression of cell cycle regulatory genes was analysed in the CPTIO-inducible system. Germinated seeds were treated with CPTIO for 2 d and then shifted to H_2O, NAA, or SNP. Figure 4A shows that at To (2 d of CPTIO treatment), there was a low expression of *CYCD3*;1. Conversely, *CYCD3*;1 was induced by either NAA or SNP after 4 h of treatment. The transcript level of *CDKA1* was constitutively expressed up to 6 h of treatment (Fig. 4A, B). The expression of the tomato CDK inhibitor *KRP2* gene was also included in this study. *KRP2* expression was high in the inactive pericycle of CPTIO-treated roots (To) and increased when the seedlings were shifted to water. This increase may be due to stress conditions for the roots during the transfer from CPTIO treatment to H_2O. Nevertheless, the NAA and SNP treatments inhibited *KRP2* expression under the same conditions. *KRP2* was strongly down-regulated by SNP and NAA after 2 h and 6 h of treatment, respectively (Fig. 4A, B).

Discussion

Auxin has been known for a long time to be the main plant hormone involved in LR development. It was recently shown that NO is required for the auxin-mediated LR formation (Correa-Aragunde *et al*., 2004). NO is produced in the pericycle cells that will give place to an LR, indicating that NO is required during the early stages of LR development. The data support a lineal signal transduction cascade involving NO downstream of auxins (Correa-Aragunde *et al*., 2004). This communication goes further in the analysis of the NO-regulated mechanisms leading to LR promotion. Results indicate that NO promotes LR initiation in tomato by the modulation of cell cycle regulatory genes during LR primordia formation, but has no effect during LR emergence.

LR formation, as well as several processes associated with developmental patterning and organogenesis, deals with the fate of the spatial and temporal asynchrony of the initiation events. Thus, strategies for the development of synchronized biological systems would help for studying the signals and regulatory pathways governing these processes. Dealing with LR formation, Himanen *et al*. (2002) developed a synchronized system based on the depletion of endogenous auxin in *Arabidopsis*. This system allowed a detailed histological and molecular analysis of early LR initiation events. Here, an LR-inducible system is reported, based on the control of NO availability in roots by treatment with the NO scavenger CPTIO. It has been seen that the early stages of LR development in tomato are similar to those of *Arabidopsis*, described by Malamy and Benfey (1997). Based on this analogy, microscopical analysis indicates that the treatment with 1 mM CPTIO blocked primordia formation in a stage below stage III (data not shown). Therefore, a CPTIO-dependent inducible system is proposed as a complementary tool for the study of early events leading to LR development.

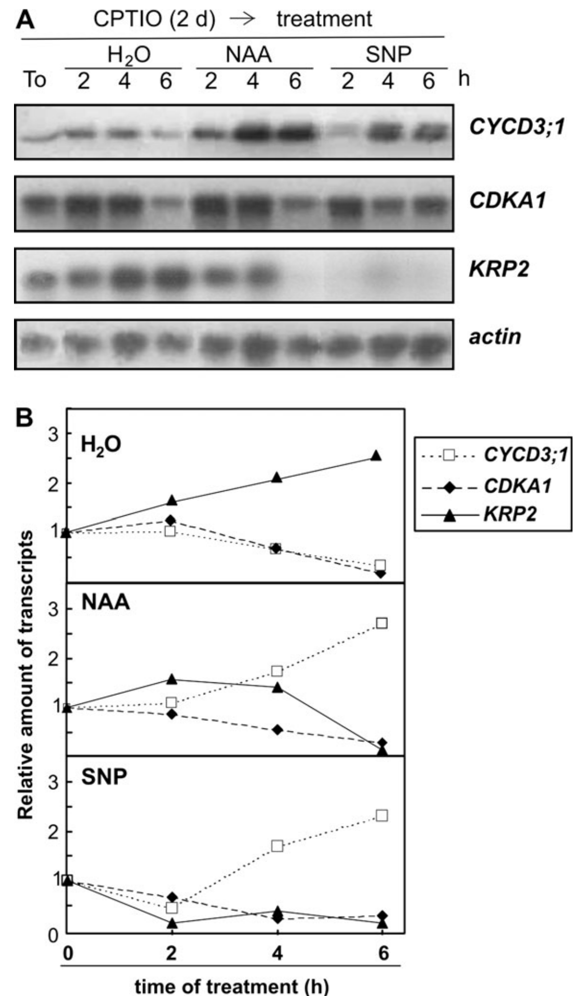

Fig. 4. Expression analysis of cell cycle regulatory genes in CPTIO-synchronized tomato seedlings. (A) Germinated tomato seeds were incubated in 1 mM CPTIO for 2 d (To) and then shifted to water or 0.1 μM NAA or 200 μM SNP for 2, 4, and 6 h. Total RNA was extracted and was used for reverse transcription with an oligo dT primer and M-MLV reverse transcriptase. The cDNA was used for semi-quantitative PCR. The PCR reaction products were loaded on an agarose gel, transferred to Nylon membranes and hybridized with specific ^{32}P-radiolabelled cDNA probes. (B) Quantitative time-course analysis of *CYCD3*;1, *CDKA1*, and *KRP2* transcript levels. The data were obtained by densitometric analysis of the gel blots corrected for loading differences by using actin transcript levels. The values represent relative transcript levels with respect to To.

In this report, it is shown that NO, as well as auxin, induces the transcription of *CYCD3*;1 while it keeps *CDKA1* transcript levels high, in both NPA- and CPTIO-dependent inducible systems (Figs 3B, 4). NO also induced *CYCA2*;1 in the non-inducible system (Fig. 3A). CYCD3 is a rate-limiting factor during the G_1-to-S phase transition

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since overexpression of CYCD3 in *Arabidopsis* or tobacco stimulates cells to exit the G_1 phase (Nakagami *et al*., 2002; Dewitte *et al*., 2003). Transcription of D-type cyclins is activated by many extracellular signals and growth regulators such as sugar availability and hormones (auxin, cytokinin, gibberellins, brassinosteroids) (Dewitte and Murray, 2003). *CYCD3*;1 was found to be elevated in *Arabidopsis* mutants with a high level of cytokinin and to be induced by cytokinin application in both cell cultures and whole plant (Riou-Khamlichi *et al*., 1999). On the other side, the treatment of tobacco BY2 cells with cytokinin induces NO release (Tun *et al*., 2001). Probably, NO is acting in a common signalling cascade downstream of auxin and cytokinin to induce *CYCD3;*1 gene expression and, consequently, cell cycle activation.

KRPs were reported to be involved in preventing the formation of active CDK/cyclin complexes that regulate the G_1-to-S phase transition (Wang *et al*., 1998; Verkest *et al*., 2005). The binding of KRPs to CDK/CYCD complexes might prevent the phosphorylation of Rb, since overproduction of a tobacco KRP protein in *Arabidopsis* completely complements the phenotype that is obtained by overproduction of *CYCD3*;1 (Jasinski *et al*., 2002). Moreover, overexpression of a *KRP* gene in trichomes is able to restore the phenotype induced by CYCD overexpression (Schnittger *et al*., 2003). During LR formation in *Arabidopsis*, *KRP1* and *KRP2* genes are down-regulated by NAA. Thus, it was suggested that *KRP1* and *KRP2* prevent the activity of CDK/cyclin complexes, which regulate the G_1-to-S phase transition in the pericycle of *Arabidopsis* (Himanen *et al*., 2002). The results presented here show that tomato *KRP2* behaves similarly to the *Arabidopsis KRP* genes during LR formation and that it is strongly down-regulated by the presence of not only auxin but also NO in roots.

The results presented in this work suggest that the G_1-to-S transition phase could be a target point for the NO-regulated LR initiation. Nevertheless, positive modulation of regulators affecting one phase of the cell cycle may be masked by compensatory changes in another. It was previously argued that it is difficult to distinguish the phase-specific effects of G_1 cyclin expression without considering the effects of cyclins in the overall cellular growth (Cooper, 1998). In a recent report, Koroleva *et al*. (2004) showed that, in contrast with animal D cyclin, cyclin D1 could promote both G_1-to-S phase and G_2-to-M phase progression in plants. Schnittger *et al*. (2002) also showed that D-type cyclins could have an additional function at the G_2-to-M transition. Therefore, further studies involving specific expression of cyclin and *CDK* genes at the different cell cycle phases and how they are affected by NO would be necessary to confirm the transition phase(s) that is (are) regulated. In addition, more evidence is needed to confirm that the treatment with CPTIO synchronize pericycle cells in the G_0 phase. Flow cytometry analysis is a useful strategy that provides information about the cell cycle, and consequently to study how different stimuli (i.e. drug treatment or transfected genes) affect cell cycle progression. However, flow cytometry measurements cannot be applied in intact roots. The aim was to study the action on cell cycle regulatory genes at the organ level. However, in a very recent report, Otvos *et al*. (2005) showed that NO promotes cell division and embryogenic cell formation in leaf protoplast-derived cells of alfalfa. NO donors were able to stimulate BrdU incorporation in DNA while NO synthase inhibitors diminished it. Additionally, NO was able to activate the somatic embryogenesis marker receptor kinase (SERK) protein, a putative marker of the embryonic capability of alfalfa cells. In cell suspensions, NO transiently induces *CYCA2*;1 and *CYCD3*;1 mRNA expression (Otvos *et al*., 2005). This study strongly supports the data presented here. An intriguing point is that while authors found that the effect of NO in protoplasts only occurs in the presence of auxin, the induction of *CYCD3*;1 by NO in tomato roots (Fig. 3B) as well as LR formation (Correa-Aragunde *et al*., 2004) occur even in the presence of the auxin transport inhibitor NPA. This apparent discrepancy points out that whole plant responses to hormones are probably more complex and do not necessarily reflect those produced in cell culture.

The transcript profile presented in this work allows a simple model to be proposed that involves NO in cell cycle activation during LR initiation. In CPTIO or NPA treatment, pericycle cells display low levels of *CYCD3*;1 and high levels of the CDK inhibitor *KRP2*. In these treatments, *CDKA1* transcripts remain at high levels indicating that pericycle cells are competent for cell division. When auxin or NO concentrations in roots increase, *KRP2* transcript levels drastically decrease accompanied by the accumulation of *CYCD3*;1, favouring the association with CDKA1 and the progression from G_1-to-S phase. This study constitutes the first report that involves the NO-induced activation of the cell cycle in the process leading to LR formation through the modulation of *CYCD3*;1 and an acute decline of *KRP2*. However, it should be noteworthy that non-localized or non-specific activation of the cell cycle is not sufficient to induce the formation of LRs. It was reported that overexpression of cyclins leads to an increased growth rate without a differential increase in the initiation of LRs (Doerner *et al*., 1996; Cockcroft *et al*., 2000), probably because its expression is induced in the whole plant. Moreover, LR formation requires the induction of genes involved in root meristem identity. To date, several regulatory genes are known to be required for the development of root meristems (Celenza *et al*., 1995; Xie *et al*., 2000; Aida *et al*., 2004; Hawker and Bowman, 2004). Since NO can increase LR number, it suggests that NO is specifically stimulating some pericycle cells triggering cell cycle activation and LR formation. Probably, NO takes part in a signalling cascade downstream of auxin,

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present in competent pericycle cells, that finally activates cell cycle regulatory genes. In addition, NO should be able to modulate the expression of genes associated with root meristem identity. It will be interesting to explore whether NO is involved in the regulation of those genes and how the target pericycle cells are specified.

Studies on cell cycle transition have been focused in learning about the mechanisms of action of cell cycle regulatory components and understanding how cell proliferation, growth and development are integrated (Gutierrez *et al*., 2002). The results presented in this study open a wide field of research for the role of NO on cell cycle regulation during organogenesis. It will allow going further into a more complete understanding of the components that control plant cell differentiation and proliferation.

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