Abscisic acid-induced nitric oxide and proline accumulation in independent pathways under water-deficit stress during seedling establishment in *Medicago truncatula*

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Abstract

Nitric oxide (NO) production and amino acid metabolism modulation, in particular abscisic acid (ABA)-dependent proline accumulation, are stimulated *in planta* by most abiotic stresses. However, the relationship between NO production and proline accumulation under abiotic stress is still poorly understood, especially in the early phases of plant development. To unravel this question, this work investigated the tight relationship between NO production and proline metabolism under water-deficit stress during seedling establishment. Endogenous nitrate reductase-dependent NO production in *Medicago truncatula* seedlings increased in a time-dependent manner after short-term water-deficit stress. This water-deficit-induced endogenous NO accumulation was mediated through a ABA-dependent pathway and accompanied by an inhibition of seed germination, a loss of water content, and a decrease in elongation of embryo axes. Interestingly, a treatment with a specific NO scavenger (cPTIO) alleviated these water-deficit detrimental effects. However, the content of total amino acids, in particular glutamate and proline, as well as the expression of genes encoding enzymes of synthesis and degradation of proline were not affected by cPTIO treatment under water-deficit stress. Under normal conditions, exogenous NO donor stimulated neither the expression of P5CS2 nor the proline content, as observed after PEG treatment. These results strongly suggest that the modulation of proline metabolism is independent of NO production under short-term water-deficit stress during seedling establishment.

Key words: ABA, abiotic stress, amino acid, germination, NO, PEG.

Introduction

Water-deficit stress is the major factor limiting crop production, notably by causing deleterious effects on plant metabolic processes including nutrient uptake and photosynthesis (Pinheiro and Chaves, 2011). To cope with stress, young seedlings and plants have developed complex cellular signalling mechanisms to sense and to respond to unfavourable environmental situations (Nakashima *et al.*, 2009). The phytohormone abscisic acid (ABA) has been shown to play an important role in the perception and transduction of drought-induced signals, by inducing drought-stress-regulated genes in order to confer plant tolerance to water-deficit stress (Shinozaki and Yamaguchi-Shinozaki, 2007). These adaptive...
responses to drought stress induce distinct physiological and biochemical changes such activation of antioxidative enzymes and metabolite accumulation.

It is well known that proline largely accumulates in higher plants and algae subjected to abiotic stresses such as osmotic stress (Verdooy et al., 2006), salinity (Kheder et al., 2003), heavy metals (Tripathi and Gaur, 2004; Zhang et al., 2008), freezing (Zhao et al., 2009), and water-deficit stress (Vendruscolo et al., 2007). Under stress conditions, proline accumulation can result from enhanced biosynthesis and/or reduced degradation of proline. In plants, proline synthesis is mainly catalysed by Δ-1-pyrroline-5-carboxylate synthetase (P5CS; EC 2.7.2.11/1.2.1.41), which converts glutamate into Δ-1-pyrroline-5-carboxylate (P5C) in the cytoplasm or chloroplast. P5C reductase (P5CR) further reduces P5C to proline. In Medicago truncatula, two P5CS genes, P5CS1 and P5CS2, have been reported to be differentially regulated according to organs and osmotic stress responses (Armengaud et al., 2004). Very recently, a novel gene of M. truncatula, P5CS3, has been isolated and proved to play a predominant role in stress-induced proline accumulation during symbiotic nitrogen fixation (Kim and Nam, 2013). In addition, proline may be also synthesized in mitochondria from ornithine by ornithine-δ-aminotransferase (OAT; EC 2.6.1.13), catalysing the formation of P5C, which is further reduced to proline by P5CR. Proline degradation, occurring in the mitochondria, is mediated by the action of proline dehydrogenase (ProDH; EC 1.5.99.8), which produces P5C from proline, and of P5C dehydrogenase which converts P5C to glutamate (Szabados and Savouré, 2010). Proline has been reported to act as an osmoprotectant and antioxidant by scavenging free radicals, to protect the structure of biological macromolecules during dehydration, and to store and transfer reductants, thus conferring plant tolerance to environmental stresses (Ashraf and Foolad, 2007). However, the precise role of proline accumulation under abiotic stress remains still controversial.

Nitric oxide (NO), a product of nitrogen metabolism, is known to be a stress regulator involved in signal transduction pathways (Besson-Bard et al., 2008). In plants, NO synthesis has been reported to be mainly mediated by (i) the reduction of nitrite by nitrate reductase (NR; Kaiser et al., 2002; Rockel et al., 2002), mitochondrial electron transfer (Planchet et al., 2005), or a non-enzymatic way (Bethke et al., 2004) and (ii) the oxidation of arginine by a putative nitric oxide synthase (NOS)-like enzyme (Guo et al., 2003). However, some uncertainties remain about the nature and the timing of NO synthesis by these various mechanisms. NO has been shown to play multiple biological roles in plants, in particular in developmental processes such as germination, root organogenesis, stomatal closure, flowering, and senescence (Lamattina et al., 2003). NO has been also demonstrated to be involved in plant adaptive responses to environmental constraints such as drought (García-Mata and Lamattina, 2001; Arasimowicz-Jelonek et al., 2009), heat (Song et al., 2006), salt (Zhao et al., 2007), metal toxicity (Rodriguez-Serrano et al., 2006; Ma et al., 2010), and UV-B radiation (Shi et al., 2005). More precisely, NO acts downstream of ABA in adaptive responses to water-deficit stress by inducing stomatal closure (Desikan et al., 2002). Several reports have demonstrated that exogenous application of NO as a pretreatment alleviated water-deficit-induced membrane permeability and lipid peroxidation in cucumber roots (Arasimowicz-Jelonek et al., 2009) and enhanced plant growth and maintenance of a higher relative water content in plants under drought stress (García-Mata and Lamattina, 2001; Zhao et al., 2001; Tian and Lei, 2006; Hao et al., 2008). Exogenous application of NO has been also reported to induce proline accumulation in plants under drought stress (Farooq et al., 2009), osmotic stress (Lei et al., 2007) and copper stress (Zhang et al., 2008), but the relationship between endogenous NO production and proline accumulation remains still unknown during early phases of plant development under stress.

The current study group previously reported that water-deficit stress induced a ABA-dependent proline accumulation in the model legume M. truncatula during post-germinative growth (Planchet et al., 2011a, b). The present study investigated whether and how intracellular and extracellular NO participates in seedling adaptive responses after short-term water-deficit stress and regulates the proline metabolism in M. truncatula seedlings, in order to improve knowledge on the water-deficit signalling network through NO production and proline accumulation.

### Materials and methods

#### Germination and seedling growth conditions

Seeds of M. truncatula cv. Jemalong were germinated in darkness at 21 °C in Petri dishes (diameter 9 cm) on Whatman paper soaked with 4 ml modified Murashige and Skoog medium (MS medium containing macro- and micronutrients, buffered with MES) for 40 h. Afterwards (t=0), seedlings were transferred in Petri dishes onto MS, with or without NO-donor diethylamine NONOate sodium (DEA, 500 μM, Sigma-Aldrich, St Quentin Fallavier, France), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 250 μM, Sigma-Aldrich) or with polyethylene glycol 8000 (PEG, Sigma-Aldrich) at –0.75 MPa (prepared in MS medium) ± cPTIO. The DEA concentration of 500 μM was selected following different DEA dose-dependent experiments (Supplementary Table S1, available at JXB online). Into each Petri dish, 30 seedlings were grown in darkness (21 °C) and were thereafter harvested at different times following the onset of treatment (2, 6, and 24 h). Cotyledons were discarded and embryo axes (hypocotyl plus radicle) were immediately frozen in liquid nitrogen before being stored at −80 °C. Moreover, the fresh weight (FW) of axes was also determined after 24 h of treatment and the dry weight (DW) was obtained after 48 h of desiccation in an oven at 80 °C. The water content (FW–DW) per axis was calculated based on dry weight. The elongation of embryo axis was measured using ImageJ software after 24 h of growth. The percentage of germinated seeds (designated by the seed-coat rupture) was observed during 10 d (nutrient solution changed every 3 d) in normal growth conditions (MS) with DEA (500 μM) or cPTIO (250 μM), and with PEG (−0.50 MPa) ± cPTIO (250 μM).

#### Endogenous NO detection by fluorescence

For fluorometric NO determination, the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) was used. Roots of intact seedlings were preincubated with 10 μM DAF-2DA in MES buffer (5 mM, pH 6) for 30 min at 21 °C in darkness and then rinsed twice with MES buffer during 10 min to remove excess fluorophore. DAF-2DA fluorescence (495 nm excitation and 515 nm emission wavelength) was observed by microscopy (DM4500B, Leica) connected to a L5 fluorescence detection system. Fluorescence quantification was done using ImageJ software. Before DAF-2DA treatment, seedlings were incubated for 2, 6, or
24 h with DEA (500 μM) or ABA (100 μM, Sigma-Aldrich) in MS medium or with PEG (−0.75 MPa) ± cPTIO (250 μM) for NO scavenging. For NR inactivation, seedlings were grown after germination on nitrate-free MS medium (MS medium in which nitrate was replaced by 5 mM NH₄Cl). Inhibition of NOS activity and ABA biosynthesis in seedlings were performed by addition of N(G)-nitro-l-arginine methyl ester (L-NAME, 500 μM, Sigma-Aldrich) and norflurazon (Norf, 100 μM, Sigma-Aldrich), respectively, for 24 h.

RNA extraction and reverse transcription

For total RNA extraction, frozen embryo axes were crushed in liquid nitrogen with a mortar and pestle. The homogenous powder was treated with TRI Reagent (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. cDNA was obtained by reverse transcription of 2 μg total RNA using 200 U of RT-MMLV (Promega, Madison, WI, USA) and 2 μg random primer (Invitrogen, Breda, The Netherlands) with 40 μl of a recombinate ribonuclease inhibitor (RNasin, Promega). The reaction mixture was incubated for 1 h at 37 °C in a total volume of 50 μl.

Real-time quantitative PCR

Amplification was performed using a Light Cycler ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA, USA). Each reaction was performed with 4 μl of a 1:1 (v/v) dilution of first cDNA strands using SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer’s instructions, with 200 nM each primer in a total volume of 20 μl. The primers used for quantitative RT-PCR were MtOAT₁ (5′-GGCAAGTCAACACATGACACAA-3′), MtOAT₂ (5′-TGTGGATCTCTATCCGCACCTTA-3′), MtP5CS₁ (5′-AAAGGGTGATATTACGCCGAGTG-3′), MtP5CS₂ (5′-CCGAA GACACGGCTTGAA-3′), MtP5CS₂ (5′-GAGAGGAAGCAAGCGGA CAATG-3′), MtP5CS₂ (5′-CAGATCCTTGTTGTTGATA-3′), MtPr₁ (5′-CAAGCTCACCAGTGTAAGA-3′), and MtPr₁ (5′-ACAGTGTCCTATAGCCGATGA-3′). The cycling program consisted of incubation at 50 °C for 2 min and at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the PCR amplification was checked with a heat-dissociation protocol (from 65 to 95 °C) after the final cycle of PCR. Each measurement was carried out with at least two biological repeats, using triplicate PCR reactions to determine C_t values. The ratio was calculated using an equation with normalization by a reference gene (18S), using primers Mt18S (5′-GAAACGGCTACCACATCCAAG-3′) and Mt18S (5′-GAAACGGCTACCACATCCAAG-3′) (Livak and Schmittgen, 2001).

Amino acid extraction and analysis by HPLC

Total amino acids were extracted from embryo axes in 96% (v/v) ethanol for 1 h at 4 °C. After centrifugation (9800 g, 4 °C, 15 min), the ethanol fraction was removed and the same process was then repeated with deionized water. The ethanol and water fractions were combined. After evaporation of the extract under vacuum, organic residues were dissolved in deionized water and extracted with the same volume of chloroform. After centrifugation (12 000 g, 4 °C, 15 min), the aqueous phase, containing amino acids, was vacuum dried. Amino acids were then redissolved in deionized water. Samples were passed through a nylon syringe filter (0.45 μm) and then analysed by HPLC (Waters Corporation, MA, USA). The determination of amino acids was using the Waters AccQTag method, which uses Waters AccQFluor Reagent (patent pending) to derivatize the amino acids. The reagent is a highly reactive compound, 6-aminoquinolinyl-N-hydroxy-succinimidyl carbamate (AQC), which forms stable derivatives with primary and secondary amino acids including proline. Derivatives were separated by reversed-phase HPLC (C₁₈ column) and quantified by fluorescence detection. Each sample was analysed for 1 h. The amounts of all amino acids determined by HPLC were summed to determine total amino acid content. Measurements were repeated four times with independent biological samples.

Statistical analysis

All data are presented as mean±SE of values from at least three independent experiments. A one-way ANOVA (analysis of variance) test was also performed. P-values <0.05 were considered statistically significant.

Results

Accumulation of NO in M. truncatula seedlings under water-deficit stress

To investigate whether M. truncatula seedlings produced NO in response to water-deficit stress, detection of endogenous NO from seedling axes was achieved using DAF-2DA fluorescence microscopy. NO fluorescence intensity increased gradually and significantly with the duration of water-deficit stress (Fig. 1A). After 24 h of stress, NO accumulation was ~2.2-fold more than under normal conditions. As negative control, addition of the NO scavenger cPTIO to the PEG treatment strongly reduced NO fluorescence, showing the specificity of NO detection. Moreover, NO fluorescence from axes incubated with a NO donor (DEA) increased gradually with time. The amount of NO emission was substantially similar between the PEG and DEA treatments.

To identify whether NR could be involved as a potential source of NO, seedlings were grown specifically on ammonium medium (nitrate free) in order to inhibit NR activity. Under water-deficit stress, NO content in ammonium-fed axes was strongly reduced, with almost 80% inhibition in NO production (Fig. 1B). To check whether NOS could participate to this PEG-induced NO production, a NOS inhibitor (L-NAME) was added to the medium. In this condition, NO production was reduced by ~39% compared with the PEG-only condition after 24 h (Fig. 1B).

ABA-induced NO production under water-deficit stress

This study group previously reported that water-deficit stress induced ABA accumulation in embryo axes of M. truncatula seedlings (Planchet et al., 2011a). Thereby, in order to investigate whether PEG-induced NO production was mediated by ABA, NO fluorescence was quantified in embryo axes after 24 h of treatment with ABA (Fig. 2). Endogenous NO accumulation increased after exogenous ABA application but to a lesser extent than with PEG treatment. The presence of a NO-scavenger (cPTIO) strongly inhibited the ABA-induced NO production.

In order to validate that PEG induced NO production through ABA, an inhibitor of ABA biosynthesis (Norf) was added to the PEG solution. Interestingly, NO production with PEG + Norf was strongly reduced, by ~70%, compared to PEG after 24 h. Application of Norf alone under normal conditions did not modify the basal NO fluorescence. ABA with Norf induced NO accumulation in a similar way to exogenous ABA. These data indicate that PEG induced NO accumulation mainly through a ABA-dependent pathway.
Influence of NO on germination and physiological adaptations in response to water deficit

The aforementioned results indicate that endogenous NO could take part in the water-deficit adaptive responses in *Medicago* seedlings. However, it is not known whether water-deficit-induced endogenous NO would play similar roles to exogenous NO application under normal conditions. Thus, the involvement of intracellular and extracellular NO accumulation on seed germination and physiological adaptations of seedlings was tested. The germination rate of *Medicago* seeds largely decreased after PEG treatment: only 12% of seeds showed seed coat rupture, against 68% under normal conditions after 10 d of germination (Fig. 3A). The presence of a NO scavenger (cPTIO) increased the germination rate to 40% under water-deficit stress. It should be noted that, under normal conditions, cPTIO did not modify the germination rate compared to normal conditions. The application of a NO donor (DEA) in the growth medium induced a nonsignificant slight decrease in germination rate (63%) compared to normal conditions, and the germination rate did not appear to be dependent on the DEA concentration (Supplementary Table S1 available at JXB online). While PEG inhibited both the fresh weight and the elongation of the embryo axis after 24 h (Fig. 3B and C), the scavenging of endogenous NO by cPTIO had no significant effect on these parameters. Also, cPTIO addition to normal conditions did not modify these parameters. Application of exogenous NO (DEA) induced a significant dose-dependent reduction in axis fresh weight and also elongation compared to normal conditions (Fig. 3B and C; Supplementary Table S1 available at JXB online). Water content was largely reduced under water-deficit stress (loss of 48%) in comparison to normal conditions, but was slightly and significantly improved with a NO scavenger (gain of 6% compared to PEG treatment; Fig. 3D). Exogenous NO application alone did not alter water content.

Effect of NO on amino acid accumulation under water-deficit stress

In order to check whether exogenous NO influenced glutamate metabolism differently to intracellular NO produced under water-deficit stress, the content of total amino acids, glutamate, and proline was quantified. As shown in Fig. 4A, PEG induced a slight increase in the total amino acid content of embryo axes with stress (2.1-fold more after 24 h compared to normal conditions). cPTIO seemed to have no effect on PEG-induced amino acid accumulation, and exogenous NO (DEA) did not modify amino acid accumulation in comparison to control conditions. Analysis of individual amino acids
showed that the content of glutamate, the main precursor of proline, was higher after 24 h of PEG treatment (3.1-fold more compared to control conditions) and remained constant, which indicated glutamate homeostasis under stress (Fig. 4B). PEG-induced glutamate accumulation appeared to be independent of the NO scavenger. Once again, DEA induced a steadily decrease of glutamate content in time and in a similar way to normal conditions. While proline content decreased with time under normal conditions, PEG induced a significant accumulation of proline (3.4-fold more after 24 h compared to normal conditions: Fig. 4C). cPTIO with PEG did not interfere in PEG-induced proline accumulation after 24 h, and the presence of DEA contributed to a slight decrease of proline accumulation with time, similarly to normal conditions.

**Influence of exogenous and endogenous NO on genes of proline metabolism during short-term water-deficit stress**

Proline is synthesized from glutamate via P5CS and also from arginine/ornithine via OAT. In response to water-deficit stress, the expression of P5CS2 was strongly upregulated after 6 and 24 h (~5.3- and 6.5-fold more than under normal conditions, respectively) whereas P5CSI expression was inhibited (Fig. 5A and B). The combination of cPTIO and PEG did not modify the PEG-induced down- and upregulation of P5CSI and P5CS2 expression, respectively. Interestingly, with the exception of P5CS2 after 2 h of treatment, NO released from DEA was unable to stimulate the expression of either of the P5CS genes in comparison to water-deficit treatment. OAT expression was not stimulated in presence or absence of NO with PEG treatment (Fig. 5C) or after exogenous NO application, suggesting that the synthesis of proline from glutamate, via P5CS2, appears to be the predominant pathway, especially under water-deficit stress in *M. truncatula* seedlings.

Proline catabolism was also tested through expression of ProDH. Proline accumulation was correlated to a strong downregulation of ProDH expression during short-term water-deficit stress (Fig. 5D). It should be noted that the pattern of ProDH expression induced by PEG was similar to that in absence of intracellular NO (with cPTIO). Exogenous NO also resulted in the inhibition of ProDH expression, but to a lesser extent than with PEG.

**Discussion**

**Roles of NO in seed germination and physiological responses of Medicago seedlings during water-deficit stress**

The present work established that endogenous NO accumulation was induced in a time-dependent manner in embryo axes
Exogenous application of NO via sodium nitroprusside, the most commonly used NO donor, has been reported to stimulate germination in Arabidopsis, lettuce, lupin, grass, and apple (Beligni and Lamattina, 2000; Kopyra and Gwozdz, 2003; Libourel et al., 2006; Sarath et al., 2006; Gniazdowska et al., 2010). Otherwise, cPTIO has been shown to reduce the stimulatory effects of the NO-donor compound on germination under normal conditions (Beligni and Lamattina, 2000; Sarath et al., 2006). Contrary to these reports, the present results have revealed that endogenously produced NO exerts an inhibitory effect on M. truncatula seed germination, because removal of intracellular NO with cPTIO improved seed germination with PEG treatment. However, application of a NO donor (DEA) did not modify the germination rate, indicating that the endogenous or exogenous origin of NO seems to influence its action. Interestingly, oxygen uptake has been reported to increase in seeds during imbibition (Logan et al., 2001). Since NO competes with oxygen for binding to cytochrome c oxidase (complex IV), thus affecting the respiratory chain and the oxidative phosphorylation (Millar and Day, 1996; Yamasaki et al., 2001), the present work hypothesizes that, under water-deficit stress, the removal of NO by cPTIO would allow better respiratory activity and, consequently, a fully active metabolism, which could be responsible for the higher germination rate with PEG plus cPTIO compared to normal conditions. The contradictory findings by different laboratories about the effects of NO on germination could be due to the plant species, growth medium, experimental systems, and/or the exogenous NO donor used in each experiment. Indeed, some non-NO-releasing compounds from sodium nitroprusside solution (e.g. cyanide) could be responsible for stimulatory effects on seed germination by acting on biological tissues (Bethke et al., 2006). Furthermore, it is well known that ABA inhibits germination and is essential for the control of seed dormancy (Finkelstein et al., 2002). Interestingly, in the present system, exogenous ABA induced NO production, and this study group has previously reported that water-deficit stress induced ABA-dependent inhibition of germination (Planchet et al., 2011a). Therefore, it is suggested that cross-talk between ABA- and NO-signalling pathways is involved in the water-stress-induced inhibition of germination of M. truncatula seeds (Fig. 6).

This study found that NO is involved in abiotic stress signalling pathways and alleviates detrimental stress effects. This protective effect of NO in response to water-deficit stress has been recently described by Shi et al. (2012), who overexpressed a rat neuronal NOS gene (nNOS) in Arabidopsis. An increase in endogenous NO in the transgenic plants overexpressing rat nNOS was accompanied by reduction of stomatal aperture and maintenance of higher relative water content under cucumber roots (Arasimowicz-Jelonek et al., 2009) and rice leaves (Xiong et al., 2012). Although the source of NO production in plants is subject to debate (Moreau et al., 2010), in the present study the strong inhibition of NO accumulation observed in seedling roots grown exclusively on nitrate-free medium with PEG showed clearly that NR was the main source of NO in M. truncatula seedlings under water-deficit stress. Another study has reported the involvement of both NR and NOS-like enzyme in roots of cucumber seedlings after dehydration (Arasimowicz-Jelonek et al., 2009).

Fig. 4. NO influence on total free amino acid, glutamate, and proline content of Medicago truncatula embryo axes subjected to water-deficit stress. Amino acid (A), glutamate (B), and proline (C) contents in axes of seedlings grown with normal growth medium (MS) ± DEA (500 μM), or with PEG (~0.75MPa) ± cPTIO (250 μM); Measurements were made at the indicated time points. Data are mean±SE of four independent experiments.
drought stress. From the present results, NO quenching with PEG did not result in excessive water loss and the presence of exogenous NO under normal conditions did not induce a gain in water content. In addition, under these two conditions, early root growth appeared to be modulated in the same way. Inhibition of primary root growth has been also demonstrated after exogenous NO application under normal conditions in Arabidopsis (Fernández-Marcos et al., 2011). NO-induced early root growth inhibition was due to root apical meristem defects, reduction of cell elongation in the elongation-differentiation zone, and effects on auxin transport (Fernández-Marcos et al., 2011, 2012). Interestingly, the present study showed that PEG-induced ABA production led to NO accumulation in M. truncatula axes under water-deficit stress, and this study group has previously demonstrated ABA-induced inhibition of cell expansion in the radicles of post-germination M. truncatula seeds (Gimené-Gilles et al., 2009). It may be concluded that this primary root inhibition is mediated through a ABA-dependent NO pathway (Fig. 6) but not completely, because the scavenging of endogenous NO under water-deficit stress contributed to a reduction of embryo axis elongation.

**No relationship between ABA-induced NO and proline accumulation during short-term water-deficit stress**

The expression of many stress-induced genes requires ABA for induction, particularly genes involved in proline biosynthesis (Strizhov et al., 1997; Szabados and Savouré, 2010). Nevertheless, depending on the stress, it has been shown that proline accumulation can also occur independently of ABA (Savouré et al., 1997), demonstrating that the involvement of ABA in proline biosynthesis is still unclear. However, in a previous paper, the current study group showed that proline accumulation and regulation of gene expression related to proline metabolism in response to PEG was mediated through a ABA-dependent pathway (Planchet et al., 2011a). The present study has shown that water-deficit-induced intracellular NO production was mediated through ABA and that this endogenous NO production was concomitant with rapid proline accumulation and P5CS2 expression in primary roots of water-deficit stressed Medicago seedlings. Only a few studies have reported concomitant NO and proline production, in Chlamydomonas reinhardtii under excess copper (Zhang et al., 2008), in cucumber and rice under drought stress (Arasimowicz-Jelonek et al., 2009; Xiong et al., 2012), and in Arabidopsis under cold acclimation (Zhao et al., 2009). Furthermore, Shi et al. (2012) showed under normal growth conditions that transgenic lines overexpressing nNOS accumulated more NO and produced more proline compared to wild type. However, no direct link between these two molecules has been established from these studies. The present study found no relationship between proline accumulation and NO production. During PEG treatment, NO removal by cPTIO resulted in a high proline content which was almost
Water deficit stress

\[ \text{NCED} \rightarrow \text{ABA} \]

\[ \text{NR (NOS)} \]

\[ \text{P5CS2} \rightarrow \text{ProDH} \]

\[ \text{NO} \]

\[ \text{Proline} \]

Adaptive responses

Fig. 6. Model of ABA, NO, and proline signalling pathways in Medicago truncatula seedlings submitted to water-deficit stress. Water-deficit stress has been reported to induce ABA accumulation through NCED expression, a gene involved in the ABA synthesis (Planchet et al., 2011a). Adaptive responses to water-deficit stress could be initiated (i) mainly by a NR-dependent NO accumulation by ABA leading to an inhibition of germination, reduction of water content, and axis elongation or (ii) by a NO-independent proline accumulation via ABA through the expression of P5CS2 and inhibition of ProDH. Broken line indicates predicted pathways.

Production and proline accumulation could be explained by the application of commercially available NO donors. It is well known that several metabolic changes are affected by the NO forms generated from NO donors (Arasimowicz-Jelonek et al., 2011), by the processes of NO donor decomposition which depends on external factors (Floryszak-Wieczorek et al., 2006; Ederli et al., 2009), and by the concentration of NO donors (Lamattina et al., 2003). Therefore, caution is needed when interpreting results obtained, according to the distinction made between the physiological and metabolic effects due to exogenous or endogenous NO. This idea is even stronger given that in vivo NO concentrations remain difficult to determine after NO exogenous application.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Table S1. Dose-dependent effects of DEA on growth parameters of M. truncatula seedlings.

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Several days of a stress treatment stimulated proline production in presence of a NO-releasing chemical in C. reinhardtii cells treated with copper (Zhang et al., 2008) and in wheat seedlings under osmotic stress (Lei et al., 2007). However, in agreement with the current results, an exogenous NO donor alone is not suitable to induce proline accumulation in C. reinhardtii cells (Zhang et al., 2008) or in rice leaves (Xiong et al., 2012) and can also induce a significant decrease of proline content in response to dehydration in cucumber roots (Arasimowicz-Jelonek et al., 2009) or to salt treatment in Chinese cabbage leaves (Lopez-Carrion et al., 2008). All these discrepancies about the relationship between NO


