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ORIGINAL ARTICLE

Polyamines modulate nitrate reductase activity in wheat leaves: involvement of nitric oxide

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Abstract In the present work, the effect of polyamines (PAs) on nitrate reductase (NR) activity was studied in wheat leaves exposed to exogenously added PAs while assessing the nitric oxide (NO) involvement in the regulation of the enzyme activity. A biphasic response was observed along the time of treatment using 0.1 mM of putrescine (Put), spermidine (Spd) or spermine (Spm). At 3 h, Spd and Spm significantly reduced NR activity by 29 or 35%, respectively, whereas at 6 h, the activity of the enzyme decreased by an average of 25%. At 21 h, Put increased NR activity by 63%, while Spd and Spm elevated the enzyme activity by 114%. NR activity, that was reduced by 0.1 mM Spm at 3 and 6 h, returned almost to control values when c-PTIO (an NO scavenger) was used, confirming that NO was involved in the inhibition of NR activity. Nitric oxide was also mediating the PA-increase of the enzyme activity at longer incubation times, evidenced when the raise in NR activity produced by 0.1 mM Spm at the longest incubation time returned to the value of the control in the presence of cPTIO. Neither the protein expression nor the nitrate content were modified by PAs treatments. The involvement of PAs and NO in the regulation of NR activity is discussed.

Keywords Nitrate reductase · Nitric oxide · Polyamines · Wheat

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Abbreviations

Put	Putrescine
Spd	Spermidine
Spm	Spermine
SNP	Sodium nitroprusside
NR	Nitrate reductase
L-Arg	L-Arginine
D-Arg	D-Arginine

Introduction

Polyamines (PAs) are probably one of the oldest groups of substances known in biochemistry (Galston and Kaur-Sawhney 1990). Polyamines are organic polycations, nitrogen-containing compounds of low molecular weight, whose content in cells are maintained by biosynthesis, degradation and transport (Igarashi and Kashiwagi 2000).

The diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm) are widespread in almost all cells and free-living microbes (Agostinelli et al. 2010) except for some archaeal methanogens and halophiles (Fuell et al. 2010).

Putrescine is the major diamine in plants and a direct substrate for the synthesis of Spd and Spm (Slocum and Flores 1991). Amongst eukaryotes, plants are unique in having an alternative biosynthetic route to Put from arginine (Arg) through arginine decarboxylase (ADC), acquired from the cyanobacterial ancestor of the chloroplast. Putrescine is also synthesized from ornithine (Orn) by Orn decarboxylase (ODC). Spd synthase catalyses Spd synthesis from Put and decarboxylated *S*-adenosylmethionine (SAM)(dcSAM), whereas Spm is synthesized from

Spd and dcSAM, via a reaction catalysed by Spm synthase (Bagni and Tassoni 2001).

Polyamines are involved in a variety of fundamental cellular processes, including transcription, RNA modification, protein synthesis and the modulation of enzyme activities (Tabor and Tabor 1984). They are preferentially detected in actively growing tissues (Takahashi and Kakehi 2010) and have been implicated in the control of cell division and embryogenesis (Kakkar et al. 2000; Handa and Mattoo 2010), fruit development and ripening (Kumar et al. 1997), senescence or signalling (Takahashi et al. 2003) and responses to biotic and abiotic stresses (Alcázar et al. 2010; Cona et al. 2006; Groppa et al. 2007, 2008). Specific receptors for PAs have not been still discovered in plants, for what it is particularly interesting to investigate how PAs perform diverse functions in plant cells.

The possible linkage between PAs and NO metabolism have started to be explored a few years ago, when Tun et al. (2006) reported that PAs induced rapid NO biosynthesis in specific tissues of *Arabidopsis thaliana* seedlings, mainly in the elongation zone of root tips and in the veins and trichomes of primary leaves. These authors have also demonstrated that 2-aminoethyl-2-thiopseudourea, an inhibitor of mammalian NO synthase (NOS), inhibits spermine-induced DAR-4M fluorescence, suggesting the presence of an unidentified pathway for NO production in response to PAs. Polyamine levels also correlate with nitric oxide (NO) because L-arginine is a common precursor in their biosynthesis (Gao et al. 2009). Spermine increased endogenous NO content and reduced wheat root growth rates, and this response was partially abolished by adding 2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-1-1-oxy-3-oxide (cPTIO), a commonly used NO scavenger (Groppa et al. 2008).

In the past years, the involvement of the highly diffusible gaseous free radical NO in several fundamental processes in plants have entirely changed our recognition of this molecule from just an air pollutant to an essential signalling molecule. Nitric oxide plays a key role as an intra- and intercellular messenger, inducing various processes in plants, including the expression of defence-related genes and programmed cell death, stomatal closure, seed germination, cadmium toxicity and root development (Wendehenne et al. 2001; Neill et al. 2003; Lamotte et al. 2004; Correa-Aragunde et al. 2006; Groppa et al. 2008; Besson-Bard et al. 2009) or in postranslational modifications of proteins, a subject scarcely studied in plants yet (Alvarez and Radi 2003; Bartesaghi et al. 2007). Nitric oxide biosynthetic pathways can be classified as either oxidative or reductive in operation. The well-documented routes via nitrate reductase (NR, EC 1.7.1.1) and mitochondrial or plasma membrane-associated NO production are all reductive reactions, whereas NO production from L-arginine or polyamines are oxidative routes (Besson-Bard et al. 2008).

In plants, NO synthesis via the oxidation of L-arg by an enzyme(s) homologous to the snail nitric oxide synthase (NOS) has been documented (Guo et al. 2003; Neill et al. 2003). However, though several studies using pharmacological tools have demonstrated NOS-like activity in plants, the identity of the enzymes involved remains unknown. The only postulated plant NOS (AtNOA1/RIF1) has recently been shown to lack of NOS activity (Moreau et al. 2008). Instead, it is a chloroplast-targeted GTPase essential for proper ribosome assembly (Flores-Pérez et al. 2008).

The best-characterized production pathway for NO in plants is mediated by a key enzyme of nitrogen metabolism, NR, which is localized in the cytosol. NR catalyses the transfer of two electrons from NAD(P)H to nitrate to produce nitrite, which is further reduced to $NH4^+$ by nitrite reductase (NiR, EC 1.7.2.1) (Kaiser et al. 1999). This enzyme is regulated at multiple levels ranging from transcription to post-translational modification and protein degradation (Kaiser et al. 2002). Alternatively, NR catalyses one-electron reduction of nitrite to form NO, using NAD(P)H as an electron donor (Yamasaki et al. 1999; Meyer et al. 2005), constituting an alternative physiological function for NR in plants. Although NR has been studied extensively as a key enzyme of nitrogen metabolism, its NO-producing ability has been almost disregarded for years. Various factors are known to affect NO production via NR. First, NO synthesis by NR requires relatively low nitrate and high nitrite concentrations because the affinity for nitrite is high compared with the inhibition constant of nitrate (Rockel et al. 2002) and, therefore, NO production depends on nitrite accumulation. Second, post-translational modifications of the NR enzyme affect NO production both in vitro and in vivo. Phosphorylation of a conserved serine in the NR enzyme by NR-kinase prepares NR for binding to 14-3-3 proteins. This modification ultimately leads to inactivation and proteolytic degradation of NR in a magnesium- and calcium-dependent manner (Lillo et al. 2004). In this regard, it has been demonstrated that PAs can substitute for divalent cations in modulating the interaction of 14-3-3 proteins with nitrate reductase (Provan et al. 2000; Athwal and Huber 2002; Shen and Huber 2006).

Due to their chemical attributes, polyamines can interact with different classes of macromolecules, alter structural states and influence metabolic activity by modifying transcription or translation events. The identification of the targets is a first step for developing strategies to elucidate the mechanisms involved. In previous works we have already studied the relationship between PAs and nitric oxide in connection with wheat root growth (Groppa et al.

2008) and the regulation of NR by NO in wheat leaves (Rosales et al. 2011). In this occasion, we investigated the role of PAs in the regulation of NR activity in wheat leaves segments, assessing the involvement of NO in this regulation.

Materials and methods

Chemicals

NADH, Put, Spd, Spm, hexanediamine, cPTIO, L-Arg, D-Arg, casein, PVP were from Sigma Chemical Company (Saint Louis, MO, USA). All chemicals were of analytical grade.

Plant material and treatments

Wheat seeds (*Triticum aestivum* L., provided by Nidera) were germinated and grown at 26/20 ± 3°C (day and night), with a 16-h photoperiod under fluorescent white light (175 mmol m^2 s) in a controlled-environment growth chamber. Plants were daily watered with a nutrient solution (Hoagland and Arnon 1950). At the end of the illumination period in the growth chamber, leaf segments (8 mm length) from 12-day-old plants were placed in flasks containing 0.1 mM Put, Spd or Spm dissolved in distilled water and incubated for 3, 6, 9, 16 or 21 h in a rotatory shaker under continuous illumination. Some determinations were only made for 3 and 21 h. Controls were incubated in distilled water. When indicated, cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide, 200 µM), D-Arg (2 mM) or L-Arg (2 mM) were added to the incubation medium.

Determination of NR activity

NR activity was measured according to Yaneva et al. (2002) and Savidov et al. (1997). Wheat leaf segments were homogenized in a medium containing 5 mM EDTA, 5 mM GSH, 1% (w/v) casein, 0.1% (w/v) insoluble PVP and 50 mM HEPES pH 7.5 and centrifuged for 15 min at 17,000*g*. The assay mixture for measuring NR activity contained 200 µmol KNO_3, 0.2 µmol NADH and 100 µL of the homogenate. After incubation at 30°C for 20 min, the reaction was stopped by the addition of 50 µL 1 M zinc acetate. The mixture was centrifuged 5 min at 7,600*g* and the supernatant was used to determine nitrite production by reading the absorbance at 540 nm after the addition of 1% sulphanilamide in 1.5 M HCl and 0.01% *N*-(1-naphthyl)-ethylenediammonium dichloride. Nitrate reductase activity was also measured directly in a reaction tube by incubating a sample of a crude homogenate from control wheat segments, taken directly from the plants, with 0.1 mM of Put, Spd or Spm. The reaction mixtures were incubated under continuous illumination. NR activity was determined at 3, 10, 20 and 30 min after PAs addition. Nitrites are themselves competitive inhibitors of NR, so NO_2^- contents in leaves were measured immediately after PAs' addition. It was verified that NO_2^- levels were not significantly different from the control without PAs' addition.

Western blot analysis of NR expression

Leaves were homogenized and extracted with 50 mM HEPES–KOH pH 7.5, 1 mM EDTA, 10 mM FAD, 1 mM DTT, 1% (w/v) insoluble PVP, 5 mM ascorbate and protease inhibitor cocktail (Sigma). The extract was centrifuged at 17,000*g* for 15 min at 4°C and the protein concentration in the supernatant was determined according to Bradford (1976). Proteins were separated on a 10% SDS–PAGE in a Mini PROTEAN III equipment (Bio-Rad), as described by Laemmli (1970). Following electrophoresis at 4–8°C, proteins were transferred to PVDF membrane (GE Healthcare, Amersham Hybond P). Membranes were then blocked with 3% (w/v) BSA dissolved in PBS, incubated overnight with the primary antibody against NR dissolved in blocking buffer (1/5,000) and washed several times with PBS. Immunodetection of NR was carried out using a rabbit serum antibody raised against NR from Arabidopsis (kindly provided by Dr. Steven Huber, Department of Plant Biology, University of Illinois). Bands were revealed using a goat anti-rabbit IgG peroxidase-conjugated secondary antibody (Dako Cytomation), and 3,3-diaminobenzidine (DAB) was used as substrate for the staining procedure.

Measurement of nitrate contents

Wheat leaf segments were dried at 85°C until constant weight. The dried material (25 mg) was grounded to powder and incubated in 10 ml of distilled water for 2.5 h. Nitrate was measured colorimetrically after a reaction with salicylic acid (Cataldo et al. 1975).

Analysis of polyamines

Wheat leaf segments were homogenized with 5% (v/v) $HClO_4$, maintained 30 min on ice and centrifuged at 3,000*g* for 10 min. The supernatants were derivatized using the dansylation method described by Smith and Meeuse (1966) and 1,6-hexanediamine was used as an internal standard. Standards of Put, Spd and Spm were dansylated simultaneously. The dansylated derivatives were extracted with ethylacetate (1 ml). Polyamines were separated and identified by TLC performed on high-resolution silica gel

plates (JT Baker, silica gel plates IB 2-F) using a *n*-hexane:EtOAc (1:1) solvent system. Dansylated polyamines were identified by comparing Rf values of dansylated standards. Silica plates were observed under UV light and bands corresponding to the PAs in the samples and standards were scraped off the plates and eluted with EtOAc (1 ml). Fluorescence measurements were made at 365 nm excitation and 510 nm emission in an spectrofluorometer (Aminco Bowman).

Statistics

All determinations were performed from three independent experiments. Analytical measurements were done three times for all parameters in each experiment, with a minimum of three replicates. Figures show mean values ± SE. Differences among treatments were analysed by one-way ANOVA. Asterisks indicate statistically significant differences ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$) according to Tukey's multiple range test.

Results

Polyamines and L-Arg modify NR activity

In order to study the effect of Put, Spd and Spm on NR activity, leaf discs were exposed to 0.1 mM of each polyamine for 3, 6, 9, 16 and 21 h. A biphasic response was observed for the three PAs (Fig. 1). At 3 h of exposure, Spd and Spm significantly reduced NR activity by 29 or 35%, respectively, whereas at 6 h, the three PAs reduced the enzyme activity at an average of 25%. At the longest incubation time (21 h), Put increased NR activity 63% over the control, whereas Spd and Spm both increased the enzyme activity by 114%.

Considering that L-Arg is the substrate for Put and NO biosynthesis, the effect of the exogenous addition of this aminoacid was evaluated in relation to NR activity at 3 or 21 h. L-Arg enhanced NR activity by 35% only at 21 h of exposure (Fig. 2).

"In vitro" NR activity is reduced by PAs

In order to study the direct effect of PAs on NR activity, an in vitro measurement of the enzyme activity was carried out using crude wheat leaf homogenates and adding directly Put, Spd or Spm at 0.1 mM into the reaction solution. Thirty minutes after the addition of Put, NR activity was reduced by 25% with respect to the control (Fig. 1 inset). No significant differences in the enzyme activity were observed respect to the controls when Spd or Spm were used.

Spermine inhibition of NR activity is mediated by NO

Considering that NO was involved in Spm-induced wheat root growth inhibition (Groppa et al. 2008), and NO

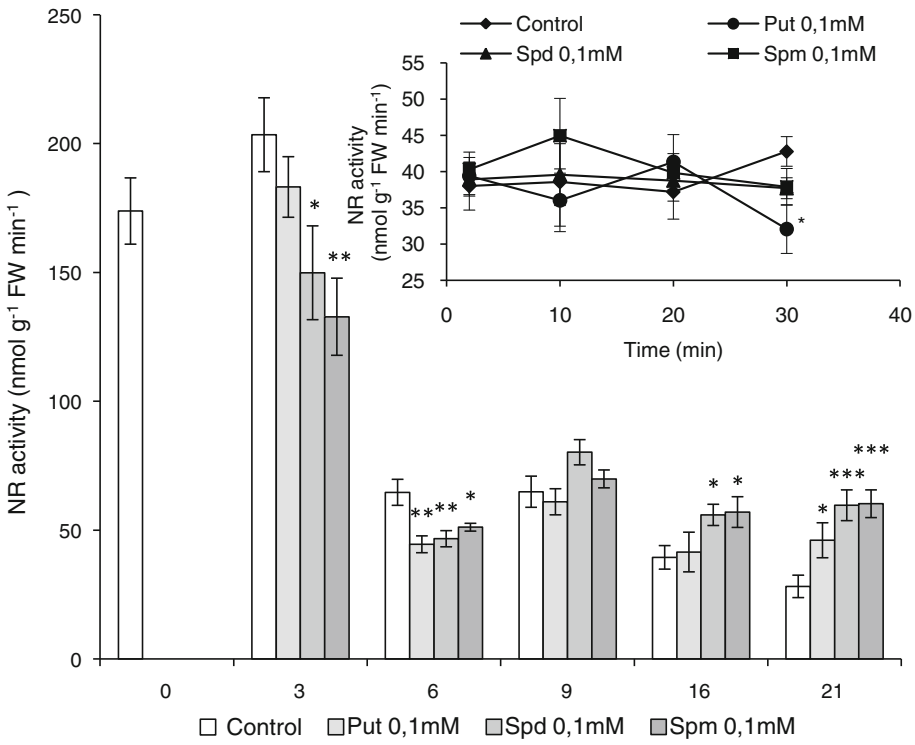

Fig. 1 NR activity in wheat leaf segments treated with 0.1 mM PAs at 3, 6, 9, 16 and 21 h of exposure, as described in "Materials and methods". Inset: In vitro NR activity. A crude homogenate from control wheat leaf segments were used for the assays with the addition of 0.1 mM Put, Spd or Spm. Figures show mean values ± SE and corresponds to three different experiments with five replicated measurements. *Asterisks* indicate statistically significant differences ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$) according to Tukey's multiple range test

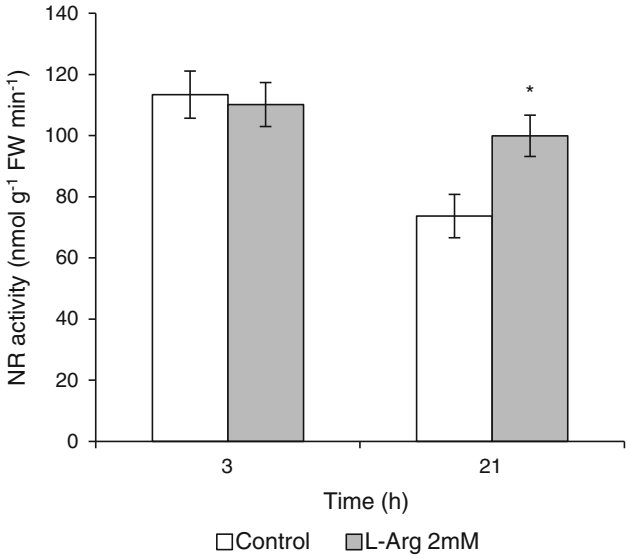

Fig. 2 NR activity of wheat leaf segments, at 3 and 21 h, after the addition of 2 mM L-Arg. NR activity was assayed as described in "Materials and methods". Figures show mean values ± SE and corresponds to three different experiments with three replicated measurements. *Asterisks* indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$) according to Tukey's multiple range test

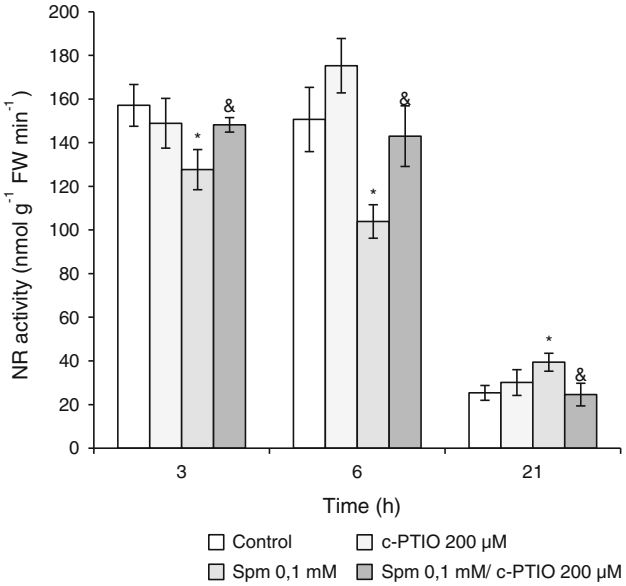

Fig. 3 NR activity of wheat leaf segments treated with 0.1 mM Spm and/or the NO scavenger cPTIO (200 µM) during 3, 6 and 21 h. NR activity was assayed as described in "Materials and methods". Figures show mean values ± SE and corresponds to three different experiments with three replicated measurements. *Asterisks* indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) according to Tukey's multiple range test and indicates statistically significant differences ($P < 0.05$) between Spm + cPTIO versus Spm

reduced NR activity in wheat leaves (Rosales et al. 2011), we decided to study if NO was mediating the decay and/or the increase in NR activity in wheat leaves. The NO scavenger cPTIO was used at 200 µM to trap the NO that could be in the incubation medium, only at 3, 6 or 21 h of incubation with 0.1 mM Spm. NR activity, that was reduced by 20 or 32% by 0.1 mM Spm at 3 and 6 h, respectively, returned almost to control values when NO was trapped using 200 µM of the scavenger, thus confirming that NO was involved in the inhibition of NR activity (Fig. 3). Nitric oxide was also mediating the increase of the enzyme activity at longer incubation times. The rise in NR activity produced by 0.1 mM Spm at 21 h was not observed in the presence of cPTIO (Fig. 3). When used alone, cPTIO did not produce any significant effect.

D-Arg and L-Arg effects on PAs content

Both isomers of the aminoacid arginine, D-Arg and L-Arg, were used to measure Put, Spd and Spm content in wheat leaves (Fig. 4). L-Arg induced a rise in Put content of about 150% at 3 h and 83% at 21 h, whereas D-Arg decreased Put content significantly at both times (63 and 68%, at 3 and 21 h, respectively) (Fig. 4a). Spermidine content was reduced 35 with L-Arg and 42% with D-Arg at 21 h of exposure (Fig. 4b). Spermine content decreased 50% at 3 h with L-Arg, but its content was not affected using D-Arg (Fig. 4c).

Effect of PAs on the NR protein content

Considering that NR activity was modified in a different way by the three PAs at short or long times, we decided to test if this change was accompanied by a modification in the NR protein content. Putrescine or Spm at 0.1 mM were used at 6 or 21 h of exposure. Despite NR activity was either reduced at shorter times or increased at longer times by the three PAs, neither Put nor Spm modified the content of the NR protein, as was evidenced by the western blot analysis (Fig. 5 b, c). The antibody used was raised against the NR protein of *Arabidopsis thaliana*, so it was tested that the antibody recognized the wheat protein as well (Fig. 5a).

Nitrate contents

In view of that the three PAs modified NR activity along the exposure time, we measured nitrate contents at 3 and 21 h using only 0.1 mM Spm. Nitrate contents were not modified by this PA at any of the sampling times (Fig. 6).

Discussion

In the present work we studied PAs effect on NR activity, trying to elucidate if PAs can regulate NR function directly

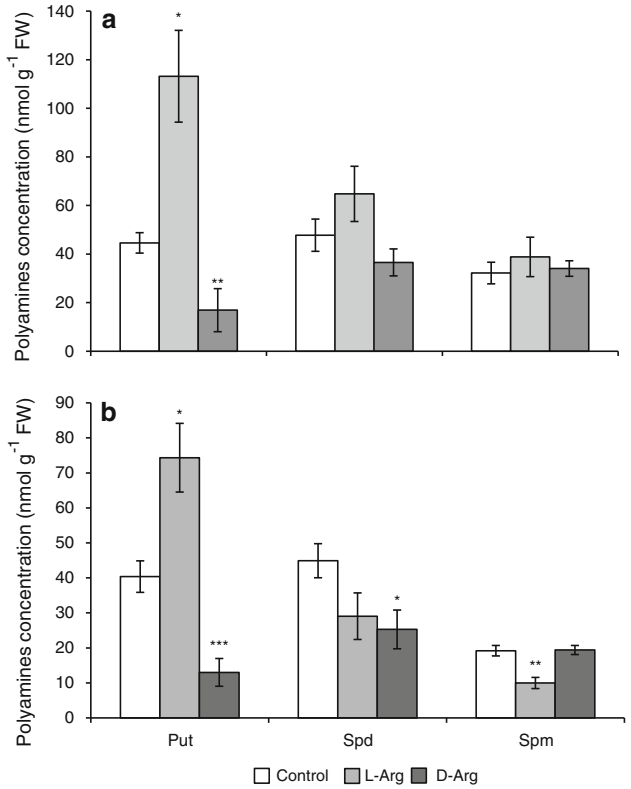

Fig. 4 Polyamine levels in wheat leaf segments treated with 2 mM D-Arg and 2 mM L-Arg at 3 and 21 h according to the description in "Materials and methods". **a** 3 h, **b** 21 h. Figures show mean values ± SE and corresponds to three different experiments with three replicated measurements *Asterisks* indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) according to Tukey's multiple range test

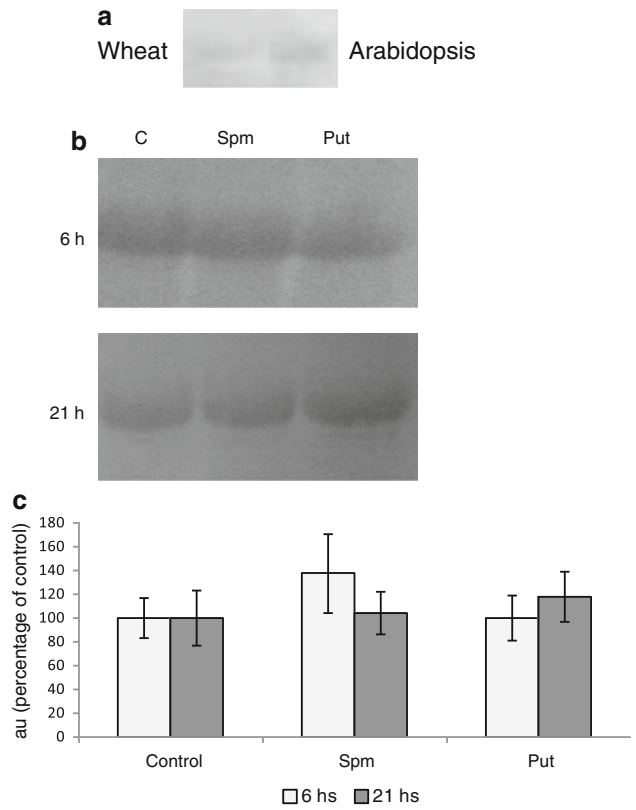

Fig. 5 **a** Western blot analysis showing NR protein expression in wheat leaf segments exposed to 0.1 mM Spm for 6 and 21 h. The experiment was repeated three times and a representative image is presented. **a** Bands corresponding to *Arabidopsis* or wheat NR protein revealed with the antibody raised against Arabidopsis NR **c** relative amount of proteins in **b** considering control homogenates as 100 au

or indirectly by modifying NO formation, with the aim to get deeper knowledge about PAs' involvement in protein modulation and nitrogen metabolism.

Since the discovery of NO_3^- as a signalling molecule, researchers have been trying to elucidate the N-signalling pathway in plants. Because NR is an enzyme involved not only in NO_3^- reduction but also in NO formation in higher plants, we attempted to show whether nitrogen metabolites, like PAs, are involved in NR regulation through the signalling molecule NO.

In a previous work, we reported that NO was involved in the regulation of NR activity in wheat leaves (Rosales et al. 2011), whereas NO was shown to be mediating PA-induced root growth inhibition in wheat plants (Groppa et al. 2008).

A biphasic time-dependent response was observed on NR activity when leaf segments were exposed to PAs, which, with the exception for Put at 3 h, reduced NR activity at 3 and 6 h, but markedly increased the enzyme activity at longer times (21 h). Spermine-dependent reduction in NR activity was almost completely abolished using cPTIO, a NO scavenger, in the incubation medium, indicating that NO was mediating the inhibitory response, as was observed in Spm-treated wheat roots (Groppa et al. 2008) and in wheat leaves exposed to sodium nitroprusside (SNP) (Rosales et al. 2011).

In the present days, one of the most puzzling issues in plant NO biology is the mechanism(s) for NO formation. Although it is still possible that a unique plant NOS might exist, the loss of two strong candidates for catalysis of an arginine-dependent NO pathway (NOS1 and NOA1/RIF1) in plants has left a wide hole in the field (Crawford 2006; Zemojtel et al. 2006; Gas et al. 2009; Moreau et al. 2010). As an enzymatic source of NO in plants, NR is now the only protein whose NO-producing activity has been confirmed (Yamasaki and Sakihama 2000). However, Tun et al. (2006) have recently added another possible source of NO linked to PAs, showing that the very rapid induction of NO release by Spm argued in favour of the presence of an unknown polyamine-induced enzyme which directly converts PAs to NO and other products. Moreover, whereas up-regulation of arginase activity reduced the release of NO in *Arabidopsis* mutants, NO production was rescued by

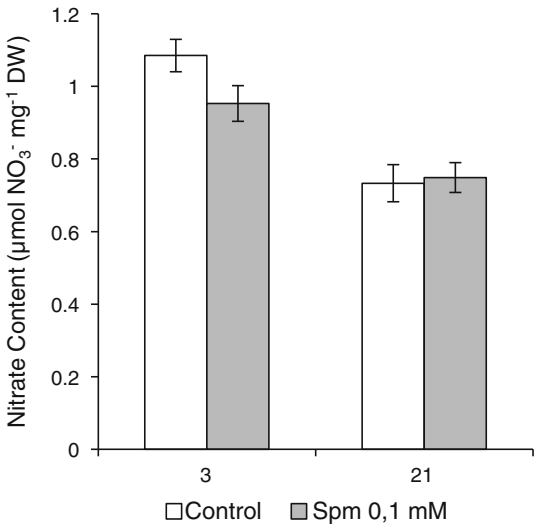

Fig. 6 Nitrates content in wheat leaf segments exposed to 0.1 mM Spm for 3 and 21 h. Figures show mean values ± SE and corresponds to three different experiments with three replicated measurements

providing Spm to the plants, suggesting that the PA synthesis from arginine is involved in the production of NO (Flores et al. 2008).

It is unclear in what way PAs could regulate NO-generating enzymes. However, there is evidence that in the presence of Mg^{2+}, Spd or Spm, the phosphorylated form of NR interacts with 14-3-3 proteins, and NR activity is then inhibited (Athwal and Huber 2002; Bridges and Moorhead 2005; Shen and Huber 2006). After 21 h of PA exposure, NR activity was significantly increased to different extents according to the PA used, Spm being the one which produced the major rise. This response was also mediated by NO, as was suggested when cPTIO almost completely reversed the NR-enhanced PA-mediated activity. In our previous work (Rosales et al. 2011), NR activity was highly reduced both at 3 and 21 h of treatment, possibly due to a higher and constant NO concentration released from SNP in the medium, compared with PAs-released NO in this work. In the present experiments, at 21 h PA concentration inside the tissues could be lower compared with that obtained at 3 h of exposure, due to an enhanced PA degradation occurring in the incubation medium, that could have led to lower PA-derived NO levels. The minor NO levels released from PAs at 21 h could be showing a different modulation of NR activity than the observed at 3 h of incubation, when NO was at higher levels. This could be the reason for the opposite effect displayed by NO on NR activity at short and long times. In wheat leaves, a competition between NR and H^+-ATPase for 14-3-3 proteins could be displacing the equilibrium in favour of one or the other depending on PA or NO levels in the medium, thus inducing the inhibition or activation of NR (Athwal and Huber 2002; Provan et al. 2000). Shi et al. (2008) demonstrated that Put enhanced tolerance to hypoxia by increasing the transcript levels of NR and its cofactor-binding domain genes, thereby stimulating the activities of NR and nitrate reduction to maintain the redox and energy status through an increase in ATP concentration.

The involvement of NO in the activation of NR activity has been reported recently in roots of Chinese pakchoi cabbage (Du et al. 2008), where NR activity was significantly enhanced by the addition of the NO donors SNP or diethylamine NONOate. These authors also found that NR protein content was not affected by SNP and suggested that the stimulating effect of NO on NR activity might be due to an enhancement of electron transfer from haem to nitrate through activating the haem and molybdenum centres in the NR (Du et al. 2008). In concordance with these results, no changes in protein content were observed after PAs' exposure at any time of our experimental conditions.

By the time that NR activity was significantly stimulated by 0.1 mM Spm, nitrate content did not change respect to the control. In contrast, in SNP-treated wheat leaf segments, NO_3^- increased along with the decrease in NR activity at 21 h of exposure (Rosales et al. 2011). Jin et al. (2009) demonstrated that the addition of the NO donors SNP and NONOate stimulated NR activity of tomato roots fed with 0.5 mM nitrate, whereas the reverse was true for the roots fed with 5 mM nitrate, suggesting that NO mediates the NR activity in plant roots differently depending on the level of nitrate supply. Tun et al. (2006) reported that Spd and Spm increased NO biosynthesis in the elongation zone of *Arabidopsis* root tips and in primary leaves, but they did not measure NR or NOS activity to establish the enzymatic source of NO. It could not be discarded that NO produced by any of the mentioned enzymes was performing a signalling role regulating its own forming enzymes.

Putrescine and Spm treatment resulted in a high NR activity in wheat leaves at 21 h and an enhanced NO formation was indirectly proved, since cPTIO addition prevented this effect. As expected, the isomer L-Arg, precursor for both NO and Put biosynthesis (through the enzymes NOS and ADC, respectively), L-Arg increased the biosynthesis of Put at 3 and 21 h. Since exogenous addition of Put increased NR activity, it is possible that the addition of the precursor L-Arg with the concomitant increase of endogenous Put levels has produced the same response.

It might be possible that PAs were participating in the regulation of NR activity in a dual manner: at short times, inhibiting NR by both increasing NO and by stimulating 14-3-3 protein interaction with NR. At longer times, PAs could be modulating the association of 14-3-3 proteins with the H^+-ATPase, thus activating NR activity, and this action could be prevailing over the effect of the increased NO level.

The results obtained in the present work lead us to think that there are two essential issues to be clarified in the near future. The top priorities are the identification of the enzyme/s involved in NO generation in plants and/or the demonstration that the L-Arg-dependent pathway for NO production is molecularly distinct from the pathways in animals. It would be also valuable to elucidate if PAs are a minor or a major pathway for NO synthesis.

Second, PA-related NO signalling has temporal and spatial arrangements that determine the specificity of the biological effect. Establishing this concept will notably require the identification of proteins that interact with PAs (like ATPase or 14-3-3 or others still unknown), as well as the NO target proteins, and the analysis of the dynamics of formation and localization of the resulting complexes under physiological conditions. Nitrates, NO and PAs might be key players in the integration of N signals in plants. The elucidation of these crossroads will be of enormous importance in N metabolism.

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