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Nitric oxide is required for the auxin-induced activation of NADPH-dependent thioredoxin reductase and protein denitrosylation during root growth responses in arabidopsis

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- **Background and Aims** Auxin is the main phytohormone controlling root development in plants. This study uses pharmacological and genetic approaches to examine the role of auxin and nitric oxide (NO) in the activation of NADPH-dependent thioredoxin reductase (NTR), and the effect that this activity has on root growth responses in *Arabidopsis thaliana*.
- **Methods** Arabidopsis seedlings were treated with auxin with or without the NTR inhibitors auranofin (ANF) and 1-chloro-2, 4-dinitrobenzene (DNCB). NTR activity, lateral root (LR) formation and S-nitrosothiol content were measured in roots. Protein S-nitrosylation was analysed by the biotin switch method in wild-type arabidopsis and in the double mutant *ntra ntrb*.
- **Key Results** The auxin-mediated induction of NTR activity is inhibited by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CPTIO), suggesting that NO is downstream of auxin in this regulatory pathway. The NTR inhibitors ANF and DNCB prevent auxin-mediated activation of NTR and LR formation. Moreover, ANF and DNCB also inhibit auxin-induced DR5::GUS and BA3::GUS gene expression, suggesting that the auxin signalling pathway is compromised without full NTR activity. Treatment of roots with ANF and DNCB increases total nitrosothiols (SNO) content and protein S-nitrosylation, suggesting a role of the NTR-thioredoxin (Trx)-redox system in protein denitrosylation. In agreement with these results, the level of S-nitrosylated proteins is increased in the arabidopsis double mutant *ntra ntrb* as compared with the wild-type.
- **Conclusions** The results support for the idea that NTR is involved in protein denitrosylation during auxin-mediated root development. The fact that a high NO concentration induces NTR activity suggests that a feedback mechanism to control massive and unregulated protein S-nitrosylation could be operating in plant cells.

Key words: *Arabidopsis thaliana*, auxin, denitrosylation, nitric oxide, NTR, reactive nitrogen species, root growth, thioredoxin reductase.

INTRODUCTION

Nitric oxide (NO) is a free radical molecule, produced enzymatically and non-enzymatically in plants, which is involved in different signalling cascades of phytohormones, as well as in biotic and abiotic stress responses (Lamattina *et al.*, 2003). One of the more important modes of action of NO is the S-nitrosylation of proteins. S-nitrosylation has emerged as a relevant post-translational modification (PTM) of proteins given its high reactivity and specificity. It occurs under physiological conditions, influencing the function and activity of a large number of proteins. S-nitrosylation consists of the incorporation of NO moieties by covalent bonding to reactive Cys residues in proteins (Lindermayr and Durner, 2009). Many attempts have been made to establish a consensus sequence of S-nitrosylation in proteins without success. Structural analysis of identified S-nitrosylated proteins showed that Cys residues prone to nitrosylation are flanked by acidic or basic residues. Moreover, a hydrophobic environment, low pKa values and solvent exposure of Cys residues are thought to favour S-nitrosylation

(Marino and Gladyshev, 2010). In animals, the transnitrosylase complex shuttles NO from nitric oxide synthase (NOS) isoforms to target proteins via direct NOS–substrate interaction and sequence motif recognition (Kim *et al.*, 2005; Jia *et al.*, 2014). In contrast, studies of the enzymatic transnitrosylation complex have not been reported in plants.

Denitrosylation has been demonstrated to be an enzymatic and selective modification. Elucidation of the molecular mechanisms of denitrosylation in plants is important to understand how signal transduction pathways are regulated by and how plants modulate nitrosative stress. Two systems have emerged as physiologically relevant denitrosylases in plant cells. The first one is the nitrosogluthione reductase (GSNOR) system conserved in bacteria, plants and humans (Sakamoto *et al.*, 2002; Díaz *et al.*, 2003). When synthesized, NO rapidly reacts with glutathione (GSH) to form nitrosogluthione (GSNO), a potent transnitrosylating agent that can act also as a mobile reservoir of NO. GSNOR catalyses the NADPH-dependent reduction of GSNO to form glutathione sulfonamide as a major

product (Jensen *et al.*, 1998). Therefore, GSNOR indirectly should affect the level of *S*-nitrosylation in proteins. *Arabidopsis thaliana* overexpressing GSNOR displays lower levels of nitrosothiols (SNO), suggesting that regulation of GSNO levels modulates *S*-nitrosylation (Lee *et al.*, 2008). GSNOR is responsive to pathogen attack, wounding, cadmium stress, and salicylic and jasmonic acids (Díaz *et al.*, 2003; Feechan *et al.*, 2005; Barroso *et al.*, 2006; Rustérucci *et al.*, 2007). The redox system formed by NADPH-dependent thioredoxin reductase (NTR) and thioredoxin (Trx), the NTR–Trx system, is an additional mechanism that controls the *S*-nitrosylation process. Trxs are small ubiquitous proteins with a disulfide active-site that maintains the reduction of Cys residues in target proteins (Meyer *et al.*, 2008). Progression in the redox-based modification of Cys thiols in proteins involves SNO, sulfenic acid (SOH), disulfide (S–S), and sulfinic (SO₂[−]) to the irreversible sulfonic acid (SO₃[−]). Trxs are able to catalyse the denitrosylation of low-molecular-mass molecules and protein-SNO (Sengupta *et al.*, 2007). While in animals, yeast and bacteria there are only two Trxs, plants contain several isoforms, which are classified into *f*, *h*, *m*, *o*, *x*, *y* and *z* types. *Arabidopsis* has at least 20 Trx isoforms, distributed in the cytosol (type *h*), chloroplast (types *f*, *m*, *x*, *z* and *y*) and mitochondria (type *o*) (Meyer *et al.*, 2008). Furthermore, 20 other Trx-related proteins have been identified (Meyer *et al.*, 2008). In contrast, only three genes encoding NTR have been identified in the *Arabidopsis* genome, termed NTRA, NTRB and NTRC (Serrato *et al.*, 2004). NTRA and NTRB show dual localization in mitochondria and cytosol, although NTRA is the predominant isoform in cytosol and NTRB in mitochondria (Reichheld *et al.*, 2005). NTRC, an NTR with a joint Trx domain at the C terminus, which was first reported as a chloroplast-localized enzyme (Serrato *et al.*, 2004), is also present in plastids from non-photosynthetic tissues (Kirchsteiger *et al.*, 2012).

As stated above, auxin is the main phytohormone involved in plant root development. Auxin promotes the production of NO in roots, which is required for auxin-mediated root organogenesis (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2004). Moreover, participation of the NTR–Trx system and GSH in auxin signalling and transport was demonstrated using the triple *Arabidopsis* mutant *ntra ntrb cad2* (Bashandy *et al.*, 2010). Recently, cytosolic ascorbate peroxidase1 (APX1) was shown to be denitrosylated and partially inactivated by auxin in *Arabidopsis* roots, leading to an increase in H₂O₂ concentration and modulation of root branching (Correa-Aragunde *et al.*, 2013). In this study, the participation of the NTR–Trx system in auxin-mediated root development and its participation in denitrosylating activity were studied.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col-0) ecotype seeds were surface-sterilized in 30% bleach and 0.02% Triton-X100 for 15 min, rinsed three times with sterile water and stratified at 4 °C for 2 d in the dark. The *Arabidopsis thaliana* mutant *ntra ntrb* was generated in the Columbia ecotype. Seeds were placed on ATS medium supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar (Wilson *et al.*, 1990) and placed vertically in a

growth chamber at 25 °C with a light intensity of 100 μmol photons m^{−2} s^{−1} and 16 : 8-h (light–dark) cycles. For root growth measurements, plants were grown for 4 d and then transferred to auxin naphthyl acetic acid (NAA) or sodium nitroprusside (SNP) provided by Sigma (St Louis, MO, USA) for another 4 d. Lateral root (LR) formation was quantified manually using magnifying glasses. For NTR activity assays, plants were grown for 7 d and then subjected to the different treatments for 1 d.

GUS staining

Histochemical assays for GUS activity were performed as described by Jefferson *et al.* (1987). Five days after germination BA : GUS or DR5 : GUS seedlings were treated with NAA and the NTR inhibitors auranofin (ANF) (Alexis Biochemicals, San Diego, CA, USA) or 1-chloro-2,4-dinitrobenzene (DNCB) (Fluka AG, Buchs, Switzerland) and then submerged in GUS staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in 100 mM sodium phosphate (pH 7.5). Tissues were incubated at 37 °C for 3–5 h. Images were taken under a magnifier.

Determination of S-nitrosothiol content

SNO content was measured using the Saville–Griess assay (Park and Kostka, 1997) with modifications. Briefly, *Arabidopsis* roots were powdered in a mortar in liquid nitrogen and then homogenized in 100 mM phosphate buffer, pH 7.4, containing 1 mM PMSF and incubated on ice for 20 min. After centrifugation (10 000 g, 15 min, 4 °C), 50 μL of supernatant (approximately 80 μg of protein) was incubated with the same volume of 1% (w/v) sulfanilamide with or without the addition of 4 mM HgCl₂ for 15 min in the dark. Then, 50 μL of 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride (NED) was added and incubated for 10 min. SNO content was measured at an absorbance of 540 nm. SNO content was calculated by using a nitrite standard curve.

NTR activity assay

NTR activity was measured with the thioredoxin reductase assay kit according to the manufacturer's instructions (Sigma). Root protein extracts, approx. 100 μg of protein, were assayed in 100 mM phosphate buffer, pH 7.0, 10 mM EDTA and 200 μM NADPH. Reactions were initiated by the addition of 300 μM of the substrate 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB; Sigma). The assay is based on the reduction of DTNB with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which can be measured by absorbance at 412 nm. One mole of Trx reduces 1 mol DTNB to produce 2 mol TNB with a molar extinction coefficient of 13 600 M^{−1} cm^{−1}. Thus, a molar extinction coefficient of 27 200 M^{−1} cm^{−1} was applied for quantification of consumed DTNB (Shahpiri *et al.*, 2008). NTR activity results from total reductase activity minus activity with the addition of the inhibitor ANF. For NTR inhibition, 5 μM ANF was used and samples were incubated for 15 min before measurement of activity.

Biotin switch

Arabidopsis roots were homogenized in HEN buffer (25 mM Hepes, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) containing complete protease inhibitor cocktail (Sigma). Samples were centrifuged at 4 °C for 20 min and protein concentration in the supernatant was measured using the Bradford method. For *in vitro* S-nitrosylation, protein extracts were incubated with 500 μ M of the nitrosylating agent nitrosocysteine (CysNO) in the dark for 30 min with frequent vortexing. Protein extracts were incubated with 30 mM methyl-methanethiosulfate (MMTS; Sigma) and 3.3 % SDS in HEN buffer at 50 °C for 20 min to block free Cys. Proteins were precipitated with 2 volumes of cold acetone and resuspended in HEN buffer with 1 % SDS. After the addition of 20 mM ascorbic acid and 1 mM biotin-HPDP (Pierce Chemical), the mixture was incubated for 1 h at room temperature in the dark. Proteins were then subjected to immunoblot analysis using an anti-biotin antibody.

RESULTS

NO and auxin induce NTR activity in roots

To test the effect of auxin and NO on NTR activity in roots, 7-d-old arabidopsis plants were treated with the NO donor SNP or with the synthetic auxin NAA for 1 d. Total reductase activity was measured in arabidopsis root extracts using DTNB as substrate. Specific NTR activity was calculated from the difference between total reductase activities and the activity in the presence of the NTR inhibitor ANF, as previously used for measurement of NTR activity in plants (Alkhalifioui *et al.*, 2008; Shahpiri *et al.*, 2008; Smiri *et al.*, 2013). The inhibitory effect of ANF on root NTR activity was previously analysed in arabidopsis. ANF inhibits almost 50 % of total reductase activity in roots (Supplementary Data Figure S1). Figure 1A and B show that auxin and NO induce NTR activity in a dose-dependent manner. NTR activity increases two-fold in roots treated with 10 μ M NAA with respect to untreated roots (Fig. 1A). Western blot analysis showed that NTR protein levels in roots were not significantly altered by the NAA treatment (Fig. S2). Treatment with 10 μ M of the NO donor SNP triggers, in turn, a stronger response, reaching up to seven-fold induction of NTR activity in roots (Fig. 1B). The effect of SNP is due to the release of NO as the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CPTIO) abolished the induction of NTR activity by SNP (Fig. 1C). CPTIO was also able to repress the NAA-promoted induction of NTR activity, suggesting that NO is required for the auxin-mediated induction of NTR activity (Fig. 1C).

With the aim of dissecting the connection between auxin, NO and cellular reductases during root branching, arabidopsis seedlings were incubated with NAA or SNP in the presence or absence of the NTR inhibitors ANF and DNCB. In mammalian systems, ANF and DNCB have been widely used as inhibitors of NTR (Ishikawa *et al.*, 1999; Rigobello *et al.*, 2004). Figure 2A shows the induction of LR formation by NAA treatment, as was extensively reported elsewhere (Bhalerao *et al.*, 2002; Marchant *et al.*, 2002). Both ANF and DNCB were able to block, in a dose-dependent manner, the effect of NAA on LR formation (Fig. 2). We further examined the effect of NTR

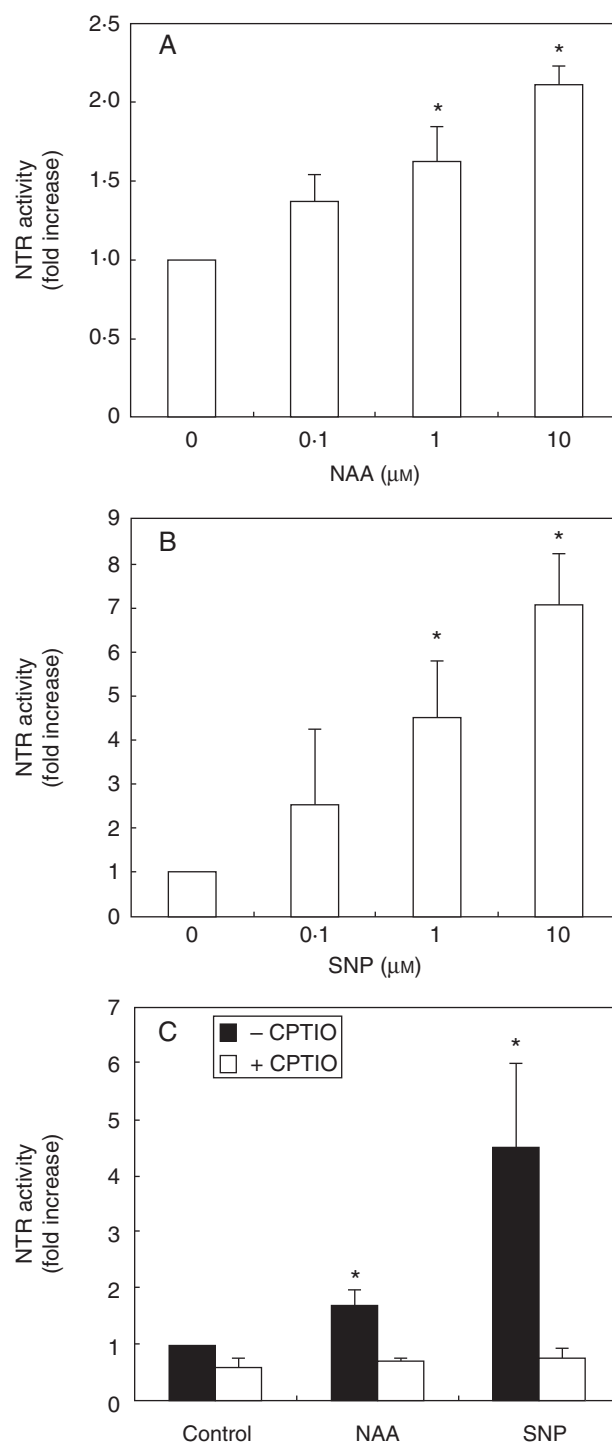


FIG. 1. NADPH thioredoxin reductase (NTR) activity is induced by auxin and NO in Arabidopsis roots. Arabidopsis plants were grown in ATS-agar for 7 d and then transferred to plates containing ATS-agar with different concentrations of the auxin NAA (A) or the NO donor SNP (B) for an additional day. NTR activity was measured in root extracts using the substrate DTNB in the absence or presence of 5 μ M of the NTR inhibitor ANF. (C) NTR activity in roots treated or not (control) with 1 μ M NAA or 1 μ M SNP in the absence or presence of 0.5 μ M CPTIO. Values are the difference between the total activity minus the activity in the presence of 5 μ M ANF and expressed as fold increase with respect to control. The actual value of NTR activity in control root extracts without ANF is $6.92 \pm 1.03 \mu$ M DTNB consumed $\text{min}^{-1} \text{mg}^{-1}$. Asterisks indicate a statistical difference with respect to control (*t*-test, $P > 0.05$).

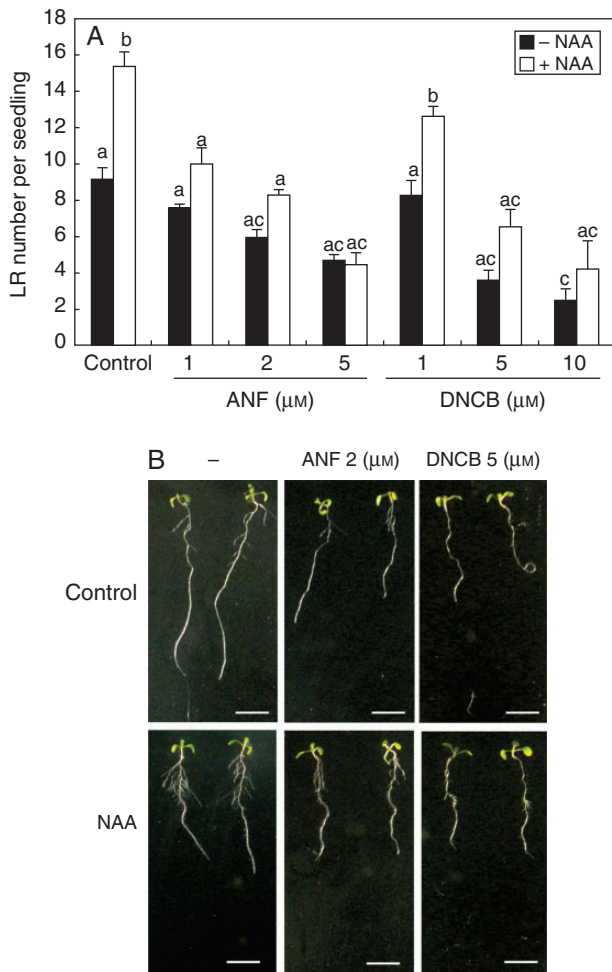


FIG. 2. NTR activity inhibition provokes impairment of lateral root (LR) formation. (A) Arabidopsis plants were grown for 4 d in ATS-agar and transferred to treatments with 0.1 μM NAA with or without (control) different concentrations of ANF or DNCB, as indicated, for a further 4 d. LR number was counted with a magnifier. Values are mean and bars are standard error of five independent experiments ($n = 5$). (B) Photographs of seedlings after 4 d of treatment. Scale bars = 1 cm.

inhibitors on auxin signalling through analysis of the arabidopsis auxin responsive reporter lines BA3 : GUS and DR5 : GUS. Figure 3 shows that, as reported, NAA induces the expression of β-glucuronidase (GUS) under the auxin response promoters BA3 or DR5. However, NAA-induced GUS expression was affected in the presence of either of the NTR inhibitors ANF or DNCB (Fig. 3). Together, these results suggest strongly for the participation of NTR activity in auxin signalling during LR development in arabidopsis.

NTR activity influences the level of nitrosylated proteins

Studies in animals have shown that Trx is the major denitrosylating agent and, together with NTR, controls the level of protein nitrosylation in cells (Benhar et al., 2008; Holmgren, 2008). Thus, it was hypothesized that NTR is involved in protein denitrosylation in plants and thereby that treatment with NTR inhibitors would enhance the level of nitrosylated

proteins. The Griess–Saville method was used to detect S-nitrosothiol content in roots of arabidopsis plants treated with 1 μM NAA with and without ANF. Figure 4A shows that the treatment with NAA did not significantly change the SNO content in roots. The treatment with ANF, however, increased SNO content in roots in a dose-dependent manner and independently of the auxin treatment.

The biotin switch is a three-step method that allows labelling with biotin of the NO-modified Cys residues in proteins (Jaffrey and Snyder, 2001). Arabidopsis root extracts were treated with either ANF or DNCB for 2 h and then *in vitro* nitrosylated with CysNO for 30 min. The nitrosylated proteins were detected by the biotin switch method followed by immunoblot with the anti-biotin antibody. Figure 4B shows that treatment with the inhibitors ANF or DNCB increases the amount of the *in vitro* S-nitrosylated proteins in a dose-dependent manner, supporting that NTR functions in protein denitrosylation in arabidopsis roots.

The level of S-nitrosylated proteins is impaired in the arabidopsis mutant *ntra ntrb*

A genetic approach with the arabidopsis *ntra ntrb* double mutant was used to analyse the role of NTR in the regulation of the level of S-nitrosylated proteins. The *ntra ntrb* plants showed a 50% reduction of total reductase activity with respect to wild-type seedlings (Supplementary Data Fig. S3). Root extracts from wild-type and mutant seedlings were *in vitro* S-nitrosylated with CysNO for 30 min and the level of S-nitrosylated proteins was detected by the biotin switch method using ascorbate as a specific reductant of S-nitrosothiols. Figure 5A shows that the double mutant *ntra ntrb* displays an increased level of S-nitrosylated proteins with respect to the wild-type roots. The difference of signal between the wild-type and *ntra ntrb* mutant was not detected when dithiothreitol was used as reductant, which is able to reduce oxidized Cys (disulfides, sulfenic acid and S-nitrosothiols) (Fig. 5B). This genetic approach further supports the participation of the NTR–Trx system in modulation of the S-nitrosylation pattern in root proteins.

DISCUSSION

The NTR–Trx redox system in plants has been proposed to function in oxidative stress physiology, playing an antioxidant role (Vieira Dos and Rey, 2006). Indeed, in nuclei from wheat seeds cells that suffer high oxidative stress, this system supports the peroxiredoxin-dependent scavenging of H₂O₂ (Pulido et al., 2009). In addition, the NTR–Trx system has an important role as a modulator of plant growth and development, although this function remains poorly known. Given the complexity of the *h*-type Trx isoforms in plants, the role of the NTR–Trx system in growth and developmental processes was addressed by analysing NTR-deficient mutants as arabidopsis contains only two genes encoding non-plastidial NTRs, termed NTRA and NTRB (Serrato et al., 2004). Although the arabidopsis *ntra ntrb* mutant shows an almost wild-type phenotype, a more detailed characterization revealed decreased pollen fertility, slower plant growth and wrinkled seed phenotype (Reichheld et al., 2007).

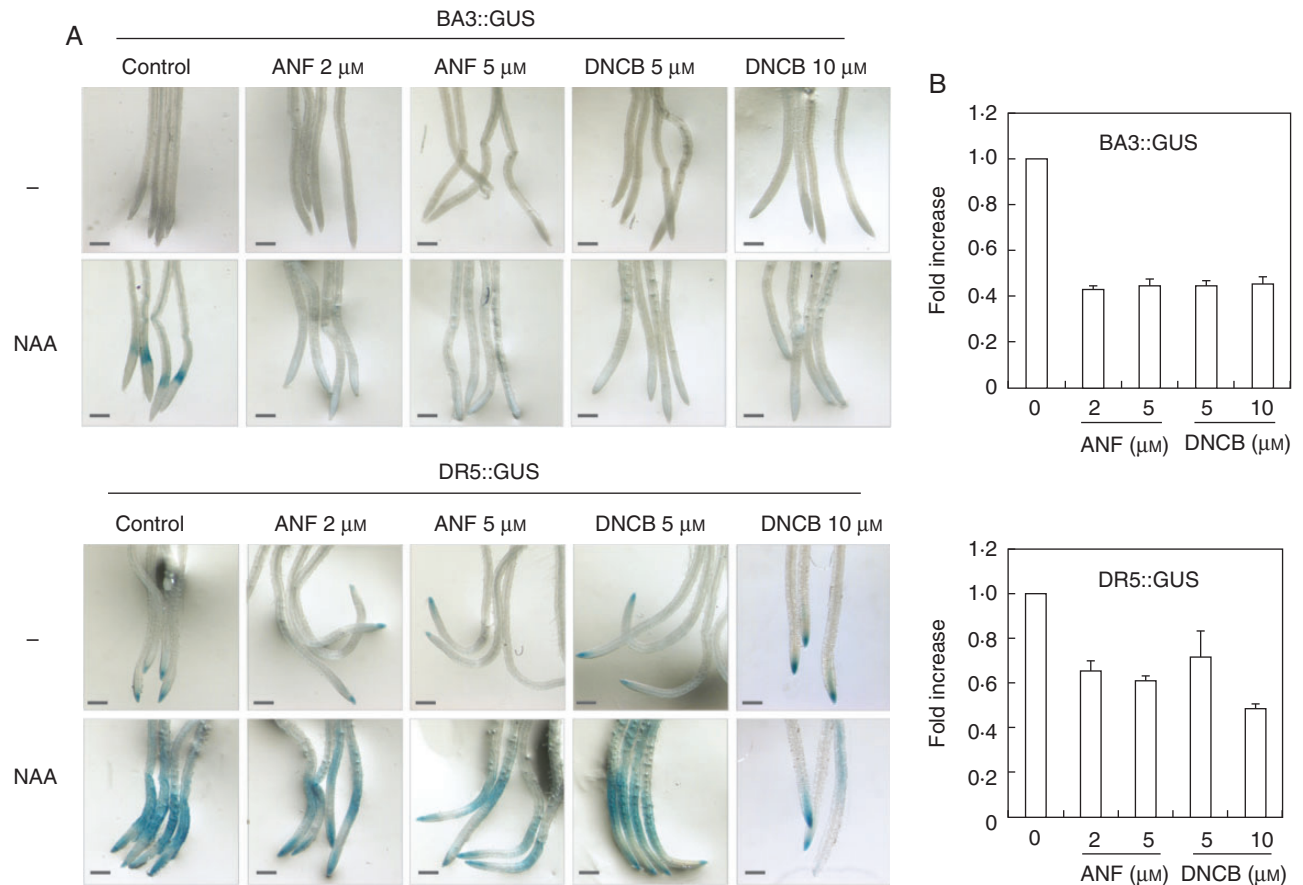


FIG. 3. Inhibitors of NTR activity interfere with auxin response. *Arabidopsis thaliana* transgenic plants expressing the BA3::GUS and DR5::GUS genes were grown in ATS-agar for 4 d. Plants were incubated in ATS solution containing 0.5 μM NAA or not (–) and with or without (control) the NTR inhibitors either ANF or DNCB, at the indicated concentrations, for 18 h. Plants were incubated at 37 °C in the presence of the substrate X-Glu for 2 h. Pictures were taken under a magnifier. Scale bars = 2 mm. (B) Densitometric analysis of roots treated with NAA with or without the inhibitors ANF and DNCB using Image J software. Bars denote standard error of at least two independent experiments ($n = 5$).

Furthermore, the GSH deficiency in the *ntra ntrb* mutant background resulted in an altered root meristem, indicating the coordinated action of both redox systems, GSH and NTR–Trx, in this developmental process (Reichheld *et al.*, 2007). The triple mutant *ntra ntrb cad2*, which combines the deficiency of NTR with the deficiency of γ -glutamylcysteine synthetase, the first enzyme of GSH synthesis, shows loss of apical dominance and reduced LR number, processes in which auxin plays an important role. Indeed, the *ntra ntrb cad2* mutant has altered auxin signalling, decreased auxin levels and impaired auxin transport capacities (Bashandy *et al.*, 2010), thus explaining the impairment of developmental processes controlled by auxin. These results suggest participation of the NTR–Trx and GSH systems in auxin metabolism or signalling, although the redox-sensitive targets of this pathway are not known. Here we have addressed this question using both biochemical and genetic approaches. The results show that auxin treatment induces NTR activity in roots of 7-d-old *Arabidopsis* plants. The NTR activity inhibitors ANF and DNCB blocked auxin-induced LR formation and gene expression, supporting a role of NTR in the auxin signal transduction pathway. Moreover, our results show that the NO donor SNP is also able to induce NTR activity, while depletion

of endogenous NO results in blockage of the auxin-induced NTR activity, suggesting that NO is required for the auxin-mediated increase in NTR activity.

In animal cells, one of the physiological functions proposed for the NTR–Trx system is protein denitrosylation (Nikitovic and Holmgren, 1996; Benhar *et al.*, 2008, 2010; Wu *et al.*, 2011). Treatment with NTR inhibitors increases the level of *S*-nitrosylated proteins in murine macrophage cells, thus lending support to the function of the NTR–Trx system in the control of *S*-nitrosylation (Tello *et al.*, 2009). In plants, participation of the NTR–Trx system in protein denitrosylation was first described in the salicylic acid (SA)-dependent defence response against pathogen attack. In response to SA, the non-expressor of pathogenesis-related 1 protein (NPR1) is denitrosylated by Trx, which favours an oligomer to monomer conversion that promotes NPR1 translocation to the nucleus (Tada *et al.*, 2008). Kneeshaw *et al.* (2014) showed that cytosolic Trxh5 exhibits protein denitrosylating activity during SA-dependent plant immunity, discriminating among different SNO protein targets. In another study, the use of the NTR inhibitor DNCB in plant protoplasts resulted in an increase of NO donor-induced protein SNO content while no effect was observed with ANF

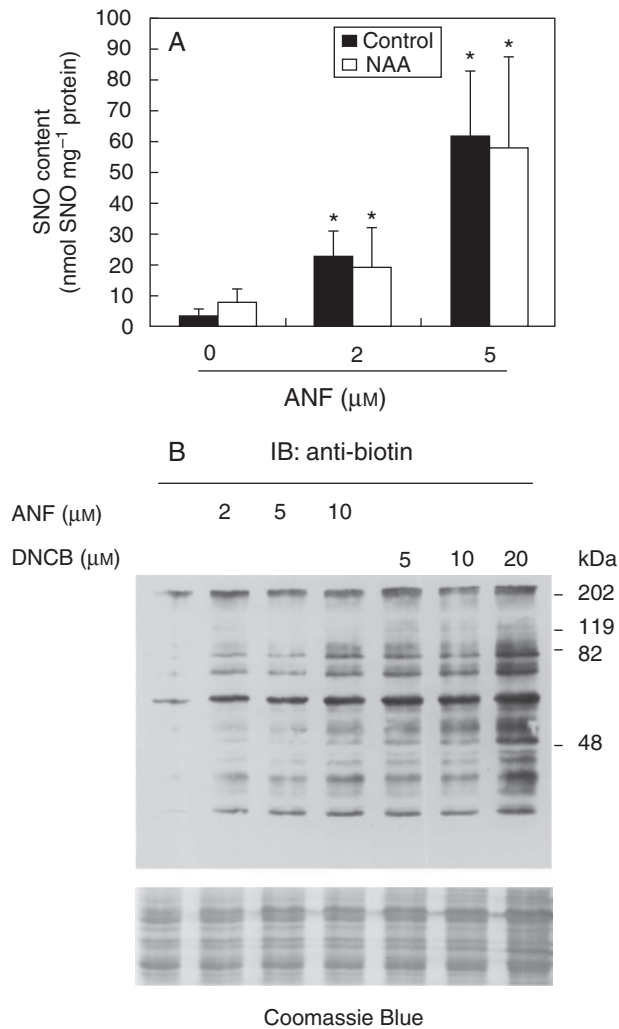


FIG. 4. NTR inhibition produces an increase in the content of *S*-nitrosothiol and *in vitro* protein *S*-nitrosylation. (A) Arabidopsis plants were grown in ATS-agar for 7 d and then transferred to plates containing ATS-agar supplemented or not (control) with 1 μM NAA in the absence or presence of auranofin (ANF) at the indicated concentrations. *S*-nitrosothiol content in root extracts were measured by the Saville–Griess method. Bars indicate standard error ($n = 3$). Asterisks indicate significant differences for the ANF treatment with respect to untreated (t -test, $P < 0.05$). (B) Arabidopsis root extracts (60 μg) were treated with different concentrations of the NTR inhibitors ANF and DNCB for 2 h, and then *in vitro* *S*-nitrosylated with 500 μM CysNO for 30 min. *S*-nitrosylated proteins were detected by the biotin switch method using anti-biotin antibody. Molecular weight markers, in kDa, are indicated on the right.

(Kneeshaw *et al.*, 2014). Here we show that the treatment of arabidopsis roots with ANF and DNCB increases SNO content and protein *S*-nitrosylation independently of auxin treatment. Moreover, the arabidopsis *ntra ntrb* mutant shows an increase of *S*-nitrosylated proteins in roots, as compared with the wild-type. Together, these results suggest that NTR activity is able to promote protein denitrosylation and, as a consequence, is an active component modulating the level of the *S*-nitrosylated proteins in plant roots. In mammalian systems, ANF was shown to inhibit NTR at nanomolar concentrations. At higher concentrations (1–50 μM) ANF inhibits also glutathione reductase (GR) enzyme (Gromer *et al.*, 1998). Thus, although these drugs are

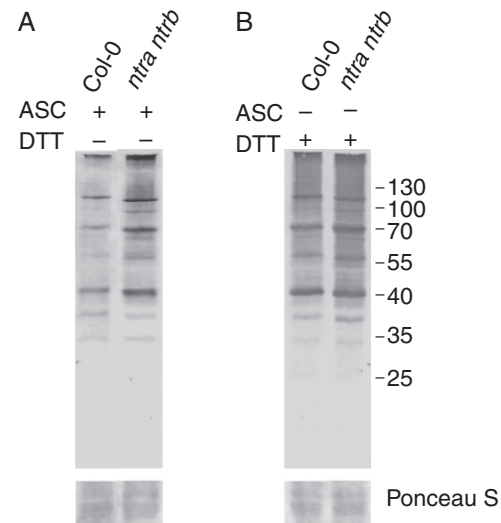


FIG. 5. The arabidopsis mutant *ntra ntrb* has increased levels of *S*-nitrosylated proteins. Arabidopsis root extracts of wild-type or *ntra ntrb* mutants were *in vitro* *S*-nitrosylated with 500 μM CysNO for 30 min. (A) *S*-nitrosylated proteins were detected by the biotin switch method using 10 mM ascorbate (ASC) as a specific reductant of *S*-nitrosothiols. (B) Total oxidized Cys were detected by the biotin switch method using 20 mM dithiothreitol (DTT). Biotinylated Cys were detected by an anti-biotin antibody.

commonly used as NTR inhibitors we cannot discard a effect on GSH metabolism or other cellular reductase activities in plants.

The auxin-dependent induction of NTR activity may promote denitrosylation/reduction of specific target proteins required for the hormone signal transduction pathway. The specific Trxs involved in this pathway remain unknown. Recent results have demonstrated that cytosolic APX1 is denitrosylated and partially inhibited by auxin treatment. Denitrosylated APX1 is less active than the *S*-nitrosylated form of the enzyme, and therefore denitrosylation probably contributes to the increase in H₂O₂ concentration in auxin-treated arabidopsis roots (Correa-Aragunde *et al.*, 2013). Treatment of roots with ANF resulted in increased levels of *S*-nitrosylated APX1 and activity, suggesting that the NTR–Trx system is involved in the denitrosylation of APX1 and modulation of its activity (Correa-Aragunde *et al.*, 2013). Accordingly, APX1 was shown to be a target of *h*-type Trxs (Marchand *et al.*, 2004; Yamazaki *et al.*, 2004; Gelhaye *et al.*, 2006). Therefore, the NTR–Trx redox system seems to play an important function in the modulation of auxin response in plants, in agreement with previous reports (Bashandy *et al.*, 2010), but new proteomic studies are required to identify more targets of Trx-mediated protein denitrosylation/reduction during auxin signalling, including transport and control of hormone levels.

Our results indicate that auxin and NO induce NTR activity and that this activity correlates with protein denitrosylation. Auxin promotes the increase of NO levels in roots enzymatically through nitrate reductase (NR) and NOS-like activities (Kolbert *et al.*, 2007; Flores *et al.*, 2008; Mendez-Bravo *et al.*, 2010; Wang *et al.*, 2010). Moreover, the auxin-mediated increase of NO concentration could be derived from protein denitrosylation. In animal systems, this was described as a novel cellular mechanism of NO production generated from

intracellular *S*-nitrosothiol storage. Chvanov *et al.* (2006) described the rapid Ca²⁺-dependent NO release from *S*-nitrosothiols in isolated pancreatic acinar cells stimulated by acetylcholine. In addition, extracellular ATP stimulates production of NO in human monocytes from intracellular *S*-nitrosothiol groups rather than *de novo* synthesis (Hewinson *et al.*, 2008). In plants, the critical role played by the *S*-nitrosothiol pool in the regulation of N assimilation in arabidopsis has been reported (Fruingillo *et al.*, 2014). Thus, *S*-nitrosothiols, in fact, provide a rapid source of NO available in cells, rather than *de novo* NO biosynthesis by the action of NOS or NR. The auxin-mediated increase of NO in roots may promote *S*-nitrosylation of several targets required for hormone response, such as the auxin receptor transport inhibitor response 1 (TIR1). Terrile *et al.* (2012) showed that TIR1 *S*-nitrosylation enhances TIR1–Aux/indole-3-acetic acid (IAA) interaction, facilitating Aux/IAA degradation and subsequently promoting auxin-dependent gene expression. In addition, the fact that high NO concentration induces NTR activity suggests that a feedback mechanism to control massive and unregulated protein *S*-nitrosylation could be operating in plant cells.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: effect of auranofin on thioredoxin reductase activity in arabidopsis roots. Figure S2: NTR protein levels in auxin-treated arabidopsis roots. Figure S3: western blot and NTR activity in the *ntra ntrb* mutant.

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