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RESEARCH PAPER

Auxin induces redox regulation of ascorbate peroxidase 1 activity by S-nitrosylation/denitrosylation balance resulting in changes of root growth pattern in *Arabidopsis*

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Abstract

S-Nitrosylation of Cys residues is one of the molecular mechanisms driven by nitric oxide (NO) for regulating biological functions of key proteins. While the studies on S-nitrosylation of Cys residues have served for identifying SNO proteomes, the physiological relevance of protein S-nitrosylation/denitrosylation remains poorly understood. In this study, it is shown that auxin influences the balance of S-nitrosylated/denitrosylated proteins in roots of *Arabidopsis* seedlings. 2D-PAGE allowed the identification of ascorbate peroxidase 1 (APX1) as target of auxin-induced denitrosylation in roots. Auxin causes APX1 denitrosylation and partial inhibition of APX1 activity in *Arabidopsis* roots. In agreement, the S-nitrosylated form of recombinant APX1 expressed in *Escherichia coli* is more active than the denitrosylated form. Consistently, *Arabidopsis apx1* mutants have increased H_2O_2 accumulation in roots, shorter roots, and less sensitivity to auxin than the wild type. It is postulated that an auxin-regulated counterbalance of APX1 S-nitrosylation/denitrosylation contributes to a fine-tuned control of root development and determination of root architecture.

Key words: *Arabidopsis*, ascorbate peroxidase 1, auxin, nitric oxide, S-nitrosylation, root.

Introduction

Redox signalling is pivotal in stress perception, photosynthesis regulation, pathogen defence responses, and plant growth and developmental processes (Apel and Hirt, 2004; Gapper and Dolan, 2006; Eckardt, 2010; Tognetti *et al*., 2012). Nitric oxide (NO) is a free radical active in preserving cellular reduction/oxidation homeostasis. As a consequence, NO is involved in numerous physiological processes in the plant life (Lamattina *et al*., 2003; Leitner *et al*., 2009). Regarding plant growth, NO is required during adventitious and lateral root formation, root hair elongation, and gravitropic responses, all processes controlled by the phytohormone auxin. Auxin induces NO production in roots via nitrate reductase and nitric oxide synthase-like activities (Pagnussat *et al*., 2002; Correa-Aragunde *et al*., 2004; Hu *et al*., 2005; Lombardo *et al*., 2006; Kolbert *et al*., 2007).

As a diffusible free radical, NO reacts with sulphhydryl groups in Cys residues and transition metals to form S-nitrosothiols and metal nitrosyls, respectively. Protein S-nitrosylation represents a ubiquitous mechanism for post-translational modification that is emerging with relevant biological functions as O-phosphorylation does (Lane *et al*., 2001; Mannick and Schonhoff, 2002). Proteomic assays in plants have detected S-nitrosothiol modification of enzymes, ion channels, and transcription factors (Lindermayr *et al*., 2005; Abat *et al*., 2008; Romero-Puertas *et al*., 2008). Several target proteins of NO are related with the enzymes involved in reactive oxygen species production and metabolism. S-Nitrosylation of peroxiredoxin II E inhibits hydroperoxide-reducing peroxidase activity (Romero-Puertas *et al*., 2007). In peroxisomes, the H_2O_2-degrading enzymes catalase and

glycolate oxidase are S-nitrosylated proteins and its activities are inhibited by NO donors (Ortega-Galisteo *et al*., 2012). In addition, NADPH oxidase have been described as a new target of S-nitrosylation involved in plant immunity (Yun *et al*., 2011). The regulation of NO level is tightly mutually linked with H_2O_2; NO regulates the levels of H_2O_2 and vice versa (Neill *et al*., 2002). Both NO and H_2O_2 are involved in programmed cell death and pathogen defence responses (Delledonne *et al*., 1998), stomatal closure (Bright *et al*., 2006), and lateral root growth (Wang *et al*., 2010a,b).

Auxin is the main hormone controlling root growth. The primary mechanisms controlling auxin action involve auxin biosynthesis, conjugation, catabolism, and transport. The auxin receptors are the F-box TIR1 and its paralogues AFB1, AFB2, and AFB3, subunits of SCF E3-ligase complex. Auxin binds to SCFTIR1 and triggers the ubiquitination of the auxin repressors Aux/IAA proteins and its degradation by the proteasome. The degradation of Aux/IAA leads to the release of the auxin transcription factors ARFs with the consequent induction of auxin-responsive genes (Calderon-Villalobos *et al*., 2010). Very recently, it has been demonstrated the S-nitrosylation of the auxin receptor TIR1 (Terrile *et al*., 2012).

This work studied whether auxin and NO could regulate the balance of S-nitrosylated/denitrosylated proteins that take part of the auxin signalling during root growth. Through biotin switch assays, it was shown that auxin treatment affects the level of S-nitrosylated proteins. Ascorbate peroxidase 1 (APX1) was found to be denitrosylated and its enzyme activity partially inhibited in auxin-treated *Arabidopsis* roots. APX1 is a cytosolic enzyme involved in H_2O_2 degradation using ascorbate as the electron donor. *APX1* is highly expressed in *Arabidopsis* roots (Fourcroy *et al*., 2004). To date, studies on the APX1 role in *Arabidopsis* have been focused on the reactive oxygen species-scavenging mechanism triggered by stress conditions (Davletova *et al*., 2005; Vanderauwera *et al*., 2011; Maruta *et al*., 2012). In this report, the participation of APX1 in auxin-mediated root growth process is investigated.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col-0) ecotype seeds were surface-sterilized in 30% (v/v) bleach and 0.02% (v/v) Triton-X100 for 15 min, rinsed with sterile water, and stratified at 4 °C for 2 days. *Arabidopsis* mutant *tir1-1 afb2-3* is in the Columbia ecotype and *apx1* is in Wassilewskija (WS) ecotype. Seeds were plated on ATS medium 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 under 100 µmol photons m^{-2} s^{-1} and a 16/8 light/dark cycle. For growth measurements, seedlings were grown for 4 days and then transferred to the auxin naphthyl acetic acid (NAA) or sodium nitroprusside provided by Sigma (St Louis, MO, USA). The density of lateral roots and the length of the primary roots were determined after 4 days of growth. For APX activity assays and 2D analysis, seedlings were grown for 7 days and then transferred to the treatments for 1 day.

Detection of H_2O_2

Roots were incubated in 15 µM of the peroxide-sensitive dye 2,7′-dichlorodihydrofluorescein diacetate (H2DFDA) (Molecular Probes, Eugene, OR, USA) in 10 mM MES buffer (pH 6) and 10 mM KCl for 20 min followed by 20 min wash in fresh buffer. Roots were visualized under bright field and epifluorescent microscopy (Nikon).

APX and TrxR activity assays

Root proteins were extracted with 50 mM phosphate buffer (pH 7.5), 1 mM EDTA, and 1 mM ascorbic acid. APX activity was measured following the H_2O_2-dependent oxidation of ascorbic acid at 290 nm (extinction coefficient = 2.8 mM^{-1} cm^{-1}) as described by Amako *et al*. (1994). To measure the activity of S-nitrosylated recombinant APX1, protein extracts from bacteria expressing APX1 were treated with the S-nitrosylating agent nitrosocysteine (CysNO) for 20 min. Then proteins were desalted by Micro Bio-Spin columns with Bio-Gel P-6 (Bio-Rad). Thioredoxin reductase (TrxR) activity was measured 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 and followed at 412 nm. For inhibition experiments of TrxR activity, samples were incubated with 5 µM auranofin (ANF) for 15 min previous to the measurement of TrxR activity.

Biotin switch

The biotin switch was performed according to Jaffrey and Snyder (2001) with minor modifications. *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). For *in vitro* S-nitrosylation, protein extracts were incubated with CysNO in the dark for 1 h. Samples were incubated with 30 mM methyl methanethiosulphate (MMTS, Sigma) and 3.3% (w/v) SDS in HEN buffer at 50 °C for 20 min and precipitated with 2 volumes of acetone. Samples were resuspended in HEN buffer, 1% (w/v) SDS, 20 mM ascorbic acid, and 1 mM biotin-HPDP (Pierce Chemical) and incubated for 1 h. Proteins were then subjected to immunoblot using an anti-biotin antibody (Sigma).

Purification of S-nitrosylated proteins

Purification of S-nitrosylated proteins was performed according to Forrester *et al*. (2009). For proteomic assays, the starting material was 8 mg protein. Biotinylation of S-nitrosylated proteins was carried out as described above. Samples were incubated with 30 µl of neutravidin agarose resin (Thermo Scientific) per milligram of initial protein with three volumes of neutralization buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.5%, v/v, Triton X-100, pH 7.5) and overnight at 4 °C. The resin was washed four times with 1 ml neutralization buffer containing 600 mM NaCl. Bound proteins were eluted with HEN/10 buffer containing 1% (v/v) of β-mercaptoethanol.

2D-PAGE and MS analyses

Purified proteins were resuspended in a buffer containing 8 M urea, 2% (w/v) CHAPSO, 20 mM DTT, 2% (v/v) carrier ampholytes (pH 4–7), and 0.002% (w/v) bromophenol blue. IEF was performed as applying a voltage gradient up to 10,000 V with a limiting current of 50 µA per strip. The strips were loaded onto the second dimension and run at 15 mA/gel. Spots were excised and subjected to in-gel trypsin digestion. Mass spectrometric data were obtained using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker), in the mass spectrometry facility at the CEQUIBIEM mass spectrometry centre, Argentina.

Cloning and expression of AtAPX1 in Escherichia coli

The ORF of *AtAPX1* was amplified from PUNI51 vector containing the full-length cDNA provided by the *Arabidopsis* Biological Resource Center. Primers were designed with an *Eco*RI site at the

5′-end (5′-CGAATTCATGACGAAGAACTACCCAACCGTGAG CGAAGATTACAAGAAGGCTGTTGAG-3′) and with a c-Myc tag and *Xho*I site followed by a stop codon at the 3′ end (5′-GCTCGAGTCACAGGTCCTCCTCTGAGATCAGC TTCTGCTCAGCATCAGCAAACCCAAG-3′). The amplified product was ligated in pET24b vector (Novagen) and transformed BL21 Rosetta strain. Soluble expressed proteins were extracted by sonication in 50 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM ascorbic acid, and 1 mM PMSF. Recombinant *APX1* was detected by immunoblot using anti c-Myc antibody (Sigma) or cytosolic APX antibody (Agrisera, Sweden).

Homology modeling and alignment

Structural models were built with the program @TOME (Pons and Labesse, 2009). The figures were drawn using Web Lab ViewerLite 3.20 software (Molecular Simulations). Cytosolic APX sequences were aligned using Clustal X software (version 1.81) and edited with GeneDoc software (version 2.5.010).

Densitometry and statistical analysis

Densitometry analyses of gel bands were performed with Matrox Inspector v2.2 (Matrox, Quebec, Canada). Root fluorescence was quantified by optical densitometry using ImageJ software. Statistical analysis was performed employing SigmaStat (Jandel Scientific, San Rafael, CA, USA), using ANOVA and Tukey tests for multiple comparison analyses and the t-test for pair-wise comparisons.

Results

Detection of S-nitrosylated proteins in auxin-treated Arabidopsis roots

The effect of NO and auxin on root growth was studied in *Arabidopsis* seedlings with the NO donor sodium nitroprusside and the synthetic auxin NAA. As previously reported (Casimiro *et al*., 2001), Fig. 1A and B show that the treatment with 0.1 μM NAA inhibits primary root elongation and stimulates lateral root formation. Similarly but to a lesser extent, the treatment with 10 μM sodium nitroprusside induces 2-fold increase in lateral root density and inhibit primary root growth (Fig. 1A and B). The treatment of *Arabidopsis* with the NO scavenger CPTIO inhibits the NAA-mediated lateral root formation, suggesting that NO is required for the auxin

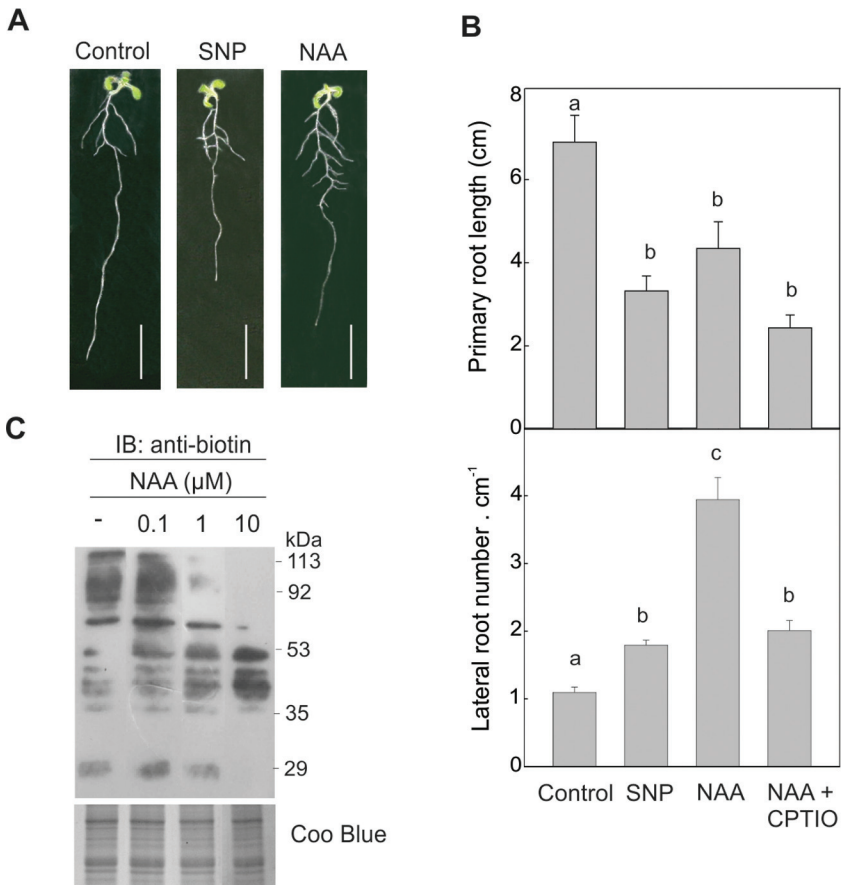

Fig. 1. Auxin affects lateral root density, primary root length, and the pattern of S-nitrosylated proteins in *Arabidopsis*. (A) Four-day-old *Arabidopsis* seedlings treated with 0.1 μM naphthyl acetic acid (NAA) with or without 0.5 mM of the NO scavenger CPTIO or 10 μM of the NO donor sodium nitroprusside (SNP) for 4 days; bar, 1 cm. (B) Primary root length and lateral root density; data represent mean and SE of three experiments ($n = 6$); letters represent significant difference (ANOVA, $P < 0.05$). (C) Effect of auxin in the pattern of S-nitrosylated proteins; 7-day-old *Arabidopsis* seedlings treated with NAA for 1 day; root extracts were S-nitrosylated *in vitro* with 0.5 mM CysNO for 1 h; S-nitrosylated proteins were detected by the biotin switch method using an anti-biotin antibody (IB: anti-biotin); Coo Blue, Coomassie blue staining.

response (Fig. 1B). As was previously described, NO production is stimulated by auxin in roots of several plant species (Correa-Aragunde *et al*., 2004; Kolbert *et al*., 2007; Terrile *et al*., 2012).

To analyse the effect of auxin in the pattern of S-nitrosylated protein in roots, *Arabidopsis* seedlings were treated with increasing concentrations of NAA and then root extracts were S-nitrosylated with the nitrosylating agent nitrosocysteine (CysNO). Fig. 1C shows that the treatment with NAA modifies the pattern of S-nitrosylated proteins in roots. NAA treatment increases the level of S-nitrosylated protein among 35–53 kDa whereas diminishes the level of S-nitrosylated proteins with molecular weights higher than 53 kDa and below 35 kDa. This result suggests that while some proteins are favoured to be S-nitrosylated other target proteins seem to be denitrosylated by the auxin treatment. To improve the analysis, S-nitrosylated proteins were separated in a 2D-PAGE and identified by MS.

APX1 is denitrosylated by auxin treatment in Arabidopsis roots

To identify the S-nitrosylated proteins affected by auxin treatment, approximately 8 mg of root protein extracts from H_2O (control)- or NAA-treated *Arabidopsis* were subjected to the biotin-switch method, purified by neutravidin affinity chromatography, and separated by 2D-PAGE. Fig. 2A shows more than 40 polypeptides clearly detected in the range pH 4–7 in control roots. Supplementary Fig. S1 (available at *JXB* online) shows the comparative intensity of spots in the 2D-PAGE from the control and NAA treatments. The most abundant 15 spots were chosen for MS. Four out of 15 gave protein identification (Fig. 2A, right). The other 11 spots were unable to be identified. The analysis of the proteins identified reveals that three of them have already been detected as S-nitrosylated targets in other biotin-switch proteomic assays in *Arabidopsis* (Supplementary Table S1). Enolase (2-phospho-D-glycerate hydrolase), triose-phosphate isomerase, and ascorbate peroxidase 1 (APX1) have been already identified as target proteins for S-nitrosylation in *Arabidopsis* (Lindermayr *et al*., 2005; Romero-Puertas *et al*., 2008; Fares *et al*., 2011). In contrast, a copper-binding MLP-like protein is now reported as a new target of S-nitrosylation in *Arabidopsis* roots (Fig. 2A, right). The four identified proteins diminished its S-nitrosylation levels in NAA treatment (Supplementary Fig. S1).

APX1 was chosen to continue the study since many reports have pointed out the correlation between oxidative stress and auxin signal transduction (Vernoux *et al*., 2000; Bashandy *et al*., 2010; Wang *et al*., 2010a,b; Tognetti *et al*., 2012). However, the information about the mechanism of how auxin regulates the redox changes leading to root development is scarce. Fig. 2A (centre) shows that the level of S-nitrosylated APX1 diminished 35% in NAA-treated roots with respect to control. Consequently, this study was interested in knowing the connection between auxin actions on root growth through the control of APX1 activity.

To confirm the S-nitrosylation of APX1, *Arabidopsis* root proteins were subjected to the biotin switch method and biotinylated proteins were purified by a neutravidin affinity chromatography. APX1 was detected by immunoblot using an anti-APX antibody. Fig. 2B shows that, under these conditions, the S-nitrosylated forms of APX1 are found as high-molecular-weight forms, probably including the homodimer form of APX1. Cytosolic APX is a dimer consisting of identical subunits with a molecular mass of 28 kDa (Mittler and Zilinskas, 1991). The NAA treatment does not induce a significant reduction of the level of S-nitrosylated APX1 (Fig. 2B). However, when one of the enzymatic activities responsible of protein denitrosylation the thioredoxin reductase (TrxR)-thioredoxin (Trx) system (Benhar *et al*., 2009) is inhibited with the TrxR inhibitor ANF, the level of S-nitrosylated APX1 increases up to 5-fold with respect to the control (Fig. 2B). The inhibitory effect of ANF on the plant TrxR activity was confirmed in *Arabidopsis* roots (Supplementary Fig. S2). To check the effect of NO in the transition of the APX monomer to the high-molecular-mass form of APX, protein root extracts were treated *in vitro* with CysNO. Fig. 2C shows that the treatment with CysNO stabilizes the high-molecular-mass form of APX1. Furthermore, the addition of the thiol reducing agent DTT to root extracts switches the high-molecular-mass form of APX1 to the monomer (Fig. 2C).

APX1 contains five Cys that could be potential target of S-nitrosylation (Fig. 3A). Cys32 and Cys168 are almost conserved among all described cytosolic APXs in plants (Fig. 3B and Supplementary Fig. S3). Cys32 is located near the ascorbate binding site (Fig. 3C; Mandelman *et al*., 1998) while Cys168 is located near the haem-binding motif. The distance between SH group of Cys168 and Fe ion of the haem group is 6.72 Å (Fig. 3C). The Cys32 was already reported to be S-nitrosylated in *Arabidopsis* (Fares *et al*., 2011). Local hydrophobicity within primary or tertiary protein structure might promote S-nitrosylation since it increases thiol nucleophilicity and concentrate nitrosylating equivalents (Hess *et al*., 2005; Marino and Gladyshev, 2010). The hydrophobicity value by Kyte-Doolittle scale showed that it is considerably high (value of 0.615) in the sequence flanking Cys32 in APX1, while the average of Cys168 is rather low (–1.308; Supplementary Fig. S4). At structural level, both Cys32 and Cys168 are surrounded by hydrophobic amino acids within a 6 Å radius from the sulphur atom (Supplementary Fig. S4). When longer distances (up to 8 Å) from the sulphur atom of Cys32 and Cys168 are considered, charged amino acids appear (Supplementary Fig. S4). These characteristics are shared among most CysNO of S-nitrosylated proteins reported so far (Marino and Gladyshev, 2010).

To study the effect of auxin on APX1 activity in roots, *Arabidopsis* seedlings were treated with increasing concentrations of NAA. Fig. 3D shows that APX activity is inhibited up to 30% in NAA-treated *Arabidopsis* roots. APX1 activity restores to control levels when NAA-treated roots are incubated in the presence of ANF. Monomeric APX1 protein levels are unaffected by these treatments (Fig. 3D, top), while the dimeric form could not be detected in this assay. NAA treatment was unable to inhibit APX1 activity in the *Arabidopsis* auxin-receptor mutant *tir1 afb2*, indicating that

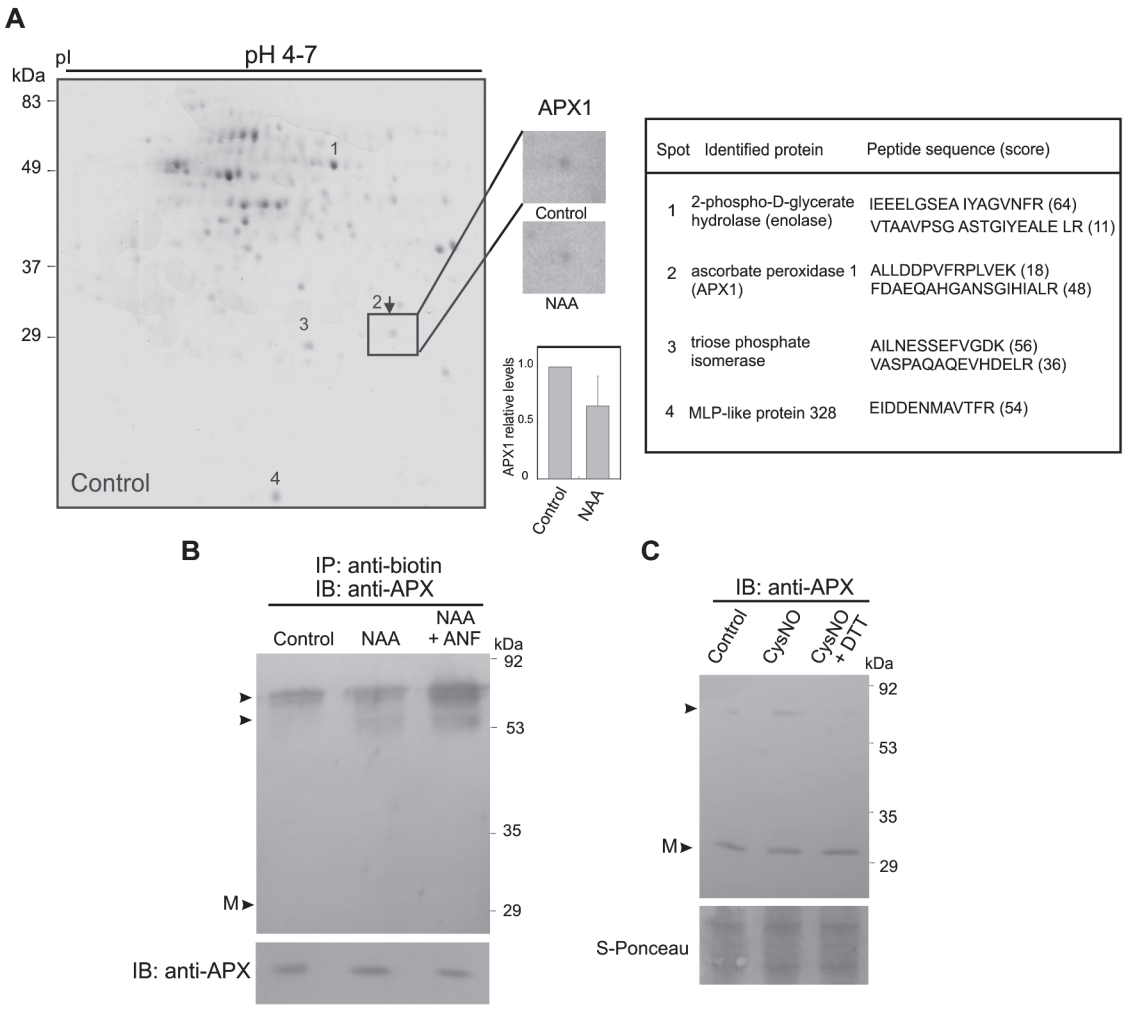

Fig. 2. Ascorbate peroxidase 1 (APX1) is S-nitrosylated in *Arabidopsis* roots. (A) Seven-day-old *Arabidopsis* seedlings treated with H_2O (control) or 1 μM naphthyl acetic acid (NAA) for 1 day; root extracts were subjected to the biotin switch method; biotinylated proteins were purified with neutravidin beads and analysed by 2D-PAGE; the experiment was repeated three times with similar results; numbers indicate identified proteins by MS. (B) Four-week-old seedlings treated with H_2O (control), 1 μM NAA, or 1 μM NAA plus 2 μM auranofin (ANF); root extracts were subjected to the biotin switch method; biotinylated proteins were purified with neutravidin beads (IP: anti-biotin), and APX1 was detected by immunoblot (IB: anti-APX); to verify protein loading, APX1 was detected by immunoblot before neutravidin purification. (C) Root extracts treated with 0.5 mM CysNO with or without 50 mM DTT; APX1 was detected by immunoblot (IB: anti-APX). Arrowheads indicate high-molecular-weight forms of APX1; M, monomer; Ponceau red staining was used to confirm equal loading.

an active TIR1/AFB2-mediated auxin signalling is required for the auxin-induced inhibition of APX1 activity (Fig. 3D, right).

S-Nitrosylated recombinant AtAPX1 is more active than the denitrosylated form

The ORF of *AtAPX1* was cloned into pET24b expression vector with a c-Myc tag in the C-terminal. *E. coli* BL21 was transformed with pET24b-*APX1* and induced with IPTG. Fig. 4A shows that the maximal APX1 activity was reached at 5 h of induction with 0.1 mM IPTG. The expression of IPTG-induced recombinant APX1 was verified by immunoblot and the biological activity of the recombinant APX1 was analysed in bacteria by examining the tolerance to oxidative stresses (Supplementary Fig. S5). Supplementary Fig. S5B shows that bacteria expressing recombinant APX1 are more tolerant to treatment with either NaCl or H_2O_2 than bacteria carrying the empty vector.

Soluble proteins from bacteria expressing APX1 were S-nitrosylated *in vitro* with CysNO, and then the CysNO was removed by gel filtration to prevent APX1 inhibition by reversible binding of NO to the haem group. Fig. 4B shows that the treatment with 50 μM CysNO increases APX1 activity 1.45-fold with respect to untreated APX1. Addition of 1 mM DTT to CysNO-treated extract abolishes any increase of APX1 activity (Fig. 4B). Treatment with CysNO was able to S-nitrosylate the monomeric form of APX1 (Fig. 4C). This study did not observe the high-molecular-form of recombinant APX in the bacterial extracts, probably due to the reducing environment of bacterial cytoplasm. Very low levels of S-nitrosylated APX1 detected in samples treated

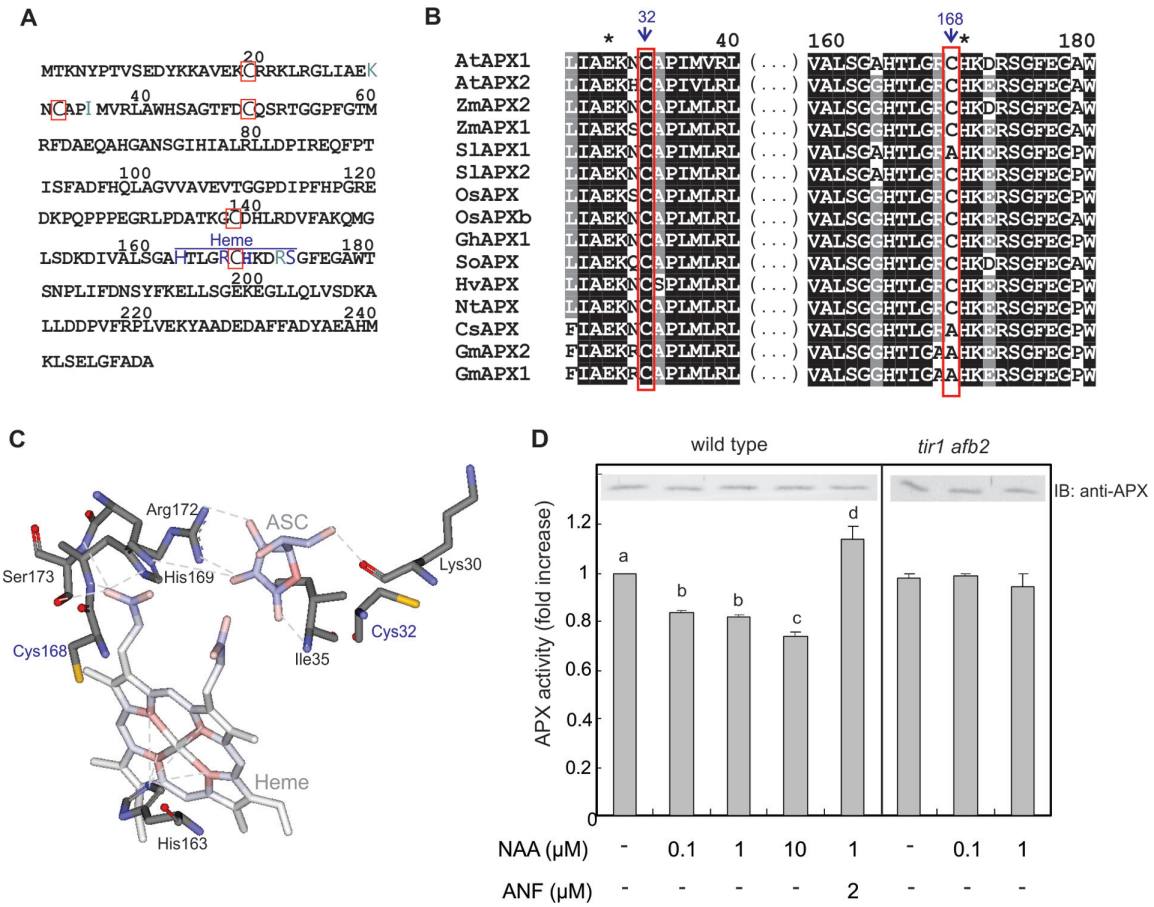

Fig. 3. Structure and activity of ascorbate peroxidase 1 (APX1) from *Arabidopsis* roots. (A) Amino acid sequence of *Arabidopsis* APX1; amino acids that make contact with haem or ascorbic acid (ASC) are depicted in blue or green, respectively. (B) Cytosolic APX alignment showing the conservation in Cys32 and Cys168; black boxes indicate identical amino acids among all sequences; grey boxes indicate identical residues among 12 or 13 sequences. (C) Model of *Arabidopsis* APX1 according to the coordinates of APX from *Glycine max* (PDB code 2VCF); Cys32, Cys168, and amino acids that make contact to haem and ASC are shown. (D) APX activity from roots of *Arabidopsis* wild type and *tir1 afb2* mutant treated with H_2O (control) or different concentrations of naphthyl acetic acid (NAA) or 1 μM NAA plus 2 μM auranofin (ANF) for 1 day; values are fold increase with respect to control; APX activity=1 corresponds to 8.58 μmol ascorbic acid (ASC) min^{-1} (mg protein)$^{-1}$; bars, 1 SE (n = 3); different letters indicate significant difference among treatments (ANOVA, $P < 0.05$). APX1 protein levels were detected by immunoblot.

with 0.5 mM Cys, indicate that endogenous S-nitrosylation of APX1 could occur (Fig. 4C). Supplementary Fig. S6 also shows that the addition of the thiol-reducing agent DTT after S-nitrosylation with CysNO results in the removal of the S-NO bonds.

To detect the S-nitrosylated APX1 high-molecular-weight form, recombinant APX1 was purified from bacteria extracts, S-nitrosylated *in vitro* with CysNO, and subjected to the biotin switch method. S-Nitrosylated APX1 was recognized by an anti-biotin antibody. This strategy allows detecting the S-nitrosylation of the high-molecular-weight APX1 form (Fig. 4D).

Arabidopsis mutant in APX1 results in changes in root architecture

To analyse the role of APX1 in root development, this study used the *apx1 Arabidopsis* mutant. Immunoblot confirmed the absence of APX1 in the homozygous line (Fig. 5A). To analyse the level of H_2O_2 production in *apx1* plants, the peroxide-sensitive dye 2′,7′- dichlorodihydrofluorescein diacetate (H2DFDA) was used. Fig. 5B and C shows that the treatment with auxin induces H_2O_2 production in *Arabidopsis* roots. Under basal conditions, roots of *apx1* mutants accumulate 2-fold-more H_2O_2 than to the wild type (Fig. 5C). To analyse the root phenotype of *apx1* mutant, *Arabidopsis* seedlings were treated with different concentrations of NAA for 4 days. Fig. 5D shows that increasing concentrations of exogenous NAA inhibited primary root elongation in the wild type. Intriguingly, the effect of auxin in the *apx1* background is not statistically significant, suggesting that the mutant is less sensitive to auxin. When lateral root density was quantified, the *Arabidopsis apx1* background was shown to be less sensitive than wild type too. Increased concentrations of NAA did not increase lateral root density in *apx1* as much as in the wild type (Fig. 5D).

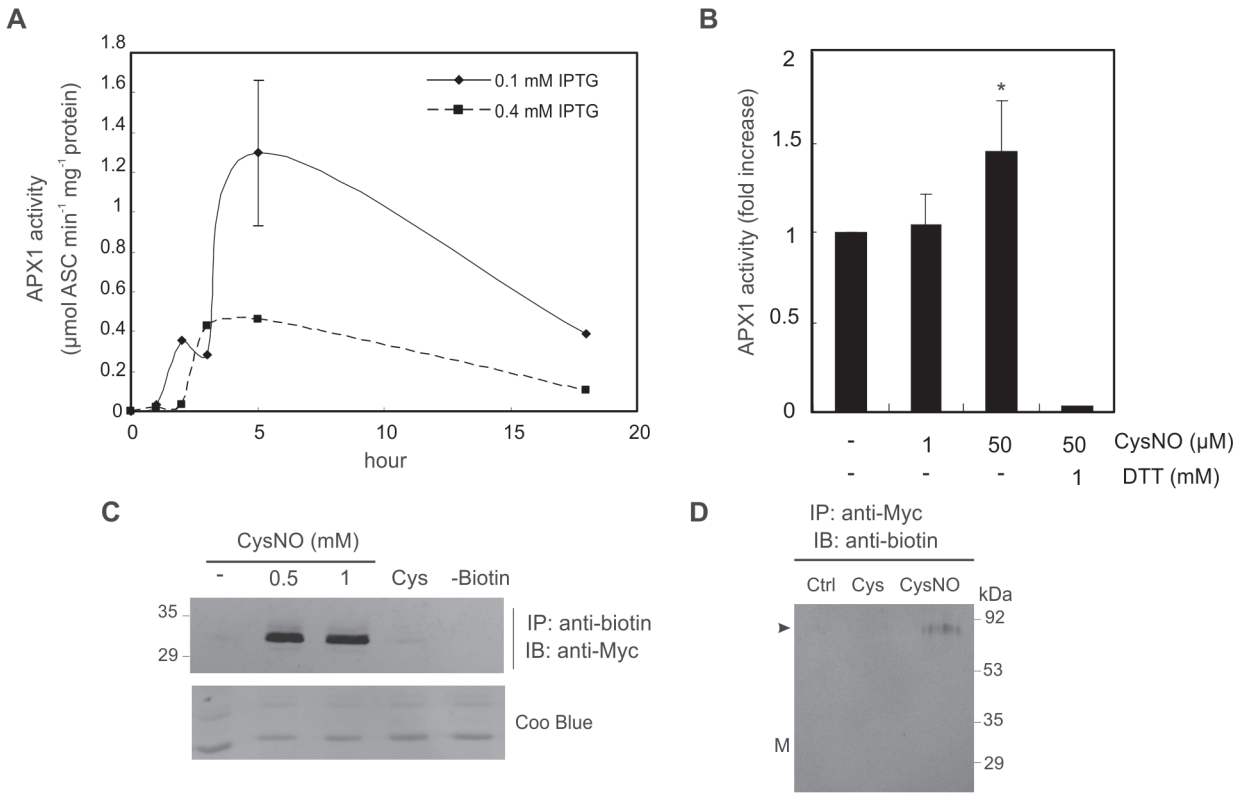

Fig. 4. Expression analysis of the recombinant APX1 in *Escherichia coli.* (A) APX1 activity in *E. coli* expressing pET24b-APX1. (B) APX1 activity from bacterial extracts treated with CysNO in the presence or absence of 1 mM DTT and desalted by bio-gel p6 filtration; APX1 activity = 1 corresponds to 0.706 μmol ascorbic acid (ASC) min^{-1} (mg protein)$^{-1}$. (C) *In vitro* S-nitrosylation of APX1. Protein extracts from bacteria expressing APX1 were S-nitrosylated with CysNO for 1 h. Samples were subjected to the biotin switch method and biotinylated protein were purified with neutravidin beads (IP: antibiotin). The monomeric form of APX1 was identified by immunoblot (IB: anti-Myc). The proteins that were not retained by the column were loaded on gels and stained with Coomassie Blue (Coo Blue) as loading control. (D) APX1 purified using an anti-Myc agarose column (IP: anti-Myc), subjected to the biotin switch method, and detected by immunoblot (IB: anti-biotin).

Discussion

Reactive oxygen species are involved in auxin-induced root growth in Arabidopsis

Auxin is the main plant morphogen that regulates developmental processes, including embryo morphogenesis and root, shoot, and flower development. Although the described auxin signalling pathway is apparently complete (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005), the modulator pathways remain to be integrated in a more comprehensive model. Redox molecules have been involved in auxin transport, metabolism, and signalling during plant development and stress responses (Gapper *et al.*, 2006; Feher *et al.*, 2008; Tognetti *et al.*, 2012). The results presented here indicate that auxin treatment increases H_2O_2 accumulation in *Arabidopsis* roots and that this accumulation may be due to the auxin-mediated regulation of APX1 activity through its partial denitrosylation.

The participation of H_2O_2 in the auxin signalling was already reported in other developmental processes. H_2O_2 plays a role as a downstream component in the auxin-mediated signalling pathway leading to root gravitropic response in *Arabidopsis* (Joo *et al.*, 2001). According to this, the impairment of auxin signalling in the *tir1 afb2 Arabidopsis* mutants resulted in a reduced accumulation of H_2O_2 and superoxide anion (Iglesias *et al.*, 2010). Wang *et al.* (2010b) showed that the *Arabidopsis* carrying a mutation in the prohibitin gene *PHB3*, defective in H_2O_2-induced NO accumulation, was altered in auxin-induced lateral root formation. Alterations in the redox intermediates ASC and GSH are associated with changes in the quiescent centre (Kerk and Feldman, 1995; Jiang *et al.*, 2003) and are involved in the G1 to S transition in the plant cell cycle (Potters *et al.*, 2010). Altogether, the current knowledge indicates that a fine-tuned and spatial-temporal regulation of redox molecules is required for initiation and maintenance of cell division during root development.

Auxin treatment induces denitrosylation and inhibition of APX1 activity in Arabidopsis roots

APX is a haem-containing enzyme that reduces H_2O_2 to water with ascorbate as specific electron donor (Asada, 1992; Shigeoka *et al.*, 2002). APX1 is a cytosolic enzyme as well as APX2 and APX6. Expression of *APX1* in leaves is relatively high with respect to *APX2* and *APX6* (Panchuk *et al.*, 2005) and also in roots according to the Genevestigator

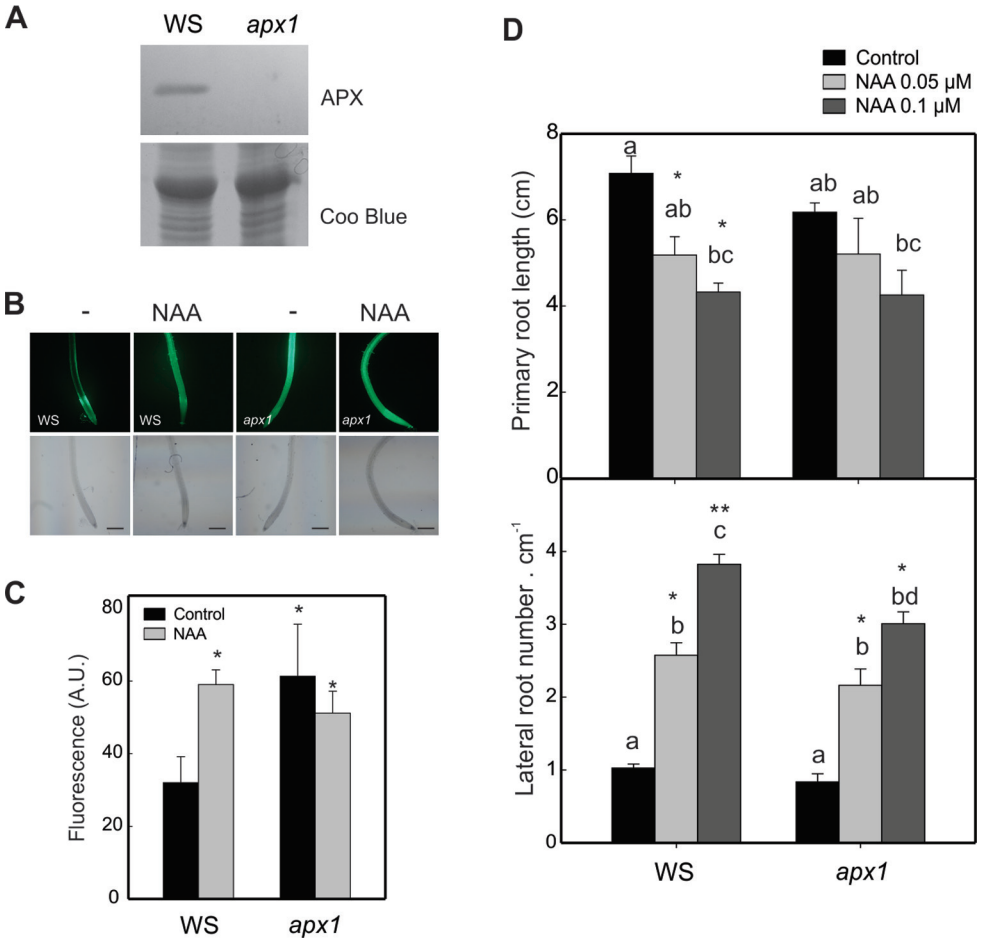

Fig. 5. Root development and H_2O_2 accumulation are affected in *apx1 Arabidopsis* mutant. (A) Detection of cytosolic APX1 in wild type (WS) and *apx1* mutant seedlings using anti-APX. (B) Production of H_2O_2 in *apx1* mutant; *Arabidopsis* seedlings were treated with H_2O (control) or 0.1 µM naphthyl acetic acid (NAA) for 4 days; roots were then incubated in 15 µM of the peroxide-sensitive dye H2DFDA for 20 min; bar, 1 mm. (C) Densitometric analysis of H_2O_2 production using ImageJ software; asterisks denote statistical difference from the respective control value (t-test; $P < 0.05$) (D) Four-day-old seedlings were treated with 0.05 or 0.1 µM NAA for 4 days. Primary root length and lateral root density were measured; data are mean and SE of three experiments ($n = 6$); different letters indicate significant difference (ANOVA, $P < 0.05$); asterisks denote statistical difference from the respective control value (t-test; *, $P < 0.05$; **, $P < 0.01$).

database (Hruz *et al.*, 2008). The current work demonstrates the S-nitrosylation of APX1. High-molecular-weight forms of APX1 were detected to be S-nitrosylated *in vivo*. Even though this study cannot discard the possibility of interaction between APX1 and other proteins (i.e. Trxs) and/or macromolecules, one of these forms may be the homodimer of APX1, since it also appears when the recombinant APX1 purified from bacterial extracts is S-nitrosylated. The intensity of the bands corresponding to the high-molecular-weight forms of APX1 in *Arabidopsis* extracts are relatively low compared to monomeric APX1 form. It cannot be discarded to a low recognition of the monomeric form by the specific APX1 antibody. Blue native gels should probably help to get more insights about the identity of the high-molecular-S-nitrosylated complex.

Auxin induces denitrosylation and partial inactivation of APX1 in roots. Two cellular systems have emerged as physiologically relevant denitrosylases: the nitrosoglutathione reductase (GSNOR) and the Trx system. The Trx system is involved in auxin signalling, transport, and homeostasis (Bashandy *et al.*, 2010). APX1 has been clearly described as target of plant Trxs and Trx-SH_2 causes the inactivation of APX1 (Marchand *et al.*, 2004; Gelhaye *et al.*, 2006). According to this, the treatment with the TrxR inhibitor ANF augments the level of S-nitrosylated APX1 and increases its activity in roots. In mammalian systems, ANF was shown to inhibit TrxR at nanomolar concentrations. At higher concentrations (1–50 µM) ANF could also inhibit glutathione reductase (Gromer *et al.*, 1998).

Few examples of protein denitrosylation have been reported in plants so far. Denitrosylation of the salicylic acid (SA) regulator NPR1 catalysed by Trx facilitates its monomerization, nuclear translocation, and activation of genes coding for pathogenesis-related proteins (Tada *et al.*, 2008). On the other hand, NO production is induced by SA treatment (Zottini *et al.*, 2007) and required for the expression of SA-regulated pathogenesis-related protein genes (Klessig *et al.*, 2000). The proposed model for the SA-mediated

regulation of NPR1 is then through the opposing and balancing action of GSNO and Trx on NPR1 (Tada *et al*., 2008; Vlot *et al*., 2009). In a similar way, auxin treatment induces NO production in roots and at the same time provokes protein denitrosylation. Taking all available data, it is suggested that a counterbalance effect of NO production and APX1 denitrosylation are coordinately operating downstream auxin in root developmental processes.

S-Nitrosylated APX1 is more active than denitrosylated form

The stimulation of APX activity by S-nitrosylation may explain the effect of NO donors on APX activity described in several plant organs and physiological conditions. For example, three isoforms of APX are activated by NO donors in soybean root nodules (Keyster *et al*., 2011). NO protects against UV irradiation by stimulating the activity of the antioxidant enzymes catalase and APX (Shi *et al*., 2005). In addition, NO induces S-nitrosylation of APX in *Anticaria toxicaria* seeds, triggering a positive effect on APX activity during seed desiccation (Bai *et al*., 2011). Also reported is the reversible *in vitro* inactivation of APX by NO donors in tobacco crude extract (Clark *et al*., 2000). Since NO binds to and reversibly inhibits some mammalian haem-containing enzymes, the effect of NO donors on tobacco APX activity was attributed to the binding of NO to the metal Fe-group (Clark *et al*., 2000). Thereby, two mechanisms might be operating on NO-mediated regulation of APX activity: (i) S-nitrosylation of Cys residue/s and (ii) binding of NO to the haem group. Proteins that bind transition metals as Fe^{2+} and Cu^{2+} close to a S-nitrosylated target thiol can catalyse the transnitrosylation to reactive Cys residues (Hess *et al*., 2005). In APX1, the proximity of Cys168 to the haem group is 6.72 Å and makes Cys168 a strong candidate for S-nitrosylation through transnitrosylation reaction.

APX1 is involved in auxin-mediated root growth

Cytosolic APX1 was characterized as a central component regulating redox homeostasis in *Arabidopsis*. Knockout *apx1 Arabidopsis* plants have an increased H_2O_2 accumulation in leaves under high light conditions and higher sensitivity to paraquat (Davletova *et al*., 2005). Regarding the growth behaviour, *apx1* plants have delayed development phenotype, late flowering, and perturbed stomatal responses (Pnueli *et al*., 2003). The current data show that primary root elongation and lateral root formation is altered in response to auxin in the *apx1* mutant. This mutant has increased levels of H_2O_2 in roots (Suzuki *et al*., 2013; this work). However, *Arabidopsis apx1* root phenotype differs from exogenous application of H_2O_2 to roots. Exogenous H_2O_2 results in inhibition of primary root elongation and induction of lateral root formation, reminiscent of the phenotype found in auxin-treated roots (Wang *et al*., 2010a). Thus, APX1 may participate in the appropriate redistribution of H_2O_2 accumulation during root growth and lateral root development in *Arabidopsis*. The implicances of the crosstalk between auxins and NO in the regulation of APX1 activity by S-nitrosylation/denitrosylation in roots and the effect on the H_2O_2 accumulation and signalling began to be elucidated. A finely tuned and controlled counterbalance of the redox cell status seems to be a hub in auxin signalling control of root architecture.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Bidimensional PAGE of S-nitrosylated proteins in *Arabidopsis* roots.

Supplementary Fig. S2. Effect of auranofin (ANF) on TrxR activity in *Arabidopsis* roots.

Supplementary Fig. S3. Alignment of plant cytosolic APXs and the conservation of Cys residues in APX sequences.

Supplementary Fig. S4. Microenvironment of Cys32 and Cys168 in *Arabidopsis* APX1.

Supplementary Fig. S5. Expression of recombinant APX1 in bacteria.

Supplementary Fig. S6. Recombinant APX1 is specifically S-nitrosylated *in vitro*.

Supplementary Table S1. Protein and peptide sequences identified from selected spots by MALDI-TOF/TOF MS.

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