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Research paper

# Priming with NO controls redox state and prevents cadmium-induced general up-regulation of methionine sulfoxide reductase gene family in *Arabidopsis*

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ABSTRACT

In the present study we evaluated the pre-treatment (priming) of *Arabidopsis thaliana* plants with sodium nitroprusside (SNP), a NO-donor, as an interesting approach for improving plant tolerance to cadmium stress. We focused on the cell redox balance and on the methionine sulfoxide reductases (MSR) family as a key component of such response. MSR catalyse the reversible oxidation of MetSO residues back to Met. Five MSRA genes and nine MSRB genes have been identified in *A. thaliana*, coding for proteins with different subcellular locations. After treating 20 days-old *A. thaliana* (Col 0) plants with 100 μM $CdCl_2$, increased protein carbonylation in leaf tissue, lower chlorophyll content and higher levels of reactive oxygen species (ROS) in chloroplasts were detected, together with increased accumulation of all MSR transcripts evaluated. Further analysis showed reduction in guaiacol peroxidase activity (GPX) and increased catalase (CAT) activity, with no effect on ascorbate peroxidase (APX) activity. Pre-exposition of plants to 100 μM SNP before cadmium treatment restored redox balance; this seems to be linked to a better performance of antioxidant defenses. Our results indicate that NO priming may be acting as a modulator of plant antioxidant system by interfering in oxidative responses and by preventing up-regulation of MSR genes caused by metal exposure.



## 1. Introduction

Cadmium is one of the most phytotoxic heavy metals found in the environment and is considered among the main abiotic stress factors that cause injury to plants. Over 25000 tons of Cd are released per year, half of them into rivers through rock decomposition, and into the air through volcanoes and wildfire. The rest is introduced in the environment by industrial processes, phosphate fertilizers and waste disposal practices [1]. Several studies suggest that oxidative stress is involved in Cd toxicity [2].

Plants possess a complex machinery of enzymes and non-enzymatic antioxidants compounds that work in concert to keep the levels of ROS under control and to protect plant cell from oxidative damage [3]. During abiotic stress, ROS-antioxidant interaction modulates the appropriate induction of acclimation processes or, alternatively, execution of cell death programs [4,5].

External application of natural or synthetic compounds can induce plants to a physiological state of greater tolerance against environmental stresses; this phenomenon is known as “priming” [6]. Priming with different compounds such as $H_2O_2$, $H_2S$, β-aminobutyric acid (BABA) and polyamines has already been communicated [7]. Nitric oxide (NO) was also pointed out as an effective priming molecule that may contribute to cope with oxidative stress because of its involvement in the regulation of antioxidants and other defense enzymes [8], and several reports highlight the protective action of sodium nitroprusside (SNP) acting as a NO donor [9]. Moreover, it has been recently described that primed plants have the ability to reduce the harmful effects of ROS produced under different abiotic stresses, either by enhancing the capacity of the antioxidant machinery or by scavenging ROS [10].

NO is a small, gaseous, and universal plant-signalling molecule. Several reports have shown that increased NO levels, in different forms and doses, are likely to help the plant to trigger competent mechanisms against the harmful effects of cadmium stress [11];

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among them, the restoration of the cell redox balance has been proposed. NO protective function against oxidative stress involves the arrest of lipid oxidation, ROS scavenging and modulation of antioxidant enzymes [12–15].

Proteins are amongst the main cellular macromolecules that may undergo modifications under oxidative stress conditions triggered by cadmium [16–19]. Within proteins, sulphur-containing aminoacids are major sites of oxidation [20]. Methionine (Met) residues are oxidized to methionine sulfoxide (MetSO) by addition of oxygen to the sulphur atom, leading to the formation of equal amounts of epimers Met-S-(SO) and Met-R-(SO) [21]. This oxidative transformation can be reversed by members of the methionine sulfoxide reductase (MSR) family.

MSR are thioredoxin/glutaredoxin-dependent enzymes found in most aerobic organisms [22]. The reversible oxidation and reduction of Met plays a key role in the cell metabolism. Inadequate reversion of Met-SO to Met may cause loss of protein functionality or formation of aggregates that can be toxic to plant cells [23]. The MSR family is composed by two sets of unrelated classes of monomeric stereospecific reductases. One MSR set is specific for the reduction of Met-(S)-SO and is referred to as MSRA (EC 1.8.4.11). The other one is specific for the reduction of Met-(R)-SO and is referred to as MSRB (EC 1.8.4.12) [24].

*Arabidopsis thaliana* possesses multiple members of these two MSR families involving 5 MSRA and 9 MSRB genes, these genes encode proteins distributed in various subcellular compartments [25].

MSR are considered alternative redox-regulation compounds implicated in Cd tolerance of *Brassica juncea,* a plant known to accumulate high levels of heavy metals [26]. Likewise, it has been reported that MSRB are required for cadmium resistance in *Escherichia coli* [27], and similar observations were made in *Schizosaccharomyces pombe* cells [28]. However, the relevance of MSR under cadmium stress in plants still remains undeciphered.

The functional role of *Arabidopsis thaliana* priming with SNP, a NO-donor, was examined in the present work. Special attention was paid upon priming effects on MSR gene expression and redox balance.

## 2. Materials and methods

### 2.1. Plant material and growing conditions

Seeds of *Arabidopsis thaliana* Col 0 were germinated and grown in 150 $cm^3$ pots containing a mixture (1:1:1) of soil, perlite and vermiculite. After stratification at 4 °C for 3 days, pots were transferred to a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h light (170 μmol photons $m^{-2}$ $s^{-1}$). Plants were watered with diluted (1/4) Hoagland's nutrient solution [29]. After 20 days of growth, plants were divided into two groups: the first group was subjected to the priming procedure by receiving 100 μM sodium nitroprusside (SNP) in the watering solution that day (considered day 0) and 2 and 4 days later (days 2 and 4); the second group kept on receiving diluted Hoagland's nutrient solution. At day 4, half of the plants of each group (primed and non-primed) received 100 μM $CdCl_2$ in the watering solution. In that way, four treatments were obtained: control or "C" (no priming, no Cd-exposition); "Cd" (no priming, Cd exposition); "SNP" (priming with sodium nitroprusside, no Cd-exposition) and "SNP + Cd" (priming with sodium nitroprusside, Cd-exposition). At day 7, leaves were harvested in order to perform the determinations described below.

### 2.2. Carbonylation of soluble proteins

Carbonylation of soluble proteins was evaluated and taken as evidence of oxidative protein modification. Protein extracts were prepared by homogenizing 0.2 g of leaf tissue in 0.8 mL of 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1% (v/v) Triton X-100. After centrifuged at 26000 g for 15 min at 4 °C, supernatants were derivatized with 2,4-dinitrophenylhydrazine (2,4-DNPH) [30]. Samples (50 μg protein) were subjected to electrophoretic analysis using 10% (w/v) SDS-PAGE, then electrotransfered to polyvinylidene fluoride membrane (PVDF) and incubated with anti-DNP as primary antibody. Bands were subsequently visualized using a secondary rabbit antibody conjugated with horseradish peroxidase (HRP) and stained using 3,3′-diaminobenzidine (DAB) as substrate, as described by Pena et al. [31]. Ponceau S staining was used as loading control. Membranes were photographed with a Fotodyn, analyzed with Gel-Pro software and expressed as arbitrary units (assuming control value equal to 100 units), based on absolute integrated optical density of each line.

### 2.3. Chlorophyll content and ascorbic and dehydroascorbic acid determination

Chlorophyll was extracted by homogenizing and boiling 60 mg of fresh weight (FW) of leaves in 2 mL of 96% ethanol. After centrifugation for 10 min at 3000 g, chlorophyll content was determined spectrophotometrically on the ethanolic supernatant at 654 nm, as described by Wintermans and De Mots [32].

Extracts for ascorbic (Asc) and dehydroascorbic acid (DHAsc) determination were prepared by homogenizing 0.1 mg of FW leaf in 0.5 mL of 10% (w/v) tricloroacetic acid (TCA). Determinations were performed as described by Law et al. [33].

### 2.4. Antioxidant enzymes activity

Extracts for catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and guaiacol peroxidase (GPX, EC 1.11.1.7) activities were prepared from 80 mg (FW) of leaves homogenized under ice-cold conditions in 0.8 mL buffer containing 50 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 1% (w/v) PVP and 0.5% (v/v) Triton X-100 at 4 °C. Homogenates were centrifuged at 26000 × *g* for 30 min and the supernatant fraction was used for assays. CAT activity was determined by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM $H_2O_2$. The pseudo-first order reaction constant (k' = k x [CAT]) of the decrease in $H_2O_2$ absorption was determined and catalase content in pmol $mg^{-1}$ protein was calculated using $k = 4.7 \times 10^{-7}$ $M^{-1}$ $s^{-1}$ [34]. APX activity was measured immediately in fresh extracts as described by Nakano and Asada [35], using a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM $H_2O_2$, 0.5 mM ascorbate and 0.1 mM EDTA. Hydrogen peroxide-dependent oxidation of ascorbate was followed by decrease in absorbance at 290 nm ($\varepsilon = 2.8$ $mM^{-1}$ $cm^{-1}$). GPX activity was determined by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol ($\varepsilon = 26.6$ $mM^{-1}$ $cm^{-1}$) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 10 mM guaiacol and 10 mM $H_2O_2$. Absorbance was determined in a FlexStation 3 Multi-Mode Microplate Reader.

### 2.5. Reactive oxygen and nitrogen species detection

The production and localization of ROS (primarily $H_2O_2$/peroxides) was assessed in detached *A. thaliana* leaves after Cd and/or

**Table 1**
Primers designed for *Arabidopsis thaliana* to amplify several cDNA fragments.

| Gene | Sense and antisense primers | Amplicon size (pb) | Accession number (GenBank ID) |
|---|---|---|---|
| *MSRA1* | 5′- GAGCGTGGAATTAGCGTACC<br>5′- TTGCTGGTGATGTTCCTCAG | 390 | NM_125558 |
| *MSRA2* | 5′- GTGGAGCTTGCTTTCCAGAG<br>5′- TGCCGGTAAGATCTCAGTCA | 350 | NM_120828 |
| *MSRA3* | 5′- AGCTGGCGTTTCAGAGAGTC<br>5′- CCTGCTCGGGTGTGTAGAAG | 269 | NM_120829 |
| *MSRA4* | 5′- TCTGTACTGGCACTACGGGA<br>5′- TCTTAAACCCATGCGACCCC | 328 | NM_118645 |
| *MSRA5* | 5′- CTTAGGTGATCACGCCGAGT<br>5′- GTTGCTCCCTTTCTTTGCTG | 208 | NM_127359 |
| *MSRB1* | 5′- CGGCGAAAACCATTATAAGC<br>5′- GGTGTTAGCCGTTTCTTCCA | 282 | NM_104245 |
| *MSRB3* | 5′- CGGTTGTGTAGGATGCAATG<br>5′- TTCGGCCAAGATTTACAAGG | 279 | NM_116718 |
| *MSRB4* | 5′- GCCGGGAACAGAAGAATATG<br>5′- TAACACAAAGGCGTTCATCG | 268 | NM_116719 |
| *MSRB5* | 5′- GGAATCTTCGATTGCGTAGG<br>5′- TTGACCACCACAGAAAGTGC | 375 | NM_116721 |
| *MSRB6* | 5′- TGGCGTACGGTTCTATCTCC<br>5′- TCGTCAGTTGGTGTTGGAAA | 313 | NM_116722 |
| *MSRB7* | 5′- TCCAGCAACTGGATCTTTCC<br>5′- CTTCTTCCACCTGCTTCTGG | 269 | NM_118303 |
| *MSRB8* | 5′- CGTGCTGTTCTGTCTCCTGA<br>5′- TGGCCTAGATGTCCATCACA | 271 | NM_118304 |
| *MSRB9* | 5′- AATCATGCCAACATCAGCAA<br>5′- TTAATGGCACCAGGGATAGC | 272 | NM_118305 |
| *Tubulin4* | 5′- GCGAACAGTTCACAGCTATGTTCA<br>5′- GAGGGAGCCATTGACAACATCTT | 212 | NM_123801 |

SNP treatments using 25 μM of 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) as fluorescent cell permeable probe, prepared in 10 mM Tris-HCl (pH 7.4) [36]. Leaves were vacuum infiltrated 3 times during 15 s, incubated in the dark for 30 min and washed three times with distilled water. Fluorescence was visualized using an Olympus FluoView®300/BX61 confocal laser scanning microscope. Intensity fluorescence measurements for quantification and colocalization were done in single cortical CLSM sections using the Fiji (*Fiji Is Just ImageJ)* software (http://fiji.sc). Mesophyll tissue was analyzed and linear adjustments in pixel values were made when measuring signal intensities and the corrected total cell fluorescence (CTCF) was calculated using this formula: CTCF = integrated density - (area of selected cell x mean fluorescence of background readings). From each treatment, fluorescence intensity from 10 individual zones was measured and the mean values were calculated. Control values were considered as 100%. To determine colocalization, Coloc2 plugin was used to determine Manders co-localization coefficients [37].

Nitric oxide formation in leaves after each treatment was monitored using 10 μM of the fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA) [38] prepared in buffer 10 mM Tris–HCl (pH 7.4). Detached leaves were vacuum infiltrated 3 times during 15 s, incubated in the dark for 30 min and washed three times with distilled water. Fluorescence was visualized using an Olympus DP73 microscope. Intensity fluorescence measurements for quantification were done using the Fiji (*Fiji Is Just ImageJ)* software (http://fiji.sc). From each treatment, fluorescence intensity from 10 individual zones was measured and the mean values were calculated.

### 2.6. RNA isolation and RT-PCR analysis of MSR expression

Total RNA was extracted from 50 mg of homogenized leaf tissue

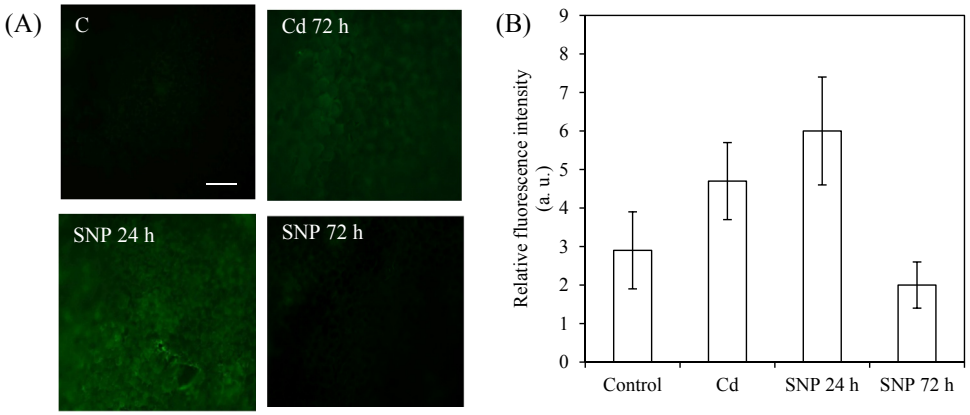


**Fig. 1.** Detection (A) and quantification (B) of NO by epifluorescence microscopy in detached leaves labelled with 10 μM DAF-2DA fluorescent marker. (A) Images were obtained overlaying 495 nm emission and 510 nm excitation to detect NO (green); representative images are shown as recorded in repeated experiments. Bar = 50 μm. (B) Means of four different experiments are shown; bars indicate SEM. a.u = arbitrary units.

using Power Plant RNA isolation Kit with DNAase (MoBio Laboratories), according to manufacturer's instructions. A determined amount of RNA (1–2 μg) was reverse transcribed for 1 h at 42 °C using Thermo Scientific RevertAid Reverse Transcriptase. The cDNA obtained was subjected to a PCR using Thermo Scientific Taq Polimerase and specific primers designed for each MSR sequence (Table 1); *Tubulin 4* was chosen as internal control. PCR conditions were as follows: 3 min 95 °C; 35 cycles of 3 s 95 °C, 30 s 50 °C, 35 s 72 °C; and 10 min 72 °C. For quantification, gels were photographed with a Fotodyn and band intensities were determined with Gel-Pro software.

To rule out changes in MSR expression related to possible by-products of SNP degradation others than NO, treatments with 50 μM $NaNO_2$ and 10 μM $K_3[Fe(CN)_6]$) were included.

### 2.7. Protein concentration

Protein concentration was determined according to Bradford [39] using bovine serum albumin as standard.

### 2.8. Statistics

Values are expressed as means ± SEM. Differences among treatments were analyzed by 1-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

## 3. Results

### 3.1. Nitric oxide-loading by SNP

Nitric oxide release from SNP when applied in the watering solution was corroborated on detached leaves using the fluorescent probe DAF-2DA. Representative images obtained under epifluorescence microscopy and fluorescence quantification are shown in Fig. 1 (A and B, respectively). It may be noticed an increased fluorescence after 24 h of SNP treatment revealing NO presence by this time; however, NO signal was almost absent after 72 h of SNP treatment, when all analytical determinations were performed. Fig. 1 also shows that cadmium itself led to a considerable fluorescence increase, which exceeded that of control in about 65% after 72 h of metal treatment.

### 3.2. SNP prevented ROS increase induced by Cd in chloroplasts

Histochemical staining for $H_2O_2$/peroxides was performed in leaves using the $H_2$DCFDA probe. Representative images of confocal laser microscopy and fluorescence quantification are shown in Fig. 2 (A and B, respectively). Stacks of optical section through the leaves revealed staining associated with chloroplasts, where colocalization analysis of ROS-probe and chlorophyll fluorescence showed Manders coefficients' M1 and M2 values of 0.939 and 0.980, respectively. The fluorescence intensity was faint in chloroplast of mesophyll cells of untreated plants. However, ROS-derived fluorescence increased by three times respect to control values in chloroplasts under cadmium treatment. This striking difference in ROS accumulation between control and Cd-treated plants dropped almost to control levels when plants were primed with SNP before cadmium application. SNP treatment did not increase ROS production in mesophyll cells.

### 3.3. Oxidative stress induced by Cd was prevented by SNP

After cadmium treatment, the level of protein carbonylation in leaves increased 40% respect to control plants (Fig. 3). In contrast, SNP treatment reduced protein oxidation levels (Fig. 3). A similar

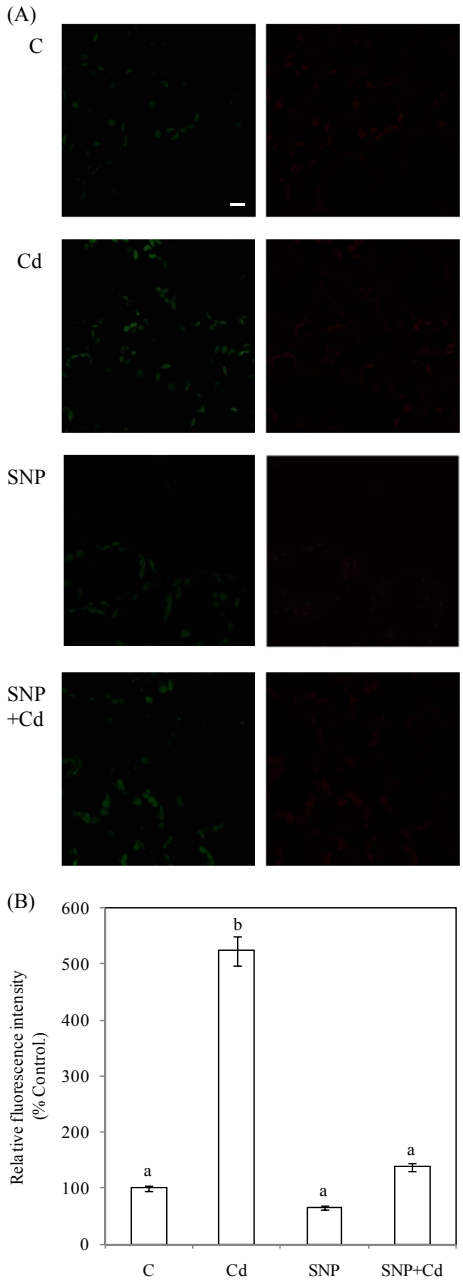


**Fig. 2.** Detection (A) and quantification (B) of $H_2O_2$/peroxides by CLSM in detached leaves using the $H_2$DCFDA fluorescent marker stain. (A) Mesophyll cells are shown in field of view; images were obtained overlaying 488 nm emission and 633 nm excitation to detect ROS (green, left panel) and chloroplast autofluorescence (red, right panel), respectively. Representative images are shown as recorded in repeated experiments. Bar = 50 μm. (B) Means of four different experiments are shown; bars indicate SEM.

result was obtained when plants were treated with SNP before cadmium administration, where carbonyl group content decreased 40% respect to control.

Additionally, decreased chlorophyll contents were found in Cd-treated plants. After 3 days of metal treatment, chlorophyll content decreased by 20% with respect to controls (Fig. 4). Previous SNP treatment, however, increased chlorophyll contents and prevented chlorosis in cadmium-treated plants. Plants subjected to SNP treatment alone showed also greater chlorophyll content than controls (Fig. 4). On the other hand, cadmium treatment decreased

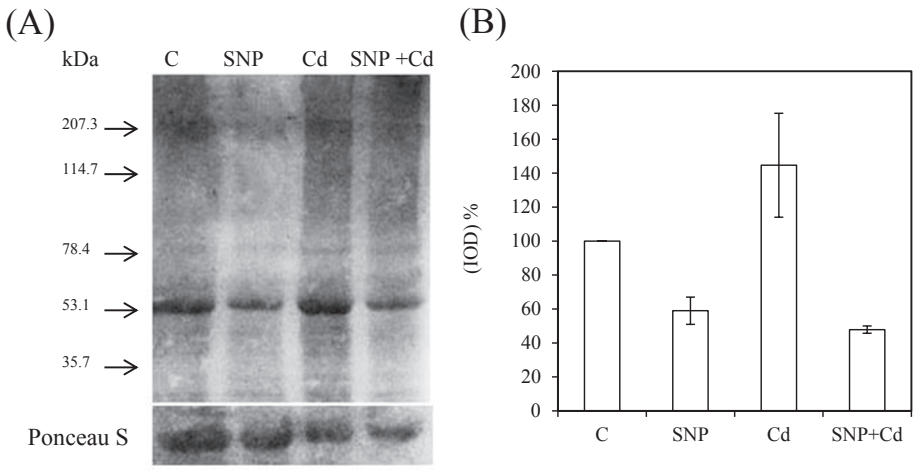


**Fig. 3.** Detection (A) and quantification (B) of oxidative-modified proteins obtained from detached leaves. (A) DNPH-derivatized proteins (50 μg) were subjected to one-dimensional SDS-PAGE (10% w/v). Western blotting was performed using anti-DNP antibody, bands were visualized as described in Material and Methods. A representative photograph of the membranes as recorded in repeated experiments is shown. The Ponceau S-stained membrane showing the Rubisco large subunit is included as loading control. The position of molecular mass markers (in kDa) is shown on the left. (B) Quantification of oxidized proteins expressed in arbitrary units (assuming control value equal to 100%), based on absolute integrated optical density (IOD) of each lane. Means of four biological replicates are shown; bars indicate SEM.

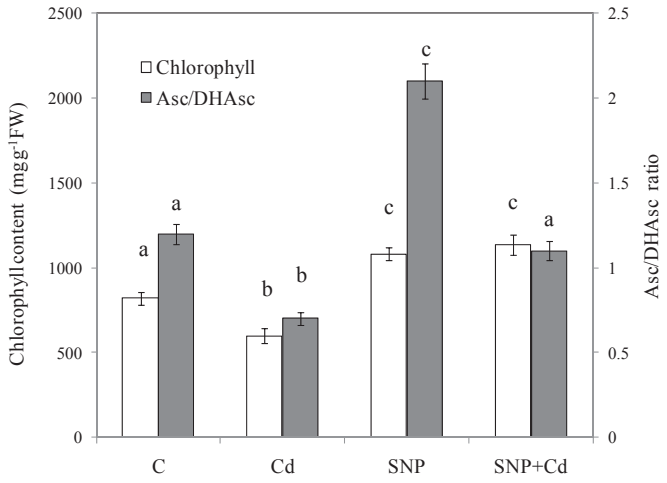


**Fig. 4.** Chlorophyll content and ascorbic/dehydroascorbic acid (Asc/DHAsc) ratio. Means of three different experiments with five replicated measurements each are shown; bars indicate SEM. Different letters indicate significant difference ($P < 0.05$) according Tukey's multiple range test.

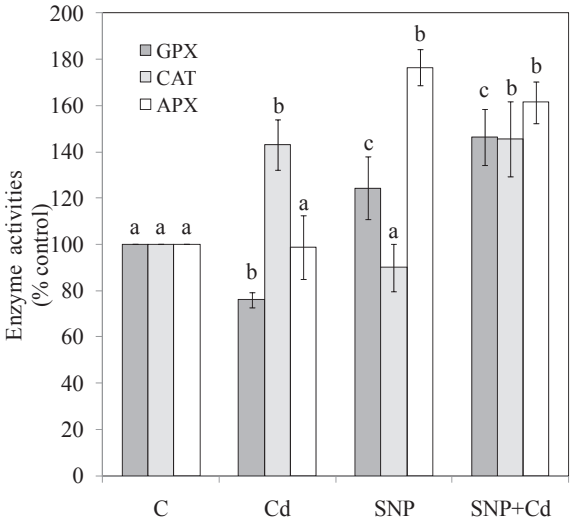


**Fig. 5.** ROS-detoxifying enzymes activities. Enzymatic activities were assayed as indicated in Materials and methods. GPX and APX were measured as U $mg^{-1}$ protein (one unit of APX forms 1 μmol of ascorbate oxidized per min per mg of protein under the assay conditions; one unit of GPX forms 1 μmol of guaiacol oxidized per min per mg of protein under the assay conditions); CAT was determined as pmol $mg^{-1}$ protein. Means of three different experiments with five replicated measurements each are shown; bars indicate SEM. Different letters indicate significant difference ($P < 0.05$) according Tukey's multiple range test.

Asc/DHAsc ratio by 41% respect to control. On the contrary, SNP treatment increased this ratio 75% over the control values, while when used as a priming compound previous to Cd treatment, Asc/DHAsc ratio remained similar to the control (Fig. 4).

Treatments' effects on antioxidant defence system were assessed by testing the activity of the ROS-scavenging enzymes catalase, guaiacol peroxidase and ascorbate peroxidase. After three days of Cd exposure, GPX activity was 25% decreased and almost no effect on APX activity could be detected, but CAT activity increased 40% respect to control values. Exposure to SNP, on the other hand, induced GPX and APX activity (25% and 70% over control values, respectively), but slightly diminished CAT activity. NO-priming through SNP treatment before Cd exposure led to increased GPX, CAT and APX activities (50%, 45% and 60% over control values, respectively) (Fig. 5).

### 3.4. MSRs were up regulated by cadmium and SNP modulated this response

Cadmium treatment significantly increased mRNA accumulation of all MSRs analyzed; transcripts accumulation increase was approximately twice for *MSRA2*, *A3*, *A5*, *B1*, *B3*, *B4*, *B7*, *B8* and *B9*; three times for *B5* and *B6*; and almost four times for *A1* and *A4* (Fig. 6 and Fig. 7). On the other side, the levels of most MSR transcripts remained similar to those of controls in SNP-treated plants; only *MSRB7* was up-regulated (Fig. 7). NO-priming through SNP treatment before Cd exposure prevented this notorious up-regulation and resulted in MSR transcript levels comparable to those of controls (Figs. 6 and 7).

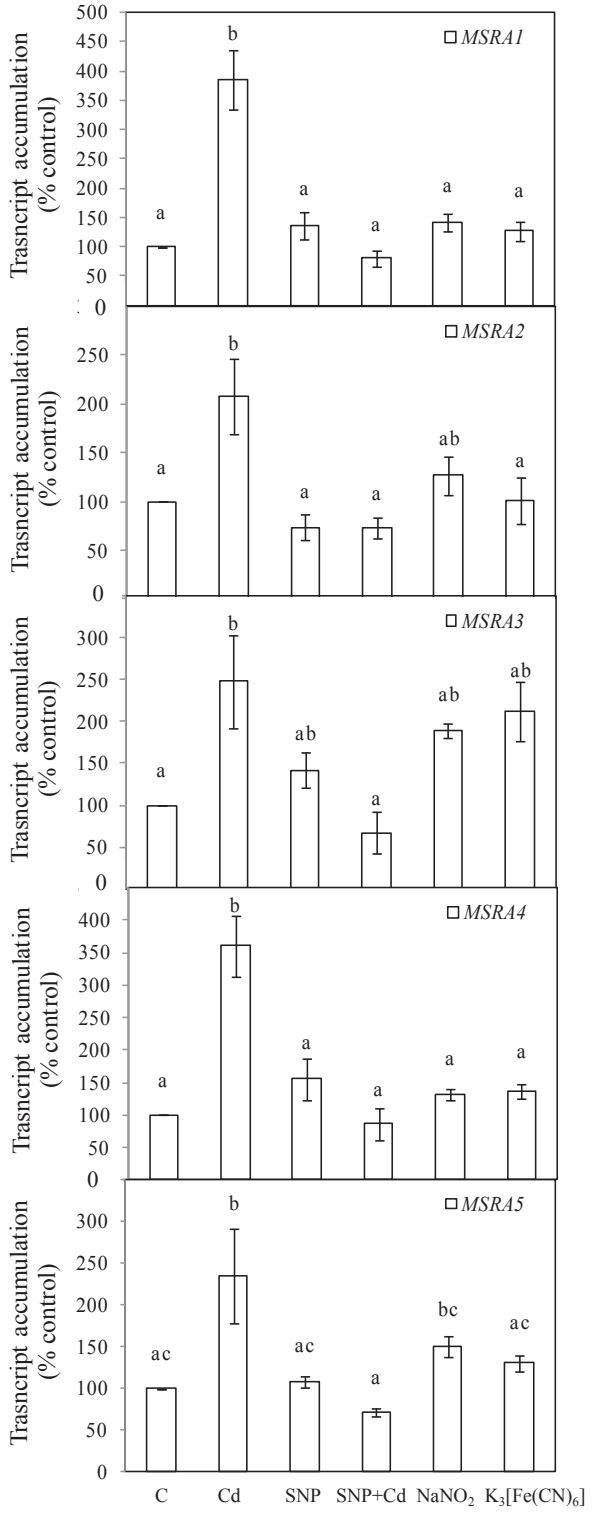


**Fig. 6.** Semiquantitative RT PCR analysis in RNA samples obtained from leaves using specific primers for MSRA1-5. *Tubulin4* transcript was used as housekeeping gene. Data are expressed as percentage of control (assuming a control value equal to 100). Means of three different experiments with five replicated measurements each are shown; bars indicate SEM. Different letters indicate significant difference ($P < 0.05$) according Tukey's multiple range test.

## 4. Discussion

The degree of protein oxidation was determined in order to assess cell redox status. Increased protein carbonylation is considered a reliable marker of oxidative stress. In this research, cadmium-induced oxidative stress in *Arabidopsis* leaves was demonstrated by increased protein carbonylation and enhanced ROS production, which was mainly observed at chloroplast level. Chlorosis was also evident.

Cadmium stress especially impacts on chloroplasts; this was demonstrated in this work by the significant drop of chlorophyll content, as well as of the ascorbic/dehydroascorbic acid ratio. Rubisco and rubisco activase were detected in *in vitro*-grown *Nicotiana tabacum* in response to cadmium and copper stresses [40], and similar to that observed in our experimental conditions, SNP priming prepared tobacco cells to a forthcoming metal stress.

Our results show that SNP treatment ameliorated Cd injury by reversing the metal action in terms of oxidative stress-related parameters. In this sense, the prevention of protein oxidation and of chlorosis, accompanied by a greater Asc/DHAsc ratio may account for the restoration of the cell redox balance. A similar report referred to barley plants indicates that NO released by SNP functions as an antioxidant, preventing the oxidative damage generated by cadmium [41].

The role of NO as a signalling molecule during abiotic stress linked to activation of antioxidant enzymes has been reported [12,42–44]. The balance of peroxidase activities, APX, GPX and CAT, representing the main enzymatic $H_2O_2$-scavenging mechanisms in plants, is crucial for restoring redox equilibrium [45]. Under our experimental conditions, the presence of SNP in the watering solution had no effect on CAT activity but increased APX and GPX activities. In particular, APX has a very high affinity for $H_2O_2$ and reduces it to $H_2O$, with ascorbate as specific electron donor inside chloroplasts, cytosol, mitochondria and peroxisomes, as well as in the apoplastic space [46]. The relevance of APX in ROS detoxification has already been corroborated: overexpression of this enzyme resulted in improved abiotic stress tolerance in plants [47–50]. At the molecular level, the mechanism underlying regulation of APX activity by NO was related to redox post-translational modifications [51]. Recently, Yang et al. [52] found that NO positively regulates APX activity through S-nitrosylation. This molecular mechanism could be, at least in part, responsible for keeping $H_2O_2$ homeostasis during Cd stress in plants. In this sense, the increase of APX activity we noticed under NO priming could be a key factor for the regulation of ROS levels in chloroplasts, thus serving as a hub for controlling ROS signalling [53].

Despite MSR are outside of the ascorbate-glutathione cycle, they could be considered alternative enzymes involved in maintaining cell redox balance. Recent studies focused on the characterization of MSR gene family in different plant species like tomato [54], tobacco [55,56] and maize [57]. Their response to hormones and diverse stresses in these plant species was also addressed. It has been increasingly reported that MSR system performs a crucial role, not only in the normal plant physiology but also during abiotic stress response [58–61].

Under our experimental conditions, the phytotoxic metal used as abiotic stressor (cadmium) triggered a general up-regulation of all MSR genes investigated (8 out of 9). Due to the facts that Cd induces ROS accumulation [62] and that surface-exposed Met residues in proteins might serve as sinks for ROS, MSR repair system would play a decisive role in the maintenance of cell redox homeostasis during Cd stress.

MSR system acts by reducing MetSO back to Met, resulting in ROS destruction at the expense of NADPH [63]. In *Arabidopsis* proteome, 10% of proteins are predicted to contain a MetSO residue

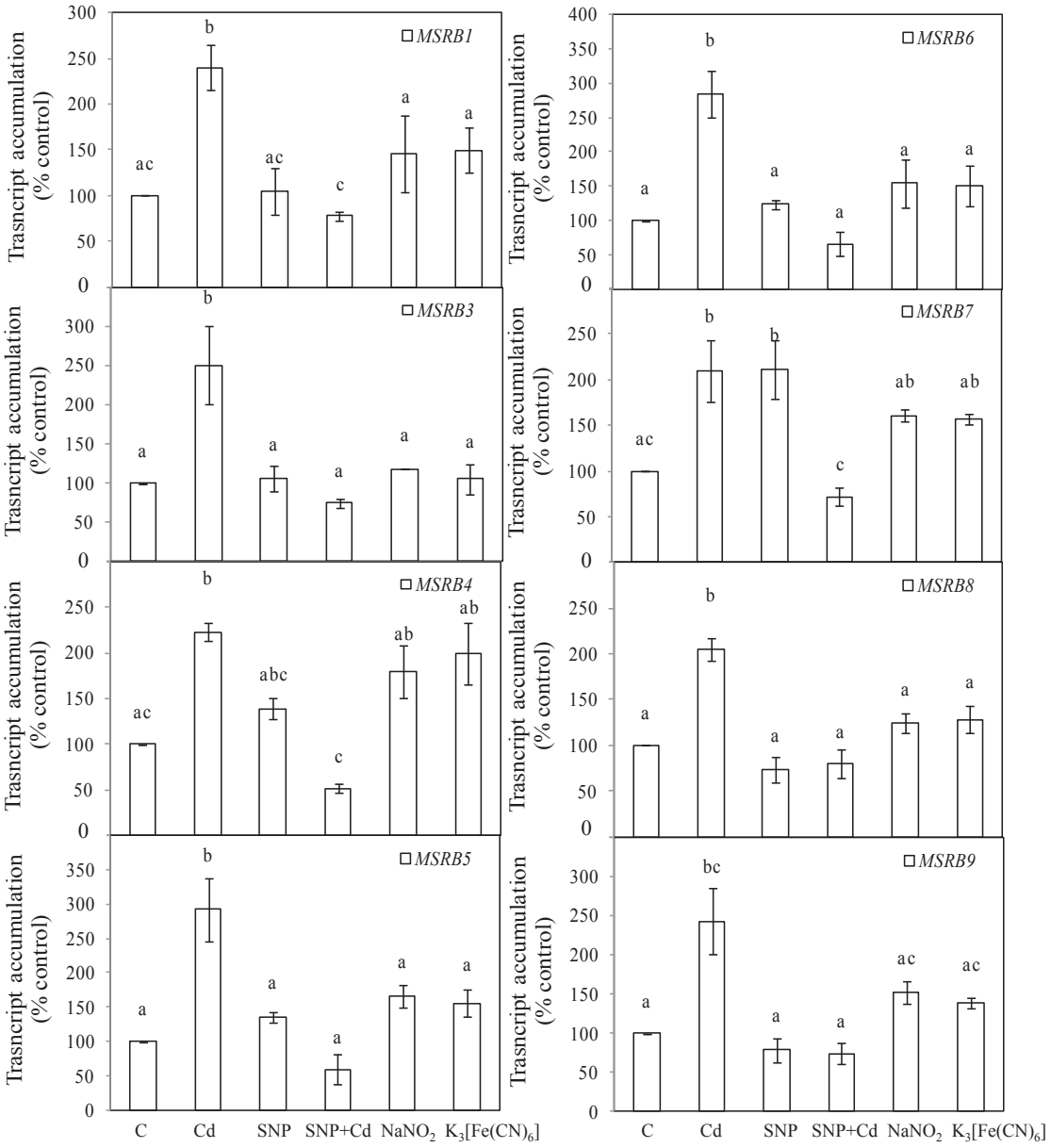


**Fig. 7.** Semiquantitative RT PCR analysis in RNA samples obtained from leaves using specific primers for MSRB1-9. *Tubulin4* transcript was used as housekeeping gene. Data are expressed as percentage of control (assuming a control value equal to 100%). Means of three different experiments with five replicated measurements each are shown; bars indicate SEM. Different letters indicate significant difference ($P < 0.05$) according Tukey's multiple range test.

[64], thus they could act as a redox buffer. Moreover, Met oxidation could function as a regulatory process in cell signalling [64]. For example, oxidation of methionine residues can inhibit protein phosphorylation, and thus altering enzyme activity, as it is documented for nitrate reductase [65]. In regard to signal transduction, reversible methionine oxidation by MSR within calmodulin may act as redox and stress sensor, modifying metabolic responses to environmental stressors [66].

The MSR genes most up-regulated by cadmium in our work were *MSRA1* and *MSRA4*. Both genes are mainly expressed in leaves [25]. Analysis of gene expression databases of *Arabidopsis* Col-0 shows that in shoot samples of 24 h hydroponically grown plants treated with 50 μM Cd, *MSRA1* –a cytosolic isoform– was the unique transcript apparently increased [67]. These results support the idea that *MSRA1* response is independent of metal concentration and metal exposure time.

An overall rise of ROS-detoxifying enzyme activities independently of their subcellular localization was detected through this research. On the other hand, MSR transcript levels could be differentially regulated by the intracellular localization of metal injury. Previous evidence suggests that the expression level of chloroplastic and non-chloroplastic MSR isoforms is dependent on both, the type and the cellular localization of the oxidative stress. We have shown that Cd enhanced ROS production mainly at

chloroplast level. As it was observed under high light or methyl viologen exposure, expression of the chloroplastic isoform *MSRA4* increased when oxidative stress took place in chloroplasts [68]. In this sense, Romero et al. [69] proposed that *PMSRA4* gene induction is the result of chloroplast-derived signalling molecules when the level of ROS increases in this organelle.

In *Chlamydomonas reinhardtii,* ROS by themselves could modulate the differential expression of MSR genes during abiotic stress [70]. One possible mechanism by which NO interferes with the up-regulation of MSR genes triggered by Cd could be associated to the high reactivity of ROS and NO/reactive nitrogen species, which show reciprocal changes after application of NO modulators in Cd-exposed plants [71]. In addition, the metal responsive element MRE1 present in MSR genes promoters [69] could act by increasing the level of gene transcription under cadmium stress. However, due to the complexity and number of MSR isoforms, and the different locations and patterns of expression in plants, it is still unclear the role and contribution of each isoform during the stress response.

As was previously described for the macroalga *Ulva fasciata*, NO does not seem to be an internal factor for regulation of MSR expression [72]. However, pre-treatment with SNP prior to cadmium exposure reversed MSR response to the metal, thus indicating that exogenous NO interferes with the signalling process that modulates MSR expression in leaves of plant subjected to Cd stress.

Since administration of others SNP releasing compounds −like $NaNO_2$ and $K_3[Fe(CN)_6]$− up regulated cytoplasmatic isoforms of MSR, whereas SNP did not, it is verified that SNP-released NO, rather than other by-products, accounts for the alleviation effect on Cd toxicity.

## 5. Conclusion

Our results indicate that NO priming interfered with the oxidative stress generated upon cadmium exposure by preventing redox imbalance, and thus, by avoiding the up-regulation of MSR genes caused by this metal. In that way, we demonstrate that the MSR family takes part of a general response pattern to oxidative stress induced by cadmium and affecting all MSR isoforms.

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