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Oxidative Stress Induced by Cadmium in Transgenic *Nicotiana tabacum* Over-expressing a Plastidial Mn-Superoxide Dismutase

Cristina Ortega-Villasante[1] • Juan Sobrino-Plata[1,4] • Ramón O. Carpena-Ruiz[2] • Nathalie Verbruggen[3] • Carolina Escobar[4] • Luis E. Hernández[1*]

[1] Laboratory of Plant Physiology, Department of Biology, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain
[2] Department of Agricultural Chemistry, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain
[3] Laboratorie de Physiologie et de Génétique Moléculaire des Plantes, Université Libre de Bruxelles, Campus-Plaine CP 242, Bd du Triomphe, B-1050 Brussels, Belgium
[4] Department of Environmental Sciences, Universidad de Castilla La Mancha, Campus Fábrica de Armas, 45070 Toledo, Spain

Corresponding author: * **luise.hernandez@uam.es**

ABSTRACT

Nicotiana tabacum transgenic plants overexpressing manganese (Mn) superoxide dismutase (SOD) targeted to plastids were studied upon 12.5 μM cadmium (Cd) application. No differences in tolerance were observed in comparison to wild type plants. Seed germination inhibition, seedling growth, and plant biomass were measured at different developmental stages. Cd analysis revealed the usual distribution of Cd between roots and shoots, 10-times higher in roots. Lipid peroxidation level, a characteristic parameter of oxidative stress, indicated that little protection was gained by overexpressing MnSOD. However, chlorophyll concentration was not affected upon Cd treatment in *35S:MnSOD* transgenic plants, suggesting that a possible protection localised at the chloroplast could indeed occur. Three antioxidant enzymes sensitive to metal stress were also analysed after *in gel* activity staining: SOD, ascorbate peroxidase (APX) and glutathione reductase (GR). As expected, MnSOD activity was remarkably higher in transgenic *N. tabacum* plants. Interestingly, Cu/ZnSOD activity decreased upon exposure of plants to 12.5 μM Cd. GR and APX activities augmented in metal-treated plants. In particular, APX activity was slightly higher in MnSOD over-expressing plants, which was possibly related with an enhanced generation of H_2O_2 through higher SOD activity.

Keywords: cadmium, heavy metals, *Nicotiana tabacum*, oxidative stress, superoxide dismutase
Abbreviations: APX, ascorbate peroxidase; **Cu/ZnSOD**, copper-zinc superoxide dismutase; **FeSOD**, iron superoxide dismutase; **GR**, glutathione reductase; **GSH**, glutathione; **MnSOD**, manganese-superoxide dismutase; **PC**, phytochelatin

INTRODUCTION

Some transition metal ions like Fe^{2+} and Cu^{+} might induce oxidative damage in biological macromolecules coupled to their auto-oxidation, through Fenton-type reactions. Other metal ions in despite of not been described under that category, such as Cd^{2+} or Hg^{2+}, can also compromise cellular redox homeostasis leading to accumulation of reactive oxygen species (ROS) and thus, appearance of oxidative stress (Lozano-Rodríguez *et al.* 1997; Piqueras *et al.* 1999; Romero-Puertas *et al*. 1999; Dixit *et al*. 2001; Schützendübel and Polle 2002). Nevertheless, the exact mechanisms that trigger ROS in plants treated with metals are still under debate (Gratão *et al*. 2005), and some efforts have been made to identify the early responses of plants where an oxidative burst occurred under Cd and Hg stress (Ortega-Villasante *et al*. 2007).

Superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are produced in normal cellular metabolism reactions, but when plants are subjected to an aggressive environment the capacity of the cellular antioxidant systems is overridden, which leads to an unbalanced accumulation of ROS and oxidative stress appears (Noctor and Foyer 1998). These defence systems are composed by redox metabolites such as ascorbate or glutathione (GSH). Both play multiple roles in cellular metabolism, and are present in tissues at millimolar concentrations. Other important components are enzymatic scavengers of ROS, such as superoxide dismutases (SODs), ascorbate peroxidases (APXs), catalases and glutathione reductase (GR); which use the referred metabolites as electron donors in the so-called ascorbate-GSH cycle (Noctor and Foyer 1998; Asada 1999). Under harmful environmental conditions, ROS are accumulated above the thresholds where detoxifying mechanisms can avoid cellular damages. Such conditions include drought, salinity, ultraviolet radiation, ozone, etc. (Mittler 2002). Thus, toxic metals are among known abiotic factors that promote alterations in plant antioxidative systems, causing in most cases oxidative damages. However, the amplitude of the responses depend greatly on several factors, such as the plant species studied, their age and physiological status, the metal and concentration used, the time of exposure, etc. (Schützendübel and Polle 2002).

One of the most characterised sources of $O_2^{\bullet-}$ is oxygen reduction in chloroplast photosystems during photoinhibition. Under certain environmental conditions, such as excess of irradiance, the O_2 generated in the chloroplasts after H_2O photolysis at photosystem II is partially reduced by reduced ferredoxine to $O_2^{\bullet-}$ (Foyer *at al*. 1994). Through the conversion of $O_2^{\bullet-}$ to H_2O_2 with the implication of SOD (EC 1.15.1.1), the level of $O_2^{\bullet-}$ is kept under control, in particular to avoid Haber-Weiss generation of $^{\bullet}OH$, an extremely reactive radical (Bowler *et al*. 1991). Other putative sources of $O_2^{\bullet-}$ exist in different cellular compartments where electron transfer occurs, principally mitochondria, peroxisomes or plasma membrane (Mittler 2002). Several SODs are distributed to different compartments, as biological membranes are impermeable to the charged $O_2^{\bullet-}$ formed in them (Alscher*et al*. 2002). Three major SOD isoforms exist in plants, which are classified according to their metal cofactor: manganese (MnSOD), iron (FeSOD) and copper/zinc (Cu/ZnSOD) isoforms (Bowler *et al*. 1992). In general, MnSOD is located in the mitochondria and peroxisomes, FeSOD is basically plastidic and Cu/ZnSODs are cytosolic,

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plastidic and possibly apoplastic (Alscher*et al*. 2002). All these enzymes are nucleus encoded and are transported to their organellar locations by means of NH_2-terminal targeting sequences. The number of isozymes, their relative abundance and biochemical properties, such as isoelectric point values, vary greatly from plant to plant (Alscher *et al*. 2002).

Biotechnological efforts are underway to improve plant stress tolerance, among them, the overexpression of SODs and other ROS scavenging enzymes (Allen *et al*. 1997). Several studies have evidenced that SOD over-expression can endow a certain level of tolerance to several stress conditions. Thus, over-expression of MnSOD targeted to the chloroplasts in *Nicotiana tabacum* led to higher tolerance to the herbicide Paraquat (methyl-viologen; Bowler *et al.* 1991). These plants were also more resistant to ozone damage (Van Camp *et al*. 1994). In addition, alfalfa plants transformed with the same construct also revealed increased tolerance to freezing and water deficit (Allen *et al*. 1997). Slooten and co-workers (1995) showed that different factors influenced the performance of the tested transgenic lines of *N. tabacum*, such as the type of stress imposed, the biochemical properties of the enzyme over-expressed, the level of transgene over-expression, vegetative status of the plant, etc. Little information is available on the responses of transgenic plants under heavy metal induced stress. Therefore, we aimed the characterisation of oxidative stress parameters in transgenic tobacco (*Nicotiana tabacum*) plants over-expressing a plastid targeted MnSOD exposed to Cd.

MATERIALS AND METHODS

Chemicals

Otherwise stated, all chemicals were analytical grade and purchased from Sigma-Aldrich (St. Louis, MI, USA).

Plant material and treatments

Seeds of wild type and MnSOD transgenic *N. tabacum* cv. PBD6 (Bowler *et al*. 1991) were surface sterilized for 10 min in 50% (v/v) commercial bleach, rinsed 3 times with sterile water and left overnight at 4°C. Subsequently seeds were germinated for one week on square Petri dishes placed vertically, containing 1/10 Murashige and Skoog medium at pH 5.7 (Duchefa Biochemie, Haarlem, The Netherlands), and supplemented with 0.1% sucrose and 0.8% (w/v) agarose (Pronadisa-CONDA, Madrid, Spain). Homogenous seedlings were carefully transferred to a hydroponic system, where plants were grown suspended for 21 days in a half strength modified Hoagland medium (see Rellán-Álvarez *et al.* 2006 for details, pH 5.7), under a 16 h light (28°C)/8 h dark (18°C) regime. To avoid nutrient depletion, the nutrient solution was removed every three-four days. When the Cd treatment was applied, the nutrient solution was changed to a complete Hoagland medium with or without 12.5 μM $CdCl_2$. Plants were kept under the same environmental conditions as described before and were harvested after two weeks of treatment (six week-old plants at harvest).

Transgenic *N. tabacum* contained an insertion of a fusion from the 35S Cauliflower Mosaic Virus promoter (35SCaMv) directing the expression of a Mn-SOD that included a plastidial signal peptide. Such transgenic plants also carried a resistance gene *nptII* that allowed us to select transformed plants in a selective medium supplemented with kanamycin (Bowler *et al*. 1991).

Biometric determinations

Root length and fresh weight (biomass) were measured as indexes of tolerance to Cd, by weighting and measuring the root length of each plant. Roots and leaves were separated and immediately frozen in liquid nitrogen. Samples coming from the same treatments and experiment were pooled and stored at -80°C until used. Growth was calculated relative to control plants. Plant length data are given as the average of at least five independent experiments ± SD.

Cd determination

Plants samples were pooled, dried for 72 h at 60°C, and ground in a mortar until a homogeneous powder was obtained. The samples were weighted and digested (0.6 mL HNO_3, 0.4 mL H_2O_2 and 1 mL H_2O), at 120°C and 1.5 atm for 30 min. After digestion, samples were filtered and the volume brought to 6 mL with deionised H_2O. Cadmium was measured by atomic absorption spectrophotometry by using an air-acetylene flame and a Cd hollow-cathode lamp (AAnalyst-80, Perkin-Elmer, Norwalk, Connecticut, USA).

Chlorophyll content

Total chlorophyll was extracted from 50 mg of frozen leaves with 10 mL 80% (v/v) acetone using a mortar and pestle. Homogenates were filtered and absorbance was measured in a spectrophotometer at 645 and 663 nm. Different chlorophyll contents were calculated according to Arnon (1949) and Wellburn (1994).

Lipid peroxidation

Lipid peroxides were estimated *in vitro* by the reaction of lipid peroxidation derivatives, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) in an acid medium, according to Buege and Aust (1978). Frozen tissue (0.2 g) was homogenised in a mortar with 1 mL of TCA-TBA-HCl reagent (15% (w/v) trichloroacetic acid (TCA), 0.37% (w/v) TBA, 0.25 M HCl and 0.01% butylatedhydroxytoluene). After homogenisation, samples were incubated at 90°C in a hot block for 30 min, placed in ice, and centrifuged at 12000 × *g* for 10 min. Absorbance was measured at 535 nm and 600 nm, the last one to correct the non-specific turbidity. Concentration was calculated directly from the extinction coefficient of $1.56 \cdot 10^5$ M^{-1} cm^{-1}.

Analysis of biothiols

Biothiols (i.e. glutathione and phytochelatins) were quantified by High Performance Liquid Chromatography (HPLC). Frozen tissue (0.1 g) was homogenized with 450 μL of 0.1 N HCl. N-acetylcysteine was also added as internal standard, at a final concentration of 100 μM. Extracts were centrifuged, for 15 min at 12000 × *g* and at 4°C, supernatant was recovered and centrifuged again. The clear supernatant was measured by HPLC following a protocol for elution and detection (Meuwly *et al*. 1995) with minor changes. A volume of 100 μL of the extract was injected in a Spherisorb precolumn coupled to a Spherisorb C18 column (250 × 4.6 mm; Waters, USA) (flux of 1 mL/min) and eluted with a gradient of solvent A (98:2 H_2O:acetonitrile (v/v) plus 0.01% TFA) and solvent B (2:98 H_2O:acetonitrile (v/v) plus 0.01% TFA). The gradient program, as for % solvent B, was: 2 min, 0%; 25 min, 25%; 26 min, 50%; 30 min, 50%; 35 min, 0%, 45 min, 0%. The chromatography was performed in the Alliance 2695 HPLC system (Waters, USA). Detection was achieved after post-column derivatisation with Ellman´s reagent: 1.8 mM 5, 5-dithio-bis(2-nitrobenzoic) acid (DTNB, absorption maximum at 412 nm), in 300 mM K-phosphate, 15 mM EDTA at pH 7.0. The reaction took place in a thermostatized 1.8 mL reactor at 37°C, as described by Rauser (1991).

Antioxidant enzymes activities in non-denaturing gels

Plant samples were frozen in liquid N_2, pooled and ground in a chilled mortar. 0.2 g of powder were homogenized with 0,8 mL of extraction buffer (0.1 M phosphate buffer, 1 mM Na-EDTA, 10 mM DTT and 2 mM ascorbate, 1% (w/v) PVPP at pH 7.2), supplemented in fresh with 50 μL proteinase inhibitor cocktail (Sigma, USA). For APX activity assay, extraction buffer pH was at 8.8 and contained 10 mM DTT and 10 mM ascorbate. Homogenate was centrifuged (14000 × *g* for 15 min at 4°C), and the supernatant was kept at -80°C in several single-use aliquots. Protein concentration of the different extracts was determined with Protein Assay (BioRad, USA). Identical amounts of protein were loaded in native PAGE (12% acrylamide) for APX and GR, and a native gradient PAGE (12-17% acrylamide) was used for SOD.

Table 1 Tolerance test in wild type (WT) and *35S::MnSOD Nicotiana tabacum* plants exposed to 12.5 µM Cd. Results are the mean of three independent assays with several replicates (up to 25 per treatment). Different letters denote significant differences with $P < 0.05$.

	Control		Cd	
	WT	***35C::MnSOD***	**WT**	***35S::MnSOD***
Root length (cm)	12.6 ± 0.9 a	13.0 ± 1.2 a	10.1 ± 1.0 a	11.0 ± 1.0 a
Root biomass (g)	0.26 ± 0.04 a	0.30 ± 0.05 a	0.12 ± 0.04 b	0.17 ± 0.03 b
Shoot biomass (g)	1.76 ± 0.33 a	2.00 ± 0.49 a	0.65 ± 0.19 b	0.66 ± 0.13 b

Table 2 Chlorophyll content in shoots (µg g^{-1}FW), lipid peroxidation measured as MDA content (nmol g^{-1} FW) and tissue Cd concentration (nmol g^{-1} FW) of wild type (WT) and transgenic 35S::MnSOD *Nicotiana tabacum* plants exposed to 12.5 µM Cd. Results are the mean of four replicates of three independent assays. Different letters denote significant differences with $P < 0.05$.

	Control		12.5 µM Cd	
	WT	***35S::MnSOD***	**WT**	***35S::MnSOD***
Chlorophyll concentration (µg g^{-1} FW)				
Chlorophyll *a*	863.1 ± 83.1 b	852.7 ± 45.6 b	663.0 ± 72.9 a	842.4 ± 98.5 b
Chlorophyll *b*	283.2 ± 15.5 d	278.2 ± 11.2 d	226.7 ± 22.2 c	282.7 ± 36.4 d
MDA (nmol g^{-1} FW)				
Roots	10.8 ± 1.3 a	9.3 ± 0.5 a	14.8 ± 2.3 b	15.6 ± 3.2 b
Shoots	6.4 ± 0.5 c	5.4 ± 0.2 c	9.9 ± 0.8 d	11.6 ± 2.1 d
Metal content (nmol/g FW)				
Roots	3.0 ± 1.1 a	1.6 ± 0.6 a	2243.6 ± 233.7 c	2516.8 ± 325.6 c
Shoots	3.1 ± 0.6 a	2.1 ± 0.7 a	322.3 ± 25.0 b	363.2 ± 9.9 b

SOD activity (EC 1.15.1.1) was assayed *in gel loading* 15 µg of total protein in each lane. Enzyme was detected following the procedure of Beauchamp and Fridocivh (1971), after incubating the gels with 1.25 mM NBT. $O_2^{\bullet-}$ was produced *in situ* by the photooxidation reaction of 50 µM riboflavine in 50 mM Na-phosphate buffered at pH 7.8.

For APX (EC 1.11.1.11) detection, 25-50 µg of total protein was separated by electrophoresis following Jiménez *et al*. (1998). After incubation of gels for 20 min with 2 mM ascorbate and 2 mM H_2O_2 in 50 mM Na-phosphate buffer at pH 7.0, the presence of APX was detected with 0.5 mM nitrobluetetrazolium and 10 mM tetramethylethylenediamine in 50 mM phosphate buffered at pH 7.8.

GR (EC 1.8.1.7) activity was revealed with the procedure developed by Sobrino-Plata *et al*. (2009). Gel slabs were incubated in GR staining solution (250 mM Tris-HCl buffer at pH 7.5, supplemented with 0.2 mg/mL thizolyl blue tetrazolium bromide, 0.2 mg/mL 2,6-dichlorophenol indophenol, 0.5 mM NADPH and 3.5 mM oxidized glutathione; GSSG). Bands corresponding to diaphorase activity were identified by the incubation of a second gel in a staining solution without GSSG.

Pictures were taken using a digital camera (Kodak 290, USA) and processed by using the Kodak 1 D Image Analysis Software ver. 3.6.

Statistical analysis

A one-way ANOVA statistical analysis was performed using the SPSS software (Release 13.0, SPSS Inc., Chicago, USA) to evaluate means ± SD of at least three independent experiments. The mean differences ($P < 0.05$) were compared utilizing Duncan's multiple range test.

RESULTS AND DISCUSSION

The over-expression of antioxidant enzymes in plants has often been a strategy to ascertain their functions under harmful environmental conditions (Allen *et al*. 1997). The tolerance gained by the over-expressed enzymes was not always satisfactory, and varied enormously when plants were subjected to different kinds of stresses (Teppermanand Dunsmuir 1990; Bowler *et al*. 1991; Gupta *et al*. 1993; Van Camp *et al*. 1994; Slooten *et al*. 1995). Additionally, very little information is available on the usage of such transgenic plants under the oxidative stress caused by heavy metals. Thus, we have studied MnSOD overexpression targeted to plastids in transgenic tobacco plants treated with Cd, to determine whether they are more resistant. Different physiological parameters typically affected by exposure to Cd stress were analysed.

Stress indexes and Cd concentration

There were no significant differences in root and shoot growth and biomass in WT and *35S::MnSOD* plants treated with 12.5 µM Cd (**Table 1**). Cadmium caused an average reduction of 35% in WT and *35S::MnSOD* plants, suggesting that over-expression of MnSOD targeted to plastids did not alleviate Cd toxicity. Similarly, the same transgenic line tested was equally sensitive than WT plants to methyl viologen (Slooten *et al*. 1995).

Photosynthetic pigments are very sensitive to heavy metal accumulation, and a common symptom of prolonged exposure to Cd is the appearance of chlorosis (Hall 2002). The determination of photosynthetic pigments concentration (i.e., chlorophyll and carotenoids) has been suggested as a simple and reliable index of heavy metal toxicity in higher plants (Krupa *et al*. 1996). Over-expression of MnSOD in plants grown in control medium had no effect on the concentration of chlorophyll *a* and chlorophyll *b* (**Table 2**). However, significant differences were observed between WT and transgenic MnSOD over-expressing plants in the presence of Cd (**Table 2**). There was a 20% decrease in WT plants, whereas plants over-expressing plastidial MnSOD had similar values than plants grown without Cd. Therefore, accumulation of recombinant MnSOD in the chloroplast seems to exert a protective barrier against the degradation of chlorophyll found in WT plants treated with Cd. This is in agreement with studies carried out with transgenic lines over-expressing MnSOD (Bowler *et al*. 1991; Slooten*et al*. 1995), where higher MnSOD activity reduced photo inhibitory effects in the chloroplast with methyl-viologen and high light treatments.

On the other hand, there was a higher accumulation of MDA upon exposure to 12.5 µM Cd. In this case, there were no significant differences between WT and MnSOD over-expressing plants (**Table 2**). Slooten and co-workers (1995) observed in the same transgenic line studied here that protection to different light irradiances and treatments with methyl viologen were dependent on plant age. Thus, cellular integrity, which was evaluated by means of ion leakage, showed that protection occurred only under extreme stress conditions or in elderly leaves, with clear symptoms of senescence (Slooten *et al*. 1995). Similar results were observed when this particular transgenic line was subjected to ozone stress (Van Camp *et al*. 1994). Therefore, it is feasible that under a prolonged exposure to Cd, larger differences between WT and *35S::SOD* plants would be observed in the induced stress.

The mechanisms of toxicity on chlorophyll accumulation and oxidation of cell components have not being com-

Table 3 Biothiols (nmol g^{-1}) in wild type (WT) and *35S::MnSOD Nicotiana tabacum* plants exposed to 12.5 µM Cd. Results are the mean of nine replicates of three independent assays. Letters of statistical analysis are significant in the same row.

	Control		12.5 µM Cd	
	WT	***35S::MnSOD***	**WT**	***35S::MnSOD***
Shoots				
Cys	116.0 ± 31.0 a	81.9 ± 12.8 b	232.7 ± 24.5 c	237.0 ± 28.5 c
GSH	933.6 ± 255.6 a	806.5 ± 46.2 a	1224.5 ± 570.5 ab	1446.0 ± 297.0 b
PCs	247.9 ± 33.4 a	231.2 ± 62.7 a	6985.8 ± 1127.5 b	6060.4 ± 822.8 b
Roots				
Cys	230.9 ± 68.4 a	244.9 ± 36.2 ab	264.3 ± 108.1 ab	310.3 ± 47.9 b
GSH	466.7 ± 34.0 a	410.1 ± 77.1 a	663.0 ± 60.5 b	642.9 ± 99.2 b
PCs	107.2 ± 79.4 a	154.3 ± 36.4 a	10821.5 ± 1382.1 b	12924.4 ± 1120.4 c

pletely elucidated (Gratão *et al*. 2005). In this respect, it would be also interesting to analyse the alteration in subcellular compartments since chlorophyll was apparently protected by the overexpressed plastidial MnSOD. On the other hand, more sensitive oxidative stress probes should be tested at cellular level, such as H_2DCFDA (Ortega-Villasante *et al*. 2005). Indeed, Cd caused an accumulation of H_2O_2 principally in pea leaf mesophyll cells as showed by DCFDA fluorescence (Rodríguez-Serrano *et al*. 2009). It is feasible that under metal stress photosynthetic active cells undergo a high rate of oxidative stress due to unpaired photochemical reactions, possibly trough a generation of ROS.

Regarding Cd accumulation in plants, there were no significant differences between WT and *35S::MnSOD N. tabaccum* plants (**Table 2**). Both genotypes accumulated much more Cd in roots than in shoots, being almost one order of magnitude this difference. Similar concentration of Cd was observed in the leaves of *N. tabacum* plants cultivated in the presence of 5 to 40 µM $CdCl_2$ (Vögeli-Lange and Wagner 1996). The amount of Cd translocated to shoots was about the expected value of 10% according to previous studies summarised by Polle and Schützendübel (2003).

Analysis of biothiols

Since glutathione (GSH) metabolism changes and synthesis of phytochelatins (PCs) are one of the main symptoms in cells after heavy metal exposure (Cobbett and Goldsbrough 2002), biothiols were analysed by HPLC with post-column derivatisation with Ellman's reagent. Cysteine (Cys) and GSH were the major peaks identified in plants grown in control nutrient solution (**Fig. 1; Table 3**). The levels of GSH were of the same order as those described by Slooten *et al*. (1995) and Vögeli-Lange and Wagner (1996) for *N. tabaccum*, although these authors only provided the concentration found in leaves. GSH concentration was similar in leaves and roots of WT and *35S::MnSOD* transgenic plants, in agreement with the previously mentioned work, where no significant differences were found between transgenic and WT plants (Slooten *et al*. 1995). After Cd exposure, the amount of Cys and GSH increased significantly in shoots and roots, and a great variety of Cd-induced new peaks appeared (i.e. phytochelatins; PCs). The concentration of PCs was particularly remarkable in roots, where their concentration was 10- to 20-fold that of GSH (**Table 3**). This response was similar in WT and *35S::MnSOD* plants, indicating that the transgene did not affect the metabolic pathway involved in the synthesis of GSH and of PCs.

The increase in GSH concentration and the appearance of PCs were coherent with known responses of plants to Cd (Vögeli-Lange and Wagner 1996; Cobbett and Goldsbrough 2002). Therefore, the increase found in tobacco upon treatment with 12.5 µM (**Table 3**) is in accordance with previous reports. The levels of GSH depleted after short-term exposure to Cd, probably caused by oxidation to GSSG as a consequence of H_2O_2 accumulation and/or due to direct interaction with the metal (Ortega-Villasante *et al*. 2005). However, under prolonged Cd exposure the levels of GSH recovered, probably to match the demands to provide precursors for PCs synthesis and to recover the GSH/GSSG

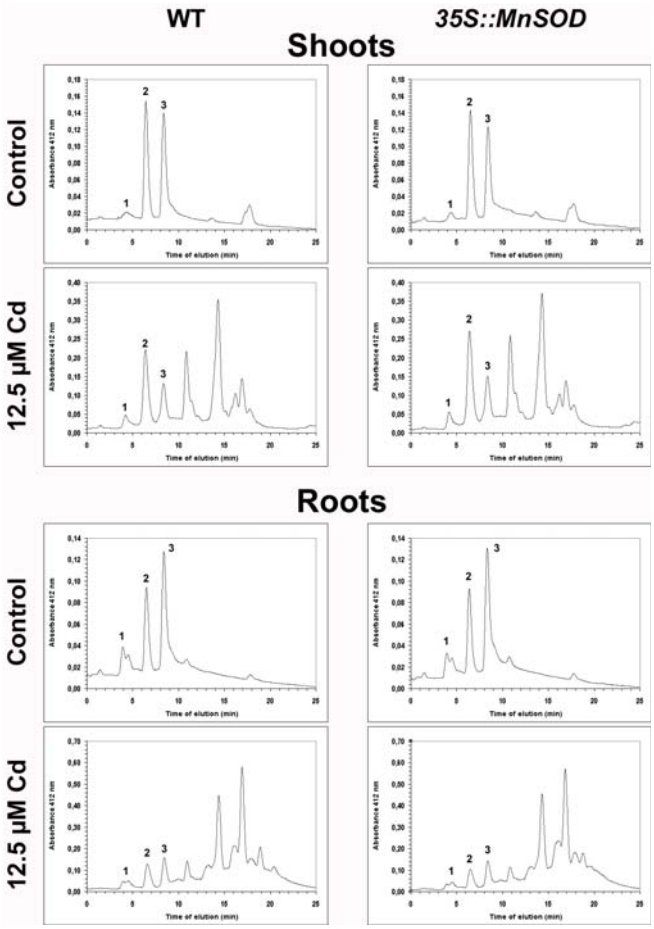

Fig. 1 Non protein thiols extracted from *N. tabacum* plants grown in control media or in the presence of 12.5 µM Cd. Peaks were identified using commercially available standards: 1, Cys; 2, GSH; 3, N-AcCys internal standard. In plants treated with Cd, further peaks corresponding to putative phytochelatins also appeared.

cellular balance (Xiang and Oliver 1998; Polle and Schützendübel 2003). This could be attained by an up-regulation of genes involved in GSH synthesis (Xiang and Oliver 1998).

Enzymatic antioxidative activities

To confirm that *35S::MnSOD* effectively had not only overexpression, but higher SOD activity, we analysed SOD activity *in gel*. Extracts from roots and shoots were prepared from WT and *35S::MnSOD* plants treated with and without 12.5 µM Cd. Thus, several isoforms of this enzyme could be detected in the samples (**Fig. 2**). In WT plants, a similar pattern of bands was described by Bowler *et al*. (1991) as the showed in **Fig. 2**: an endogenous mitochondrial MnSOD, resistant to 5 mM H_2O_2 and 5 mM KCN; two FeSODs, resistant to 5 mM KCN, and at least three Cu/ZnSODs (sensitive to both inhibitors) were found. In *35::MnSOD* transgenic plants, in addition to the previous

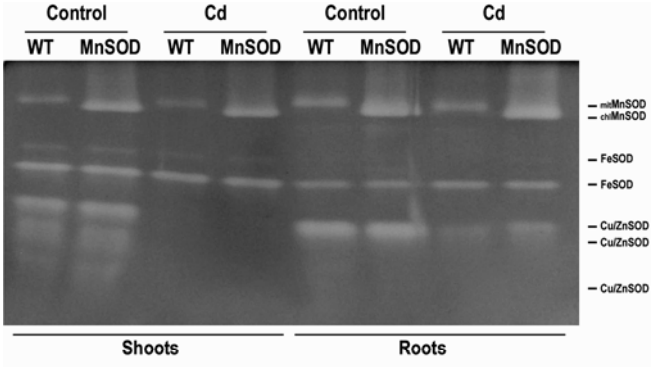

Fig. 2 SOD activity was detected *in gel* after non-denaturing PAGE. Samples were processed from shoots and roots of *Nicotiana tabacum* WT and *35S::MnSOD* plants in the absence or presence of 12.5 μM Cd. A representative gel of three independent experiments is presented.

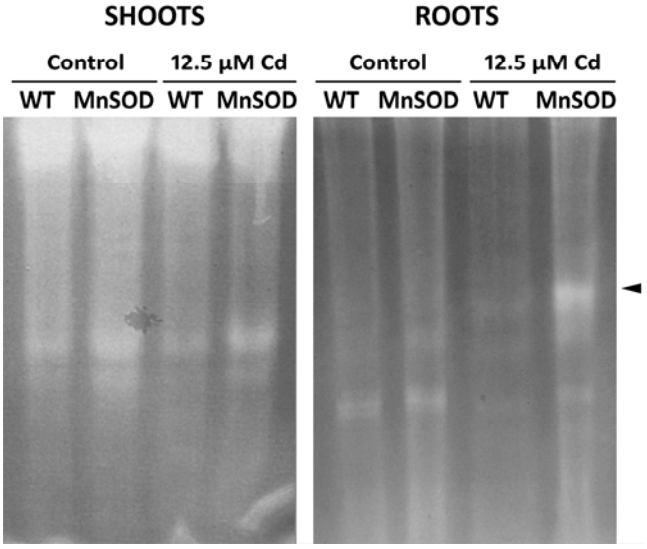

Fig. 3 APX activity was studied in a non-denaturing PAGE gel. Extracts were prepared from shoots and roots of W T and *35S::MnSOD* plants, treated with control and 12.5 μM Cd nutrient solutions. A representative gel of three independent experiments is presented. The arrow highlights the band that appears only in Cd-treated plants.

bands, there was a much stronger band close to the endogenous MnSOD. Interestingly, this protein showed slightly higher electrophoretic mobility, probably caused by its engineering. It should be remembered that a chimerical protein was constructed to target an originally mitochondrial SOD to plastids, by introducing a plastidial signal peptide (Bowler *et al*. 1991). Moreover, similar pattern of bands was observed in root extracts of transgenic plants, indicating that the recombinant MnSOD was also active in non-photosynthetic tissues (**Fig. 2**).

Exposure to Cd led to remarkable changes in SOD activity. In shoots, the bands of high mobility corresponding to Cu/ZnSOD activity were completely suppressed by Cd. This effect was observed equally in WT and transgenic plants (**Fig. 2**). However, the rest of SOD activity bands corresponding to FeSOD and MnSOD did not apparently change (**Fig. 2**). A similar behaviour was found in roots, where the intensity of Cu/ZnSODs bands decreased appreciably (**Fig. 2**). Regarding the contribution of the plastidial overexpressed MnSOD, the intensity of the band was not affected by Cd-treatment either in shoots nor roots. The rest of endogenous SOD activity bands had a similar pattern to those observed in WT plants (**Fig. 2**). Therefore, overexpression of the plastidial recombinant MnSOD did not appear to alter the response against Cd.

The inhibition of Cu/ZnSOD is a novel response of a group of SODs to Cd supply. Little variation or even slight

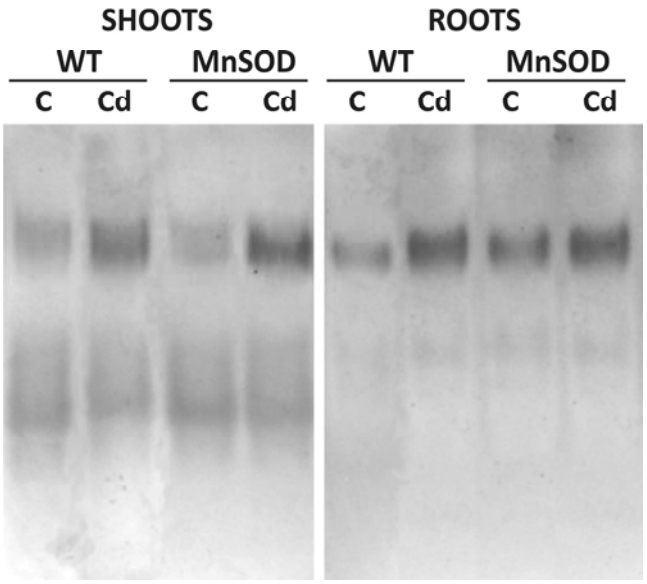

Fig. 4 GR activity was studied in a non-denaturing PAGE gel. Extracts were prepared from shoots and roots of W T and *35S::MnSOD* plants, treated with control and 12.5 μM Cd nutrient solutions. A representative gel of three independent experiments is presented.

increases have been observed in maize plants (Rellán-Álvarez*et al*. 2006), sunflower (Gallego *et al*. 1996), or tobacco BY2 cells (Piqueras *et al*. 1999). This speaks up for the poor generalization that can be outlined from particular experiment, each obtained form different plant species, ages and tissues analysed, and different growth conditions (Gratão *et al.* 2005). Indeed, since Cu/ZnSOD isoforms are sensitive to H_2O_2, it has been proposed that increased levels of H_2O_2 triggered by Cd-exposure could inhibit Cu/ZnSOD activity (Doulis *et al.* 1998).

To complete the characterization of the enzymatic systems involved in the scavenging of ROS under Cd stress, we studied ascorbate peroxidase (APX) activity *in gel*. This enzyme reaction is the following step to SOD for H_2O_2 detoxification. Some authors suggest that the equilibrium between $O_2^{\bullet-}$ and H_2O_2, and subsequently SOD and APX activities, are key factors for the overall cellular defence against ROS (Bowler *et al*. 1991; Bowler *et al*. 1992; Gupta *et al*. 1993). As shown in **Fig. 3**, a band of higher intensity appeared in the roots of transgenic plants after Cd exposure. This isoform is possibly a cytosolic isozyme, following previous observations in other species (not shown), but needs confirmation by Western-blot analysis. Interestingly, that band was almost absent in WT plant root and shoot extracts from control or Cd-treated plants (**Fig. 3**). However, in shoots APX activity did not changed appreciably between control and Cd-treated plants. Similar responses were found in alfalfa plants exposed to increasing concentrations of Cd (from 3 to 30 μM), where APX augmented in roots at the highest doses but did not change in shoots (Sobrino-Plata *et al*. 2009).

Coordinated function of SODs and APXs seems to be essential to cope with environmental stresses (Gupta *et al*. 1993). Precisely, over-expression of MnSOD or FeSOD together with APX permitted transgenic tobacco plants to cope much better to methyl viologen-mediated stress (Kwon *et al*. 2002). Thus, it is feasible that similar results would be obtained upon exposure to Cd. Indeed, this might be the case since Cd accumulated to a higher extent in roots (**Table 3**), and subsequently higher peroxidation increases occurred in this tissue (MDA concentration increased by 155% in WT roots, and in *35S::SOD* roots by 214%).

Another evidence of the redox cellular alteration caused by Cd was the increase in GR activity, antioxidant enzyme that increased its activity in WT and *35S::MnSOD* transgenic plants (**Fig. 4**). Yannarelli *et al*. (2007) also reported a

remarkable increase in root GR activity of wheat plants treated with 100 µM Cd for one and two weeks. Interestingly, a new isoform of higher electrophoretic mobility was detected under Cd stress, possibly associated which peroxisomes (Romero-Puertas *et al*. 2006). Similarly, Sobrino-Plata *et al*. (2009) observed a strong activation of root GR in alfalfa plants exposed to 3, 10 and 30 µM Cd, suggesting that the ascorbate-GSH cycle may be relevant for Cd detoxification. Finally, the over-expression of plastidial MnSOD had no effect on GR activity, as occurred in other tested responses (**Fig. 4**).

CONCLUSIONS

Over-expression of MnSOD alone had apparently little effect on the tolerance to Cd of *N. tabacum* transgenic plants. However, putative protection of chlorophyll content and enhanced APX activity in roots ensure future work. It is possible that some protection occurred at subcellular level, therefore more detailed analysis is needed with more sensitive techniques to visualise oxidative stress. On the other hand, unbalanced SOD/APX systems might be the cause for the activation of APX in the roots of *35::MnSOD* plants. Again, a more comprehensive study is needed to evaluate the importance of the proper management of the H_2O_2 generated by a higher SOD activity, and a biotechnological approach could be designed to cope effectively with Cd-mediated oxidative stress. Future work will be focused to test other transgenic plants with enhanced expression of antioxidant enzymes targeted to other organelles.

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