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Cadmium induces different biochemical responses in wild type and catalase-deficient tobacco plants

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ABSTRACT

The response of tobacco (*Nicotiana tabacum* L.) wild type SR1 and catalase-deficient CAT1AS plants was evaluated after exposure to $CdCl_2$. CAT1AS plants accumulated more Cd than SR1 plants, and this was associated with reduced growth, but higher chlorophyll content and cell viability. Despite catalase deficiency, CAT1AS plants did not accumulate more H_2O_2 than the wild line when exposed to Cd, probably due to the fact that CAT1AS plants counterbalanced their catalase deficiency by increasing the constitutive guaiacol peroxidase and ascorbate peroxidase activities and by reducing the basal NADPH oxidase-like enzyme activity. Both lines could activate their antioxidant system upon Cd stress, although the stress response pathways showed wide differences in the mineral and nitrogen metabolism, since the wild-type line had reduced nitrates and iron content, while CAT1AS maintained the same level of nitrates and Fe than that of non-treated plants, and responded with a significant increase in proline. The results showed that, unlike previous reports using other type of stress with the same line plants, catalase did not play a crucial role in protecting against Cd toxicity and CAT1AS plants, compared to SR1, were able to activate alternative defence mechanisms against Cd toxicity.

1. Introduction

Metals from anthropogenic sources frequently pollute natural systems. The biota may require some of these elements considered essentials (like Fe, Zn, Cu or Mo) in trace quantities, but at higher concentrations they may become toxic. However, metals like Cd, Pb or Al are non-essential and toxic even at very low levels.

Cadmium (Cd) is a metal widespread in soils, water and atmosphere and it has gained considerable attention over the past decade due to its increased presence in the environment (Sanità di Toppi and Gabbrielli, 1999; Benavides et al., 2005; Gratão et al., 2005; Gallego et al., 2012). Since 1980, the attention shifted to the recognition of Cd as an important environmental problem, with epidemiological studies focusing on the importance of low-level exposures in human health and its widespread toxicity amongst wildlife (Sanità di Toppi and Gabbrielli, 1999; Järup and Åkesson, 2009; Clemens et al., 2013). As a consequence of the documented adverse health effects of Cd-containing aerosols and particles, legislation and technological improvements have resulted in a steady decline in Cd emissions (Clemens et al., 2013). Cadmium is considered a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993), though it is only weakly genotoxic (Beyersmann and Hartwig, 2008).

Low Cd^{2+} concentrations in the soil solution in combination with a low diffusion coefficient for Cd^{2+} in aqueous solution suggests that transpiration driven mass-flow of the soil solution will dominate in the delivery of Cd^{2+} to plant roots (Lux et al., 2011). Cd can affect cell biochemical mechanisms and structural aspects, for example, by lowering the control of the cell redox state, so alterating photosynthesis (Qian et al., 2010), stomatal conductance and the leaf transpiration (Souza et al., 2011), water relations and mineral uptake (He et al., 2011; Gill et al., 2012), and causing oxidative stress and disruption of membrane composition and function (Cuypers et al., 2011; Azevedo et al., 2012; Gallego et al., 2012). Hence, a complex biochemical pathway within the cell can be triggered concurrently with transcription regulation of Cd-responsive genes, such as induction of antioxidant systems (Gratão et al., 2012).

The excessive production of ROS, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), is one of the main mechanisms by which plants are damaged during stress, for which they have evolved non-enzymatic (mainly ascorbate and glutathione) and enzymatic (SOD, CAT and peroxidases) protection mechanisms that

Abbreviations: APOX, ascorbate peroxidase; CAT, catalase; DAB, 3,3′-diaminobenzidine; DPI, diphenyleneiodonium; GPOX, guaiacol peroxidase; NBT, nitroblue tetrazolium; PRO, proline; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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efficiently scavenge them. Such oxidative stress has been shown to occur in plants exposed to high and low temperatures, particularly in combination with high light intensities, drought, exposure to air pollutants, ultraviolet light, metals and herbicides (Vranová et al., 2002; Mittler et al., 2004; Gill and Tuteja, 2010; Gallego et al., 2012; Iannone et al., 2012).

Several species of the genus *Nicotiana* markedly differ by their ability to accumulate Cd (Doroszewska and Berbec', 2004). Despite cadmium accumulation in different plant species preferentially occurs at root level (Wu et al., 2004; Lux et al., 2011; Clemens et al., 2013), some reports indicate that *Nicotiana tabacum* L. can accumulate relatively high Cd concentrations in leaves. Moreover, the plants used in the present study are CAT-deficient plants, which provide an additional tool to investigate the Cd-induced oxidative stress and Cd toxicity effect *in planta*, by following changes in H_2O_2 homeostasis in a non-invasive way (Dat et al., 2003). Catalase is the major scavenging enzyme in the degradation of photorespiratory H_2O_2 (Kendall et al., 1983). The role of H_2O_2 signaling during the induction of defense responses has been studied in CAT1AS plants, which have a reduced catalase activity in the peroxisomes, growing under high irradiance or pathogen attack (Willekens et al., 1995, 1997; Chamnongpol et al., 1998; Dat et al., 2003). Three different catalase (Cat1, Cat2, and Cat3) genes have been identified from *Nicotiana plumbaginifolia* that are highly similar in sequence. Catl mRNA levels are most abundant in leaves, but not in roots. Cat2 is quite constitutively expressed and Cat3 mRNA is equally expressed in root, stem, seeds and young leaf (Willekens et al., 1994). Tobacco CAT1AS transgenic plants, in a similar way to the original barley mutant RPr 79/4 (Kendall et al., 1983), presented a catalase activity of 10% of the wild type activity in the leaves, but a comparable enzyme activity in the roots of the wild type and the mutant line was observed. However, while barley RPr 79/4 line was unable to grow satisfactorily in normal air, CAT1AS plants grow less than wild type plants but well enough in our experimental conditions.

In the present work, *N. tabacum* leaves were chosen as study material, in order to better understand Cd accumulation in the aerial part of a non-hyperaccumulator plant and to evaluate the response of tobacco plants against Cd stress in relation to ROS detoxification strategies in a plant deficient in H_2O_2 scavenging.

In this context, the aim of this work was to evaluate cadmium toxicity in tobacco plants through the study of different parameters, such as growth, oxidative stress generation, membrane damage and cell death in wild type (SR1) and transgenic tobacco catalase-deficient (CAT1AS) plants, irrigated with two concentrations of $CdCl_2$ (100 and 500 μM) for 8, 13 and 25 days.

2. Materials and methods

2.1. Plant growth conditions and treatments

Seeds of *N. tabacum* var. Petit Havana SR1 wild type and *N. tabacum* CAT1AS (a transgenic line that expresses only 10–30% of wild-type catalase activity in the leaves and only 40% in the roots due to the antisense expression of the cat1 gene, kindly provided by Dr. F. Van Breusegem from Ghent University, Belgium) derived from *N. tabacum* Petit Havana SR1 (Chamnongpol et al., 1998) were germinated and grown as described previously (Chamnongpol et al., 1998), in a controlled environmental chamber with a relative humidity of 70% and temperature of 24/21 °C for 16 h light/8 h dark, with a light intensity of 120 μmol m^{-2} s^{-1} (this light intensity was chosen since the severe reduction in catalase activity had no apparent consequences under moderate light intensities, as reported by Chamnongpol et al. (1998)), and watered with Hoagland nutrient solution (Hoagland and Arnon, 1950). After 30 days of growth, 8 seedlings were transferred to plastic trays containing humus, perlite and vermiculite in 1:1:1 ratio and were grown in the same conditions described above. Since day 43, plants were treated as follows: control plants with Hoagland solution (C) and treated plants with Hoagland solution supplemented with 100 or 500 μM $CdCl_2$ (Cd 100; Cd 500). After 8, 13 or 25 days of treatment, plants were harvested and immediately used or frozen and stored for subsequent analysis.

2.2. In situ O_2^- localization

O_2^- content was estimated using a 0.05% solution of nitroblue tetrazolium (NBT), which reacts with O_2^- and produces a blue precipitate of formazan. DPI (a NADPH oxidase inhibitor) was used as a control (Bolwell et al., 1998; Frahry and Schopfer, 1998).

2.3. In situ H_2O_2 localization

H_2O_2 formation was determined by an histochemical method using 3,3′-diaminobenzidine (DAB). The appearance of brown spots is indicative of H_2O_2 formation (Thordal-Christensen et al., 1997). Ascorbic acid (an antioxidant) was used as a control.

2.4. Analysis of Cd and Fe in leaves

Cadmium and iron content were determined in tobacco leaves watered for 25 days with 100 μM or 500 μM $CdCl_2$. To analyze the metal concentrations in foliar tissues, leaves were dried for 15 days at 80 °C and the fine powder obtained (about 100 mg DW) was digested in a mixture (HNO_3:$HClO_4$ 3:1 v/v) at 170 °C, and metals determination were performed by flame atomic absorbance spectrometry Perkin Elmer AAnalyst 300.

2.5. Chlorophyll content

For chlorophyll determination, 100 mg FW of tobacco leaves were incubated in 5 ml of 96% ethanol at 50–60 °C for 1 h or until complete decolourization. Chlorophyll content was then analyzed spectrophotometrically at 654 nm on the ethanolic supernatant in a Hitachi U-2000 spectrophotometer, as described by Wintermans and De Mots (1965).

2.6. Cell death detection

2.6.1. Evans blue staining

To determine changes in cells viability upon exposure to cadmium, tobacco leaves were incubated with a 0.25% (w/v) aqueous solution of Evans blue (Baker and Mock, 1994) during 15 min at room temperature, then washed twice with distilled water and left in distilled water overnight. Then the samples were incubated 1 h at 50 °C with a methanol-SDS solution and the absorbance was measured at 595 nm.

2.6.2. Electrolyte leakage

For electrolyte leakage measurement, leaf samples were thoroughly washed with distilled water and kept in closed vials with 10 ml of deionized water. To estimate ion leakage from leaf discs (Shou et al., 2004), conductivity of each solution was measured at the initial time (T_0), after the incubation with the different treatments (T_1) and after heating at 100 °C for 1 h (T_2). The results were expressed as relative conductivity $[(T_1 - T_0)/(T_2 - T_0)] \times 100$.

2.7. Thiobarbituric acid reactive substances (TBARS) determination

The level of lipid peroxidation products in leaves was determined by estimating thiobarbituric acid reactive substances

(TBARS) as described by Heath and Packer (1968). TBARS content was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}).

2.8. Subcellular fractionation and assay of NADPH oxidation

NADPH oxidase activity was measured in the microsomal fraction and in the cytosol by monitoring NBT reduction by NADPH at 530 nm for 3 min, according to Shen et al. (2000). NADPH oxidation activity was calculated by taking the difference between the apparent reaction rates with or without SOD (75 U ml^{-1}) in the reaction mixture, using $\varepsilon = 12.8\ mM^{-1}\ cm^{-1}$.

2.9. Enzyme preparations and assays

Plant homogenates for determination of ascorbate peroxidase (APOX, EC 1.11.1.11), guaiacol peroxidase (GPOX, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) were prepared in 50 mM phosphate buffer pH 7.8 containing 0.5 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100. APOX activity was immediately determined in the supernatant according to Nakano and Asada (1981). GPOX activity was determined as described by Maehly and Chance (1954), SOD activity was assayed as described by Becana et al. (1986) and CAT activity was determined according to Chance et al. (1979).

2.10. Nitrate content

Tobacco leaves were dried at 85 °C until constant weight. The dried material (25 mg) was grounded to powder and incubated in 10 ml of distilled water for 2.5 h.

Nitrates were measured colorimetrically after a reaction with salicylic acid (Cataldo et al., 1975).

2.11. GSH and GSSG determination

The oxidized and reduced glutathione were measured by a fluorometric assay described by Hissin and Hilf (1976) with a modification reported by Cohn and Lyle (1976). Fresh plant material (300 mg) was homogenized with 3 ml of 3% sulfosalicylic acid. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the supernatants were used for the analyses

2.12. Proline determination

Proline content was determined according to Bates et al. (1973). The homogenates were done using 300 mg of plant material in 3 ml of 5% sulfosalicylic acid (w/v). The samples were incubated with ninhydrin and glacial acetic acid at 100 °C for 1 h and the reaction was stopped by placing the tubes on ice. Absorbance was measured at 520 nm and proline concentration was calculated using a standard curve made with proline.

2.13. Statistics

All data presented are the mean values of two or three independent set of experiments. Each value was presented as means ± standard errors (SE), with a minimum of three replicates. Statistical analysis was carried out by one-way ANOVA using the Tukey's test to evaluate whether the means were significantly different, taking one asterisk $^{*}p < 0.05$, two asterisks $^{**}p < 0.01$, and three asterisks $^{***}p < 0.001$ as significant.

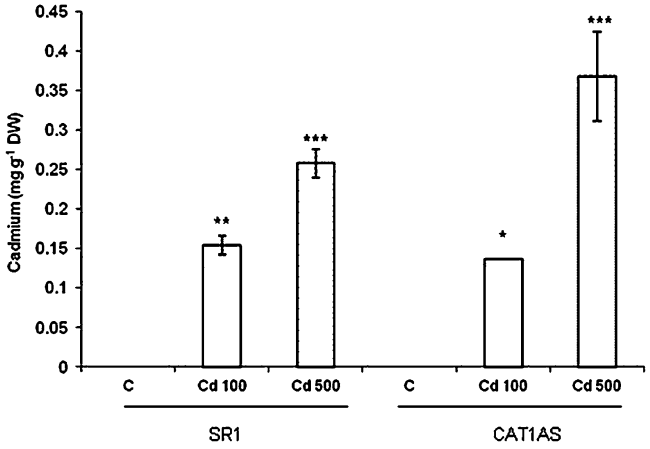

Fig. 1. Cadmium content in SR1 and CAT1AS plants exposed to 100 or 500 μM Cd for 25 days was measured as described in Section 2. Values are the mean ± SE from three independent experiments with six replicated measurements. Asterisks indicate significant differences ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) according to Tukey's multiple-range test.

3. Results

3.1. Cadmium content in plants irrigated with $CdCl_2$ for 25 days

Cadmium content was measured in the aerial part of tobacco plants exposed for 25 days at two concentrations (100 μM and 500 μM) of $CdCl_2$. As shown in Fig. 1, tobacco plants accumulated Cd in the aerial part in a concentration-dependent manner. The metal content in SR1 leaves was 0.15 and 0.26 mg g^{-1} DW for 100 μM or 500 μM $CdCl_2$, respectively; whereas CAT1AS plants showed a cadmium accumulation of 0.14 and 0.37 mg g^{-1} DW for 100 μM or 500 μM $CdCl_2$, respectively (Fig. 1).

3.2. Evaluation of symptoms of Cd toxicity in tobacco plants

3.2.1. Growth

In tobacco plants treated with 500 μM $CdCl_2$, a growth reduction was visualized from day 8 of treatment in the transgenic CAT1AS line, and from day 13 in the non-transgenic SR1 line, being clearly visible in CAT1AS by day 25. Note that transgenic non-treated plants showed a reduced size compared to control SR1 plants from the beginning of the experiment (Fig. 2). Despite chlorotic symptoms (indicated with arrows in Fig. 2) were more evident in SR1 line, the water absorption capacity of leaves were comparable in both lines, showing a little

Fig. 2. Effects of Cd on growth. Upper view of SR1 and CAT1AS plants irrigated with $CdCl_2$ by 25 days, as described in Section 2. Arrows indicate chlorotic leaves.

Table 1
Effect of 100 or 500 μM Cd on chlorophyll, nitrate and iron content at 8, 13 and 25 day of treatment.

		SR1			CAT1AS		
		C	Cd 100 μM	Cd 500 μM	C	Cd 100 μM	Cd 500 μM
Chlorophyll (μg Cla+b g^{-1} FW)	8 days	630.16 ± 9.60	630.23 ± 10.22	544.18 ± 12.42**	596.18 ± 18.62	573.04 ± 10.07	502.99 ± 17.21***
	13 days	701.14 ± 4.87	667.29 ± 29.26	490.90 ± 26.49***	640.25 ± 9.49	690.71 ± 5.02	549.12 ± 5.94**
	25 days	778.72 ± 0.38	324.15 ± 3.43***	247.30 ± 4.87***	510.84 ± 6.83	446.13 ± 4.13***	204.56 ± 13.20***
Nitrate (nmol mg^{-1} DW)	8 days	1332.41 ± 98.27	1336.11 ± 144.54	1394.85 ± 48.44	1190.73 ± 24.29	1204.36 ± 89.60	1111.67 ± 22.96
	13 days	1695.33 ± 106.34	1543.86 ± 74.88	1808.91 ± 327.11	1564.33 ± 236.72	1739.30 ± 255.95	1656.46 ± 160.58
	25 days	2839.25 ± 180.09	2067.70 ± 115.2***	982.73 ± 89.87***	1942.94 ± 77.25	2171.81 ± 97.02	1781.00 ± 175.79
Iron (mg g^{-1} DW)	25 days	0.09 ± 0.01	0.06 ± 0.01	0.05 ± 0.00*	0.07 ± 0.00	0.09 ± 0.00	0.07 ± 0.00

Data are the mean ± SE of three independent experiments, with five replicates for each treatment. Asterisks within rows indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), according to Tukey's multiple-range test.

but significant reduction only under 500 μM $CdCl_2$ (data not shown). However, all plants survived after 25 days of Cd treatment.

3.2.2. Chlorophyll, nitrates and iron content

As shown in Table 1, total chlorophyll concentration significantly declined under Cd exposure. At the highest Cd concentration, the decrease in the pigment content respect to the control was about 13% and 16% at day 8, 30% and 14% at day 13, for SR1 and CAT1AS, respectively. The maximum reduction in chlorophyll content was observed by day 25 of treatment, when the decrease was about 58% and 68% in SR1, and 13% and 60% in CAT1AS, with 100 μM and 500 μM $CdCl_2$, respectively. Notably, a strong reduction occurred at the longest exposure time in SR1 tobacco plants treated with 100 μM, but not in the transgenic CAT1AS line at this metal concentration.

Taking into account the importance of nitrogen as a key nutrient in plants, nitrate content was measured as a marker of the nitrogen status in tobacco plants under Cd stress. Both cadmium concentrations caused a decrease in nitrate content only in SR1 plants after 25 days of treatment. The reduction was about 27% and 65% for 100 or 500 μM $CdCl_2$, respectively (Table 1).

Iron content was measured as one of the markers of essential metals-uptake disturbance provoked by Cd that leads to Fe deficiency (Prasad and Hagenmeyer, 1999). The content of this essential element was measured only in tobacco plants irrigated for 25 days with 100 or 500 μM $CdCl_2$. As shown in Table 1, Fe content decreased about 44% compared to the control in SR1 plants, only with 500 μM $CdCl_2$.

3.3. Study of oxidative stress and antioxidants parameters

3.3.1. Hydrogen peroxide and superoxide anion accumulation

Hydrogen peroxide production was visualized by staining leaves with 3,3′-diaminobenzidine (DAB), a histochemical reagent that polymerizes and turns brown in the presence of H_2O_2 (Fig. 3). The staining of tobacco leaves treated for 8, 13 and 25 days with 100 or 500 μM $CdCl_2$ revealed no significant differences compared to controls on any tested day (Fig. 3). The specificity of the reaction was confirmed by infiltrating the leaves with ascorbate prior to staining with DAB (data not shown).

NBT was used to detect the blue formazan precipitation produced after the reaction with O_2^-. Along cadmium treatment there was greater O_2^- accumulation in SR1 than in CAT1AS plants particularly in controls and 100 μM $CdCl_2$ -treated plants. After 8 days of cadmium treatment there were no significant differences in the O_2^- accumulation in CAT1AS plants irrigated with the metal respect to their control, while 500 μM $CdCl_2$ inhibited the formazan formation in SR1 plants. From day 13 on, 500 μM $CdCl_2$ decreased the formation of blue formazan in both lines compared to their controls, whereas a marked inhibition of O_2^- accumulation was observed at day 25 (Fig. 3). The specificity of the reaction was confirmed by infiltrating the leaves with diphenyleneiodonium (DPI), an inhibitor of flavin-containing oxidases like NADPH oxidase, prior to staining with NBT (data not shown).

3.3.2. NADPH oxidase activity

To assess whether cadmium could restrict O_2^- and H_2O_2 generation by regulating NADPH oxidase activity, the enzyme activity was determined *in vitro*. The constitutive NADPH oxidase activity in SR1 control plants was 30%, 47% and 82% higher than in CAT1AS plants by days 8, 13 and 25, respectively. Cadmium did not affect the enzyme activity after 8 days of treatment, but at day 13, the metal significantly reduced NADPH oxidase activity in a dose-dependent manner only in wild-type SR1 plants. By the end of the treatment, both Cd concentrations significantly inhibited the enzyme activity in both lines, but most markedly in SR1 plants (Fig. 4).

3.3.3. Antioxidant enzymes activity

The activity of the main H_2O_2-detoxifying enzymes was measured to analyze the antioxidant machinery. After 8 days of treatment, CAT activity was not detectable in the transgenic CAT1AS plants, while at 13 or 25 days, CAT activity was barely detected only in untreated CAT1AS plants. Meanwhile, in SR1 plants, 500 μM Cd increased CAT activity four times at 8 days, 33% at day 13 and twice over the control at 25 days of treatment, while 25 days of exposure to 100 μM $CdCl_2$ doubled the control value of the enzyme activity.

After 13 days of exposure to 500 μM $CdCl_2$, APOX activity increased significantly to 169% and 77% over the controls in SR1 and CAT1AS, respectively. The last day of treatment, 100 μM $CdCl_2$ raised APOX activity threefold in SR1 plants, whereas in CAT1AS plants the value of the enzymatic activity augmented 56% over the control with the highest metal concentration. By this time, APOX activity was three times higher in control CAT1AS compared to control SR1 plants (Table 2).

GPOX activity of untreated CAT1AS plants was twice the value of control SR1 leaves at day 8 and 57% higher at day 25. With regard to cadmium treatment, the enzyme pattern was rather fluctuant according to Cd level along the treatment. At day 8, a strong rise of 150% over the controls was observed in GPOX activity in SR1 plants treated with 500 μM $CdCl_2$, while in CAT1AS plants, 100 μM $CdCl_2$ decreased GPOX activity by 50% below the control. At 13 days, 100 μM $CdCl_2$ reduced the GPOX activity by 20% in SR1 and 40% in CAT1AS, whereas 500 μM $CdCl_2$ increased it by 49% and 66% for SR1 and CAT1AS, respectively. The highest cadmium concentration tripled the value of the GPOX activity in SR1 plants at the end of the experiment (day 25), whereas in the transgenic plants, the enzyme activity was doubled with the two cadmium concentrations used (Table 2).

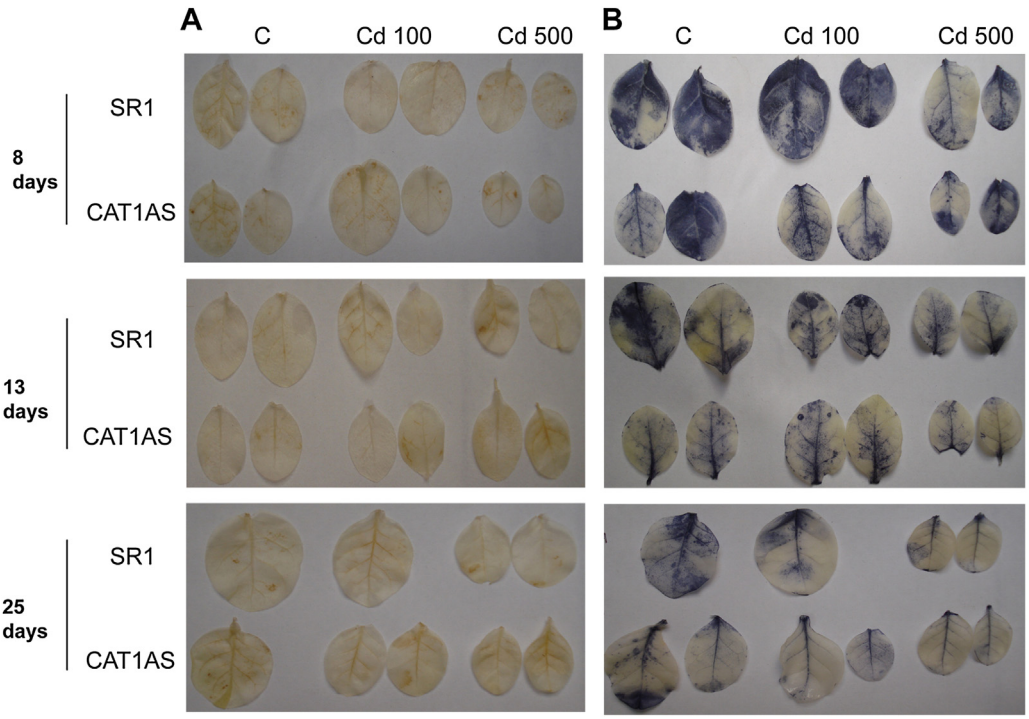

Fig. 3. H_2O_2 (A) and superoxide anion (B) accumulation in SR1 and CAT1AS plants. Plants exposed to distilled water and 100 or 500 μM Cd for 8, 13 or 25 days were vacuum-infiltrated with 0.05% nitroblue tetrazolium (NBT) or 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB) and incubated under illumination as described in Section 2. (A) H_2O_2 was revealed by the appearance of brown spots due to DAB polymerization. (B) Superoxide anion formation was revealed by the appearance of blue spots characteristics of formazan deposition.

Superoxide dismutase (SOD), an important source of superoxide anion-derived H_2O_2 in plant cells, doubled its value in SR1 plants, but decreased its activity by 40% in CAT1AS plants at 8 days of treatment with both metal concentrations. At day 13, 100 μM or 500 μM $CdCl_2$ inhibited SOD activity by 30% in SR1 plants, but 100 μM Cd slightly increased this activity (18%) in transgenic plants. At 25 days, SOD activity did not change in any of the lines exposed to cadmium compared with their controls (Table 2).

3.3.4. Study of non-enzymatic antioxidants: Proline and glutathione content

The dual role of proline as an antioxidant and as an osmotic stress indicator was a valuable marker of metal toxicity. Proline content was not modified in Cd treated-SR1 plants either at days 13 or 25 days and showed only a slight increase at day 8. However, the osmolyte content increased 47% and 83% in CAT1AS plants treated with 100 or 500 μM $CdCl_2$ at day 8, and 68% or 268% at day 13, respectively. The last day of treatment, Pro content significantly

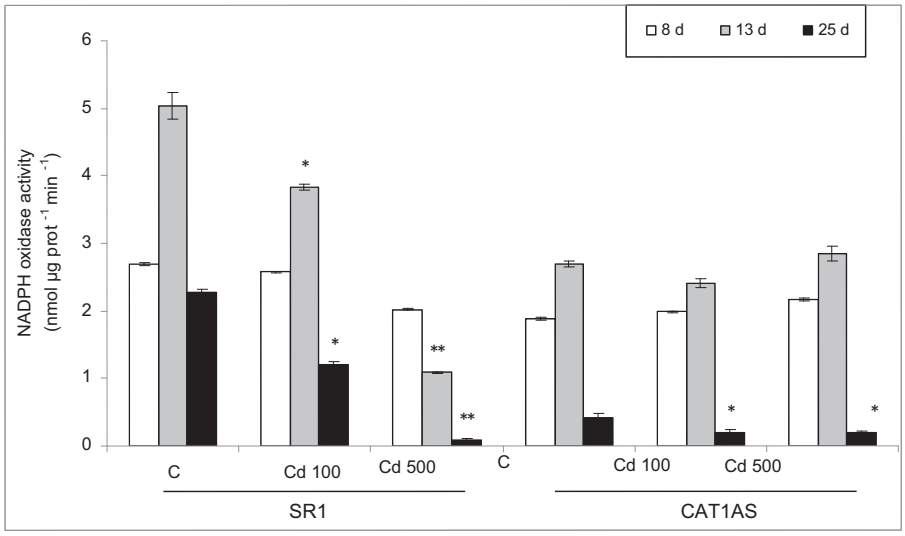

Fig. 4. NADPH dependent superoxide production in SR1 or CAT1AS plants. Plants were exposed to 100 or 500 μM Cd for 8, 13 or 25 days. The NADPH-dependent oxidase activity was performed indirectly using the SOD-inhibited reduction of NBT, as described in Section 2. Values are the mean ± SE from three independent experiments with six replicated measurements. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$) according to Tukey's multiple-range test.

Table 2
Effect of 100 or 500 μM Cd on CAT, APOX, GPOX, and SOD activities at 8, 13 and 25 day of treatment.

		SR1			CAT1AS		
		C	Cd 100 μM	Cd 500 μM	C	Cd 100 μM	Cd 500 μM
CAT	8 days	0.12 ± 0.02	0.14 ± 0.01	0.52 ± 0.02***	ND	ND	ND
(pmol g^{-1} PF s^{-1})	1 days	0.30 ± 0.01	0.25 ± 0.02	0.40 ± 0.01***	0.10 ± 0.00	ND	ND
	25 days	0.18 ± 0.01	0.37 ± 0.03***	0.39 ± 0.04***	0.01 ± 0.00	ND	ND
APOX	8 days	3.31 ± 0.40	4.21 ± 0.61	1.51 ± 0.28	1.78 ± 0.12	3.06 ± 0.68	1.76 ± 0.12
(nmol g^{-1} PF s^{-1})	13 days	9.31 ± 1.09	11.96 ± 2.64	25.00 ± 2.64***	14.90 ±2.24	17.33 ± 0.77	26.42 ± 2.70**
	25 days	4.81 ± 0.29	14.90 ± 3.69*	10.81 ± 0.54*	16.26 ±0.71	11.38 ± 0.52	25.40 ± 0.47*
GPOX (nmol g^{-1} PF)	8 days	3.24 ± 0.01	3.73 ± 0.00***	8.12 ± 0.01***	7.22 ± 0.03	3.63 ± 0.02***	7.60 ± 0.01***
	13 days	2.27 ± 0.03	1.82 ± 0.01***	3.38 ± 0.01***	2.26 ± 0.02	1.32 ± 0.01***	3.75 ± 0.01***
	25 days	2.57 ± 0.37	4.32 ± 0.46*	6.97 ± 0.16***	4.05 ± 0.55	8.51 ± 0.73***	9.28 ± 0.70***
SOD (U g^{-1} PF)	8 days	5.47 ± 0.01	11.59 ± 0.0***	11.34 ± 0.00***	11.29 ±0.00	6.41 ± 0.00***	7.21 ± 0.00***
	13 days	16.93 ± 0.35	12.20 ± 0.1***	11.54 ± 0.00***	9.76 ± 0.63	11.53 ± 0.09*	9.27 ± 0.19
	25 days	10.86 ± 0.63	9.28 ± 0.62	10.57 ± 0.75	4.90 ± 0.10	7.92 ± 1.47*	7.25 ± 0.07*

Data are the mean ± SE of three independent experiments, with five replicates for each treatment. Asterisks within rows indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), according to Tukey's multiple-range test. One unit of CAT is the amount of the enzyme that oxidized 1 μmol of H_2O_2 per minute under the assay conditions. One unit of APOX forms 1 mmol of ascorbate oxidized per minute under the assay conditions. One unit of GPOX is the amount of the enzyme that reduced 1 mmol of H_2O_2 per minute under the assay conditions. One unit of SOD is the amount of the enzyme that inhibits the reduction of NBT by 50% under the assay conditions.

increased in transgenic plants by 64% and 87% over the control upon cadmium treatment, with 100 or 500 μM $CdCl_2$, respectively (Fig. 5).

At all times tested, GSH content was higher in untreated CAT1AS than in SR1 plants, but no significant differences in GSH and GSSG content were detected in both lines exposed to cadmium at all times tested, except for a minor increase of 15% in GSH content of SR1 plants watered for 25 days with 500 μM $CdCl_2$ (Fig. 5).

3.4. Evidence of oxidative damage and cell death: TBARS content, electrolyte leakage and Evans blue staining

TBARS content, electrolyte leakage and Evans blue were measured to observe the extent of oxidative damage and cell death under different Cd treatments. As is shown in Fig. 6, there was no evidence of lipid peroxidation in any of the lines after 8 days of treatment. On the 13th day, TBARS content decreased by 60% in SR1 plants treated with 500 μM $CdCl_2$, but increased by 100% in CAT1AS plants treated with 100 μM $CdCl_2$. Unexpectedly, lipid peroxidation decreased 52% and 44% in SR1 plants and 20% and 50% in the transgenic plants by the end of the experiment, with 100 or 500 μM $CdCl_2$, respectively (Fig. 6C).

Regarding ion leakage, no symptoms of damage were observed after 8 or 13 days of treatment in both lines (Fig. 6B). After 25 days, electrolyte leakage increased 39% with 100 μM $CdCl_2$ in SR1 plants, but strikingly decreased 32% and 30% in CAT1AS plants exposed to 100 or 500 μM $CdCl_2$, respectively.

Evans blue (a dye that specifically stains dead cells) staining was measured as an indicator of cell death in tobacco plants exposed to cadmium. At 8 days, cell death was not observed in any plant line exposed to Cd. The exposure to the metal caused a 80% increase in the percentage of cell death in CAT1AS plants treated 13 days with 500 μM $CdCl_2$. However, at longer times (25 days), variations in cell death were only observed in the wild type line, which showed a rise of 61% in plants treated with 100 μM $CdCl_2$ and a decrease of 22% compared to control, in plants treated with the highest metal concentration (Fig. 6A).

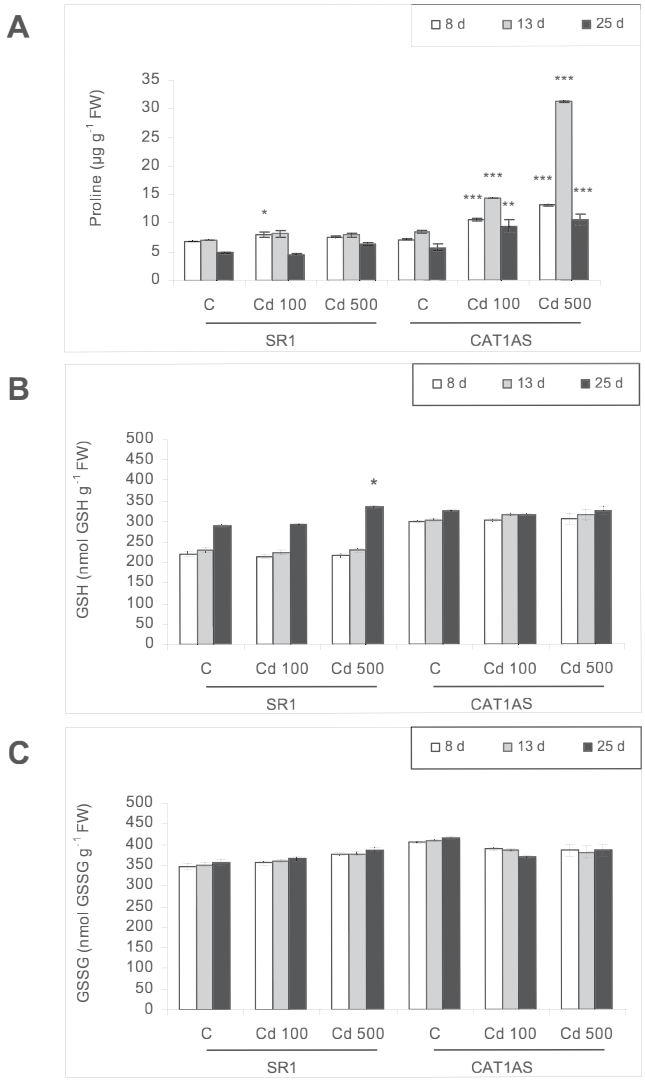

Fig. 5. Non-enzymatic antioxidants. (A) Proline, (B) GSH, (C) GSSG content were measured as described in Section 2. Values are the mean ± SE from three independent experiments with six replicated measurements. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) according to Tukey's multiple-range test.

4. Discussion

Plants have different capacity to accumulate metals, which depends on the plant species, plant age, the metal type and exposure time. In plants, metals in the soil can enter the roots through

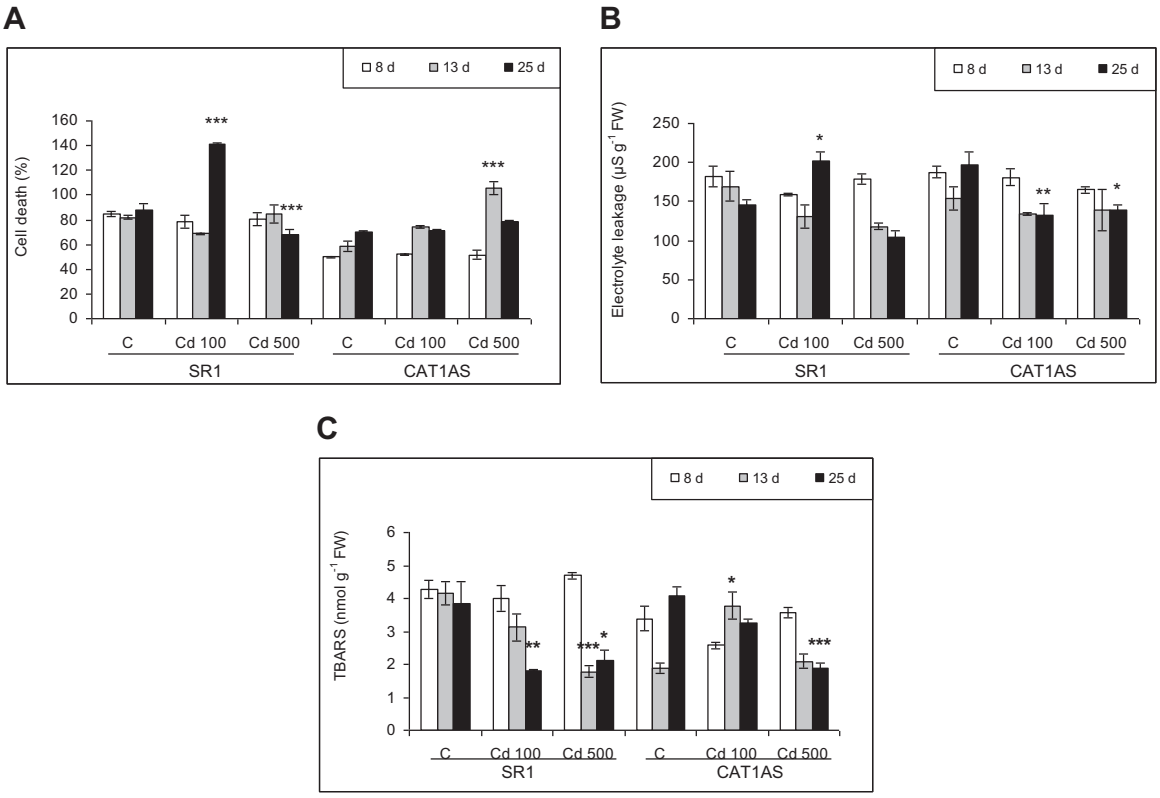

Fig. 6. Oxidative damage and cell death measured. Plants were exposed to 100 or 500 μM Cd for 8, 13 or 25 days of treatment. (A) Evans blue staining expressed as percentage of the controls, (B) electrolyte leakage expressed as relative conductivity and (C) thiobarbituric acid reactive substance (TBARS) content, as described in Section 2. Values are the mean ± SE from three independent experiments with six replicated measurements. Asterisks indicate significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) according to Tukey's multiple-range test.

symplastic or apoplastic pathways before entering the xylem and being translocated to the shoot (Lux et al., 2011). The aerial part of tobacco plants accumulated cadmium in significant amounts and this accumulation was metal concentration-dependent, being CAT1AS the line that showed the greater Cd content with the highest Cd concentration used, according to the reported high mobility in the phloem (Mendoza-Cózatl et al., 2011). Cd can accumulate in all plant parts and can trigger a series of changes that can lead to phytotoxicity (Kopittke et al., 2010; Lux et al., 2011), reported by previous findings as damage to the photosynthetic apparatus, decrease in the net photosynthetic rate, growth reduction in terms of dry mass and leaf area in *Lepidium sativum*, *Brassica juncea*, *Pisum sativum*, *Populus* and *Solanum licospersycum* L. plants (Sandalio et al., 2001; Schützendübel et al., 2002; Mobin and Khan, 2007; Gill et al., 2012; Gratão et al., 2012). On the contrary, Gonçalves et al. (2009) reported that no reduction in shoot and root dry weight was observed at any level of Cd^{2+} in hydroponically grown plantlets of potato. In tobacco plants, cadmium caused a clear growth inhibition (visualized by a small size of the plant and the leaves), that was more evident in the transgenic line treated with 500 μM $CdCl_2$, which accumulated a greater amount of the metal in their leaves, but showed a higher chlorophyll concentration and nitrates content compared to SR1 plants (Figs. 1 and 2 and Table 1). The reduced growth may be due to a cadmium alteration of photosynthesis and, consequently, to a decrease in CO_2 assimilation and photosynthates accumulation, and this could also be related to the onset of chlorosis symptoms observed (Table 1). Kendall et al. (1983) reported that the mutant line of barley RPr 79/4 was unable to grow satisfactorily in normal air but it could not be attributed to a major block in the photorespiratory carbon pathway. Studies on poplar plants exposed to Cd have demonstrated that for a short-term Cd treatment (≤14 days) a strong negative impact on proteins involved in the Calvin cycle and the light-dependent reactions of photosynthesis was observed, but for a long-term Cd exposure (up to 56 days), the impact of Cd on protein abundance was less pronounced (Kieffer et al., 2009).

Most plants can take up and utilize inorganic and organic forms of N from the soil. Nitrate (NO_3^-) is the main form of N taken up and assimilated by most crop plants in cultivated (well aerated) soils (Andrews et al., 2013). Following uptake from the soil solution, nitrate can be either stored in the vacuoles or assimilated to supply the nitrogen atom for amino acid biosynthesis. Nitrate uptake from the external medium by the root epidermal or cortical cells is the balance between concomitant influx and efflux unidirectional transports, mediated by different transporters (Tsay et al., 2007; Miller et al., 2009). The root or shoot can be the main site of NO_3^- assimilation depending on genotype and environmental conditions (Andrews et al., 2013). In many plants, nitrate assimilation occurs predominantly in the leaves, where a large part of the reducing power needed for this process directly originates from the photosystems. In SR1 tobacco plants, cadmium remarkably reduced nitrate content in leaves after 25 days of treatment with both metal concentrations, but surprisingly, nitrate content was not affected in leaves of the transgenic line. Gouia et al. (2000) reported that cadmium produced an inhibition of the nitrate uptake and of the enzymes involved in the N assimilation pathway, whereas both nitrate reductase (NR) and N content were reduced in *L. sativum* L. leaves of plants subjected to 100 mg Cd kg^{-1} soil (Gill et al., 2012). Moreover, Hernández et al. (1996) observed that cadmium caused nitrate accumulation in roots of pea plants, associated with an alteration in the nitrate translocation and a decrease in the NR activity in the stem, and severely inhibiting nitrate assimilation. This could be occurring in SR1 plants, where nitrate accumulation was reduced in leaves compared to CAT1AS. The variation in nitrates content

between SR1 and CAT1AS leaves, at comparable Cd levels, deserve to be studied. In line with the relationship between nitrates and Cd tolerance, several lines of evidence suggest that AtNRT1.8, a nitrate transporter that mediates the retrieval of nitrate from the xylem sap in *Arabidopsis* plants, could be involved in Cd^{2+} tolerance because it restricts nitrate translocation to shoots in response to Cd^{2+} (Gojon and Gaymard, 2010).

Ionic Cd^{2+} may compete with mineral nutrient cations (e.g. Ca^{2+}, Fe^{2+}, and Zn^{2+}) for transporters, by altering plasma membrane permeability, often leading to decreases in these essential elements and to disruptions in the ionic homeostasis or enzymatic activities in plants (Nazar et al., 2012). Iron was chosen to monitor mineral nutrient uptake disruption in tobacco plants exposed to Cd and a decrease of Fe^{2+} content was only observed in wild plants exposed to the higher metal concentration. Bovet et al. (2006) observed no significant differences compared to controls in Fe^{2+} content in 6 week-old *N. tabacum* plants grown for 7 days with 1 μM or 50 μM Cd. However, the author mentioned that, despite this, they observed chlorosis and necrotic effects in plants exposed to 50 μM $CdCl_2$, suggesting that a reduction in the Fe^{2+} content it is not necessary enough to monitor Cd toxic effects, in concordance with the results obtained with CAT1AS plants, where even without a detectable drop in iron content, a reduction in chlorophyll content was observed. Regarding Cd^{2+} effects on Fe^{2+} uptake, shoot Fe^{2+} content in the potato cultivar Asterix showed a continuous decrease with increasing Cd^{2+} concentrations, while, conversely, in the Macaca cultivar, shoot Fe^{2+} was reduced only at the highest Cd^{2+} level (Gonçalves et al., 2009). The marked decrease in the chlorophyll content of SR1 plants after 25 days of cadmium treatment could be associated to the reduction in Fe content, with the concomitant alteration of the photosynthetic machinery, that could limit the amount of ATP and NADPH required for nitrate uptake, thus contributing to the lower nitrate content observed in SR1 plants.

Cadmium-induced oxidative stress has been considered one of the reasons for its phytotoxicity (Kopittke et al., 2010; Lux et al., 2011; Gallego et al., 2012). Many reports have documented that Cd induces the formation of several ROS, mainly O_2^- and H_2O_2 (Benavides et al., 2005), being NADPH oxidase the enzyme most commonly accepted to be the responsible for the generation of H_2O_2 (via O_2^-) in plants after exposure to the metal (Groppa et al., 2012). In microsomes of tobacco leaves, Cd inhibited NADPH oxidase activity, and this inhibition was correlated with the reduction in O_2^- formation mainly after 25 days of cadmium treatment (Fig. 3). These results are in accordance to that found in SR1 or CAT1AS leaf discs (Iannone et al., 2010) and in sunflower leaf discs (Groppa et al., 2012), where cadmium significantly reduced the NADPH oxidase-dependent O_2^- formation in a dose dependent manner, or in *Arabidopsis thaliana* plants where the O_2^- level in the leaves decreased below their controls (Maksymiec et al., 2007). In wood and leaves (that accumulated less Cd than roots) of *Populus* × *canescens* plantlets, the O_2^- production rate increased after 24 h, and subsequently decreased to levels similar to those in controls or even less, after a prolonged Cd incubation time (He et al., 2011), in a comparable pattern than that observed in tobacco leaves.

In a natural course, the plants exposed to stress generate a large quantity of ROS, which in turn will trigger plant ROS-scavenging mechanisms, which includes antioxidant enzymes (Gratão et al., 2008; Azevedo et al., 2012; Roychoudhury et al., 2012) as well as non-enzymatic soluble compounds (Ahmad et al., 2010; Foyer and Noctor, 2012). The resistance of plants to heavy metal stress may be associated with the decreased susceptibility of enzymes to metal inhibition (Liu et al., 2004). To cope with the increase in ROS formation produced by 500 μM Cd, SR1-treated plants showed elevated enzymatic activity of CAT, GPOX and APOX at almost all times compared to controls, whereas in CAT1AS, GPOX and APOX increased over the controls only after 13 days of exposure (Table 2). Additionally, it was observed that CAT1AS plants, mostly after day 8, had increased constitutive levels of the enzymes involved in H_2O_2 detoxification, as APOX and GPOX, as well as decreased constitutive levels of NADPH oxidase or SOD activities, thus reducing the amount of H_2O_2 formed by these ways, all of which may reflect an adaptation of CAT-deficient plants to balance the constitutive H_2O_2 formation in the cytoplasm in response to stress. Moreover, under the low light conditions used in our study (120 $\mu mol\ m^{-2}\ s^{-1}$), transgenic tobacco lines with strongly reduced catalase levels showed a smaller size but were phenotypically indistinguishable from controls. This points out that under these conditions, 10% of normal catalase activity is either enough for protecting tobacco plants from H_2O_2 toxicity generated upon Cd exposure or Cat1 gene, which is the gene deficient in CAT1AS plants, is not the main gene involved in H_2O_2 detoxification upon Cd exposure. Provided that the catalase gene family is evolutionarily conserved, sequence comparison could lead to the identification of different classes of catalases (Willekens et al., 1994). The main function of Cat1 resides in the removal of H_2O_2 produced during photorespiration. When cultivated at favorable conditions of temperature, as were the conditions we used for cultivating tobacco plants, the production of photorespiratory H_2O_2 and consequently the demand for Cat1 will be directly related to the amount of light. For that reason, phenotypic effects, that were evident under high light conditions in CAT1AS plants (Dat et al., 2003), were not detected under Cd stress in plants grown under low light intensities. In addition, SOD increased its activity in CAT1AS plants at day 25 either with 100 or 500 μM $CdCl_2$, a response that was not observed in wild-type plants. The increment of antioxidant enzymes in response to Cd stress has also been described in *A. thaliana* (Smeets et al., 2008), *L. sativum* L (Gill et al., 2012) or in the citrus rootstock Citrumelo seedlings (Podazza et al., 2012), exposed for different times to Cd concentrations ranging from 5 μM to 500 μM. The importance of H_2O_2 detoxification was highlighted by Xu et al. (2008), who reported that transgenic *A. thaliana* plants constitutively overexpressing a peroxisomal APX gene (HvAPX1) from barley were shown to be more tolerant to Cd stress, a process that also resulted in a higher accumulation of Cd in the shoots. Similarly to the results obtained in our work, Willekens et al. (1997) showed that CAT1AS plants activated alternative enzymatic mechanisms of H_2O_2 detoxification to compensate the CAT deficiency after being transferred to high illumination, showing a persistent increase in the GPOX and APOX expression, but not preventing necrosis; whereas using the same transgenic plants, Chamnongpol et al. (1998) reported that H_2O_2 modulation in CAT1AS plants may constitute a strategy for disease control that is superior to chemical activators of the SAR. On the other hand, there is scarce evidence of any pleiotropic effect of the catalase deficiency in mutant line of barley RPr 79/4 on the activities of SOD and GR (Azevedo et al., 1998). CAT activity increased 5-fold only in the wild type leaves when plants were transferred from 0.7% CO_2 to air, whereas the enzyme activity decreased consistently in the roots of both genotypes after plants were transferred to normal air.

In general, the enzymatic and non-enzymatic scavengers have been viewed as independent means of eliminating the oxidant burden, but it has been suggested that other potential antioxidants, as Pro or polyamines, could be part of a dynamic interplay between various oxidant species and antioxidants in the response to stress (Groppa et al., 2003; Gill and Tuteja, 2010). Proline is considered as an inert compatible osmolyte that protects subcellular structures and macromolecules under osmotic stress (Kavi Kishor et al., 2005) and performs a ROS scavenging activity, acting as a singlet oxygen quencher (Matysik et al., 2002). Besides, Pro accumulation appeared to be a suitable indicator of heavy metal stress because this type of stress is often associated to water loss. Islam et al.

(2009) reported that *N. tabacum* L. cells exposed to Cd treatment accumulated high levels of Pro, thus relieving the inhibitory effect of Cd on cell growth. Proline increased in many different poplar species exposed to 200 μM $CdSO_4$ for 20 days (He et al., 2013), whereas in algae treated with heavy metals, Siripornadulsil et al. (2002) observed that free Pro levels correlated with GSH redox state and TBARS levels, suggesting that free Pro acts as an antioxidant in Cd-stressed cells. In the present work, Pro content increased in tobacco transgenic plants at all cadmium exposure times, while in wild plants it fairly increased only at 8 days of 100 μM Cd treatment. Proline increment in the transgenic line, can probably be associated to the less pronounced decline in chlorophyll and no increase in TBARS, electrolyte leakage or H_2O_2 at 500 μM $CdCl_2$, thus confirming a protective role of Pro against Cd-induced stress. On the other hand, total glutathione levels were higher in CAT1AS plants than in wild type plants, and they did not change under Cd stress. This could be due to the unchanged levels of H_2O_2 either in wild type or transgenic tobacco plants. The elevation of glutathione levels in the leaves of the catalase deficient mutant barley RPr 79/4 was associated with a response to the H_2O_2 produced during photorespiration (Smith et al., 1984). In tobacco transgenic line non-photorespiratory conditions were imposed, since plants were grown at normal air and with moderate illumination.

It is known that ROS can accelerate lipid peroxidation, thus affecting cell membrane fluidity and permeability due to an alteration in the composition of membrane lipids (Tian et al., 2012). Although the mechanism of Cd toxicity is far to be fully understood and this metal does not seem to directly generate free radicals, lipid peroxidation has been considered one of the primary processes responsible for Cd toxicity (Gallego et al., 1996; Gratão et al., 2012). However, SR1 or CAT1AS plants showed no evidence of lipid peroxidation even after 25 days of treatment, except for a 100% increase in CAT1AS plants exposed to 100 μM Cd at 13 days. Moreover, plants treated 25 days with the higher cadmium concentration showed a striking decrease in TBARS content and electrolyte release, with no signs of cell death. These results could indicate the existence of an adaptative mechanism to Cd stress in SR1 and CAT1AS plants, at the longer exposure time to cadmium, which could be related to an increment in the antioxidant enzymes activities in both lines and the increase in Pro in the transgenic line that would allow better tolerance to stress. Tomato cells (Msk8 line) subjected to a prolonged Cd-exposure revealed the existence of an adaptation mechanism that allowed cells to be completely recovered and with higher live cells than their controls (Yakimova et al., 2006).

The results obtained in the present work demonstrated that tobacco plants are able to tolerate Cd levels that would normally be lethal to other plant species. The increase in the antioxidant machinery observed in both SR1 and CAT1AS plants was not enough to reverse the growth inhibition produced by the metal, which could indicate that cadmium would affect other parameters not related to ROS formation, as was the alteration of iron homeostasis or disturbances in nitrogen metabolism. The transgenic line had the capacity to rise the antioxidant enzyme machinery but had also developed supplementary adaptive strategies compared to wild plants that allowed them to maintain the NO_3^- and Fe levels in concentrations similar to the untreated plants, without increasing H_2O_2 levels, probably due to the marked elevation of a soluble antioxidant such as Pro. CAT1AS plants survived after being watered with high Cd concentrations in a manner comparable to wild plants.

5. Conclusions

Although many studies have been carried out regarding Cd responses of plants, no explicit indicators are available for evaluating Cd tolerance in plants (Di Lonardo et al., 2011). The results presented in this work demonstrate that, unlike what was observed in CAT-deficient plants, when were exposed to high irradiance (Dat et al., 2003), CAT does not play a crucial role in protection against Cd toxicity and transgenic plants are able to activate alternative defence mechanisms. Additional analysis is needed for a better understanding of the mechanisms connecting antioxidant responses, ROS formation and Cd toxicity on mineral nutrition in tobacco SR1 and CAT1AS plants growing in stressful conditions.

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