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ORIGINAL ARTICLE

Reactive oxygen species formation and cell death in catalase-deficient tobacco leaf disks exposed to cadmium

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Abstract The physiological responses of tobacco (*Nicotiana tabacum* L.) to oxidative stress induced by cadmium were examined with respect to reactive oxygen species (ROS) formation, antioxidant enzymes activities, and cell death appearance in wild-type SR1 and catalase-deficient CAT1AS plants. Leaf disks treated with 100 or 500 μM $CdCl_2$ increased Evans blue staining and leakage of electrolytes in SR1 or CAT1AS plants, more pronouncedly in the transgenic cultivar, but without evidence of lipid peroxidation in any of the cultivars compared to controls. Cadmium significantly reduced the NADPH oxidase-dependent O_2^- formation in a dose dependent manner in SR1 very strongly at 500 μM (to 5% of the activity in the nontreated SR1 leaf disks). In CAT1AS, the NADPH oxidase activity was constitutively reduced at 50% with respect to that of SR1, but the magnitude of the decay was less prominent in this cultivar, reaching an average of 64% of the C at 21 h, for both Cd concentrations. Hydrogen peroxide formation was only slightly increased in SR1 or CAT1AS leaf disks at 21 h of exposure compared to the respective controls. Cd increased superoxide dismutase activity more than six times at 21 h in CAT1AS, but not in SR1 and reduced catalase activity by 59% at 21 h of treatment only in SR1 plants. Despite that catalase expression was constitutively lower in CATAS1 compared to SR1 nontreated leaf disks, 500 μM $CdCl_2$ almost doubled it only in CAT1AS at 21 h. The mechanisms underlying Cd-induced cell death were possibly not related exclusively to ROS formation or detoxification in tobacco SR1 or CAT1AS plants.

Keywords Cell death · Heavy metals · Hydrogen peroxide · *Nicotiana tabacum* · Reactive oxygen species

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Introduction

Generation of reactive oxygen species (ROS) in plants has been implicated in biotic and abiotic stresses (Apel and Hirt 2004), such as wounding (Orozco-Cárdenas and Ryan 1999), heavy metals (Gomes-Junior et al. 2007; Groppa et al. 2007; Gratão et al. 2008), illumination (Dat et al. 2003), drought, or salt stress (Demidchik et al. 2003; Mahajan and Tuteja 2005). These ROS can be generated by various processes occurring at different cellular compartments and seem to play a crucial role in several situations during plant growth and development (Foreman et al. 2003; Mithöfer et al. 2004). Despite extensive research on the source of ROS in plants, the subcellular location and the mechanism of ROS generation under abiotic stress has not been unequivocally clarified until now. The overproduction and rapid accumulation of ROS, also called "oxidative burst," is one of the earliest responses of incompatible interactions between pathogens and plants (the hypersensitive response) and is caused by the activation of a membrane bound enzyme system similar to the neutrophil NADPH oxidase and by cell wall peroxidases (Bolwell 1999; Ros-Barceló et al. 2002). There are several reports in the literature suggesting that although a superoxide-generating NADPH oxidase does exist in plants (Bolwell and Wojtaszek 1997; Torres et al. 1998; Bedard et al. 2007), the plant NADPH oxidase is most likely regulated in a different manner than the one present in mammalian macrophages (Simon-Plas et

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al. 2002; Lherminier et al. 2009). They catalyze the extracellular formation of the superoxide anion (O_2^-) from molecular oxygen, using NADPH as an electron donor (Doke and Ohashi 1988; Keller et al. 1998; Sagi and Fluhr 2001). Peroxidases and lipooxygenase have been also put forward as putative sources of ROS production during the oxidative burst (Mittler et al. 2004; Bindschedler et al. 2006; Sagi and Fluhr 2006; Van Breusegem et al. 2008). Whether one or more of these systems are producing the oxidative burst has yet to be determined. It is possible that they might be operating in tandem or have different kinetics.

Cadmium pollution of soils is of growing concern because Cd and other heavy metals are emitted by traffic, metal working industries, mining, and as a by product of mineral fertilizers (Sanitá di Toppi and Gabbrielli 1999; Benavides et al. 2005; Gratão et al. 2005). It is now unquestionable that environmental pollution is far greater than what had been previously assumed and that many heavy metals are capable of remaining in the environment for a long time, endangering the natural environment and human health (Gomes-Junior et al. 2006, 2007). Cadmium is toxic to humans, animals, and plants even at low doses (Sanitá di Toppi and Gabbrielli 1999; Semane et al. 2007) and also has genotoxic effects (Gratão et al. 2005). It has become clear that at least part of the metal-induced phytotoxicity can be attributed to oxidative stress. Cd or other heavy metals prevents the development of a normal vegetation cover, so biotechnological efforts are underway to develop more stress-tolerant species. For this purpose, it is important to completely understand the mechanisms of Cd toxicity and tolerance in plants.

Although cadmium is not a transition metal, it commonly causes oxidative stress in plants, but the way to conducting cell damage is far to be elucidated (Gallego et al. 1996; Groppa et al. 2007; Lin et al. 2007). Since H_2O_2 is a signaling molecule which triggers, among other responses, secondary pathways and programmed cell death, it has been suggested that Cd may affect the cellular redox control via H_2O_2 accumulation, thereby, inducing common plant defenses and cell death (Schützendübel et al. 2001; Gichner et al. 2004). Romero-Puertas et al. (2004) have reported that 40 μM Cd SO_4 induced a significant accumulation of H_2O_2 in pea leaves. On the contrary, we have observed that, though Cd had demonstrated to induce oxidative stress in sunflower or wheat plants (Gallego et al. 1996; Laspina et al. 2005; Groppa et al. 2001), O_2^- formation as well as H_2O_2 accumulation were reduced in Cd-treated wheat roots, and NO was the molecule involved in the response (Groppa et al. 2008).

The major sites of O_2^- and H_2O_2 formation during abiotic stress is still a matter of debate. Control of H_2O_2 levels is complex, and dissection of the mechanisms generating and relieving H_2O_2 stress is difficult, particularly in intact plants. Catalase (CAT, H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) is a tetrameric iron porphyrin protein that catalyzes the dismutation of H_2O_2 into water and dioxygen (Willekens et al. 1995). In plants, it is located in microbodies such as peroxisomes and glyoxysomes and is involved in detoxification of the H_2O_2 generated by various environmental stresses like high light (Bechtold et al. 2005), temperature (Scandalios et al. 2000), ozone (Azevedo et al. 1998), and metals (Rodríguez-Serrano et al. 2006; Groppa et al. 2007; Azpilicueta et al. 2007), as well as the H_2O_2 produced under physiological conditions. CAT1 is the dominant CAT isoform in *Nicotiana tabacum* leaves, where it serves in the removal of photorespiratory derived H_2O_2 (Willekens et al. 1997; Vranová et al. 2002) and CAT1AS plants (catalase-deficient plants) derived from *N. tabacum* Petit Havana SR1 lines with strongly reduced levels of all three catalases isoforms (Cat1, Cat2, and Cat3), were originally produced to study the role of catalases in plants (Willekens et al. 1994; Chamnongpol et al. 1996). Under photorespiratory conditions created by high light exposure, catalase-deficient plants showed no visible disorders (except for a reduced growth compared to WT plants), but in elevated light rapidly accumulated H_2O_2, developed white necrotic lesions on the leaves, stimulated salicylic acid and ethylene production, and induced the expression of acidic and basic PR proteins with a timing and magnitude similar to the hypersensitive response against pathogens (Chamnongpol et al. 1996, 1998; Willekens et al. 1997). Earlier relevant studies on catalases and abiotic stress have been done in the barley mutants RPr 79/4, which contained less than 10% of the catalase activity observed in the wild-type barley and lack the capacity to carry out photorespiration. This disturbance led the plants to develop white lesions when grown in air for a few days (Kendall et al. 1983; Parker and Lea 1983).

Most studies on the role of ROS in plant cell death have used indirect methods to generate ROS, but the catalase-deficient plants (CAT1AS) used in this study (Dat et al. 2003) provide an ideal tool to investigate the effect of changes in H_2O_2 homeostasis in a noninvasive way. In this work, we studied the injury produced by 100 or 500 μM $CdCl_2$ in CAT-deficient plants in order to study the way of free radicals and cell death generation by analyzing the formation and evolution of O_2^- and H_2O_2 and the enzymes related to their formation and detoxification.

Materials and methods

Plant growth conditions and treatments

Nicotiana 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) derived from *N. tabacum* Petit Havana SR1 (Chamnongpol et al. 1998) were used in the experiments. The seeds of the transgenic tobacco line were kindly provided by Dr. F. Van Breusegem (Ghent University, Belgium). Seeds were germinated and grown as described previously (Chamnongpol et al. 1998). All treatments were performed in a controlled environmental chamber with a relative humidity of 70% and temperature of 24/21°C for day/night period, with a light intensity of 120 mmol m^{-2} sec^{-1}. Unless otherwise indicated, all experiments were performed using the fourth–fifth leaf (counting from the bottom) of 8-week-old plants. Leaf disks (8 mm diameter) were cut with a cork borer, put in glass flasks containing 25 ml of the treatment solution (distilled water, 100 and 500 μM Cd_2Cl), and incubated in a rotary shaker for 3 or 21 h under continuous illumination. After that, leaf disks were washed with distilled water, dried with adsorbent paper, and used for analysis.

In situ O_2^- localization

Leaf disks from SR1 or CAT1AS, treated with 100 or 500 μM Cd for the specified incubation times, were immersed in a 0.05% solution of nitroblue tetrazolium (NBT) in 50 mM potassium phosphate buffer (pH 6.4), vacuum-infiltrated for 3 min, and illuminated for 2 h until appearance of dark spots, characteristic of blue formazan precipitates. Leaf disks were bleached by immersing in boiling ethanol. To distinguish whether the superoxide anion formation was mediated by an NADPH oxidase, inhibition studies were carried out using diphenylene iodonium (DPI). Before staining with NBT, leaves were immersed for 2 h in 20 μM DPI, infiltrated, and then treated with stressors. The inhibition of O_2^- formation by DPI is indicative of the involvement of a NADPH oxidase (Bolwell et al. 1998; Frahry and Schopfer 1998) enzyme.

In situ H_2O_2 localization

Leaf disks from SR1 and CAT1AS plants were exposed to 100 or 500 μM Cd to evaluate "in situ" H_2O_2 production by an endogenous peroxidase-dependent staining procedure using 3,3′-diaminobenzidine (DAB; Thordal-Christensen et al. 1997). Leaf segments were immersed in a 1 mg/ml DAB solution, pH 3.8, vacuum-infiltrated for 3 min, and incubated at room temperature for 2 h in light until appearance of brown spots to evidence H_2O_2 formation. Leaves were bleached in boiling ethanol. Ascorbic acid was used as antioxidant to confirm that brown spots correspond to H_2O_2 formation.

Cell death detection: Evans blue staining and electrolyte leakage

To determine if 100 or 500 μM $CdCl_2$ or H_2O_2, produce changes in cell viability, leaf disks were infiltrated once with a 0.25% (*w*/*v*) aqueous solution of Evans blue (Baker and Mock 1994) for 15 min at room temperature and then washed twice for 15 min with distilled water to remove the excess dye. Finally, leaf disks were incubated in distilled water overnight. For quantitative assessment, blue precipitates were quantified by solubilization with 1% (*w*/*v*) SDS in 50% (*v*/*v*) methanol at 50°C for 1 h, and the absorbance was measured at 595 nm.

Cell death was also estimated by measuring ion leakage from leaf disks according to the method of Shou et al. (2004). First, conductivity of treatment solutions (T0) was measured by using a conductivity meter (Hanna Instruments). After that, 200 mg of leaf disks were floated on 30 ml of treatment solutions during 3 or 21 h under continuous illumination. Following incubation, the conductivity of the solution was measured (T1), and then, leaf disks were boiled in the solution at 100°C for 1 h. After the solution was cooled, the conductivity was measured again (T2), and the results were expressed as relative conductivity [(T1-T0)/(T2-T0)]×100.

Thiobarbituric acid reactive substances determination

Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) measured by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh leaf disks (0.3 g FW) were homogenized in 3 ml of 20% (*w*/*v*) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 20 min. To 1 ml of the aliquot of the supernatant, 1 ml of 20% (*w*/*v*) TCA containing 0.5% (*w*/*v*) TBA and 100 μl of 4% (*w*/*v*) butyl hydroxytoluene in ethanol were added. The mixture was heated at 95°C for 25 min and then quickly cooled on ice. The contents were centrifuged at 1,100 g for 3 min, and the absorbance was measured at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Subcellular fractionation and assay of NADPH oxidation

Plant material was frozen in liquid nitrogen and grounded to a fine powder in a prechilled mortar and pestle. Samples (1 g FW) were homogenized in 3.5 ml of HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH buffer (pH 7.8), containing 250 mM sucrose, 0.5 mM PMSF, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM DTT, and 0.5 mM $MgCl_2$ (reaction buffer). The homoge-

nates were centrifuged at 600 g for 15 min. To obtain microsomes, the 600 g supernatant was centrifuged at 12,000 g for 20 min, and the resultant supernatant was centrifuged at 140,000 g for 1 h. From this last centrifugation, a pellet (microsomal fraction) and a supernatant (cytosolic fraction) were obtained. The final pellet was resuspended in 500 µL of the reaction buffer and was used to determine the NADPH oxidation rate. The NADPH-dependent O_2^--generating activity in isolated PM microsomes was performed indirectly using the superoxide dismutase (SOD)-inhibited reduction of nitroblue tetrazolium (NBT) as described by Van Gestelen et al. (1997) in a reaction mixture containing the reaction buffer, 100 µM NBT and 0.01% Tritonx-100, in a final volume of 1 ml. NBT is rapidly converted to monoformazan by two molecules of O_2^-. After incubating the samples for 2 min at room temperature, reactions were initiated by the addition of 100 µM NADPH, and the reduction of NBT was monitored at 530 nm for 3 min. NADPH oxidation activity was calculated by taking the difference between the apparent reaction rates with or without SOD (75 units per milliliter) in the reaction mixture, using an extinction coefficient of 12.8 $mM^{-1}cm^{-1}$. This reduction is detected spectrophotometrically (Aminco DW2000 spectrophotometer, SLM Instruments, Urbana, IL, USA) at 530 nm. DPI was used to inhibit NADPH oxidase-like enzyme activity in control microsomes.

Enzyme preparations and assays

Extracts for determination of ascorbate peroxidase (APOX, EC 1.11.1.11) and guaiacol peroxidase (GPOX, EC 1.11.1.7) were prepared using 0.2 g FW of leaf disks in 1.5 ml of 50 mM potassium phosphate buffer pH 7.8 containing 1 mM EDTA and 1 g polyvinylpyrrolidone (PVP). Because APOX is labile in the absence of ascorbate, 5 mM ascorbate was included for the extraction of this enzyme. The homogenates were centrifuged at 27,000 g for 30 min, and the supernatant fraction was used for the assays. APOX activity was measured immediately in fresh extracts as described by Nakano and Asada (1981) using a reaction mixture (1 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate, and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε, 2.8 $mM^{-1}cm^{-1}$).

GPOX activity was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 1 mM guaiacol, 0.1 mM H_2O_2, and 100 µl enzyme preparation in a final volume of 1 ml. Activity was measured by following the increase in absorbance at 470 nm due to the formation of tetraguaiacol (ε, 26.6 $mM^{-1}cm^{-1}$) (Maehly and Chance 1954).

Homogenates for SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities were prepared under ice-cold conditions using 0.3 or 0.6 g FW of leaf disks, homogenized in 3 or 2 ml of extraction buffer (containing 50 mM phosphate buffer (pH 7.8), 0.5 mM EDTA, 1 g PVP, and 0.5% (*v*/*v*) Triton X-100 at 4°C), respectively. The homogenates were centrifuged at 27,000 g for 30 min, and the supernatant fraction was used for the assays.

Total SOD activity was assayed by the inhibition of the photochemical reduction of NBT, as described by Becana et al. (1986). The reaction mixture contained 100–300 µl of the plant extract and 3.5 ml of a O_2^- generating solution, which contained 14.3 mM methionine, 82.5 µM NBT, and 2.2 µM riboflavin. Extracts were brought to a final volume of 0.3 ml with 50 mM potassium phosphate (pH 7.8) and 0.1 mM EDTA. Test tubes were shaken and placed at 30 cm from a light bank consisting of six 15-W fluorescent lamps. The reaction was allowed to run for 10 min and stopped by switching the light off. The reduction in NBT was followed by obtaining the A560 values. Blanks and controls were run in the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay condition.

CAT activity was determined in the homogenates by measuring the decrease in absorbance at 240 nm (Hitachi U-2000) 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 ($k'=k\times[CAT]$) of the decrease in H_2O_2 absorption was determined, and catalase content in picomole per gram FW was calculated using k=4.7 107 $M^{-1}s^{-1}$ (Chance et al. 1979).

Western blot analysis of CAT

Soluble protein extracts were prepared from leaf disks and 25 µg of total protein were electrophoresed on 12% SDS-PAGE in Mini PROTEAN III equipment (Bio-Rad), as described by Laemmli (1970). Following electrophoresis at 4–8°C, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences). For the CAT immunodetection, the membrane was incubated at 4–8°C overnight with a polyclonal antibody raised in rabbit against a cottonseed CAT (kindly provided by Dr Trelease from Arizona State University). Bands were detected with an anti-rabbit IgG peroxidase conjugated secondary antibody (Sigma), and DAB was used as substrate for staining procedure. Membranes were photographed with Fotodyn equipment and analyzed with GelPro software.

Statistics

All data presented are the mean values of two independent set of experiments. Each value was presented as means±

standard errors of the mean (SE), with a minimum of three replicates. Statistical analysis was carried out by one-way analysis of variance using the Tukey test to evaluate whether the means were significantly different, taking $p<0.05$ as significant.

Results

Evidence of oxidative damage and cell death: thiobarbituric acid reactive substances content, electrolyte leakage, and Evans blue staining

Tobacco leaf disks from SR1 or CAT1AS plants were treated with 100 or 500 μM Cd for 3 or 21 h. TBARS were measured as an index of lipid peroxidation, whereas, electrolyte leakage or Evans blue staining were evaluated as markers of membrane damage and cell death, respectively. Oxidation of membrane lipids was not evident either in wild type or in transgenic plants under Cd exposure at any time (Fig. 1). At 3 h of exposure, almost no leakage of electrolytes was observed for 100 or 500 μM of $CdCl_2$ for both cultivars, except for significant increase (an average of 43% over the nontreated disks) in CAT1AS disks at the highest Cd concentration. The leakage of electrolytes increased 47% and 72% in SR1-treated leaf disks after 21 h of treatment, with 100 or 500 μM Cd, respectively (Fig. 2), whereas, the increase in Cd-treated CAT1AS leaf disks reached 61% for 100 μM and 117% for 500 μM $CdCl_2$ (Fig. 2). The exogenous addition of 10 or 50 mM H_2O_2, one of the ROS suspected to be responsible for membrane damage, did not produce toxicity symptoms at 3 h of exposure for SR1, but increased the leakage of electrolytes by 46% for CAT1AS leaf disks only at the highest concentration (Fig. 2). As it is well known, H_2O_2 has a short life in aqueous solutions, so a high H_2O_2 concentration was used to cover 21 h of exposure. A significant increase of 136% and 207% over the controls was observed in the electrolyte leakage in SR1 plants and 58% and 118% in transgenic plants, with 10 or 50 mM of H_2O_2 at 21 h of treatment, respectively (Fig. 2). Exogenously added catalase (1,000 U ml^{-1}) almost completely reversed the Cd-induced leakage of electrolytes, suggesting that H_2O_2 was involved in Cd-induced membrane damage and electrolyte leakage (data not shown).

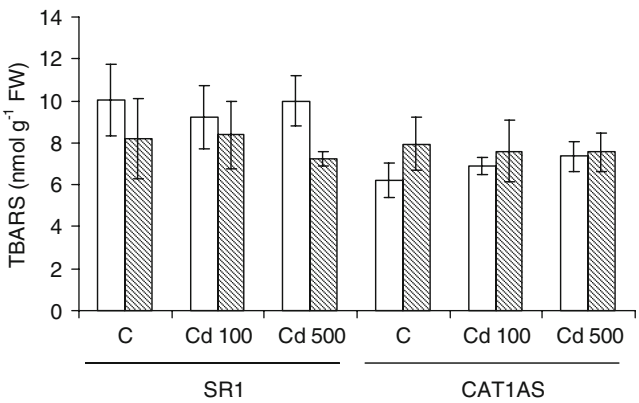

Fig. 1 Thiobarbituric acid reactive substances (TBARS) content of tobacco SR1 or CAT1AS plants. Leaf disks were exposed to either 100 or 500 μM $CdCl_2$ for 3 (*open bars*) or 21 h (*filled bars*) as described in Materials and methods. *Values* are the means of two different experiments with three replicated measurements and *bars* indicate SE. The *single asterisk* indicates significant differences ($p<0.05$) according to Tukey's multiple range test

In a parallel experiment, the time course of cell death in tobacco leaf disks exposed to 100 or 500 μM Cd was visualized using Evans blue, a dye that specifically stains dead cells. The exposure of SR1 or CAT1AS plants to $CdCl_2$ resulted in an increased staining of leaf disks due to the presence of dead cells, which was 34% and 69% over C in the SR1 leaf disks and 60% and 91% for CAT1AS treated disks for 100 μM and 500 μM $CdCl_2$ at 3 h of exposure, respectively; whereas, at 21 h, the magnitude of the increase reached 84% and 116% for SR1 and 113% and 148% for CAT1AS leaf disks, with 100 and 500 μM of the metal, respectively (Fig. 3).

Assessment of hydrogen peroxide and superoxide anion accumulation

NBT and DAB staining

Regarding O_2^- formation, NBT was used to detect the formazan precipitation produced after the reaction with O_2^-. At 3 h, nontreated or 100 μM Cd-treated SR1 or CAT1AS leaf disks showed a comparable O_2^--dependent formazan deposition, only with a slight inhibition of formazan deposition in CAT1AS disks (Fig. 4); whereas, a marked inhibition of O_2^- formation was evident in SR1 or CAT1AS plants treated with 500 μM $CdCl_2$ compared to nontreated leaf disks. At 21 h, CAT1AS leaf disks showed a lower blue staining than SR1 leaf disks compared to C disks with 100 μM $CdCl_2$, while the blue color due to formazan deposition completely disappeared with 500 μM $CdCl_2$. In order to confirm the specificity of the reaction, DPI, an inhibitor of flavin-containing oxidases, including NADPH oxidase, was used at 50 μM and almost a complete reversion of the staining was observed in non-treated or Cd-treated leaf disks (Fig. 4), more markedly at 21 h of treatment.

Hydrogen peroxide production was visualized by staining leaf segments with DAB, a histochemical reagent that polymerizes and turns brown in the presence of H_2O_2. Leaf disks from SR1 or CAT1AS plants were vacuum-infiltrated with DAB after the respective treatment with 100 or

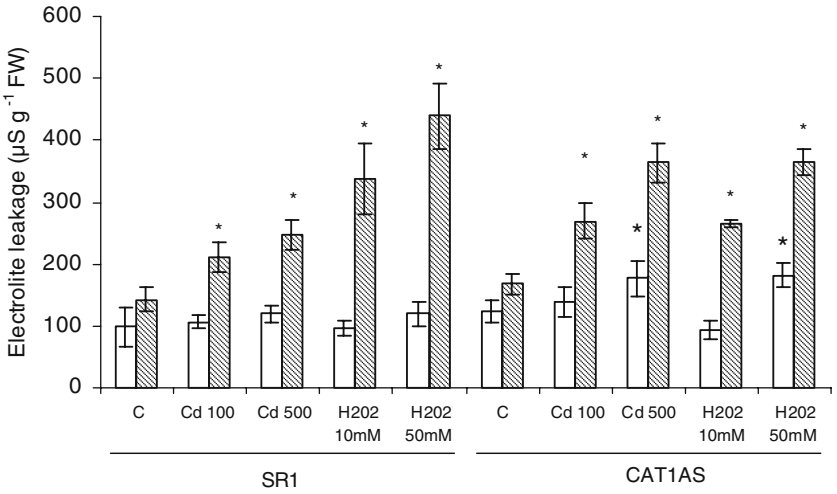

Fig. 2 Cell death measured as electrolyte leakage in leaf disks of tobacco SR1 or CAT1AS plants expressed as relative conductivity. Leaf disks were exposed to either 100 or 500 µM $CdCl_2$ and 10 or 50 mM H_2O_2 for 3 (*open bars*) or 21 h (*filled bars*) as described in Materials and methods. The showed results are expressed as relative conductivity by applying the formula [(T1-T0)/(T2-T0)]×100. *Values* are the means of two different experiments with three replicated measurements, and *bars* indicate SE. The *single asterisk* indicate significant differences ($p<0.05$) according to Tukey's multiple range test

500 µM Cd. No differences with respect to the controls were observed in SR1 or CAT1AS leaf disks at 3 h of the metal exposure, either with 100 or 500 µM $CdCl_2$ (data not shown). At 21 h, Cd increased DAB staining more in CAT1AS than in SR1 plants (Fig. 5), but little differences between cultivars were observed. Brown spots were detected (see black arrows) in certain places of the leaf disks, though the differences were slightly appreciable. Hydrogen peroxide accumulation could be prevented to different extents by infiltrating leaf disks with ascorbic acid previously to DAB staining, which demonstrated the specificity of this reaction for H_2O_2 (data not shown; Fig. 5).

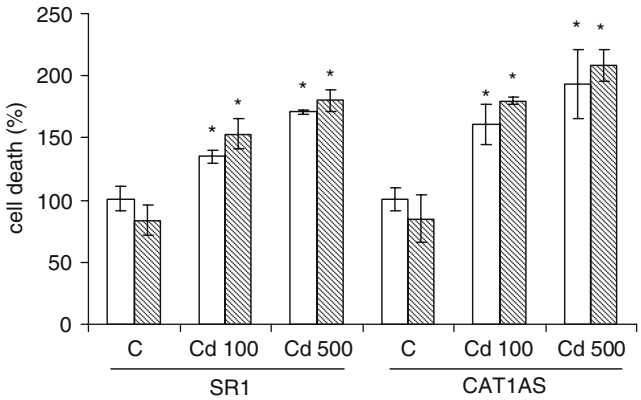

Fig. 3 Cell death estimated as Evans blue staining in leaf disks of tobacco SR1 or CAT1AS plants, expressed as percentage of the controls. Leaf disks were exposed to either 100 or 500 µM $CdCl_2$ for 3 (*open bars*) or 21 h (filled bars) as described in Materials and methods. *Values* are the means of two different experiments with three replicated measurements, and *bars* indicate SE. The *single asterisk* indicate significant differences ($p<0.05$) according to Tukey's multiple range test

NADPH oxidase activity

To further evaluate if cadmium may be restraining O_2^- and H_2O_2 generation by acting on a NADPH oxidase-like enzyme, the activity of the enzyme was assayed at 21 h of exposure by measuring superoxide dismutase inhibitable and NADPH-dependent reduction of NBT by O_2^- in the microsomes obtained from 100 or 500 µM $CdCl_2$-treated leaf disks. The CAT-deficient cultivar CAT1AS showed half of the SR1-constitutive oxidase activity-dependent on NADPH (Fig. 6). Cadmium significantly reduced the NADPH oxidase-dependent O_2^- formation in a dose dependent manner in SR1, and this reduction was very strong at 500 µM of the metal when the enzyme activity was depleted to 5% of the activity of the enzyme in nontreated SR1 leaf disks. In the transgenic cultivar, the magnitude of the decay was less pronounced and reached an average of 64% for both Cd concentrations (Fig. 6) compared to the nontreated CAT1AS plants. DPI, which has been suggested to interact with flavin cofactors on enzymes, showed an almost complete enzyme inactivation in the microsomes from control SR1 or CAT1AS leaf disks when used in the reaction medium at 200 µM (about 50% inhibition at 100 µM, data not shown).

Superoxide dismutase activity

Superoxide dismutase activity, known to be an important source of H_2O_2 derived from O_2^- in plant cells, was

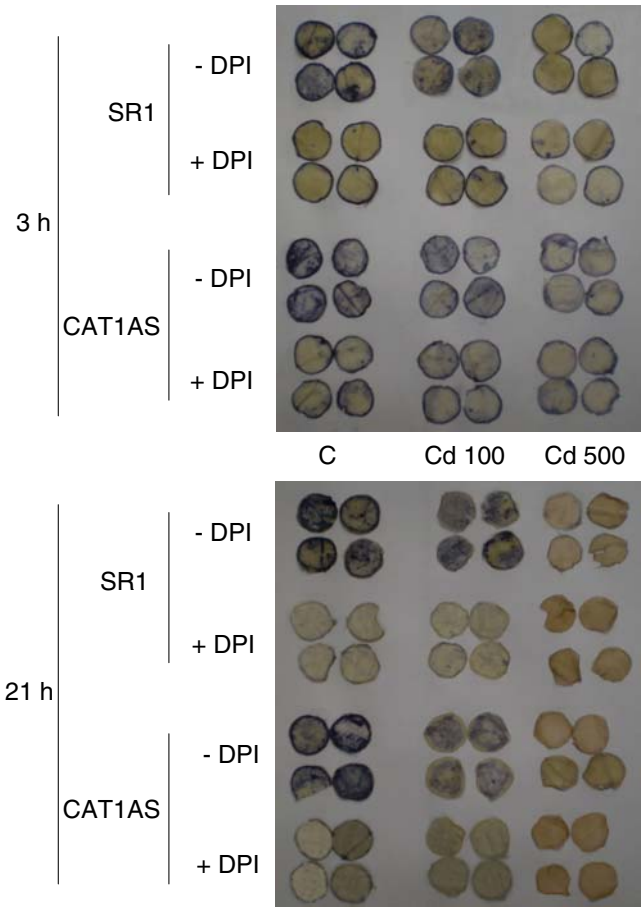

Fig. 4 Accumulation of O_2^- in leaf disks of tobacco SR1 or CAT1AS plants. Leaf disks were exposed to either 100 μM or 500 μM $CdCl_2$ for 3 or 21 h, and then, leaf disks were vacuum-infiltrated for 3 min, and O_2^- formation was revealed staining the disks with 0.05% nitroblue tetrazolium (NBT), incubating the disks 2 h under illumination, until appearance of *blue spots*, as described in Material and methods

increased 26% by 100 μM and 44% by 500 μM $CdCl_2$ in CAT1AS at 3 h ($p<0.01$), whereas, the activity of the enzyme increased about six times by the highest Cd concentration at 21 h of exposure in the transgenic genotype ($p<0.01$) but did not change in the SR1 cultivar (Table 1) at any exposure time. CAT1AS leaf disks treated with 100 μM Cd did accumulate less superoxide at 21 h as revealed by NBT staining, probably due to the higher SOD activity detected in transgenic leaf disks treated with the metal at this time (Table 1).

Hydrogen peroxide removal: Catalase, ascorbate peroxidase, and guaiacol peroxidase activities

The effect of $CdCl_2$ on CAT, APOX, and GPOX activities was analyzed in order to verify that transgenic plants presented lower CAT activity than wild-type plants and to assess if CAT-deficient plants used other H_2O_2-detoxifying

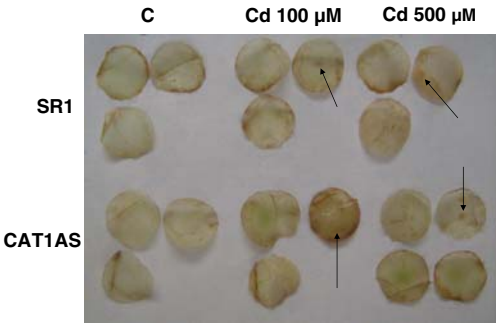

Fig. 5 Accumulation of H_2O_2 in leaf disks of tobacco SR1 or CAT1AS plants. Leaf disks were exposed to either 100 or 500 μM $CdCl_2$ for 3 or 21 h, and then, leaf disks were vacuum-infiltrated for 3 min, and H_2O_2 was revealed using 1 mg ml^{-1} diaminobencidine (DAB), incubating the disks for 2 h under illumination, as described in Material and methods

enzymes to compensate CAT deficiency. Catalase activity in nontreated CAT1AS leaf disks was 39% of that of SR1 plants (Table 1). Cadmium (500 μM) reduced CAT activity to 41% with respect to the nontreated plants only at 21 h of treatment ($p<0.01$) in SR1 cultivar. In CAT1AS plants, none of Cd concentrations used modified the enzyme activity at 3 or 21 h, compared to nontreated transgenic plants.

The constitutive CAT expression was reduced by about 64% in nontreated CATAS1 plants compared to nontreated SR1 plants at 3 h of exposure (Fig. 7). Only 500 μM Cd^{2+} was used to check the reduction in the protein expression. At 3 or 21 h of exposure, the metal did not modify the protein expression in SR1 leaf disks but increased CAT

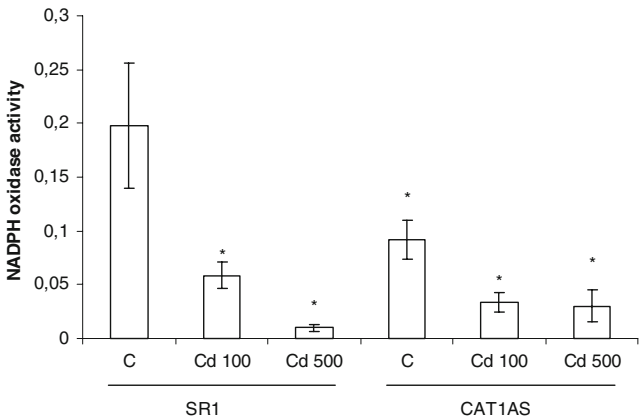

Fig. 6 NADPH-dependent superoxide production in SR1 or CAT1As tobacco leaf disks. Leaf disks were exposed to either 100 or 500 μM $CdCl_2$ for 21 h. The NADPH-dependent O_2^--generating activity in isolated PM microsomes was performed indirectly using the SOD-inhibited reduction of NBT, as described in Materials and methods. *Values* are the means of three different experiments with three replicated measurements, and *bars* indicate SE. The *single asterisk* indicates significant differences ($p<0.05$) according to Tukey's multiple range test

Table 1 Effect of 100 or 500 μM Cd_2Cl on CAT, APOX, GPOX, and SOD activities at 3 and 21 h of treatment

		SR1			CAT1AS		
		C	Cd 100	Cd 500	C	Cd 100	Cd 500
CAT (U mg^{-1} PT)	3 h	0.48±0.06a	0.48±0.03a	0.40±0.02a	0.19±0.06a*	0.21±0.02a	0.22±0.02a
	21 h	0.63±0.10a	0.56±0.05a	0.26±0.04b	0.24±0.07a*	0.26±0.01a	0.25±0.06a
APOX (U mg^{-1} PT)	3 h	19.14±1.29a	17.01±2.48a	11.8±0.85b	16.73±1.45a	14.94±1.83a	6.39±0.02b
	21 h	24.97±3.73a	20.54±1.37a	5.53±0.12b	37.48±3.86a*	35.43±3.03a	12.46±0.45b
GPOX (U mg^{-1} PT)	3 h	1933.90±347.1a	1790.63±213.74a	1537.98±142.02a	1403.08±127.51a	1139.37±209.10a	486.01±131.53c
	21 h	1634.91±268.5a	968.35±126.14b	568.36±1.74c	1449.71±265.19a	763.14±8.96b	326.89±1.76c
SOD (U mg^{-1} PT)	3 h	17.95±5.39a	21.61±9.01a	18.16±1.82a	15.91±1.13a	20.10±1.03b	22.99±2.17b
	21 h	24.45±4.23a	25.85±6.16a	22.33±2.89a	10.93±1.55a*	15.67±1.99b	66.04±3.98c

Data are the mean±SEM of two independent experiments, with five replicates for each treatment. Different letters within rows indicate significant differences (b, $p<0.05$; c, $p<0.01$), according to Tukey's multiple range test. Asterisks denote significant differences between SR1 and CAT1AS controls. Enzymatic activities were assayed as described in "Materials and methods" section. 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

protein expression by about 70% in the transgenic cultivar at 21 h (Fig. 7). Considering that CAT1AS plants presented a diminished CAT protein expression and enzyme activity, we decided to evaluate if other H_2O_2-removing enzymes, like GPOX or APOX, were compensating for CAT deficiency by increasing their own activities to remove H_2O_2. At 21 h, APOX activity was increased approximately to 50% in nontreated CAT1AS compared to nontreated SR1 leaf disks, while GPOX activity remained similar in SR1 or CAT1AS nontreated leaf disks. Regarding stress treatments, the lower Cd level did not modify APOX activity at 3 or 21 h in SR1 or CAT1AS disks. At 3 h, 500 μM $CdCl_2$ decreased APOX activity 39% in SR1 and 62% in CAT1AS; whereas, at 21 h, the enzyme activity decreased 78% in SR1 and 67% in CAT1AS disks. At 3 h of Cd treatment, GPOX activity was inhibited 65% in CAT1AS leaf disks only by 500 μM $CdCl_2$. At 21 h, the lower concentration of the metal caused a decay of 41% and 47% of the enzyme activity in SR1 and CAT1AS disks, respectively, compared to controls; while 500 μM $CdCl_2$ strongly diminished GPOX activity to 35% and 23% of the control in SR1 and CAT1AS disks, respectively ($p<0.01$; Table 1).

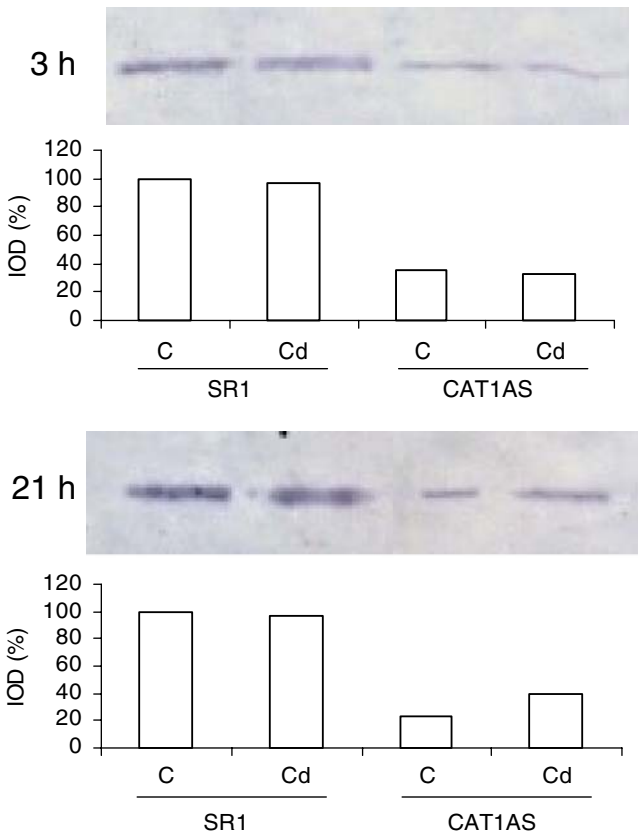

Fig. 7 Catalase protein expression in leaf disks of tobacco SR1 or CAT1AS plants determined by Western blot protein analysis and densitometry. Leaf disks were exposed to 500 μM $CdCl_2$ for 3 or 21 h. CAT expression was revealed after incubating the membrane at 4–8°C overnight with a polyclonal antibody raised in rabbit against a cottonseed CAT. The experiment was repeated three times and a representative image is presented

Discussion

To scavenge ROS, plants possess a well-organized antioxidant defense system. The cooperative function of the components of this system plays an important role in maintaining the physiological redox status. The steady-state level of ROS within cells is determined by an interplay

between the activity of ROS generating mechanisms, such as the NADPH oxidase complex and some peroxidases and the activity of ROS-detoxifying enzymes such as SOD, APOX, or CAT. Several environmental stress conditions are thought to generate oxidative stress in plants (Mittler 2002; Van Breusegem et al. 2008). Hence, we addressed the question of whether the increased sensitivity of CAT-deficient plants to photorespiration, salinity, or high light intensities (Kendall et al. 1983; Azevedo et al. 1998; Chamnongpol et al. 1996; Willekens et al. 1997; Dat et al. 2003) is also mirrored in an increased susceptibility to other abiotic stress conditions, in particular, to heavy metals like cadmium.

The induction of oxidative stress by Cd has been considered to be partially responsible for Cd phytotoxicity (Gallego et al. 1996; Mithöfer et al. 2004; Groppa et al. 2001, 2007). NADPH-oxidizing activities are most commonly accepted as responsible for ROS formation in plants (Van Gestelen et al. 1997; Foreman et al. 2003), and divalent cations, such as Cd, are known to trigger the oxidation of NADPH leading to O_2^- production (Kawano 2003). Cadmium-induced oxidative stress mediated by an NADPH oxidase-like enzyme has been reported by several authors (Garnier et al. 2006; Olmos et al. 2003; Romero-Puertas et al. 2004). However, in microsomes of tobacco leaves of SR1 or CAT1AS plants, Cd inhibited this potential source of O_2^- anions, and this inhibition correlated to the reduced NBT-dependent formazan deposition observed, as occurred at 21 h, when this inhibition was more evident in SR1 Cd-treated leaf disks compared to CAT1AS (Fig. 4). Maksymiec et al. (2007) found a similar result in *Arabidopsis thaliana* plants exposed to Cd, where O_2^- level in the leaves decreased below the control levels. The basal NADPH oxidase activity was constitutively reduced (about 50%) in the transgenic cultivar, but the magnitude of the inhibition of the enzyme activity induced by 500 μM $CdCl_2$ was more severe in SR1-treated leaf disks. A transcript accumulation of a tobacco homolog (NtrbohD) of a mammalian NADPH oxidase was observed in the CAT1AS plants exposed to high light (Dat et al. 2003), where cell death was attributed to the accumulation of H_2O_2 in CAT1AS plants. These results showed that the effect of Cd on ROS formation was different from that observed by Dat et al. (2003) in CAT1AS plants exposed to high light.

Although NADPH oxidase activity and O_2^- formation were inhibited by Cd in wild type and transgenic plants, tobacco leaf disks showed a slight increase of H_2O_2 compared to controls, evidenced by DAB deposition. This result suggested that H_2O_2 formed under Cd stress should not be originating only in the microsomes but from another subcellular compartment, as suggested by Heyno et al. (2008), who reported that Cd inhibited O_2^- production in isolated plasma membranes from soybean but stimulated it in potato tuber mitochondria. Another possibility is the lack of availability of other crucial ions like Cu, for the optimal function and integrity of the membranes, due to the excess Cd in the cell, which resulted in the inhibition of the NADPH-dependent O_2^- formation, as was suggested by Quartacci et al. (2001). In *Solanum lycopersicum* plants, H_2O_2 generation by a cell wall peroxidase was demonstrated to be involved in the cell death response controlled by the Pto-Fen complex rather than by NADPH oxidases (De Biasi et al. 2003). In the same way, in *Arabidopsis* leaves infected by *Pseudomonas syringae*, a CaPO2 peroxidase, but not a NADPH oxidase, was involved in ROS generation during the defense response to pathogen invasion (Choi et al. 2007).

Cd-induced damage has been frequently attributed to an increased formation of H_2O_2, as has been observed in *Arabidopsis* leaves (Cho and Seo 2005; Zawoznik et al. 2007) or pea plants (Cho and Seo 2005; Rodríguez-Serrano et al. 2006; Yokimova et al. 2006), suggesting that Cd-induced phytotoxicity can be induced by H_2O_2 accumulation and subsequent oxidative stress. In transgenic tobacco CAT1AS plants with reduced CAT activity, high levels of H_2O_2 can accumulate under photorespiratory conditions (Willekens et al. 1997; Dat et al. 2003) and such a perturbation in H_2O_2 homeostasis induced cell death (Dat et al. 2003). The increase in the electrolyte leakage correlated to cell death in Cd-treated plants only at the highest metal concentration, either in the wild type or in the transgenic cultivar at 21 h of treatment, but the magnitude of the damage was significantly higher in the transgenic cultivar. At 3 h, though cells did not release electrolytes, Cd-treated SR1 or CAT1AS leaf disks displayed a cultivar-, dose-, and time-dependent reduction of cell viability (evidenced by Evans blue staining), as shown in Bright Yellow-2 tobacco cells exposed to millimolar concentrations of $CdCl_2$ (Garnier et al. 2006). At the longer exposure time, an evident injury to membranes with increased electrolyte leakage but without lipid peroxidation was observed in Cd-treated SR1 or CAT1AS leaf disks, in accordance to Montillet et al. (2005), who demonstrated that lipid peroxidation does not always accompany cell death processes in CAT1AS tobacco plants.

The involvement of H_2O_2 in Cd-induced electrolyte leakage was confirmed by using H_2O_2 as exogenous stressor, which significantly increased the leakage of solutes out of the cells (Fig. 2). However, the level of H_2O_2 detected by DAB formation under Cd stress was too low to justify the extent of the cell damage revealed by Evans blue or by ion leakage, leading us to presume that Cd toxicity was mediated by other mechanisms besides H_2O_2. Moreover, different cell death signaling pathways should be operating in SR1 or CAT1AS plants that deserved to be studied. Recent discoveries of promoter regions and cis-regulatory elements, specific for distinct types of ROS, further support the notion for different ROS-signaling

pathways (Ho et al. 2008) that might be occurring in tobacco SR1 or CAT1AS plants.

Even though the toxic action of cadmium is far from being completely understood and this metal does not appear to generate free radicals directly, lipid peroxidation has long been considered as one of the primary processes responsible for cadmium toxicity (Pal et al. 1993; Gallego et al. 1996; Gratão et al. 2005). However, Cd-treated SR1 or CAT1AS leaf disks did not show any evidence of lipid peroxidation even after 21 h of treatment. Gomes-Junior et al. (2006) observed that 0.5 mM $CdCl_2$ induced an elevation in the amount of TBARS in coffee cell suspensions only after 24 h of $CdCl_2$ exposure, but lower Cd concentrations (0.05 mM) or shorter exposure times did not affect the oxidation of lipids in cell cultures. Maksymiec et al. (2007) reported that only a slightly inductive effect of Cd on MDA formation was found in Cd-treated *Arabidopsis* leaves after 144 h of exposure, whereas, Razinger et al. (2008) showed that the significant lipid peroxidation observed in duckweed treated with 10 mM $CdCl_2$ provide evidence that lipid damage could be an important disruptive action of cadmium. These results show that considerably longer exposure times or unusually higher Cd concentrations than those used in our experiments can produce almost negligible or significant peroxidation in other plant species, respectively. On the other hand, many authors do not agree on cadmium-induced lipid peroxidation as a basis for cell damage as many toxic events occur prior to enhanced lipid peroxidation and these are not prevented by antioxidant agents (Casalino et al. 2000). This could be occurring with Cd-treated SR1 or CAT1AS leaf disks, where lipid peroxidation remained unaltered after 21 h of Cd treatment, but other symptoms of Cd toxicity, as electrolyte leakage, were clear.

Cd-treated CAT1AS leaf disks showed a higher SOD activity at both times of exposure and Cd levels, and this increase was of high magnitude at 21 h under 500 μM $CdCl_2$. An increase in Mn-SOD protein expression was also observed in CAT1AS leaves exposed to high light (Dat et al. 2003), whereas, Azevedo et al. (1998) found that in barley catalase-deficient mutants RPr 79/4, total SOD activity increased after the plants were taken out from 0.7% CO_2. On the other hand, in SR1 leaf disks, while 500 μM $CdCl_2$ did not modify CAT expression, the enzyme activity was significantly reduced (60% of the C) after 21 h of exposure. Plants contain several catalase isoforms, which are differentially expressed during development and by environmental conditions and could be differentially regulated according to the origin of the H_2O_2 (Ni and Trelease 1991). It could be occurring that in SR1, Cd was diminishing total catalase activity, probably damaging some isoforms active in H_2O_2 detoxification by enhancing its oxidation, as was observed in sunflower CAT3 isoform (Azpilicueta et al. 2007). In the tobacco transgenic cultivar, CAT protein expression was slightly enhanced by cadmium, but CAT activity was not affected as occurred in SR1. It is possible that Cd was mainly harmful for other isoforms distinct from CAT1, which is the isoform responsible for the major activity in the leaves of this transgenic tobacco line (Chamnongpol et al. 1996). In *Arabidopsis thaliana*, a marked increase of *cat1* transcripts but not in CAT activity was observed after the plants were treated with Cd (Smeets et al. 2008). In barley catalase-deficient mutants with altered photorespiration (RPr 79/4) growing under elevated CO_2, a slight reduction on catalase activity was observed both in the wild type and mutant leaves, but catalase activity increased significantly when the plants were transferred out of the elevated CO_2 (Azevedo et al. 1998). The authors considered that this should be due to an emergency reaction in response to the increased concentration of H_2O_2 during photorespiration, following the induction of *Cat1* gene (Acevedo et al. 1996).

Catalase deficiency reduces the H_2O_2-removing capacity of plant cells and consequently, may lead to a higher steady-state of the other H_2O_2 detoxifying enzymes, as APOX and GPOX (Vicente et al. 2001). An induction of peroxidase activity has been observed in mutants of *Saccharomyces cerevisiae* and *Hansenula polymorpha* that were deficient in peroxisomal catalase (Verduyn et al. 1991). Willekens et al. (1997) demonstrated that tobacco transgenic CAT1AS plants activated alternate enzymatic mechanisms for H_2O_2 scavenging to compensate for the shortage of catalase after being transferred to high illumination (300 mmol/m^2/s PPFR) for 48 h, showing a persistent increase in both glutathione peroxidase and ascorbate peroxidase expression, but without avoiding necrosis in CAT1AS plants. Also, Willekens et al. (1997) reported that the level of APOX protein was constitutively slightly higher in CAT1AS. Using the same tobacco cultivars, we did find that APOX and GPOX activities were comparable or lower, respectively, in nontreated CAT1AS compared to SR1 leaf disks at 3 h of incubation, but both enzyme activities increased constitutively in the transgenic cultivar compared to the wild type at longer incubation times. This might reflect an adaptation aimed to balance constitutive H_2O_2 formation in the cytoplasm that was not enough to avoid the damage produce by 500 μM $CdCl_2$, considering that APOX and GPOX activities were more affected in CAT1AS Cd-treated leaf disks.

Transgenic CAT-deficient plants demonstrated a higher sensitivity to membrane damage and cell death when exposed to Cd, which led us to think that the differences respect to the wild type cultivar might not be mainly related to ROS detoxification pathways, especially H_2O_2, under Cd stress. This hypothesis clearly needs to be supported by a more detailed and compartment specific analysis both of

ROS generation and detoxification rates and by analyzing other molecules, like NO, which has shown to be involved in Cd toxicity in wheat roots (Groppa et al. 2008). Mitochondrial O_2^- formation could be one of the sources of ROS that contributes to the cell damage that is currently under study.

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Conflict of interest The authors of the manuscript declare that they have no conflict of interest with the institution that sponsored the research.

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