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Endogenous salicylic acid potentiates cadmium-induced oxidative stress in *Arabidopsis thaliana*

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Abstract

To better understand the role of endogenous salicylic acid (SA) in plants exposed to abiotic stresses known to generate oxidative damage, the response to cadmium treatment of a wild *Arabidopsis thaliana* ecotype and a SA-deficient transgenic line was investigated. After 5 days of Cd treatment, chlorophyll content was significantly reduced and TBARS significantly increased in wild type seedlings but not in the SA-deficient line. Leaves of wild type plants exposed to the metal showed accumulation of H_2O_2 and increased oxidized glutathione content, resulting in decreased GSH/GSSG ratios. After Cd treatment, transgenic plants displayed a significantly decreased SOD activity (about 50% of control) which may have contributed to prevent H_2O_2 increase. The activity of several H_2O_2-detoxyfing enzymes diminished in wild type seedlings exposed to the metal (27–35% decrease). In SA-deficient plants ascorbate peroxidase and glutathione peroxidase activities slightly decreased, but guaiacol peroxidase and catalase activities significantly increased. CAT1 isoform was found to be particularly abundant in wild type *Arabidopsis* plants exposed to Cd, but scarcely expressed and almost unaffected by the metal addition in the transgenic line. Even when protective effects of SA have been extensively reported for several plant species, our results suggest that endogenous SA may function in *A. thaliana* as a signaling molecule necessary to generate, to sustain or to amplify Cd-induced oxidative stress.

Keywords: Oxidative stress; Salicylic acid; Catalase; *Arabidopsis thaliana*

1. Introduction

As far as agriculture intensity increases, environmental stresses are gaining prevalence. Cadmium is one of the most aggressive and persistent heavy metals that may be present in natural environments as a by-product of human activities; among them, application of phosphoric fertilizers constitutes the major Cd input into agricultural soils [1]. It is easily taken up by roots and translocated to other parts of the plant, being toxic to living cells at very low concentrations [2]. Plants affected by Cd showed impaired photosynthesis [3,4], altered mineral nutrition [5,6] and water imbalance [7].

The activation of the cellular antioxidant metabolism belongs to the general stress responses induced by heavy metals [8]. It was recently demonstrated that oxidative stress of *Arabidopsis thaliana* seedlings exposed to cadmium is caused by H_2O_2 accumulation [9], which in turn may be responsible for lipid peroxidation and oxidative damage to proteins and DNA.

Salicylic acid (SA) has been identified as a key signaling molecule involved in plant defense responses against pathogen attack. There is strong evidence that SA mediates the oxidative burst which precedes the hypersensitive response and the development of systemic acquired resistance [10]. In addition, it has been shown to play a major role in plant responses to several abiotic stresses, such as UV irradiation and ozone exposure [11,12]; also in chilling tolerance [13] and heat acclimation [14].

The role of SA under heavy metal stress is not fully understood. It was shown that exogenous applied SA

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; GlPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GuPX, guaiacol peroxidase; NBT, nitroblue tetrazolium; PR-1, pathogenesis related protein 1; ROS, reactive oxyigen species; SA, salicylic acid; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; wt, wild type

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ameliorated the lipoxygenase-mediated damaging effects of Pb and Hg on membranes in two cultivars of rice [15]. Metwally et al. [16] found that SA-pretreatment of barley seedlings alleviated Cd toxicity, but not by acting at the level of antioxidant defenses. On another hand, Drazic and Mihailovic [17] reported that simultaneous addition of SA and Cd to soybean seedlings mitigated several Cd-toxic effects such as leaf desiccation and altered ionic homeostasis. They suggested that this could be part of a general antistress response which includes the regulation of K and Mg distribution.

The use of SA-deficient transgenic plants expressing the bacterial salicylate hydroxylase gene (*NahG*) enables focusing on the role of endogenous SA in the complex biochemical network triggered by abiotic stressors, a point scarcely surveyed up to now. The purpose of this work was to compare the response of wild type and SA-deficient *A. thaliana* plants to Cd treatment, with special regard to its effects on the antioxidant defense system and on H_2O_2 metabolism.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of an *A. thaliana* L. wild type line (Columbia-0) and of a transgenic line expressing the bacterial salicylate hydroxylase gene (NahG) were surface sterilized in 20% (v/v) commercial bleach for 10 min, followed by four washes with sterile distilled water. The seeds were sown for germination onto agar plates containing basal Murashige-Skoog medium, supplemented with 3% sucrose (w/v). The plates were placed at 4 °C in the dark for 48 h and then transferred to growth chambers under 16 h light/8 h dark photoperiod (175 μmol m^{-2} s^{-1}) at 22–24 °C and 70% relative humidity for a week. One-week old seedlings were transferred to plastic trays (12–15 seedlings/tray) filled with a mixture of vermiculite and perlite (3:1) and watered with half-strength Hoagland solution, keeping the same growth chamber conditions. After 10 days, wild type and transgenic seedlings were watered for five consecutive days with half-strength Hoagland solution with or without $CdCl_2$ 0.5 mM, to obtain treated and control plants, respectively. Five replicated extracts were prepared to represent each treatment for the determination of enzymatic activities and glutathione contents. For $O_2^{\bullet -}$ and H_2O_2 analysis, intact leaves were used and five replicated test vials were run. The whole experiment was carried out twice.

Oxidative damage was assessed at days 2 and 5. Accumulation of $O_2^{\bullet -}$ and H_2O_2, and enzymatic and non-enzymatic parameters involved in antioxidant defense system were investigated at day 5 after the beginning of Cd treatment. A western blotting analysis to evaluate the abundance of CAT1 protein – a catalase isoform known to be highly expressed in photosynthetic tissues – was also performed using plant extracts prepared at day 5 of Cd treatment.

2.2. Assessment of oxidative damage

Oxidative damage in leaves was estimated by measuring lipid peroxidation and chlorophyll content. The level of lipid peroxidation was determined based on the measurement of thiobarbituric acid reactive substances (TBARS). Briefly, 0.2 g of fresh tissue were ground in 2 ml of 20% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000 × *g* for 10 min. To a 0.5 ml aliquot of the supernatant, 0.5 ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA and 100 μl of butyl-hydroxitoluene were added. The mixture was heated at 95 °C for 30 min and cooled immediately, and the absorption of the supernatant was measured spectrophotometrically at 532 nm. The value for non-specific absorption at 600 nm was substracted. TBARS concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} [18].

Chlorophyll was extracted from 0.1 g leaf samples with 3 ml of 96% ethanol. After heating for 15–20 min at 55 °C (until complete leaf discoloration), absorbance at 654 nm was measured and total chlorophyll content was calculated, according to Wintermans and de Mots [19].

2.3. Determination of superoxide anion and hydrogen peroxide

In situ accumulation of superoxide anion ($O_2^{\bullet -}$) was evaluated at day 5 after the beginning of Cd treatment by infiltrating excised leaves with nitroblue tetrazolium (NBT), based on the technique described by Jabs et al. [20]. Leaves were vacuum infiltrated with a solution containing NaN_3 (10 mM) and NBT (0.1% w/v), applying three 20-seconds pulses, and then incubated for an hour at room temperature under light. Leaves were then cleared with ethanol 96° and photographed. Control experiments adding SOD (to verify the specificity of $O_2^{\bullet -}$ staining) were included.

Intracellular accumulation of hydrogen peroxide was measured using the molecular probe 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA), as described by Maxwell et al. [21]. Excised leaves (approximately 20 mg) were placed in glass vials containing 3 ml of hydrolyzed dichlorofluorescein diacetate solution, at a final concentration of 15 μM. Blanks without plant material (to substract for unspecific dichlorofluorescein oxidation) were also prepared. After an incubation of 1 h, the fluorescence of the solution was measured in an Aminco Bowman spectrofluorometer, with excitation and emission wavelengths of 488 and 520 nm, respectively. Hydrogen peroxide concentrations were calculated based on the fluorescence of a calibration curve prepared with the probe and increasing concentrations of H_2O_2.

2.4. Determination of GSH and GSSG

Reduced (GSH) and oxidized (GSSG) glutathione were determined based on the use of the fluorescent probe *o*-phtalaldehyde (OPT), as described by Senft et al. [22]. Total GSH was measured as the fluorescent product of the reaction with OPT. Total GSSG was also measured with OPT after derivatization of the samples with *N*-ethylmaleimide (NEM), which alkylates free thiol, for 30 min. Fluorescence was quantified in an Aminco Bowman spectrofluorometer, with excitation and emission wavelengths of 350 and 420 nm,

respectively. Calibration curves with commercial GSH and GSSG were performed.

2.5. Enzyme preparations and assays

Extracts for determination of glutathione peroxidase (GlPX), guaiacol peroxidase (GuPX), catalase (CAT) and superoxide dismutase (SOD) activities were prepared from leaf samples (0.15–0.40 g in 1.2 ml), homogenized under ice-cold conditions in 50 mM phosphate buffer (pH 7.4) containing EDTA (1 mM), PVP (0.5 g) and Triton X-100 (0.5% v/v), and frozen at −20 °C until use. Ascorbate peroxidase (APX) activity was measured immediately on fresh extracts (0.3 g in 1.2 ml), to which 5 mM ascorbic acid had been added during homogenization, as described by Nakano and Asada [23].

GlPX activity was determined based on the decrease in the absorbance at 340 nm due to NADPH consumption coupled to GSH regeneration, in the presence of H_2O_2 and glutathione reductase [24]. GuPX activity was estimated by measuring the increase in the absorbance at 470 nm due to tetraguaiacol formation in the presence of guaiacol and H_2O_2 [25]. CAT activity was assayed based on the decrease in the absorbance at 240 nm due to the degradation of H_2O_2, as described by Chance and Maehly [25].

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of NBT, as described by Giannopolitis and Ries [26], in a reaction mixture which contained the $O_2^{\bullet -}$-generating solution and 250 to 350 μl aliquots of plant extract. One unit of SOD was defined as the amount of enzyme required to cause a 50% inhibition of NBT reduction, under the assay conditions.

2.6. Salicylic extraction and quantification

Total salicylic acid content (free plus bound) was determined in 0.5-g leaf samples based on Verberne et al. protocol [27]. Leaf tissue collected from control and treated *Arabidopsis* plants after 5 days of Cd exposure was ground in liquid nitrogen, homogenized in 1 ml methanol 90% v/v, vortexed and sonicated for 3 min at 30-s intervals. After centrifugation at 10,000 × *g* for 15 min, supernatants were transferred and pellets reextracted with 0.5 ml methanol 100%; sonication and centrifugation steps were repeated. The combined supernatants were speed-vacuum evaporated under heat (Savant Speedvac Plus). The residue was dissolved in 0.25 ml TCA 5% (w/v) and partitioned twice using 0.8 ml ethyl acetate/cyclohexane (1:1). The combined upper layers containing free SA were evaporated at medium drying speed. Aqueous lower phases containing bound SA were subjected to acid hydrolysis by adding HCl 8 M (0.3 ml) and heating at 80 °C for 1 h. The released free SA was then extracted with the organic mixture and concentrated as described above. An internal standard of 2,3-dihydroxybenzoic acid (2,3-DHBA) was used to estimate SA recovery. Quantification of SA and 2,3-DHBA was performed by HPLC (Thermo Separation Products Spectra Series P-100) under fluorescence detection (Linear LC304), using a C18 reverse phase column (Phenomenex Luna, 5 μm, 250 mm × 4.6 mm) and a mobile phase consisting of sodium acetate buffer 0.2 M pH 5.5 (90%) and methanol (10%). Excitation and emission wavelengths: 294 and 426 nm (SA), 305 and 437 nm (2,3 DHBA).

2.7. Determination of Cd content

Dry plant material (leaves) was powdered and mineralized in the presence of strong acids. After proper dilution, Cd concentration was determined using an inductively coupled plasma atomic absorbance spectrophotometer (ICP-AAS, Baird 2070), following standard AOAC (Association of Official Analytical Chemists) protocol for trace elements analysis.

2.8. Western blot analysis of CAT1

Soluble protein extracts were prepared at day 5 after the beginning of Cd treatment. Thirty micrograms of total protein were electrophoresed on 12% SDS-PAGE in a mini PROTEAN III equipment (Bio-Rad), as described by Laemmli [28]. Following electrophoresis at 4 °C, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences). To immunodetect CAT1, the membrane was incubated at 4 °C overnight with a polyclonal antibody raised in rabbit against two peptides deduced from the amino acid sequence of a *Solanum tuberosum* L. cDNA (NCBI accession no. AY442179), developed and kindly provided by Dr Isabel Santos (University of Porto, Portugal). Bands were detected with an anti-rabbit IgG peroxidase conjugated secondary antibody (Sigma), and 3,3′-diaminobenzidine (DAB) was used as substrate for staining procedure.

2.9. Statistics

Data shown in tables and figures are mean values of two independent experiments, with five replicates each. Standard errors of the means (S.E.M.) are presented. Differences among treatments were analyzed by unpaired *t*-test or one-way ANOVA followed by Tukey's multiple range test using InStat™ software (Graph Pad Software, San Diego, CA), taking $P < 0.05$ as significant.

3. Results

3.1. Evidence of oxidative damage

No visible symptoms of plant decay could be appreciated after two days of watering *A. thaliana* seedlings with Hoagland solution containing 0.5 mM $CdCl_2$. However, three days later, chlorosis became evident and chlorophyll content was significantly reduced in wild type plants but not in the SA-deficient transgenic line (Fig. 1). By this time, TBARS level was found to be significantly increased in wild type plants, while in transgenic plants this parameter remained unaffected (Fig. 2).

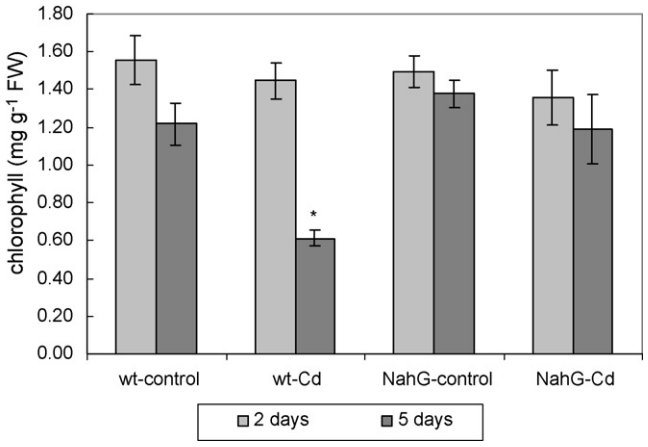

Fig. 1. Effect of cadmium treatment on chlorophyll content. Values are the means of two independent experiments with five replicated measurements, and bars indicate S.E.M.. Asterisks indicate significant differences between control and Cd-treated plants for the same genotype and sampling time ($P < 0.05$).

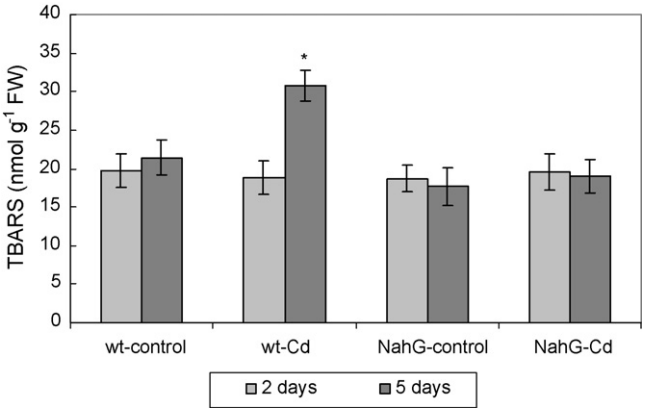

Fig. 2. Effect of cadmium treatment on TBARS concentration. Values are the means of two independent experiments with five replicated measurements, and bars indicate S.E.M.. Asterisks indicate significant differences between control and Cd-treated plants for the same genotype and sampling time ($P < 0.05$).

3.2. Assessment of hydrogen peroxide and superoxide anion accumulation

At day 5 of Cd treatment, excised leaves of wild type plants showed an important accumulation of hydrogen peroxide; which was not observed in transgenic plants (Table 1). SOD activity, thought to be an important source of H_2O_2 in plant cells, was significantly reduced in transgenic plants exposed to the metal, while in the wild genotype this enzyme activity remained similar to controls (Table 1). Despite this, Cd-treated NahG plants did not accumulate in their leaves more superoxide anion than the rest of the treatments; on the contrary, these leaves showed a NBT-staining even weaker (Fig. 3).

3.3. Non-enzymatic antioxidant defenses and SA and Cd content

As Table 1 shows, in both *Arabidopsis* genotypes Cd altered not only total glutathione pools in the leaves but also the balance between GSH and GSSG. After 5 days of treatment, while in wild type plants total glutathione increased by about 36%, mainly at the expense of its oxidized form (which itself increased by 75%), SA-deficient plants showed a more discrete total glutathione increase (16%) but entirely due to the significant rise observed for the reduced form (GSH). In this way, GSH/GSSG ratio decreased for wild type plants and increased for SA-deficient plants (Table 1).

For the wt genotype, a significant increase in total SA content (free plus bound) respect to untreated plants was observed after Cd treatment; this increment was not significant in transgenic plants. No significant differences in Cd contents between wild type and transgenic plants were found at the end of the experiments (Table 1).

3.4. Enzymatic antioxidant defenses

Four H_2O_2-degrading activities in *Arabidopsis* leaves were tested at day 5 of Cd treatment: ascorbate peroxidase (APX); unspecific peroxidases that use guaiacol as electron donor (GuPX); glutathione peroxidase (GlPX) and catalase (CAT). It may be noticed in Fig. 4 that leaves of wild type seedlings diminished all these activities, ranging the rate of decrease between 27 and 35%. In transgenic plants, however, the decreases observed did not exceed a 17%, and were detected only for APX and GlPX. By opposite, GuPX and CAT showed significant increases in their activities (169 and 166% of the control, respectively) after Cd treatment (Fig. 4).

Table 1
Effect of cadmium treatment on hydrogen peroxide and SA accumulation, and other parameters involved in antioxidant plant defense system (day 5)

	wt	wt-Cd	NahG	NahG–Cd
H_2O_2 (nmol/g FW/min)	12.80 ± 2.13 c	39.17 ± 5.34 a	19.72 ± 2.87 b	20.39 ± 3.40 b
SOD activity (U/g FW)	7.25 ± 0.38 a	6.60 ± 0.45 a	7.17 ± 0.36 a	3.80 ± 0.25 b
GSH (μg/g FW)	77.99 ± 6.00 b	91.37 ± 7.27 ab	83.22 ± 4.82 b	103.73 ± 5.55 a
GSSG (μg/g FW)	38.66 ± 2.26 b	67.56 ± 5.96 a	43.74 ± 2.08 b	44.55 ± 6.04 b
GSH/GSSG ratio	2.02	1.35	1.90	2.33
Total SA content (ng/g FW)[a]	72 ± 7 b	235 ± 13 a	41 ± 6 c	52 ± 7 c
Cd content (mg/kg DW)	6.6 ± 0.5 b	105 ± 7 a	6.2 ± 0.7 b	112 ± 13 a

Data are the mean ± S.E.M. of two independent experiments, with five replicates for each treatment. Different letters within rows indicate significant differences ($P < 0.05$), according to Tukey's multiple range test.

[a] A recovery rate of 72 and 64% was calculated for free and bound SA, respectively.

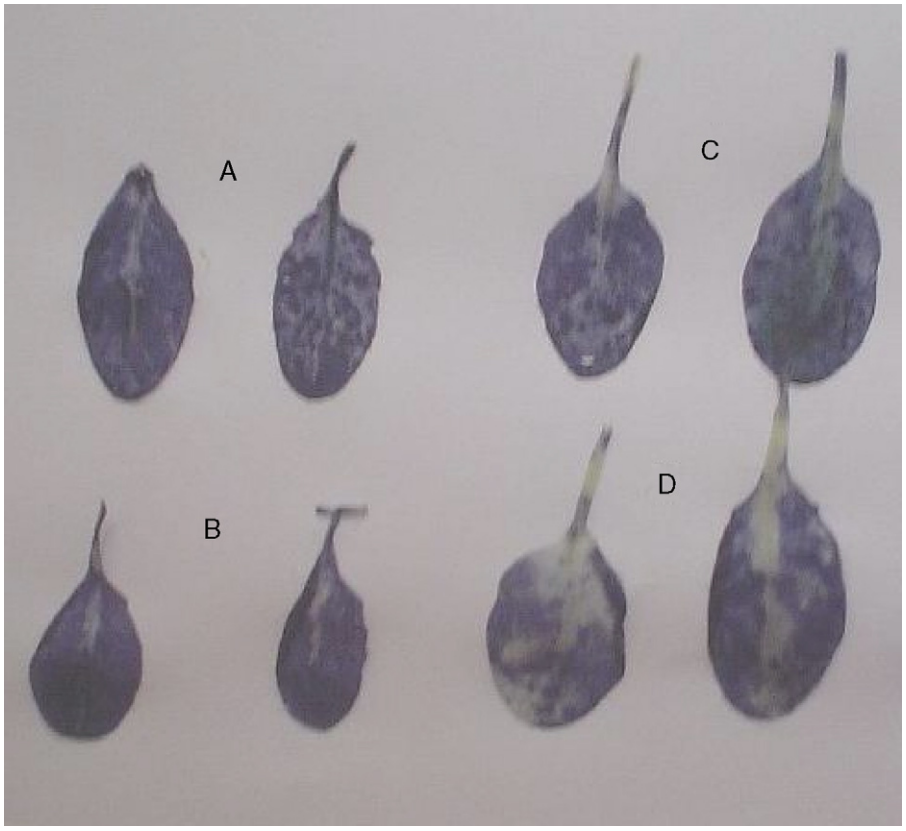

Fig. 3. Superoxide anion accumulation after cadmium treatment, as evidenced by nitroblue tetrazolium staining. Two representative leaves of each treatment are shown. (A) Wild type, control; (B) wild type, Cd; (C) NahG, control; (D) NahG, Cd.

3.5. Catalase western blotting

A representative nitrocellulose membrane showing immunoblotting of CAT1 isoform of catalase is presented in Fig. 5. It may be noticed the important increase in the concentration of this protein, reflected by the intensity of the DAB-stained band that took place in extracts obtained from wild type plants after exposure to the metal. On the contrary, in soluble protein extracts obtained from SA-deficient *Arabidopsis* plants, the intensity of this band was found to be lower than that observed for control wild type extracts, and almost unaffected by Cd treatment.

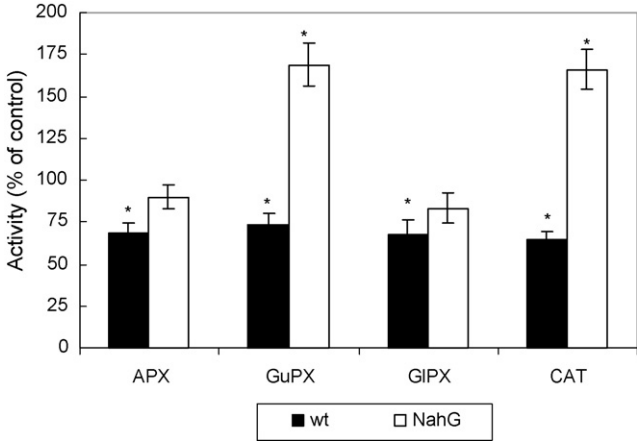

Fig. 4. Effect of cadmium treatment on the activity of four antioxidant enzymes. Activities are expressed as percent of control values. Means were obtained from two independent experiments with five replicated measurements, and bars indicate S.E.M.. Asterisks indicate significant differences respect to the corresponding control ($P < 0.05$). APX: ascorbate peroxidase; GuPX, guaiacol peroxidase; GlPX, glutathione peroxidase; CAT: catalase.

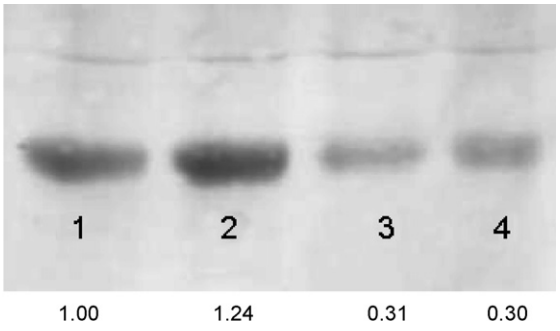

Fig. 5. Western blotting of CAT1 isoform after cadmium treatment. Total protein samples (30 μg) were electrophoresed on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. CAT1 was immunodetected with a polyclonal antibody and revealed with IgG peroxidase-conjugated secondary antibody, using DAB as substrate, as described in Materials and methods. Membrane was photographed with a Fotodyn, analyzed with GelPro software and expressed in arbitrary units (assuming wt-control value equal to 1 unit), based on absolute integrated optical density of bands. The figure shows results which are representative of those obtained in three independent experiments. Line 1: wild type, control; line 2: wild type, Cd; line 3: NahG, control; line 4: NahG, Cd. AU, arbitrary units.

4. Discussion

Although several reports show that exogenous applied SA can alleviate oxidative damage caused by biotic and abiotic stressors on different plant species, the results presented here indicate that endogenous SA acts as a potentiating agent of Cd induced oxidative stress in *Arabidopsis* seedlings. Transgenic plants expressing the bacterial salicylate hydroxylase gene and thus incapable to accumulate SA showed no evidence of being affected by Cd exposure after 5 days of metal treatment, while wild type plants did, as it was reflected by increased TBARS and reduced chlorophyll contents.

Leaf Cd concentrations at the end of the experiments were determined by an ICP-AAS procedure and no significant differences between genotypes could be found (Table 1). Therefore, the differential behavior above reported cannot be attributed to differences in Cd absorption or translocation rates. On another hand, negligible levels of this heavy metal (below 7 mg/kg DW), probably coming from Hoagland's salts impurities, were detected in both wild type and transgenic control seedlings. Although it was reported that Cd contamination may result in plant toxicity even at low doses, no signs of oxidative damage could be observed in these *Arabidopsis* control seedlings, at least until day 15 (end of experiments).

Our results are in line with those reported by Borsani et al. [29], who analyzed the behavior of wild type and SA-deficient *Arabidopsis* seedlings faced to osmotic stress. These authors found lower increases in TBARS contents and lower decreases in GSH/GSSG ratios for *Arabidopsis* SA-deficient plants, and corroborated that this was not a consequence of the antioxidant properties of cathecol, the main degradation product of SA in transgenic plants. They also reported for both genotypes pronounced expression of a glutathione peroxidase gene after exposure to 200 mM NaCl. We observed that in the transgenic line, peroxidases activities (including that of glutathione peroxidase) were less affected by Cd treatment, and that H_2O_2 concentration remained similar to control levels. The existence in *A. thaliana* of a glutathione *S*-transferase protein with glutathione peroxidase activity, showing high affinity for the long chain fatty acid hydroperoxides typically formed as products of membrane oxidation, was already reported [30].

All antioxidant enzymes activities decreased by about 30% in wt plants under Cd treatment. ROS are known to cause oxidative modifications on protein structure and to generate reactive aldehydes as by-products of lipid peroxidation, giving rise to the production of carbonyl groups in the protein molecule [31], which may result in impaired functionality. It has also been proposed that metal toxicity in plants may result from the binding of metals to protein sulphydryl groups, inhibiting enzyme activities or altering protein structure [32]. On another hand, it has been demonstrated that oxidatively modified proteins are selected targets for proteases [33].

Cho and Seo [9] have recently demonstrated that *A. thaliana* resistance to high Cd concentrations is closely related to its capability to prevent hydrogen peroxide rises, which in turn mainly depends on an effective glutathione-ascorbate pathway. We found that APX activity was little affected by Cd treatment in the SA-deficient line; on another hand, the rise in GSH/GSSG ratio observed gives proof of a rapid turnover of oxidized to reduced glutathione.

In contrary to what it could be expected, we found no correlation between SOD activity and H_2O_2 accumulation rates. As can be observed in Table 1, Cd-treated wild type plants displayed similar SOD activities than controls but accumulated significantly higher amounts of H_2O_2, while SOD activities in Cd-treated transgenic seedlings halved those of controls but did not alter H_2O_2 contents. This suggests that actual hydrogen peroxide concentrations in the leaves of *Arabidopsis* plants exposed to Cd mainly depends on the maintenance of an effective H_2O_2-removal machinery (not observed in the wt plants); even when a reduced SOD activity may have contributed to prevent H_2O_2 rises.

It is noteworthy that $O_2^{\bullet -}$ markedly accumulated in *Arabidopsis* leaves in all cases except for transgenic Cd-treated plants, where NBT staining was slightly weaker (Fig. 3). Since this response cannot be explained by an improved SOD activity (in fact SOD activity was reduced), an inhibitory effect of Cd on NADPH oxidase – enzyme involved in $O_2^{\bullet -}$ generation – seems feasible. Some results obtained by our group in experiments performed with sunflower plants tend to support the hypothesis of a Cd-mediated NADPH oxidase inhibition (manuscript in preparation).

Rodríguez-Serrano et al. [34] found that in pea roots Cd down-regulated CuZn-SODs activity, whereas Fe-SOD and Mn-SOD were not affected. However, at transcriptional level, Fe and Mn-SODs were strongly up-regulated. Likewise, there are reports showing decreased SOD activity after Cd treatment in wheat [35] and bean [36], while for other plant species an opposite effect was observed [37–39].

Catalase is essential to prevent peroxisomal H_2O_2 accumulation coupled to the photorespiration pathway under elevated light intensities, and represents one of the primary enzymatic defenses against oxidative stress induced by senescence, chilling, dehydration, osmotic stress, wounding, paraquat, ozone and heavy metals [40]. It has been shown that SA specifically binds and inhibits CAT activity during development of systemic acquired resistance [41]. For protein-enriched fractions obtained from *Arabidopsis* leaves, Sánchez-Casas and Klessig [42] reported the presence of a salicylic acid binding protein responsible for a 47% inhibition in CAT activity in the presence of 1 mM SA. We observed a significant decrease in CAT activity in wild type *Arabidopsis* seedlings exposed to Cd (Fig. 4), together with a sharp rise in CAT1 isoform expression (Fig. 5). This suggests a possible postranslational inactivation of this protein, whose transcription and translation was probably enhanced to compensate functionality impairment.

Several genes involved in protein denaturation and refolding were found to be activated in *A. thaliana* plants upon Cd exposure [43]. Romero-Puertas et al. [44] reported enhancement of oxidized proteins in pea plants after Cd treatment, finding that glutathione reductase, Mn-superoxide dismutase

and CAT were among the oxidized proteins identified. We observed that in the SA-deficient genotype, expression of CAT1 isoform was much lower and slightly affected by Cd addition. However; CAT activity did not decrease, by opposite it greatly increased (166% over control). One possibility that would explain these findings is involvement of other catalase(s) isoform(s) in H_2O_2-detoxification in NahG plants. The CAT multi-gene family in *Arabidopsis* includes three genes (*CAT1*, *CAT2* and *CAT3*) encoding individual subunits which associate to form at least six different isozymes [45]. It was reported that neither steady state of *Arabidopsis CAT2* mRNA abundance [46] nor CAT activity [47] increased in response to ozone-induced oxidative stress. However, using a fluorescent differential display technique, Lang et al. [48] recently demonstrated that in the heavy metal hyperaccumulator *Brassica juncea*, *CAT3* mRNA expression strongly increased after Cd treatment, especially in the leaves.

There are some reports showing increases of SA and their precursors (benzoic acid and *O*-hydroxycinamic acid) in roots or/and leaves of Cd-treated plants, but also oxidative damage [16,49]. For wt plants we observed a significant increase (app. 3.3 times) in total SA leaf content respect to the untreated group at day 5 of Cd treatment. Since it was reported that the *PR-1* gene expression is induced by SA [50] and can therefore be considered a molecular marker for SA accumulation [29], we investigated the level of *PR-1* transcripts after a RT-PCR procedure in *Arabidopsis* leaf samples taken 36 h after the beginning of Cd treatment, and found increases of about 17–23%, but only within the wt group (unpublished results). This suggests that enhancement of SA biosynthesis may be among the earlier responses to heavy metal exposure.

Yang et al. [51] proposed a model to explain different roles of SA in mediating defense response and cell death – events certainly coupled to oxidative stress – by introducing the notion of ''SA-insensitive'' and ''SA-sensitive'' plants, and included *Arabidopsis* within the latter group. It is possible that after Cd treatment NahG plants, with a SA-hydroxilase activity unable to completely remove SA, had reached ''optimal'' SA levels which probably provide protection against oxidative stress, whereas in wt plants higher SA increases become toxic and result in plant cell injury.

Based on the results herein presented, we conclude that impairment of H_2O_2-detoxifying enzymes, together with a GSH/GSSG imbalance are key components of the oxidative stress cascade generated in *A. thaliana* seedlings exposed to Cd, which seem to be coupled to increases (probably just transient) in SA biosynthesis.

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