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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