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Sunflower cotyledons cope with copper stress by inducing catalase subunits less sensitive to oxidation

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ABSTRACT

Copper is an essential trace element for living organisms, in excess, can be toxic to the cell because of its capacity to generate reactive oxygen species (ROS). Catalase (CAT) catalyzes the dismutation of hydrogen peroxide into water and dioxygen and in plants it is located in peroxisomes and glyoxysomes. Different metals can induce changes in CAT activity, but the mechanism underlying its changes is unclear. After 4 h of treatment with 5 and 10 μM $CuCl_2$ a decrease in the specific CAT activity was detected in sunflower cotyledons of post-germinative heterotrophic seedlings. At 8 h of treatment, 5 μM Cu^{2+} produced an induction of CAT activity while only a complete recovery to control values was observed for 10 μM Cu^{2+} treated seedlings. These activity variations were not related to the level of CAT protein expression, but they did correlate with the oxidative state of the CAT protein. This indicates that the mechanism of CAT inactivation by Cu^{2+} involves oxidation of the protein structure. The level of the mRNA of *CATA3* and *CATA4* increased with the presence of the metal after 4 h of exposure. These CAT genes code for the synthesis of CAT subunits less sensitive to oxidation, which would prevent the copper-induced oxidative inactivation of CAT.

Introduction

Copper is considered an essential trace element for the living organisms. As part of the plant cell components it plays a vital role as a prosthetic group of many proteins involved in redox reactions, like plastocyanin, cytochrome *c* oxidase or superoxide dismutase, and it is also required by the ethylene receptor for proper signaling [1,2].

At the same time that the amount of copper available to the organism is critical for its life, an excess of this metal can be toxic for the cell. Copper is a redox active metal that has the ability to catalyze, via the so-called Fenton reaction, the generation of reactive oxygen species (ROS) [3]. ROS are products obtained from the partial reduction of oxygen molecule that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical (HO•) [4]. To cope with ROS, organisms have developed a protective system that includes enzymatic (superoxide dismutase, catalase, glutathione reductase, ascorbate peroxidase) and non-enzymatic antioxidant defenses (glutathione, ascorbic acid) [4]. An increased concentration of oxidant species and/or a decrease in the antioxidant level could lead to the generation of oxidative stress.

ROS can cause oxidative modification of cellular components in plants. This type of damage will lead to an altered structure and loss of functionality of macromolecules [5]. Proteins, as well as other molecules such as lipids, are the most common cellular targets of the oxidative species; protein oxidation may result in modification of their enzymatic and binding properties and lead to diverse functional changes. Carbonylation of specific amino acid residues is the most common oxidative protein modification, and it is characterized as an irreversible and unrepairable process [6].

Catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) is one of the most important antioxidant enzymes. It is an iron porphyrin tetrameric protein found in all aerobic organisms that catalyzes the dismutation of H_2O_2 to O_2 and H_2O. In plant cells catalase decomposes H_2O_2 derived from β-oxidation of fatty acids during the heterotrophic, post-germinative growth phase of oil-rich seedlings (such as sunflower), within specialized peroxisomes called glyoxysomes. In photoautotrophic cells, catalase degrades H_2O_2 produced during the photorespiration within leaf-type peroxisomes, which are derived from glyoxysomes [7]. Moreover, catalase confers resistance to oxidative stress by detoxification of ROS that could be produced within the cell by adverse environmental conditions, such as drought [8,9], temperature [10], or UV-B radiation [11]. On the other hand, due to the fact that H_2O_2 is recognized as a second messenger and catalase acts as a key regulator of this species, this enzyme could act as mediator in the signal transduction pathway [12].

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Multiple forms of the CAT enzyme encoded by a small unlinked nuclear gene family have been reported for many higher plants. When CAT subunit polypeptides encoded by distinct genes are simultaneously expressed in the same cell, heterotetramers of CAT may be formed. In sunflower (*Helianthus annuus* L.) at least eight isoforms have been identified: CAT1–CAT8. According to studies of the composition of the protein, these eight isoforms can be divided into two groups. The first group (CAT1 through CAT5) arises as the consequence of interactions between four 55 and 59 kDa subunits in various proportions, whereas the second group (CAT6 through CAT8) contains exclusively 55 kDa subunits [13]. The biogenesis of the subunits is controlled by four different genes (*CATA1–CATA4*). *CATA1* and *CATA2* are assumed to code for subunits of 55 kDa, whereas *CATA3* and *CATA4* code for subunits of 59 kDa [8]. The presence of various isoforms may reflect the multiple functions of this enzyme, but the physiological roles of some isoforms remain yet uncertain.

Although numerous reports describe the impact of the excess of metals on plant catalase activity as part of the antioxidant response, the mechanism underlying its activity changes is unclear. Due to excess of copper is associated, at least in part, to the generation of oxidative stress, we performed this study in terms of activity, amount and oxidation of CAT protein and CAT transcript levels during the heterotrophic post-germinative stage of sunflower seeds.

Materials and methods

Plant material and growing conditions

Sunflower seeds (*H. annuus* L., cv DK3820, supplied by Dekalb, Buenos Aires, Argentina) were surface sterilized with 20% (v/v) sodium hypochlorite solution (55 g L^{-1} available chlorine) for 10 min and washed several times with distilled water. After that, seeds were soaked in demineralised water in a rotary shaker in dark conditions, at 100 rpm and 24 ± 2 °C. After 48 h of growing, seed pericarps were removed and naked seedlings with 1 cm of root length were selected and used for the assay. Eight seedlings were transferred to flasks with 30 mL of demineralised water (control) or with solutions containing 5 and 10 μM $CuCl_2$. The flasks were incubated at 24 ± 2 °C in darkness. After 4 and 8 h of incubation, germination seedlings were gently washed with distilled water; cotyledons were removed and used for the determinations. Experiments were repeated three times with five replicates per treatment.

Carbonyl group content

Protein oxidation was measured as the total carbonyl group content by reaction with 2,4-dinitrophenylhydrazine (DNPH) [14]. Extracts were prepared from cotyledon tissue (1 g), homogenized in extraction buffer (10 mL) consisting of 50 mM phosphate buffer (pH 7.4), 120 mM KCl, 1 mM EDTA and 0.1 g polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 × *g* for 20 min. Aliquots of the supernatant containing at least 0.5 mg protein were incubated with 0.03% Triton X-100 and 1% streptomycin sulfate for 15 min to remove the nucleic acids. After centrifugation at 8500 × *g* for 10 min, 500 μL of the supernatant were mixed with 500 μL of 10 mM DNPH in 2 M HCl for 1 h at room temperature. Blank samples were incubated in 2 M HCl. Proteins were precipitated with 20% (w/v) TCA, the pellets were washed three times with ethanol:ethylacetate (1:1) and finally dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3), adjusted with trifluoroacetic acid. Absorption at 380 nm was measured and carbonyl group content was calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

Determination of CAT activity

Extracts for determination of CAT activity were prepared from 0.5 g of cotyledon tissue and homogenized under ice-cold conditions in 5 mL of extraction buffer containing 50 mM K-phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP, and 0.5% (v/v) Triton X-100 at 4 °C. Homogenates were centrifuged at 10,000 × *g* for 30 min and the supernatant fraction was used for the assay. CAT activity was determined in the homogenates by measuring the decrease in absorbance at 240 nm 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 the CAT content was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1} [15].

Western blot of catalase, immunoprecipitation and immunochemical detection of catalase carbonyl groups

Extracts obtained in the same extraction buffer as described above supplemented with 2 mM cysteine, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.04% 2-mercaptoethanol (50 μg of protein) were subjected to electrophoretic analysis using 12% (w/v) SDS-polyacrylamide gel (PAGE) [16]. This gel was electrotransferred onto PVDF membranes and polyclonal antibodies raised against cotton seed catalase (generously provided by Dr Trelease) were employed to detect catalase protein. Bands were subsequently visualized using a secondary goat antibody conjugated with horseradish peroxidase and stained using 3,3-diaminobenzidine (DAB) as substrate. Membranes were photographed with Fotodyn equipment, and analyzed with GelPro software.

For the immunoprecipitation and immunochemical detection of catalase carbonyl groups, proteins (100 μg) were derivatized with DNPH as described above, but in this case proteins were dissolved in 100 mM buffer sodium phosphate (pH 7.5) and then were separated by affinity chromatography. Antibodies anti-DNP (50 μL) were linked to cyanogen bromide activated Sepharose 4% agarose matrix (100 mg) from Sigma–Aldrich (St Luis, USA). Samples were incubated overnight at 4 °C with an excess of anti-DNP-agarose resin and then centrifuged for 5 min at 10,000 × *g*. Resin beads were washed 3 times with Tris-buffered saline (TBS), with pellets resuspended in 100 mM glycine–HCl (50 μL, pH 2.5). After centrifugation, the pellets were discarded, the pH of the supernatants adjusted to 6.8 with 0.5 M Tris–HCl buffer (5 μL, pH 8.8) and used for immunodetection of catalase. DNPH derivatized proteins were separated by 12% (w/v) SDS-PAGE. After electrotransfer of the proteins to nitrocellulose membranes, the catalase was detected, photographed and analyzed as previously described.

Semiquantitative RT-PCR

Total RNA was extracted from cotyledon tissue using a modified TRIzol (Invitrogen, Carlsbad, CA) procedure. The RNAs were then treated with DNase I (Promega). They were then converted to cDNAs with random primers using the RevertAid™ M-MuLV Reverse Transcriptase (Fermentas). Primers for PCR amplifications are described in Table 1. PCRs were performed using a programmable Termocycler T 18 (Ivema) at annealing temperatures of 54 °C for 18S and 50 °C for *CATA*s. For an accurate comparison and quantification of the transcript levels, the exponential phase of PCR amplifications was determined by establishing the number of PCR cycles where the products exhibit an exponential phase: 19 cycles for 18S PCR products and 31 cycles for *CATA*s PCR products. The PCR products were electrophoresed through 1.2% agarose and visualized with ethidium bromide. Gel was photographed with Fotodyn, analyzed with GelPro software. The band intensity was