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20S proteasome and accumulation of oxidized and ubiquitinated proteins in maize leaves subjected to cadmium stress

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Abstract

In order to examine the possible involvement of the 20S proteasome in degradation of oxidized proteins, the effects of different cadmium concentrations on its activities, protein abundance and oxidation level were studied using maize (*Zea mays* L.) leaf segments. The accumulation of carbonylated and ubiquitinated proteins was also investigated. Treatment with 50 μM $CdCl_2$ increased both trypsin- and PGPH-like activities of the 20S proteasome. The incremental changes in 20S proteasome activities were probably caused by an increased level of 20S proteasome oxidation, with this being responsible for degradation of the oxidized proteins. When leaf segments were treated with 100 μM $CdCl_2$, the chymotrysin- and trypsin-like activities of the 20S proteasome also decreased, with a concomitant increase in accumulation of carbonylated and ubiquitinated proteins. With both Cd^{2+} concentrations, the abundance of the 20S proteasome protein remained similar to the control experiments. These results provide evidence for the involvement of this proteolytic system in cadmium-stressed plants.
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Keywords: *Zea mays* L.; Maize; Graminaceae; Proteasome; Protein oxidation

1. Introduction

Cadmium is a potent poison for all living cells. Although it is redox inactive, it is a well established oxidative stressor by depleting glutathione and protein-bound sulfhydryl groups, which result in production of reactive oxygen species (ROS) such as superoxide ion, hydroxyl radicals and hydrogen peroxide (Stohs et al., 2001; Romero-Puertas et al., 2004). In turn, the reactive oxygen species can cause modifications to the amino acids of proteins (Dean et al., 1997; Nyström, 2005). However, only a limited number of oxidative protein changes, such as protein disulphides or methionine sulfoxides, can be enzymatically repaired *in vivo*. By contrast, the bulk of the oxidized proteins must be degraded to prevent accumulation of the unfolded protein forms (Grune et al., 2004). The intracellular levels of oxidized proteins thus reflect the balance between the rate of protein oxidation and the rate of oxidized protein degradation.

The proteasome-ubiquitin system is the major proteolytic pathway, in both the cytoplasm and nucleus of eukaryotes (Callis and Vierstra, 2000; Vierstra, 2003). In mammalian cells, besides its function as the proteolytic core of the 26S complex, numerous studies have demonstrated its key role in degradation of oxidatively modified proteins. Interestingly, no ATP and ubiquitin requirement has ever been reported for proteolysis of oxidized proteins in various systems. Therefore, it is generally believed today that the 20S proteasome core complex is sufficient alone for degradation of oxidized proteins in the cell cytoplasm and nucleus (Shringarpure et al., 2001; Grune et al., 2003). Fur-

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thermore, cells with a non-functional ubiquitination cascade were still able to remove oxidized proteins efficiently (Shringarpure et al., 2003).

The 20S proteasome is arranged as a cylindrical stack of four heptameric rings with two inner rings of β-type subunits, and two outer rings of α-type subunits. Each β-ring contains three different proteolytically active sites, which are classified based on their specificity towards short synthetic peptides as: chymotrypsin-like, trypsin-like, and caspase-like (peptidylglutamyl-peptide hydrolyzing) (Ingvardsen and Veierskov, 2001; Orlowski and Wilk, 2000).

In plants, cadmium causes a decrease in glutathione, this being related to generation of reactive oxygen species and accumulation of oxidized proteins (Gallego et al., 2005; Rellán-Álvarez et al., 2006). While the modification of non-specific proteases activities was already reported (Palma et al., 2002), little is known about the effect of cadmium on the proteasome. This is specially relevant because of the potential role of the 20S proteasome for degradation of oxidized proteins. The aim of the present work was, therefore, to investigate if increasing cadmium concentration affects the 20S proteasome activity by oxidative modification. We also studied the quantitative and qualitative pattern of carbonylated and ubiquitinated proteins produced by the metal treatment in maize leaves.

2. Results

2.1. Effect of cadmium in maize whole plants

There was a significant decline in glutathione content in the leaves of maize plants (50% of the control value) with both Cd^{2+} concentrations used (Fig. 1). Although glutathione content decreased with metal addition, a significant increase in carbonyl group content (20% respect to control values) was also observed at the highest cadmium concentration tested (100 μM) (Fig. 1).

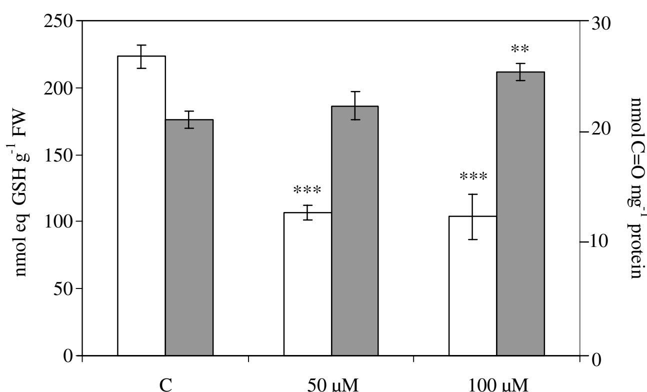

Fig. 1. Effect of cadmium on glutathione content (white) and protein oxidation level (gray). Leaves of maize (*Zea mays* L.) plants were treated with 50 and 100 μM $CdCl_2$ for 24 h in hydroponic culture. Values are means ± SEM. Significant differences $^{**}P < 0.01$ and $^{***}P < 0.001$, according to Tukey's multiple range test.

2.2. Effect of cadmium in leaf segments of maize plants

To exclude possible differences in metal absorption and distribution rates, direct treatment of leaf segments were also performed. Carbonyl group contents were determined in leaf segments as a protein oxidative damage index. Leaves treated with 50 μM Cd^{2+} for 24 h did not show increased levels of carbonylated proteins, whereas treatment with 100 μM Cd^{2+} did (19% compared to control plants) (Table 1).

Furthermore, while simultaneous treatment with 50 μM Cd^{2+} and MG132, a well known proteasome inhibitor, resulted in a significant on accumulation of oxidized proteins (20% increase), these values were not different from that observed with 100 μM Cd^{2+} alone (Table 1). Thus, the addition of MG132 in the incubation medium did not increase levels of oxidized protein with respect to control values.

The densitometric scanning of the immunodetection of carbonyl residues was in agreement with that obtained by spectrophotometric analysis. On the other hand, the qualitative pattern of carbonylated proteins (Fig. 2a) showed that 100 μM Cd^{2+} increased oxidation of proteins with a molecular weight lower than 45 kDa, while SDS–PAGE staining with Coomassie Brilliant Blue R-250 (Fig. 2b) had similar protein profiles for control and Cd-treated leaf segments.

2.3. Effect of cadmium on the ubiquitin-proteasome proteolytic system

Analysis of the ubiquitin-proteasome proteolytic system in maize leaf segments was performed following cadmium treatment for 24 h. Proteasome peptidase activities were assayed by monitoring cleavage of three different fluorogenic peptide substrates: AAF-AMC, Boc-LSYR-AMC and Clz-LLE-βNA for chymotrypsin-, trypsin-, and PGPH-like activities, respectively, in either the absence or presence of MG132, with results expressed as differences between both measurements (Fig. 3). When leaf segments were treated with 50 μM Cd^{2+}, trypsin- and

Table 1
Effect of cadmium on total carbonyl group content

Treatment	nmol C=O mg^{-1} protein
Control	51.6 ± 1.9
MG132	52.1 ± 1.5
50 μM $CdCl_2$	49.5 ± 2.2
50 μM $CdCl_2$ + MG132	62.0 ± 1.1[a]
100 μM $CdCl_2$	61.3 ± 0.8[a]

Maize plants (*Zea mays* L.) were germinated 20 d and then leaf segments were floated for 24 h in water either devoid of cadmium or containing 50 and 100 μM $CdCl_2$. When the effect of the proteasome inhibitors was investigated, 50 μMG132 was added to the incubation medium. Values are means ± SEM. Significant differences.

[a] $P < 0.05$ according Tukey's multiple range test.

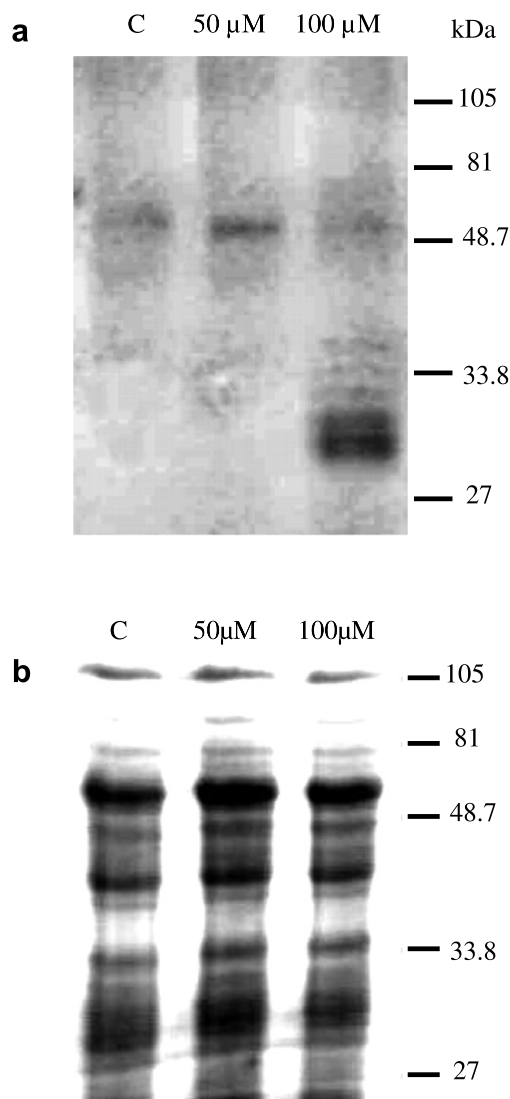

Fig. 2. Effect of cadmium on patterns of total and oxidized proteins. (a) Western blotting with anti-DNP antibody. (b) Coomassie Brilliant Blue R-250 proteins staining. Maize plants (*Zea mays* L.) were germinated 20 d with leaf segments then floated for 24 h in water either devoid of cadmium or containing 50 and 100 μM $CdCl_2$. Leaf extracts (50 μg total protein) were subjected to SDS–PAGE (12% w/v polyacrylamide), with bands visualized as described in Section 5. Gels and membranes were photographed with a Fotodyn and analyzed with GelPro software. The positions of molecular mass markers (in kDa) are shown on the right. Electrophoresis and western blot data shown are representative of three blots with a total of four to five samples/group between the three blots.

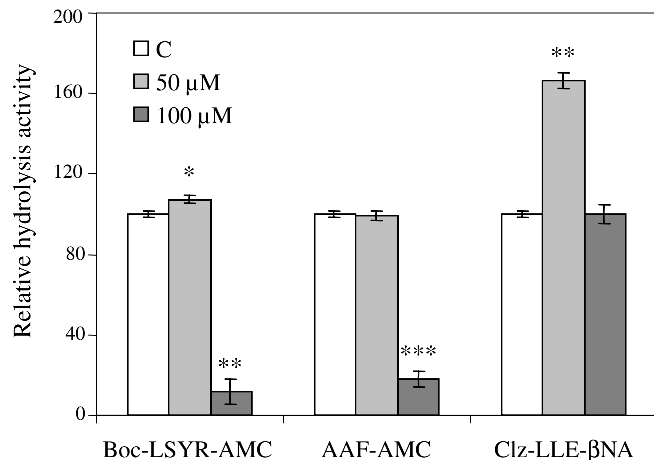

Fig. 3. Effect of cadmium on leaf proteasome activity. Maize plants (*Zea mays* L.) were treated as above (in Fig. 2). Trypsin-like, chymotrypsin-like and peptidyl glutamyl peptide hydrolase (PGPH) proteasome activities were measured in leaf extracts using three peptide substrates (Boc-LSYR-AMC, AAF-AMC and Clz-LLE-βNA, respectively) of the 20S proteasome in the absence or presence of the proteasome inhibitor MG132. Enzymatic activities were normalized for protein concentrations and expressed as percentages of activity present in the controls. Data represent mean values ± SEM. Significant differences $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were observed between control and treated plants according to Tukey's multiple range test.

PGPH-like activities increased 7% and 67%, respectively, whereas chymotrypsin-like activity remained similar to control. For the 100 μM Cd^{2+} treatment, the chymotrypsin and trypsin-like activities decreased by about 82% and 88% with respect to control, whereas the PGPH-like activity remained essentially the same. Reduction in all peptidase activities of the 20S proteasome were detected when concentration higher than 100 μM cadmium were used (data not shown).

Fig. 4 shows that the free ubiquitin and polyubiquitinated protein contents remained similar to the controls in maize leaf segments treated with 50 μM Cd^{2+}. With 100 μM Cd^{2+} treatment, however, together with an inhibition of proteasome activity, a small increase in the accumulation of ubiquitin-conjugated proteins was observed (25% respect to control).

2.4. Effect of cadmium on proteasome protein abundance and oxidation

To test whether modification of 20S proteasome activities in maize leaf segments, as induced by cadmium treatment, were due to either decomposition of the quaternary structure of the multimeric enzyme complex or to modification of amino acids, a series of non-denaturing PAGE and SDS–PAGE were performed. Western blots of a native 6% (w/v) PAGE showed a single protein band (Fig. 5a). However, the abundance of the 20S proteasome protein content in leaf segments, treated with Cd^{2+}, remained similar to the controls. Oxidation of the 20S complex induced by cadmium was also estimated using SDS–PAGE (12.5% w/v) (Fig. 5b). It was observed that both Cd^{2+} concentrations generated a significant level of oxidation of its subunits, with 100 μM resulting in more oxidation than 50 μM (Fig. 5b). SDS–PAGE analysis of the 20S proteasome before immunoprecipitation with anti-DNP also demonstrated that the antibody (anti 20S proteasome) cross-reacted with the 20S protein derivatized with 2,4 DNPH and that the individual proteasome subunits were undamaged by cadmium.

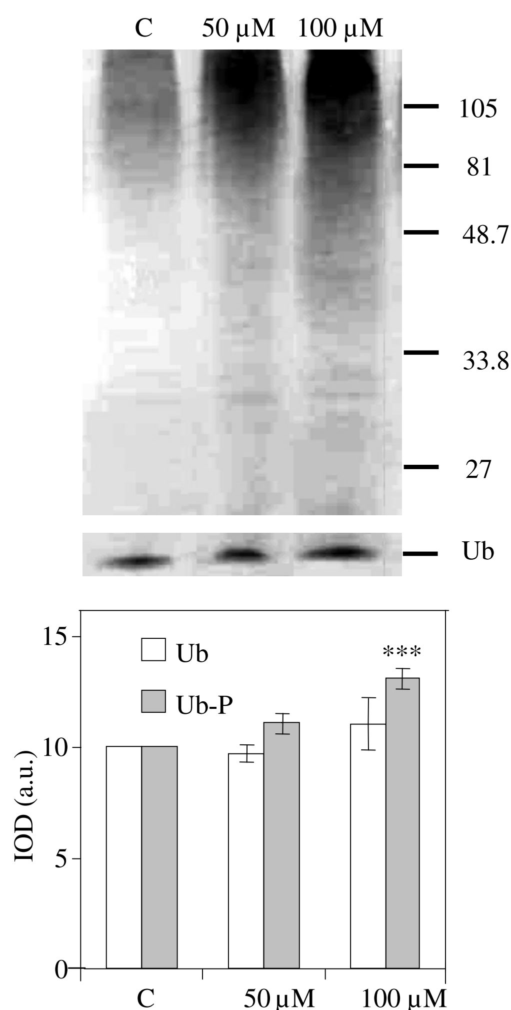

Fig. 4. Effect of cadmium on polyubiquitinated proteins accumulation. Maize plants (*Zea mays* L.) were treated as in Fig. 2. After 24 h of treatment, leaf extracts (50 μg total protein) were subjected to SDS–PAGE (10% polyacrylamide, w/v). Western blotting was performed using the anti-ubiquitin antibody and bands were visualized as described in the Experimental. Ub = free ubiquitin, Ub-P = ubiquitinated proteins. Bands were photographed with a Fotodyn, analyzed with GelPro software and expressed in arbitrary units (assuming control value equal to 10 U), based on absolute integrated optical density (IOD) of each band and line. Significant differences $^{***}P < 0.001$ according to Tukey's multiple range test. Molecular masses of standard proteins (in kDa) are indicated on the right. The western blot data shown are representative of three blots with a total of four to five samples/group between the three blots.

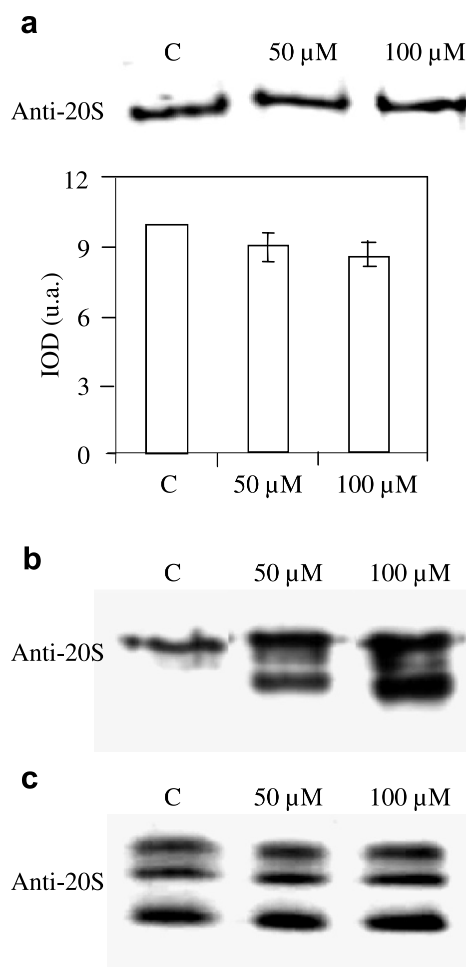

Fig. 5. Effect of cadmium on 20S proteasome protein and identification of 20S proteasome oxidized protein. (a) Western blotting of native PAGE (6% w/v). (b) Western blotting of SDS–PAGE (12.5% w/v) after derivatization with 2,4 DNPH followed by immunoseparation with anti-DNP. (c) Western blotting of SDS–PAGE (12.5% w/v) after derivatization with 2,4 DNPH. Maize plants (*Zea mays* L.) were germinated 20 d and then leaf segments were floated for 24 h in water devoid of cadmium or containing 50 and 100 μM $CdCl_2$. Western blotting was performed using the anti-20S maize proteasome antibody and bands were visualized as described in Section 5. Bands were photographed with a Fotodyn, analyzed with GelPro software and expressed in arbitrary units (assuming control value equal to 10 U), based on absolute integrated optical density (IOD) of each band. Values are means ± SEM. The western blot data shown are representative of three blots with a total of four to five samples/group between the three blots.

3. Discussion

In maize plants growth inhibition, nutrient uptake reduction, disturbances in the plant–water relationships and induction of defense mechanisms, including increased levels of several antioxidant enzymes, were previously reported as consequence of cadmium stress (Pál et al., 2006).

Several studies have suggested that oxidative stress is involved in Cd^{2+} toxicity. This can occur by either inducing oxygen free radical production or by decreasing levels of enzymatic and non-enzymatic antioxidants (Benavides et al., 2005), thereby resulting in protein carbonylation (Romero-Puertas et al., 2002). In the study described herein, however, using maize leaves, although cadmium treatment decreased glutathione content, only the highest concentration assayed (100 μM) resulted in oxidized protein formation. Berlett and Stadtman (1997) indicated that protein carbonylation is an irreversible oxidative process leading to a loss of function and to an increased susceptibility to proteolytic attack. However, very severely oxidized proteins form large aggregates due to increased levels of cross-linked hydrophobic, ionic, and covalent bonds, and

thus become progressively more resistant to proteolysis (Ahmad, 1995).

Incubation of maize leaf segments with 50 μM Cd^{2+} together with the proteasome inhibitor MG132 resulted in accumulation of oxidized proteins. This indicate that, as in mammalian cells, the recognition of hydrophobic amino acid residues of oxidized proteins and their subsequent degradation by the 20S proteasome is a selective mechanism to remove oxidatively damaged proteins from the cytoplasm and nucleus, while proteases have a similar role in organelles.

Fluorogenic kinetic assays using fluorogenic substrates are extensively used to estimate proteasome activities in mammalian and plant extracts. Although the substrates are not proteasome specific, the difference observed in the presence or absence of MG132 permitted estimation of modifications in proteasome activities. AAF-AMC is frequently used as substrate to asses in plants the activity of the tripeptidyl peptidase 2 (TPP2), which is insensitive to MG132 (Book et al., 2005). Although the peptide aldehyde proteasome inhibitor MG132 inhibits some calpain type protease, Kim et al. (2003) found that most of the hydrolytic activity *in vitro* indeed comes from the proteasome.

While maize leaf segments exposed to 50 μM cadmium increased the 20S proteasome activity, neither variation in 20S protein abundance nor fragmentation were observed. Despite protein abundance remained unchanged, an increase in the 20S proteasome oxidation was observed. As suggested by Shringarpure et al. (2001), a number of chemical modifications, most of which act to relax the structure of the proteasome, can activate its proteolytic activity. This mechanism seems to be similar to that described in maize roots by sugar starvation (Basset et al., 2002). On the other hand a decline of 26S proteasome activity has been reported to occur after severe oxidative stress produced by hydrogen peroxide, but no decline in the activity of the 20S proteasome after moderate oxidative stress has been detected (Reinheckel et al., 1998).

Under our experimental conditions, the moderate level of oxidation produced by 50 μM cadmium may have caused a slight dissociation of the complex and could have been responsible for the increment in its activity, thus avoiding accumulation of carbonylated proteins. However, higher concentrations seem to result in partial inactivation of the peptidic activities, similar to what was observed under strong oxidative stress conditions in proteasome isolated from human erythrocytes treated with increasing H_2O_2 concentrations (Reinheckel et al., 1998).

Conformational changes brought about by severe oxidative modifications of specific amino acids in the 20S multicatalytic protein subunits may have resulted in inactivation of the enzyme, with a concomitant increment in the oxidized proteins. Cadmium sensitivity increased after a single amino acid substitution in the β5-subunit of neuronal cells (Li et al., 2004), while in yeast Ni^{2+} resistance was achieved by insertion of a maize cDNA encoding the α-subunit of 20S proteasome (Forzani et al., 2002). These findings reinforce the notion that a proteasome deficiency unequivocally potentiates the harmful effects of oxidative stress produced by cadmium in maize leaves.

Proteins extracted from control and cadmium treated leaf segments showed similar patterns, at least within the limits of SDS–PAGE and Coomassie staining techniques. However, immunoblots with ubiquitin antibody revealed an accumulation of ubiquitinated proteins (Ub-P) after 100 μM Cd^{2+} treatment, thus suggesting the proteasome inhibition. Proteasome dysfunction triggered by harmful conditions such as oxidative stress (Okuda et al., 1997; Lee et al., 2001) or aging (Keller et al., 2002) is likely to decrease ubiquitinated protein degradation rate, causing Ub-P accumulation. Likewise, it is known that plant exposure to heavy metal ions induces changes in the ubiquitin-dependent pathway. For example, Genschik et al. (1992) reported that $HgCl_2$ treatment increased the expression of ubiquitin genes in *Nicotiana sylvestris*, and a strong accumulation of a ubiquitin conjugating enzyme (Ubc1) was also observed in tomato after exposure to $CdCl_2$ (Feussner et al., 1997). Other types of abiotic stress like darkness, UV radiation, starvation and enhanced ozone level also influenced polyubiquitin gene expression (Ingvardsen and Veierskov, 2001).

4. Conclusions

Taken together, our findings suggest that there is a threshold response of the proteasome system to cadmium stress mediated through oxidative modification of the proteasome itself, which prevents accumulation of oxidatively damaged protein in the cell. Under low cadmium treatment, the increase in the 20S proteasome activity due to its moderate oxidation was responsible for preventing oxidized proteins accumulation. Higher cadmium concentration decreased 20S proteasome activity with a concomitant accumulation of oxidized and ubiquitinated proteins.

5. Experimental

5.1. Plant material and growing conditions

Seeds of maize (*Zea mays* L., cv DK-682) were germinated and grown in vermiculite in a controlled climate room at 24 ± 2 °C and 50% relative humidity, with a photoperiod of 16 h light (intensity, 300 $\mu mol\ m^{-2}\ s^{-1}$). Ten days old plants were removed from the pots, the roots were then carefully and gently washed and transferred to separate containers (3 L) for hydroponic culture, 20 plants per container. In the whole plant assay, the hydroponic nutrient solution was either half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950), for the controls or contained 50 and 100 μM of $CdCl_2$, respectively. The medium was continuously aerated. After 24 h treatment,

leaves were isolated and used for the determinations. For leaf segment assays, leaf segments from 20 d plants (1 cm, 0.3 g) were floated for 24 h in glass flasks with H_2O (50 mL) (control), or containing 50 and 100 μM $CdCl_2$. When the effect of proteasome inhibitors was investigated, MG132 (50 μM) was added to the incubation medium. After 24 h, leaf segments were washed with distilled H_2O and analyzed as describe below. All experiments were repeated three times with five replicates per treatment.

5.2. Glutathione assay

Non-protein thiols were extracted by homogenizing leaves (1 g) in 0.1 N HCl (10 mL, pH 2) containing polyvinylpyrrolidone (PVP) (1 g). After centrifugation at 10,000*g* for 10 min at 4 °C, each supernatant was used for analysis. Total glutathione (reduced GSH plus oxidized GSSG) levels were determined spectrophotometrically at 412 nm in the homogenates using yeast glutathione reductase, 5,5′ dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH, respectively (Anderson, 1985).

5.3. Carbonyl group determination

Extracts were prepared from leaf tissue (1 g), homogenized in extraction buffer (3 mL) consisting of 100 mM phosphate buffer (pH 7.4), 120 mM KCl and 1 mM EDTA as follows. Each homogenate was centrifuged (10,000*g* for 20 min) with the supernatant fraction used for assays. Protein oxidation was measured in term of total carbonyl group content by reaction with 2,4-dinitrophenylhydrazine (Levine et al., 1990). Extracts (50 μg protein) that were previously derivatized with 2,4 DNPH were separated by SDS–PAGE using 12% (w/v) running and 4% (w/v) stacking polyacrylamide gels, respectively (Laemmli, 1970). Two gels were run simultaneously: one for protein staining with Coomassie Brilliant Blue R-250 and the other for immunodetection. Derivatized proteins were transferred onto nitrocellulose membranes and were detected with rabbit anti-DNP primary antibody from Sigma–Aldrich (St Luis, USA). Bands corresponding to oxidized proteins were visualized by secondary goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase, using 3,3′-diaminobenzidine (DAB) as substrate.

5.4. Proteasome activity determinations

Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase (PGPH) activities of the proteasome were assessed in leaf extracts using synthetic peptide substrates linked to the fluorescence reporters Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-AMC), Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (Boc-LSYR-AMC) and Clz-Leu-Leu-Glu-β-naphthylamide (Clz-LLE-βNA), in the absence or presence of the proteasome inhibitor carbobenzoxy-Leu-Leu-leucinal (MG132) (Kim et al., 2003). Leaf extracts (1 g) were prepared in 135 mM Tris–acetate buffer (5 mL, pH 7.5), containing 12.5 mM KCl, 80 μM EGTA, 6.25 mM 2-mercaptoethanol, and 0.17% (w/v) octyl-β-D-glucopyranoside, respectively. Homogenized leaf extracts were centrifuged with supernatants used for enzymatic activity determinations. Each reaction was started by adding 100 mM HEPES–HCl buffer (100 μL, pH 7.5) and synthetic substrate (2 μL stock solution at 50 μM in dimethyl sulfoxide (DMSO)) to extract (100 μL). MG132 was added to the assay mixture at a final concentration of 100 μM. Incubations were carried out at 37 °C for 1 h. Reactions were stopped by adding 220 mM sodium acetate buffer (100 μL, pH 5.5) for chymotrypsin-like and trypsin-like activities, and EtOH (300 μL) for PGHP activity. After dilution in distilled H_2O, AMC and NA radicals released were measured fluorometrically (excitation 370 nm/emission 430 nm and excitation 333 nm/emission 450 nm, respectively). Enzymatic activities were normalized for protein concentrations and expressed as percentages of activity present in control extracts.

5.5. Western blot of 20S proteasome and polyubiquitinated proteins levels

Samples (50 μg protein) were subjected to electrophoretic analysis using 6% (w/v) native-polyacrylamide gel (PAGE) and 12.5% (w/v) SDS–PAGE for the proteasome, or 10% (w/v) SDS–PAGE for the polyubiquitinated proteins. Gels were transferred onto polyvinylidene difluoride membranes. Polyclonal antibodies, raised against the maize 20S proteasome (generously provided by Dr Brouquisse) and ubiquitin-protein conjugates (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were also employed. 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 a Fotodyn, analyzed with GelPro software and expressed as arbitrary units (assuming control value equal to 10 U), based on absolute integrated optical density of each band for 20S proteasome or each line for ubiquitinated proteins.

5.6. Immunoprecipitation and immunochemical detection of proteasome carbonyl groups

Proteins (100 μg) derivatized with DNPH as mentioned above, 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 re-suspended in 100 mM glycine–HCl (50 μL, pH 2.5). After centrifugation, the pellets were discarded, with 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 the 20S proteasome. DNPH derivatized proteins

were separated by 12.5% (w/v) SDS–PAGE. After electrotransfer of the proteins to nitrocellulose membranes, the proteasome was detected using maize anti-20S proteasome primary antibodies and goat anti-rabbit immunoglobulins-horseradish peroxidase conjugate, with DAB as substrate, respectively. Membranes were photographed with a Fotodyn equipment, analyzed with GelPro software.

5.7. Protein determination

Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as standard.

5.8. Statistics

Values are expressed as mean ± SEM. Differences among treatments were analyzed by 1-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

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