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ORIGINAL ARTICLE

Modifications in endopeptidase and 20S proteasome expression and activities in cadmium treated tomato (*Solanum lycopersicum* L.) plants

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Abstract The effects of cadmium (Cd) on cellular proteolytic responses were investigated in the roots and leaves of tomato (*Solanum lycopersicum* L., var Ibiza) plants. Three-week-old plants were grown for 3 and 10 days in the presence of 0.3–300 μM Cd and compared to control plants grown in the absence of Cd. Roots of Cd treated plants accumulated four to fivefold Cd as much as mature leaves. Although 10 days of culture at high Cd concentrations inhibited plant growth, tomato plants recovered and were still able to grow again after Cd removal. Tomato roots and leaves are not modified in their proteolytic response with low Cd concentrations (≤3 μM) in the incubation medium. At higher Cd concentration, protein oxidation state and protease activities are modified in roots and leaves although in different ways. The soluble protein content of leaves decreased and protein carbonylation level increased indicative of an oxidative stress. Conversely, protein content of roots increased from 30 to 50%, but the amount of oxidized proteins decreased by two to threefold. Proteolysis responded earlier in leaves than in root to Cd stress. Additionally, whereas cysteine- and metallo-endopeptidase activities, as well as proteasome chymotrypsin activity and subunit expression level, increased in roots and leaves, serine-endopeptidase activities increased only in leaves. This contrasted response between roots and leaves may reflect differences in Cd compartmentation and/or complexation, antioxidant responses and metabolic sensitivity to Cd between plant tissues. The up-regulation of the 20S proteasome gene expression and proteolytic activity argues in favor of the involvement of the 20S proteasome in the degradation of oxidized proteins in plants.

Keywords Cadmium · Endopeptidase · Oxidative Stress · Protease · 20S Proteasome · Tomato

Abbreviations

AMC	7-Amino-4-methyl coumarin
AOS	Active oxygen species
AP	Aminopeptidase
Cd	Cadmium
DCI	Dichloroisocoumarine
DMSO	Dimethylsulfoxide
DNPH	Dinitrophenylhydrazine
DW	Dry weight
EP	Endopeptidase
MG132	*N*-acetyl-leucyl-leucyl-norleucinal
PI1	Proteasome inhibitor 1

This paper is dedicated to Nathalie Galtier (1964–2005), who was senior researcher at the INRA Research Center, Villenave d'Ornon, France.

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PMSF	Phenylmethylsulfonylfluoride
RT	Reverse transcriptase
STI	Soybean trypsin inhibitor
TBARS	Thiobarbituric acid reactive substances

Introduction

Cadmium (Cd) is a highly toxic and persistent environmental poison for plants, yeasts and animals (Sanita di Toppi and Gabbrielli 1999). It is one of the most widespread pollutant metals, which enters the environment mainly through industrial wastes and is transferred to animal and humans through the food chain (Wagner 1993). In plants, it often damages roots, reduces nutrient and water uptake, impairs photosynthesis, induces chlorosis, inhibits growth and finally results in cell death (Kahle 1993; Das et al. 1998). Cd is known to interfere with many cellular functions mainly by complex formation with side groups of organic compounds such as proteins, lipids and nucleotides resulting in the inhibition of gene expression and metabolic activities. Although it does not directly produce hydroxyl radicals through Fenton or Haber–Weiss reactions, Cd also produces active oxygen species (AOS) and generates an oxidative stress (Sanita di Toppi and Gabbrielli 1999). AOS can lead to oxidation of side chains of amino acid residues and formation of protein–protein covalent cross-linkage, which inactivate or denature protein function (Stadtman 1993; Davies 2001). If they are not rapidly degraded, oxidatively modified proteins can undergo direct fragmentation, or form large aggregates due to covalent cross-linkage and increased surface hydrophobicity, which lead to cell death (Davies 2001; Shringarpure et al. 2001).

In plant cells, stress conditions trigger an increase in protein turnover and degradation (Brouquisse et al. 2001; Romero-Puertas et al. 2002; Smalle and Vierstra 2004; Thompson and Vierstra 2005). Proteolysis is involved in the degradation of oxidized or denaturated proteins, in the degradation of enzymes and transcription factors, which elimination allows the set up of the stress response, and in the potential regulation of specific mechanisms such as the turnover of glutathione, phytochelatins or metallothioneins (Zenk 1996; Sanita di Toppi and Gabbrielli 1999; Leustek et al. 2000; Clemens 2001; Palma et al. 2002). Cellular protein breakdown is a complex process mediated by different proteolytic systems in the vacuole and other cell organelles. Among these systems, the proteasome is active in the nucleus and the cytosol (Smalle and Vierstra 2004; Thompson and Vierstra 2005).

In animal and human cells, the proteasome has been shown to selectively recognize and degrade mildly oxidized proteins in the cytosol, nucleus, and reticulum endoplasmic, thus minimizing their cytotoxicity. From in vitro studies, it was shown that the 20S proteasome actively recognizes and degrades oxidized proteins, in contrast to the 26S proteasome, which is not very effective even in the presence of ATP and ubiquitinylation system (Shang and Taylor 1995; Obin et al. 1998). This may be explained by the fact that a mild oxidative stress rapidly inactivates both the ubiquitin activating/conjugating system and 26S proteasome activity in intact cells, but does not affect 20S proteasome activity (Shang and Taylor 1995; Obin et al. 1998; Reinheckel et al. 1998). Thus, the 20S proteasome, together with endopeptidases (EP) and aminopeptidases (AP), has been proposed to play a major role in the degradation of moderately oxidized protein during oxidative stress (Davies 2001; Grune et al. 2003).

In maize roots, the oxidative stress induced by sugar starvation was found to increase both the amount of oxidized protein, including the 20S proteasome, and the activity of the proteasome against oxidized proteins (Basset et al. 2002). Cd has also been shown to induce an oxidative stress in pea, sunflower and maize leaves, and to generate oxidized proteins, which should in turn be degraded by the proteolytic machinery (Romero-Puertas et al. 2002; Pena et al. 2006, 2007). So far, the measurement of total proteolytic activities in various plants in response to Cd has led to contrasted observations, since proteolytic activities were either induced, not modified or even decreased depending on the plant and the organ considered (Romero-Puertas et al. 2002; Balestrasse et al. 2003; Pena et al. 2006). In pea leaves, several peroxisomal AP and EP activities are increased in response to Cd (McCarthy et al. 2001). Recently, a proteomic study performed on *Arabidopsis thaliana* cells reported a 60% increase of two 20S proteasome subunits upon exposition to Cd (Sarry et al. 2006). At the same time, Pena et al. (2006) reported an increase in ubiquitin-conjugated proteins and a decrease in 20S proteasome activities in sunflower leaves subjected to Cd. An increase in proteasome activity in response to Cd in maize leaves was also reported (Pena et al. 2007). To date, there is little information on the regulation of proteases in the different plant organs in response to Cd stress, and particularly on the potential involvement of the proteasome in the proteolysis and the degradation of oxidized protein in this context.

The questions raised in this study were to determine (a) to what extend protein oxidation, protease activities, and proteasome expression, are modified in response to various Cd concentrations, and in different plants organs; (b) if plant proteasome is induced in response to Cd as it is under carbon starvation; and (c) if protein oxidation and proteolytic response are useful markers to assess the extent of Cd stress in plants.

The plant material chosen for this study was tomato (*Solanum lycopersicon* L.), which is considered to be quite tolerant to Cd. Indeed, certain tomato cell lines have the

capacity to grow for several months in the presence of up to 6 mM Cd (Chen and Goldsbrough 1994). Most of the studies of Cd effects on tomato cells were carried out with Cd concentrations ranging from 0.01 to 1 mM, during 1–12 days-treatment periods (Sanita di Toppi and Gabbrielli 1999). Thus, to get an overview of the Cd effects at the plant level, we used hydroponic culture systems, and analysed the roots and the leaves of tomato plants grown for 3 and 10 days in the presence of low, moderate and high Cd concentrations (0.3 to 300 μM).

In the present work, we first report the effects of increasing exogenous concentrations of Cd on growth and Cd storage capacity of the roots and the leaves of 3-week-old tomato plants. We describe the changes in protein oxidation, protease activities and 20S proteasome expression and activity, in the roots and leaves, as a function of Cd concentrations in order to evaluate the threshold response of proteolysis to different Cd concentrations, and the potential involvement of the proteasome in these processes.

Materials and methods

Growth conditions

Tomato seeds (*Solanum lycopersicon* L., var. Ibiza F1; Protagri Company, Tunis, Tunisia) were germinated in vermiculite and grown hydroponically in controlled conditions. The photoperiod was 16 h, with a photosynthetic photon flux density of 300–400 μmol photons $m^{-2}\,s^{-1}$. The day/night temperature was 23/18°C and the relative hygrometry was maintained close to 75%. The mineral solution was as described in Gharbi et al. (2007). The pH of the medium was checked and adjusted daily to 5.3–5.6. The culture medium was continuously sparged with air and was renewed every week. When seedlings were 3-week-old, $CdCl_2$ was added to the mineral solution at various concentrations (0.3–300 μM) except in control plants. Cd containing solutions were renewed every 3 days. At 0, 3 and 10 days of Cd exposure, the roots and the mature leaves (leaves 2 and 3) from four plants were harvested. Roots were carefully rinsed with distilled water. Roots and leaves were quickly frozen in liquid N_2, ground in powder in liquid N_2, and stored at −80°C until analysis.

For recovery experiments, plant roots were rinsed and culture medium was renewed with the mineral solution without Cd. Tomato plants were then cultured for ten additional days before root and leaf harvest.

Determination of cadmium content

Cd content in leaves and roots was analyzed by digestion of dried samples with an acid mixture ($HNO_3/HClO_4$, 3:1, v/v) as described by Van Assche et al. (1988). Metal-ion concentrations were determined by atomic absorption spectrometry (Analyst 300, flame spectrometer, Perkin-Elmer Corporation). Results are the mean of three to four replicated determinations per organ and treatment.

Preparation of clarified extracts

Two hundred to 300 mg of frozen root or leaf powder were homogenized in a mortar at 4°C with four times their fresh weight of extraction medium (50 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol and 0.5% (w/v) polyvinylpolypyrrolidone). The homogenate was centrifuged for 15 min at 27,000*g*, and the supernatant (clarified extract) was used for protein determination and protease activity measurement.

Preparation of 20S proteasome enriched fraction

Five hundred to 700 mg of frozen root or leaf powder were homogenized in a mortar at 4°C with 3 mL of extraction medium (50 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol and 0.5% (w/v) polyvinylpolypyrrolidone). The homogenate was centrifuged for 15 min at 27,000*g*. The resulting supernatant (3 mL of clarified extract) was applied to a Hiload 16/60 Superdex 200 (Amersham Bioscience) gel filtration column equilibrated with 50 mM Tris–HCl, pH 7.5, and 20 mM NaCl. The 20S proteasome was followed through its chymotrypsin-like activity. Chymotrypsin activity eluted into two different peaks centred around 700, and 400 kDa, respectively (Basset et al. 2002). The recovery of the Superdex 200 step, regarding the chymotrypsin-like activity was found to be 90–93%. The fractions of the two activity peaks were pooled separately (pool1: 550–900 kDa proteins; pool2: 250–500 kDa proteins), and used for chymotrypsin-like activity measurement in the presence or absence of either proteasome inhibitors, or anti-20S proteasome antibodies, as mentionned below. Fractions corresponding to the pool1 contained only 20S proteasome as checked with anti-20S proteasome antibodies (Basset et al. 2002), and constituted the proteasome enriched fraction. The column was calibrated with the following markers: Blue dextran (2,000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa) and α-amylase (200 kDa).

Proteolytic activity measurements

Endopeptidase (EP) activity measurement was adapted from Brouquisse et al. (1998). One hundred microliters of clarified extract and 100 μL of azocasein (5 mg mL^{-1} in 200 mM Mes-KOH, pH 6.0) were incubated for 3 h at 37°C. The reaction was stopped by addition of 100 μL 15%

(v/v) trichloracetic acid. After 10 min on ice, the samples were centrifuged at 15,000*g* for 10 min; 250 µL of supernatant were then added to 750 µL of 1 M NaOH, and the absorbance was read at 440 nm. The extinction coefficient $E_{1\%\ \text{azocasein in 1 M NaOH}} = 37\ \text{L cm}^{-1}\ \text{g}^{-1}$ was used to calculate the azocasein degradation activity. Chymotrypsin-like activity measurement was adapted from Basset et al. (2002), using Suc-Leu-Leu-Val-Tyr-AMC (Suc-L-L-V-Y-AMC) as substrate. The assay mixture contained 10 µL of Suc-L-L-V-Y-AMC (5 mM stock solution in dimethylformamide), 100 µL of 20S proteasome enriched fraction and 90 µL of 200 mM Tris buffer, pH 8.1. After a 30 min incubation period, at 37°C, the reaction was stopped with the addition of 100 µL of 10% (w/v) SDS and 2 mL of Tris 100 mM pH 9.0. The AMC radical released was measured fluorometrically (excitation 380 nm; emission 460 nm). Activity was calcultated using AMC standard curve made in the same conditions.

Protease inhibitors were prepared as the following stock solutions: E-64 (2 mM), iodoacetamide (10 mM), STI (0.5 mM) and Na_2-EDTA (0.2 M) in water, PMSF (0.2 M) in ethanol, 1–10 phenantroline (0.2 M) and pepstatin (2 mM) in methanol, 3–4 DCI (10 mM), chymostatin (2 mM), lactacystin (1 mM), Proteasome inhibitor 1 (PI 1, Z-Ile-Glu(OtBu)-Ala-Leu-CHO, 2 mM) and MG132 (Z-Leu-Leu-Leu-CHO, 2 mM each) in DMSO. Inhibitors were first preincubated for 15 min at ambient temperature with 100 µL of either clarified extract (60–150 µg of protein), or proteasome enriched fraction (8–12 µg of protein), prior to substrate addition, and activities were measured as described above. Control assays were carried out with the corresponding solvent.

Immunoprecipitation of the proteasome

Polyclonal antibodies raised against the maize 20S proteasome (Basset et al. 2002) were used for immunoprecipitation and western-blot analysis. We observed positive cross-reaction of anti-20S proteasome antibodies with proteasome from pea, wheat, and *Arabidopsis* plants (data not shown). They were also found to cross-react with proteasome from sunflower leaves (Pena et al. 2006). Immunoprecipitation experiments were adapted from Basset et al (2002). Briefly, 100 µL of either clarified extract, or proteasome enriched fractions, were incubated 1 h at 4°C with increasing volumes of purified immune or preimmune serum. Immune complexes were incubated 1 h at 4°C with a fivefold (immunoglobulin-G binding) excess of protein A-agarose (Affi-gel, Bio-Rad) and then centrifuged for 5 min at 10,000*g*. The chymotrypsin-like activity was measured in each supernatant fraction as described above.

Extraction of total RNA and estimation of relative transcript level by RT-PCR

Total RNA was isolated from 100 mg of plant tissues using the Tri-reagent protocol (Molecular Research Center). DNAse treatment was performed to remove contaminant genomic DNA using the RQ1 DNAse-RNase free (Promega) according to manufacturers instruction.

Reverse transcription was performed using 1 µg of total RNA. Typical reactions were performed using 0.5 mM dNTPs, 0.6 µM oligo-dT, 20 units of Rnasin (Promega), 2 mM DTT, 1× reaction buffer (Promega) and 400 units of Moloney murine leukemia virus RNAseH minus reverse transcriptase (Promega). The reaction mixture was incubated at 42°C for 1 h. The enzyme was inactivated at 80°C during 5 min. Reactions were stored at −20°C until used for PCR reaction.

Semi-quantitative RT-PCR was performed using 3–5 µL of a 10× dilution of the RT reaction with 0.02 µM of each oligonucleotide primer. After an initial denaturation step of 3 min at 95°C, PCR was performed as follows: 30 s denaturation (94°C), 30 s hybridization (52°C) and 30 s elongation (72°C); the number of PCR cycles was adjusted for each gene studied to obtain a detectable signal without reaching saturation (see Table 1, 25 cycles for root and leaf cDNA). Fifteen microliters of the amplification products were loaded on a 1.5% (w/v) ethidium bromide stained agarose gel. Primers used for semi-quantitative RT-PCR are shown in Table 1. PCR fragments were cloned and sequenced. RT PCR reactions were transferred to nylon N+ Hybond membrane (Amersham) according to manufacturer instructions and hybridized with the corresponding random primed ^{32}P probe labeled using the NEB kit (Biolabs). Hybridization signals were analysed using a phosphoimager (Bio-Rad) and quantified with the Quantity One software (Biorad).

Other assays

Proteins were routinely quantified (Bradford 1976) using the Bio-Rad microassay reagent with bovine γ-globuline as the standard. Carbonyl content of soluble protein extracts were quantified by reaction with dinitrophenylhydrazine (DNPH) according to Reznick and Packer (1994). To determine the yield of protein recovery after the carbonyl derivation step with DNPH, protein were quantified before and after the derivation procedure by reading the absorption of the samples at 280 nm, as described in Reznick and Packer (1994). The amounts of protein were calculated from a bovine serum albumin (BSA) standard curve prepared in the same conditions. Protein recovery was found to be 88 ± 3%. Lipid peroxidation products were determined by

Table 1 Sets of PCR primers used to amplify gene-specific regions and corresponding size of the amplified products

cDNA	Primer sequence (5′ → 3′)		Location	Size (bp)
Lyces; *EF1α*	Sense	ATTGCTGTCAAGTTTGCTGAGA	1125–1147	130
	Anti-sense	GCTTGGTGGGAATCATCTTAAC	1126–1250	
Lyces; *α 3 proteasome*	Sense	GTGGAAATTACGGTGGTTGG	522–541	510
	Anti-sense	AGGCAAACAGAGGCACAAAC	1013–1032	
Lyces; *α 6 proteasome*	Sense	TGGTGGACTGGATGAGTCTG	476–495	508
	Anti-sense	CCATTGTGCCACACAAAATC	965–984	
Lyces; *β 1 proteasome*	Sense	GTGGCAAATAGGGCTTCAGA	201–220	403
	Anti-sense	ACCGCAGTCACAACCAGTTT	585–604	

The number of PCR cycles was 25 cycles for root and leaf cDNA

measuring the concentration of thiobarbituric acid reactive substances (TBARS), as described in Djebali et al. (2005). SDS-PAGE was performed with 12.5% (w/v) polyacrylamide gels by the procedure of Laemmli (1970). Purification of IgG fraction and Western-blot analysis were as in Basset et al. (2002).

Results

Effects of Cd treatment on root and leaf growth

To assess the impact of Cd treatment on global tomato growth, 3-week-old tomato plants were cultured for 10 days with Cd, at concentrations ranging from 0.3 to 300 µM. Both, root and leaf growths were affected for Cd concentrations above 3 µM for roots, and 1 µM for leaves (Fig. 1a). Thus, in the presence of 100 µM Cd, the growth of the roots and the leaves were 66% and 78% inhibited, respectively, compared to the control. These data are in good agreement with those previously obtained (Djebali et al. 2002). They indicate that leaf growth is more sensitive to Cd stress than that of roots. In the presence of 300 µM Cd, the growth of both organs was practically stopped, which means that a toxicity threshold was reached between 100 and 300 µM Cd with a cessation of tissue expansion.

After 10 days of Cd treatment, both root and leaf growths were reinitiated when the Cd stress was released, even for the 300 µM Cd treated plants (Fig. 1b), which indicates that tomato plants were not dead. Thus, it may be concluded that tomato growth is not affected by low or moderate Cd concentrations (≤1 µM), and that the tomato variety Ibiza can survive in the presence of Cd concentrations as high as 300 µM, for at least 10 days.

Cd accumulation in roots and leaves

Cd content was evaluated in roots and mature leaves. In both tissues, Cd accumulated in a concentration dependent

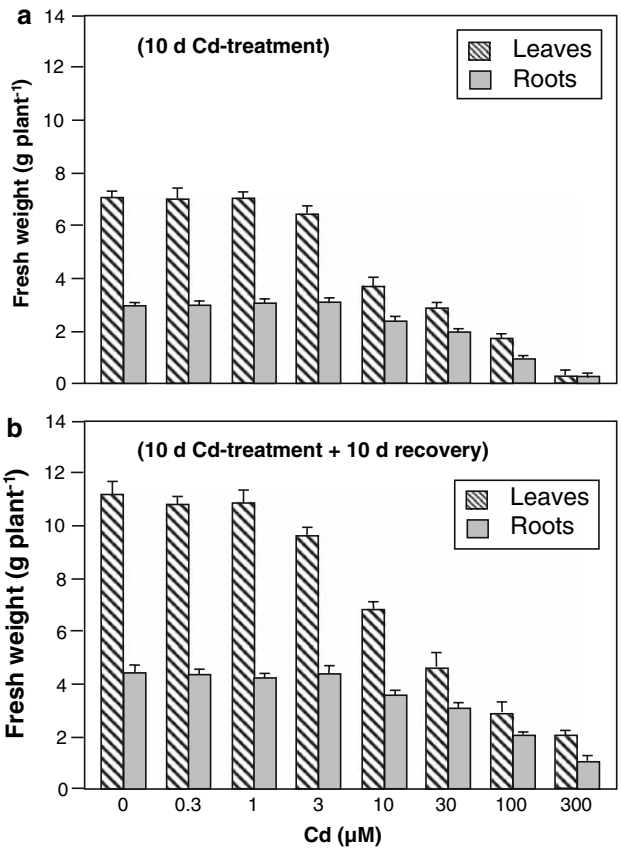

Fig. 1 Growth of roots and leaves of tomato plants as a function of Cd treatment and recovery. Three week-old hydroponically grown plants were submitted for 10 days to various Cd concentrations (**a**), and then recovered for 10 days in a medium devoid of Cd (**b**). Data are the mean of two independent experiments ± SD

manner (Fig. 2). Regardless of the Cd external concentration, most of the Cd (50–80%) accumulated in plant tissues within the first 3 days. Cd concentrations for a given treatment were four to five times higher in roots than in mature leaves. This supports previous reports indicating that more than 80% of Cd was stored in the root system in Cd exposed tomato plants (Ouariti et al. 1997). In the presence

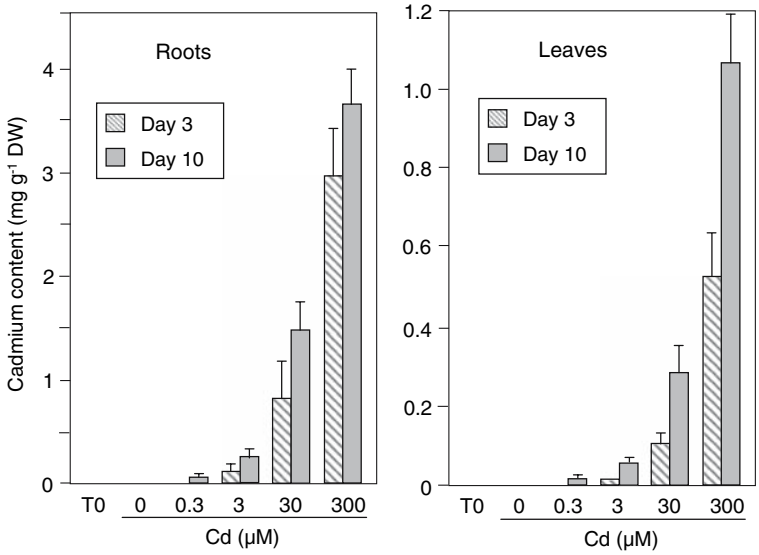

Fig. 2 Accumulation of Cd in the roots and the leaves of tomato plants as a function of time and Cd concentration in the incubation medium

of 300 μM Cd, internal Cd concentrations reached 1.1 mg g^{-1} dry weight (DW) in leaves and 3.7 mg g^{-1} DW in roots, which accounted for 0.1–0.37% of the DW, respectively. Assuming an even distribution of the Cd in the tissues, in vivo Cd concentrations in the roots and the leaves of 300 μM treated plants may be estimated close to 4 and 1 mM, respectively.

Effect of Cd treatment on protein, carbonyl-group and oxidized lipid contents

No significant change in total protein content of roots was observed after 3 days of Cd treatment, irrespective of the Cd concentration (data not shown). However, a 10-day exposure to 100 and 300 μM Cd caused a 50% increase in root total protein content (Fig. 3). In the leaves, low Cd concentration had no detectable effect on total protein content. Treatment with 100 and 300 μM of Cd had an opposite effect to that observed in roots: protein content decreased by 22 and 37%, respectively (Fig. 3).

To evaluate the possible effect of Cd on protein oxidation state, the content in carbonyl-groups was measured after derivatization with dinitrophenylhydrazine (DNPH). As reported in Table 2, a decrease in carbonyl-group content was observed in the roots of plants exposed to 30 and 300 μM Cd for 3 and 10 days. Such finding was unexpected since Cd is known to induce oxidative stress in plants (Clemens 2001; Dixit et al. 2001; Romero-Puertas et al. 2002; Ortega-Villasante et al. 2005; Garnier et al. 2006), and the extent of protein oxidation was shown to be related to the amount of Cd accumulated in the tissues (Romero-Puertas et al. 2002; Rellan-Alvarez 2006). In the leaves of non-treated plants, the content in carbonyl-groups per g FW was higher than in roots (29–33 vs. 16–18 nmol carbonyl g^{-1} FW, respectively; Table 2). It remained unchanged for treatments with Cd concentrations below 30 μM, but was doubled in leaves of 300 μM exposed plants (Table 2). This result indicates that leaf proteins are rapidly undergoing an oxidative stress at high Cd concentration. Interestingly, carbonyl-groups amounts in

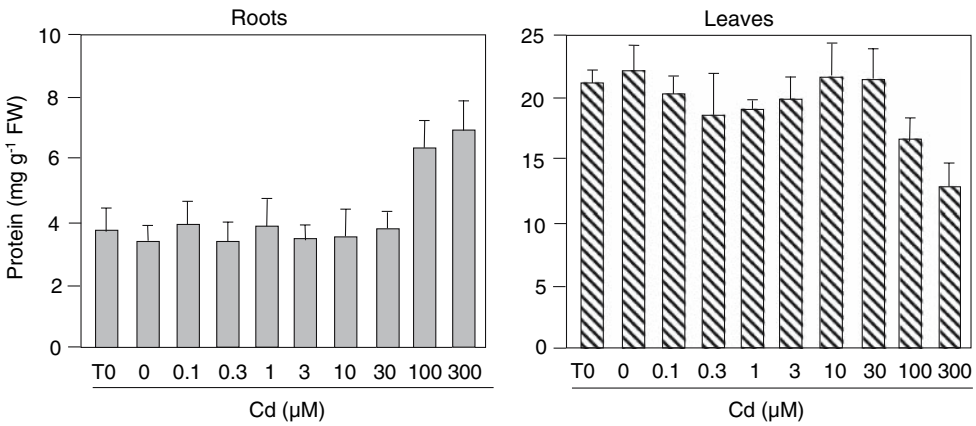

Fig. 3 Changes in protein level in roots and leaves of tomato plants submitted to Cd. Protein amounts are the mean of four independent experiments ± SD

Table 2 Effects of Cd on carbonyl-group content in the roots and the leaves of 3 and 10 day- treated tomato plants

Cd treatment (μM)	0	0.3	3	30	300
Carbonyl-group content (nmol g^{-1} FW)					
Roots					
Day 3	18.4 ± 3.8	15.2 ± 2.3	17.2 ± 3.4	11.3 ± 3.6	8.2 ± 3.5
Day 10	16.5 ± 3.4	14.8 ± 4.5	13.3 ± 3.1	6.5 ± 3.5	5.6 ± 2.0
Leaves					
Day 3	29.4 ± 10.5	30.4 ± 7.6	32.5 ± 8.7	32.0 ± 10.0	70.3 ± 7.6
Day 10	33.2 ± 8.2	28.8 ± 9.0	27.0 ± 7.2	30.8 ± 6.6	53.6 ± 3.2

Carbonyl groups were measured after derivation with 2,4 dinitrophenylhydrazine. Data are the mean of three independent experiments ± SD

leaves were higher after 3 days than after 10 days of treatment, which may suggest that a protective mechanism against either oxidative stress or oxidised protein accumulation has been set up.

As the decrease in carbonyl content in the roots subjected to Cd was unexpected, we evaluated the occurrence of an oxidative stress and, if any, its intensity. We therefore analyzed the production of lipid peroxidation products, which are well-established markers of oxidative stress in plant cells (Quartacci et al. 2001; Shah et al. 2001; Wu et al. 2003). The assay of cellular accumulation of lipid oxidation products, in the form of thiobarbituric acid reactive substances (TBARS), provides a good indication of oxidation level in biological membranes. After 10 days of Cd treatment, TBARS accumulated in the leaves in a Cd concentration-dependent manner for concentrations above 10 μM (Fig. 4). These data are in good agreement with those previously reported (Djebali et al. 2005), and confirm that oxidative stress occurs in the leaves at elevated Cd concentration. In the roots, TBARS are present at a lower level than in leaves (Fig. 4). After 10 days in the presence of 0.3 and 1 μM Cd, TBARS content was 1.4 and 1.6-fold higher, respectively, than in control roots, but at higher Cd concentration, TBARS level did not change and levelled off at about 8 nmol g^{-1} FW (Fig. 4), which is significantly lower than in control leaves.

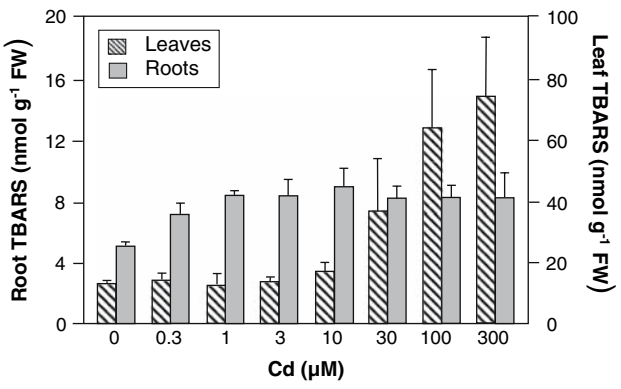

Fig. 4 Effect of Cd on thiobarbituric acid reactive substances (TBARS) content in the roots and the leaves of 10 day-treated tomato plants. Data are the mean of three independent experiments ± SD

Considered together, these results indicate that in control tomato plants, protein and lipid oxidation products are higher in leaves than in roots, two and fivefold, respectively. Upon treatment with high Cd concentrations, both carbonyl-groups and TBARS increased in the leaves, whereas they either decreased (carbonyl-groups), or remained unchanged (TBARS) in the roots. This means that, in response to a Cd-induced oxidative stress, root cells are able to limit the oxidative damage to cell components, whereas leaf cells are not. The absence of accumulation of oxidized protein in roots as compared to leaves (Table 2), may suggest that they are more rapidly degraded in roots than in leaves. We therefore focused our attention on the proteolytic response to Cd treatment in both tissues.

Effects of Cd on endoproteolytic activities

The EP activities were analyzed in roots and leaves of Cd treated tomato plants. Water-soluble activities were investigated, and azocasein was used as a global endopeptidase (EP) substrate. To assess the different types of EP, various inhibitors for each class of protease, were assayed against azocasein-degrading activities: PMSF and STI for serine protease, E-64 and iodoacetamide for cysteine EP, EDTA and 1-10 phenantroline for metallo-EP and pepstatin for aspartic EP (Table 3).

In the roots of control plants, 81% of EP activities were inhibited by PMSF, and 55% by STI, indicating that most EP is of the serine type. Cysteine and metallo-EP accounted each for less than 10% of total EP activities. No aspartic EP activity was detected. In roots exposed to 300 μM Cd, total EP activity was comparable to control (Table 3) but the effects of protease inhibitors differed significantly from control, indicating that the pattern of protease activity was dramatically modified: PMSF and STI had little effect on EP activities, indicating a global decrease in serine EP activities to less than 25%. The higher effectiveness of E-64 and EDTA indicated a significant increase in cysteine and

Table 3 Effects of protease inhibitors on the endoproteolytic activity of roots and leaves of tomato plants exposed for 10 days to either 0 or 300 μM Cd. Data are the mean of 4 independent experiments ± SD

Inhibitor	Concentration	Inhibitor			
		Roots		Leaves	
		0 μM Cd	300 μM Cd	0 μM Cd	300 μM Cd
Azocasein-degrading activity (μg azocasein min^{-1} g^{-1} FM)					
Control	–	17.5 ± 3.4	21.7 ± 5.1	13.2 ± 2.8	49.0 ± 3.7
(% of control)					
Control	–	100	100	100	100
PMSF	2 mM	19 ± 5	78 ± 5	76 ± 7	67 ± 13
STI	10 μM	45 ± 4	88 ± 4	75 ± 5	85 ± 7
E-64	100 μM	90 ± 4	59 ± 10	88 ± 2	76 ± 6
Iodoacetamide	200 μM	93 ± 2	80 ± 5	97 ± 1	80 ± 2
EDTA	10 mM	97 ± 2	56 ± 1	16 ± 10	64 ± 5
1-10 phenantroline	10 mM	96 ± 3	71 ± 5	30 ± 7	77 ± 4
Pepstatin	20 μM	100 ± 4	98 ± 2	97 ± 7	95 ± 4

metallo-EP activities from less than 10% in control plants to approximately 45% (Table 3). On the basis of PMSF, E-64, EDTA and pepstatin inhibition, serine, cysteine, metallo- and aspartic EP were found to account roughly for 20, 40, 40 and 0% of the total EP activities in the roots of Cd-treated plants.

In control leaves, total EP activity was lower than in control roots. It was significantly reduced (84–70%) in the presence of EDTA and 1-10 phenantroline, respectively (Table 3). This means that most of the EP activities are of the metallo-EP type. Serine and cysteine-EP accounted for the remaining part of the activity. Exposure to 300 μM Cd triggered a 3.7-fold increase in total EP-activity, as well as changes in the pattern of protease activity types (Table 3): serine, cysteine and metallo-EP activities each were found to represent one-third of the total activities in the leaves of Cd-treated plants.

To investigate the changes in the activity for the three main EP types, i.e. serine, cysteine and metallo-EP, as a function of Cd treatment, tomato plants were submitted to increasing concentrations of Cd in the incubation medium.

In roots of plants treated with low Cd concentrations (up to 3 μM) for 10 days, no significant change in any of the three EP types was observed (Fig. 5). At higher Cd concentrations, the activity of serine EP decreased, whereas that of cysteine and metallo-EP increased. These effects were observed between 3 and 10 days of Cd treatment, particularly at 300 μM Cd. In this latter case, after 10 days of Cd treatment, cysteine and metallo-EP activities were 4 and 13-fold as high as in control roots, respectively, and serine EP activities decreased fourfold (Fig. 5). Similarly, no change in the EP activities was observed in leaves at low Cd concentration (up to 3 μM). At high concentrations (30 and 300 μM), the three EP-types were similarly up regulated, which explains the 3.7-fold increase in total EP activity after 10 days of treatment reported in Table 3. Interestingly, the main part of the increase in EP activities was observed as soon as 3 days of treatment (Fig. 5). Metallo-EP activities were similarly increased in plants treated with 30 μM and 300 μM of Cd.

Effects of Cd on 20S proteasome activity and expression

Under oxidative stress, proteins are subjected to amino acid modifications such as carbonylation (Shringarpure et al. 2001). Oxidized proteins cannot be repaired and should be degraded by proteolysis (Palma et al. 2002; Grune et al. 2003). It has been suggested that, in plants, 20S proteasome is involved in the degradation process of oxidized proteins (Basset et al. 2002). Therefore, 20S proteasome activity and expression were analysed in roots and leaves of Cd treated tomato plants.

The chymotrypsin activity of the 20S proteasome was measured in clarified extracts, and in proteasome enriched fractions after a Superdex 200 gel filtration step (see "Materials and methods"). The use of anti-20S proteasome IgG and of proteasome inhibitors (lactacystin, PI 1, and MG132) showed that 20S proteasome accounts for approximately 23 and 25% of the chymotrypsin-like activity in root and leaf clarified extracts, respectively (Table 4). After the gel filtration step, the fractions corresponding to proteins with a molecular weight above 550 kDa and containing chymotrypsin activity (first activity peak) were pooled, and used for chymotrypsin-like activity measurement in the presence of the proteasome inhibitors. This activity was 75% (leaf) to 90% (root) inhibited by 10 μM lactacystin, 30 μM PI 1, or 30 μM MG132 (Table 4) and was therefore considered to be associated with the 20S proteasome. The fractions corresponding to the second peak of chymotrypsin activity (250–500 kDa molecular mass proteins) were

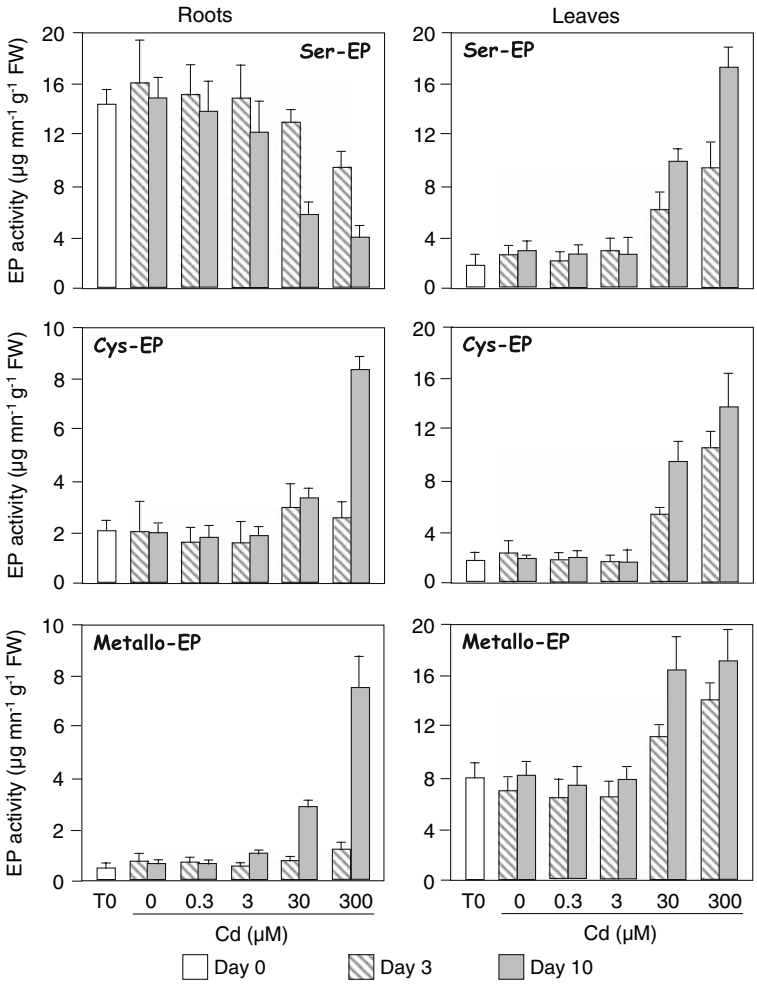

Fig. 5 Changes in serine, cysteine and metallo-endopeptidase activities in the roots and the leaves of tomato plants submitted to Cd. 2 mM PMSF, 100 µM E-64 and 10 mM 1-10 phenantroline were, respectively, used to measure the activity of serine, cysteine and metallo-EP type activities. Data are the mean of four independent experiments ± SD

Table 4 Effects of proteasome effectors on the chymotrypsin activity of clarified extracts and 20S proteasome enriched fractions from tomato roots and leaves

Inhibitor	Concentration	Effector			
		Roots		Leaves	
		Clarified extract	Proteasome fraction	Clarified extract	Proteasome fraction
(% of control)					
Control	–	100	100	100	100
Anti-20S proteasome IgG	–	78 ± 8	ND	75 ± 7	ND
Lactacystine	10 µM	76 ± 5	8 ± 4	79 ± 6	25 ± 4
PI 1	10 µM	72 ± 7	14 ± 5	70 ± 13	28 ± 5
MG 132	30 µM	85 ± 10	11 ± 5	76 ± 8	18 ± 5

Data are the mean of 2 (IgG) or 3 (inhibitors) independent experiments ± SD

ND not determined

inhibited neither by lactacystin, nor by PI 1, and did not react with anti-20S antibodies (data not shown). The total chymotrypsin activity of the 20S proteasome in the roots and the leaves was calculated on the basis of the activity in the first peak, and after taking into account a 10% loss of activity after the gel filtration step.

No major effect of Cd on the chymotrypsin activity of the 20S proteasome was observed in roots or leaves of plant treated with low Cd concentration (0.3 and 3 µM, Fig. 6a). In roots, 30 µM Cd triggered a 1.6-fold increase in chymotrypsin activity after 10 days, but 300 µM Cd led to a 1.6 and 2.3-fold increase in the proteasome activity after 3 and 10 days, respectively. To determine whether this increase was due to an increase in proteasome amount, and/or in its chymotrypsin specific activity, Western-blot analysis was performed on the same root extracts. After 3 and 10 days of Cd treatment, the proteasome protein content did not change significantly (Fig. 6b). The expression of genes

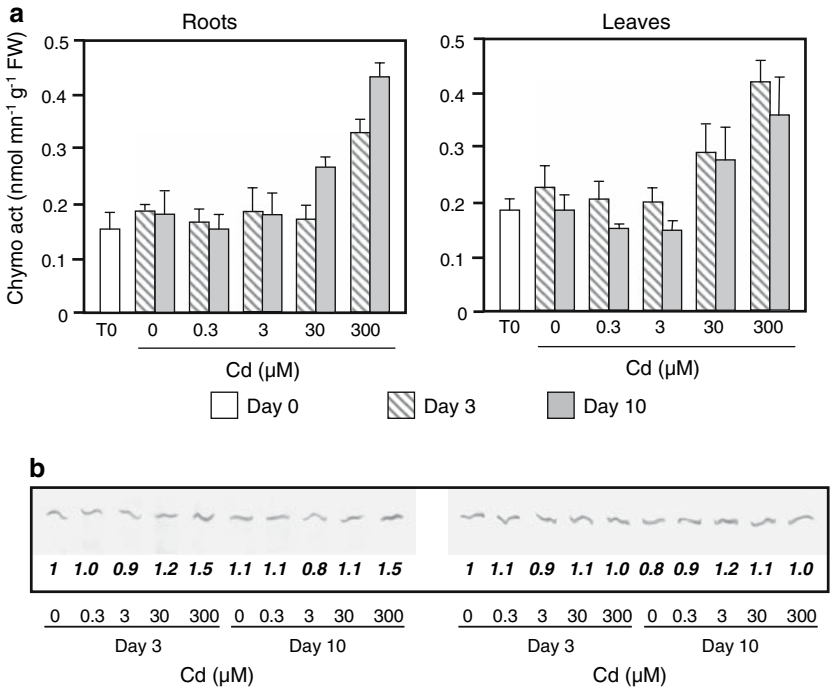

Fig. 6 20S proteasome activity and amount in the roots and the leaves of tomato plants submitted to Cd. **a** Chymotrypsin activity of 20S proteasome was determined after gel filtration and use of proteasome inhibitors, as described in "Materials and methods". Data are the mean of three independent experiments ± SD. **b** Western-blot analysis of 20S proteasome were performed after SDS-PAGE of clarified extracts (70 µg protein/track), transferred to nitrocellulose sheet and immunodetected with maize root 20S proteasome antibodies. Immunosignals were quantified using the Quantity One software (Promega). Values represent the relative intensity of each signal normalised to T0 signal. Measurements were performed in duplicate

encoding the α3, α6 and β1 subunits of the 20S proteasome was also analysed (Fig. 7). Variations of transcript levels were estimated using RT-PCR in semi-quantitative conditions (see "Materials and methods"), using *EF1α* transcript as the constitutive control. After 3 days of Cd treatment, *α3, α6* and *β1* transcript level increased 3.7, 3.9 and 6.0-fold, respectively, in the roots of 300 µM Cd-treated plants. A slight increase was also observed at 30 µM Cd. After 10 days of Cd treatment, differences in transcript levels were attenuated suggesting that Cd had a transient effect on proteasome encoding gene expression in roots.

In leaves, the chymotrypsin activity of the proteasome was maximally increased only after 3 days of treatment with 30 and 300 µM Cd (1.5 and 2.5-fold, respectively), and then levelled off, or decreased slightly, up to 10 days (Fig. 6a). Western-blot analysis showed that proteasome protein content remained constant with time irrespective of the Cd concentration (Fig. 6b). However *α3, α6* and *β1* mRNA levels increased 3.2, 2.9–4.7-fold, respectively, after 3 days of 300 µM Cd treatment (Fig. 7). A lower increase was also observed at this time point in leaves of 30 µM treated plant. In plants treated during 10 days, mRNA levels encoding the α3, α6 and β1 proteasome subunits were comparable in control plants and in Cd treated plants regardless of the concentration used.

Taken together, these data show that low Cd concentration (≤3 µM) had no effect on either proteasome gene expression or chymotrypsin activity in the roots as well as in the leaves. Higher Cd concentrations (30 and 300 µM) resulted in increased transcript level and increased activity, but, except in 300 µM Cd treated roots, no significant change was observed in 20S proteasome protein amount.

Discussion

In previous studies, it was shown that Cd induces oxidative stress and up-regulates proteolytic activity in pea leaves (McCarthy et al. 2001; Sandalio et al. 2001; Romero-Puertas et al. 2002), soybean roots (Balestrasse et al. 2003), and sunflower leaves (Pena et al. 2006). A recent proteomic study performed on *Arabidopsis thaliana* cells also reported the increase of several proteases upon exposition to Cd (Sarry et al. 2006). In the present work we aimed at evaluating the oxidation state of total protein, and the role of proteolysis in response to Cd at both realistic and classically used concentrations. We investigated and compared protein oxidation, protease activities and 20S proteasome expression in roots and leaves of tomato plants submitted to increasing Cd concentrations. As estimated by Wagner (1993), non-polluted soils contain Cd concentrations ranging from 0.04 to 0.32 µM. One micromol Cd in a soil solution may be considered as a moderate pollution, and Cd concentrations higher than 3 µM are considered as a high pollution (Sanita di Toppi and Gabbrielli 1999). Many Cd stress experiments on tomato plants have been carried out with Cd concentrations ranging from 0.01 to 1 mM, for 1–12 days (Sanita di Toppi and Gabbrielli 1999). Here, we chose to investigate a large range of Cd concentrations (0, 0.3, 3, 30 and 300 µM), to have some insight into the

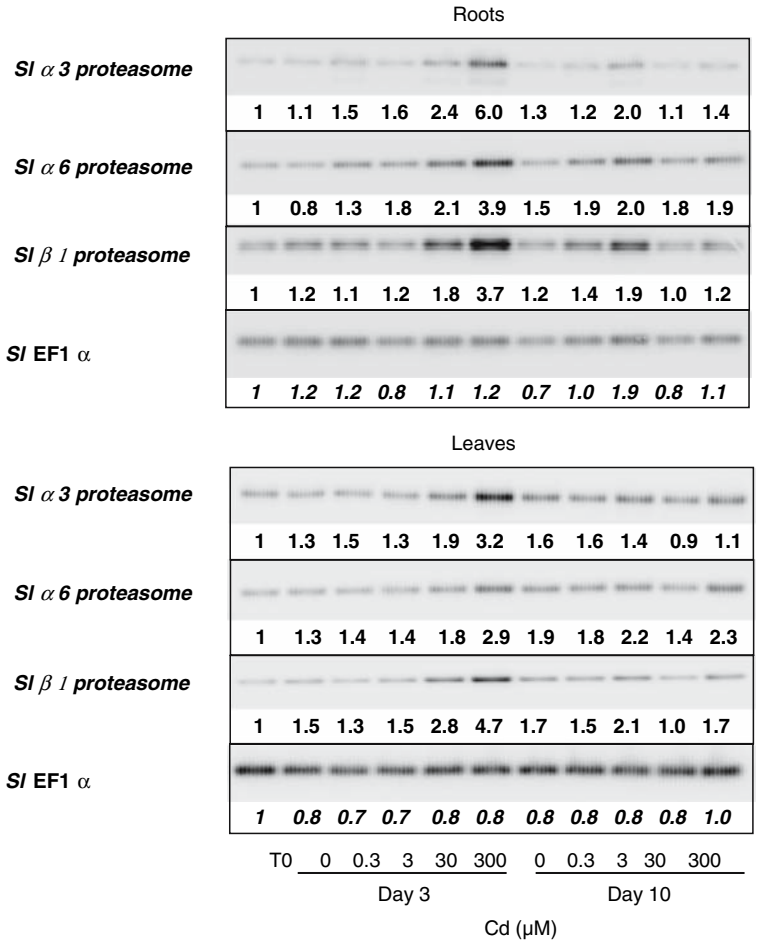

Fig. 7 20S proteasome α and β subunit transcripts in roots and leaves of tomato plants submitted to Cd. RT-PCR of α3, α6, β1 and EF1α (control) were performed using total RNA extracted from 3 and 10 days Cd treated plants. Hybridization signals were quantified using the Quantity One software (Promega). Values represent the relative intensity of each signal normalised to T0 and to the EF1α signal. Measurements were performed in duplicate

protein status and proteolytic response in low, moderately and highly Cd contaminated tomato plants.

The first characteristic of the global protein status under Cd stress in tomato plants is that protein amounts are not modified by low and medium Cd concentrations in the incubation medium (up to 3 μM Cd), whereas higher Cd concentrations induce opposite effects in roots and leaves, i.e. an increase in the roots and decrease in the leaves (Fig. 2). As the water content of both the leaves and the roots was found to decrease in response to Cd treatment (Djebali 2005), the differential modification in protein content cannot be explained by changes in water status. This rather means that protein turnover response to Cd is differently regulated in the two organs. Under Cd stress, in addition to the effects of intrinsic toxicity of Cd and the imbalance of metal uptake, an oxidative stress occurs through the production of active oxygen species (AOS; Clemens 2001; Dixit et al. 2001; Rodriguez-Serrano et al. 2006). AOS causes carbonylation of amino acids that result in loss of protein function (Grune et al. 2003). In tomato, the data here show that carbonylated protein content is higher in leaves than in roots of control plants, and that carbonyl content increases in leaves for the higher Cd concentrations (≥ 30 μM), as already observed in the leaves of pea or maize plants exposed to Cd (Romero-Puertas et al. 2002; Rellan-Alvarez et al. 2006), but decrease in roots (Table 2). This means, first, that proteins are naturally subjected to a higher oxidative pressure, or the defense mechanisms are less efficient, in relation to the intensity of the oxidative stress, in leaves than in roots. Second, if we consider that protein carbonyl content is a good indicator for oxidative stress (Levine et al. 1994), and that roots accumulated four to five times more Cd than leaves (Fig. 1), present data indicate that under Cd stress root proteins are less subjected to oxidative modifications than leaf proteins (Table 2). This is consistent with the differential lipid oxidation patterns observed in leaves and roots as a function of Cd concentration (Fig. 4) and could be explained by the differential induction of antioxidant mechanisms. Thus, in the roots of *Phaseolus vulgaris* (Chaoui et al. 1997), tomato (Djebali et al. 2002), pea (Dixit et al. 2001), and maize (Rellan-Alvarez et al. 2006) Cd enhances activities of guaiacol peroxidase and ascorbate peroxidase which contribute to the detoxification of AOS. However, such responses cannot be extrapolated since opposite results were obtained in the roots of Cd-treated pea plants (Rodriguez-Serrano et al.

2006). Another explanation might be that Cd is more efficiently complexed (i.e. to phytochelatins or metallothioneins) and/or sequestered as non-active forms (in cell walls and vacuoles) in roots than in leaves (Sandalio et al. 2001; Djebali et al. 2002; Mendoza-Cozatl et al. 2005). Taken together, these results indicate that the occurrence of a Cd-induced oxidative stress, and the resulting carbonylation of proteins, cannot be simply related to the concentration of Cd accumulated in the tissues, but also depends on the intrinsic stress response of each organ.

In addition to the involvement of protease in the general protein turnover and the set-up of the stress response, proteolysis is known to play a crucial role in the degradation of oxidized proteins, which are toxic for cell metabolism (Davies 2001; Palma et al. 2002). Total EP activities have been reported to be decreased in soybean nodules (Balestrasse et al. 2003), barely modified in pea leaves (Romero-Puertas et al. 2002), and increased in soybean roots (Balestrasse et al. 2003) and sunflower leaves (Pena et al. 2006), in response to Cd stress. However, total EP activities are probably not representative of either the modification of proteolytic machinery, or the global protein turnover. Indeed, the present work shows that serine, cysteine and metallo-EP activities were differently modified, both in roots and leaves, although total EP activities did not change in the roots (Fig. 3, Table 3). This means that the proteolytic response was probably mediated by specific proteases depending on sub-cellular compartments, organs and plant species. Previous work, showing that several peroxisomal EP and AP activities are up-regulated by Cd in pea leaves, supports this view (McCarthy et al. 2001).

In tomato, several points may be raised from the present observations. First, after 10 days, the proteolytic response seems to be modified only for Cd concentrations above 3 μM. Second, the proteases present in the roots and modified by Cd are qualitatively different from that present in the leaves, as shown by the opposite changes in serine-EP activities in response to Cd treatment. Third, the EP activities are mainly enhanced in the leaves after 3 days, whereas they are significantly modified only after 10 days in the roots. This indicates that the stress is perceived earlier and for lower Cd concentrations in the leaves than in the roots, but does not necessarily mean that Cd sensing mechanisms are different in the two organs, since the stress impact depends on the compartmentation and the chemical form of the metal. More probably, this indicates that metal scavenging or inactivating processes are more efficient in the roots than in the leaves.

Among the proteases potentially involved in the response to Cd, we focused our attention on the 20S proteasome. In eukaryotic cells the proteasome has been shown to selectively recognize and degrade mildly oxidized proteins in the cytosol, nucleus and endoplasmic reticulum (Grune et al. 2003). In mammalian cells (Figueiredo-Pereira et al. 1998) and yeast (Jungmann et al. 1993; Vido et al. 2001), Cd stress has been reported to induce proteasome, ubiquitin and ubiquitin conjugating enzyme expression. Moreover, ubiquitin conjugating enzyme (UBC1) gene is induced in tomato cell culture treated with Cd (Feussner et al. 1997), ubiquitin-conjugated proteins are increased in Cd-treated sunflower leaves (Pena et al. 2006), and the transformation of yeast cells with a maize cDNA encoding a 20S proteasome α-subunit confers resistance to Cd (Forzani et al. 2002). These reports suggest that proteasome and ubiquitin-dependent proteolysis could also be involved in the plant response to Cd stress through the degradation of oxidized proteins. However, in vitro assays have shown that while the purified 20S proteasome is able to degrade oxidized protein in the absence of ATP and ubiquitin, the 26S proteasome (with or without ATP/ubiquitin) exhibits little ability to degrade oxidized proteins (Davies 2001). Moreover, the 26S proteasome is very sensitive to direct oxidation (Reinheckel et al. 1998), and the ATP/ubiquitin 26S proteasome dependent proteolysis is depressed during even mild oxidative stress, while the activity of the 20S proteasome is unaffected (Shang and Taylor 1995; Obin et al. 1998) or increased (Basset et al. 2002). These observations led to the conclusion that 20S proteasome is mostly responsible for degrading mildly oxidized proteins during an oxidative stress, whereas the 26S proteasome would be involved in the degradation of more seriously damaged proteins during the recovery from stress (Shringarpure et al. 2001).

Our present data show that, for elevated Cd concentrations, 20S proteasome expression and chymotrypsin activity are up-regulated during the first days of Cd stress in both root and leaf tissues (Figs. 6, 7). Thus, the induction of 20S proteasome appears to be part of the proteolytic response to Cd stress. In maize roots, the 20S proteasome was postulated to be involved in the degradation of oxidized protein generated by carbon starvation (Basset et al. 2002). Considered together, these observations suggest that in plants, as in mammals and yeasts, the 20S proteasome could also be involved in the degradation of oxidized protein generated under oxidative conditions. It is possible that the up-regulation of the proteasome is not necessarily related to oxidative stress and to the degradation of oxidized proteins, since the carbonyl-group contents were found to decrease in roots at high Cd concentrations (Table 2). However, this apparent contradiction may be explained by the fact that AOS, and possibly free-Cd, but probably not carbonyl-groups per se, are the signals which change gene expression, i.e. proteasome subunit expression, in oxidative stressed plants (Apel and Hirt 2004). This means that, consequent to the AOS-induced up regulation of proteolytic processes, and possibly to the induction of efficient AOS scavenging mechanisms (see above discussion), the global carbonylated protein content can be finally decreased at high Cd concentrations.

In the leaves of sunflower plants, the proteasome level was apparently unaffected by Cd treatment while its activities were inhibited (Pena et al. 2006). In maize leaves, however, the proteasome level did not change upon Cd treatment, but both trypsin and peptidyl glutamyl peptide hydrolase-like activities were up-regulated, while the chymotrypsin-like one was not modified (Pena et al. 2007). In our experiments, the increase in 20S proteasome transcripts did not strictly correlate with the increase in activity whereas the proteasome level remained globally constant (Figs. 6, 7). Similarly, a discrepancy, although with opposite correlations, was already observed in maize roots: α and β proteasome subunits expression was down regulated, while chymotrypsin activity and proteasome amounts were increased in response to carbon starvation, (Basset et al. 2002). This activation was attributed to oxidative post-translational modifications of the 20S proteasome. As already suggested (Basset et al. 2002; Pena et al. 2007), a similar modification could explain the increase in the chymotrypsin activity in the absence of any change in the amount of 20S proteasome protein (Fig. 6). In the present case, our data indicate that Cd regulates the proteasome at both transcriptional and post-translational level. It may be thought that the up-regulation of the proteasome subunit expression is part of the acclimation response of the tomato plant to the oxidative stress. As the proteasome may be oxidized like other intracellular proteins, it is activated by mild oxidative modifications, but inactivated, and thus directed for degradation, by stronger oxidative conditions (Basset et al. 2002). Thus, the balance between up-regulation, activation and turnover probably explains the apparent discrepancies at mRNA, protein and activity levels.

The increase in EP and proteasome activities in response to Cd raises the question of a global proteolytic response to the stress. In mammals, a set of proteases have been identified acting sequentially downstream of the proteasome (tripeptidyl peptidase II, thimet oligopeptidase, leucine and proline aminopeptidase) to finish the degradation of peptides releasing free amino acids, in the cytosol (Saric et al. 2004). The presence of tripeptidyl peptidase II, and leucine and proline aminopeptidase was reported in plants (Book et al. 2005; Walling 2006). However, no thimet oligopeptidase-like protease was yet identified in plants, and the presence of the proteasome pathway remains to be demonstrated.

As observed for EP activities, the increase in 20S proteasome activity occurred earlier in leaves than in roots, which is in agreement with the above mentioned hypothesis that tomato leaves are more sensitive than roots to Cd. In addition to specific Cd neutralization mechanisms in the roots, the rapid response of the leaves to Cd might be explained by the fact that peroxisomes and chloroplasts are the main site for AOS production in green cells (Foyer and Noctor 2003), and that photosynthesis is sensitive to Cd and oxidative stress (Vassilev and Yordanov 1997; Sandalio et al. 2001; Pietrini et al. 2003; Djebali et al. 2005). The inhibition of photosynthesis dramatically enhances the production of AOS, and increases the amount of oxidatively damaged proteins which accumulation is highly toxic for cell metabolism (Apel and Hirt 2004). Efficient and rapidly inducible proteolytic machinery is thus required, and constitutes a crucial line of defence for photosynthetic cells to survive Cd and oxidative stress.

In conclusion, on the basis of the changes in protein amounts, protein carbonylation, and proteolytic activities, this work provides evidence that tomato roots and leaves are able to cope with low to moderate Cd concentrations ($\leq$3 μM) in the incubation medium, and are not modified in their proteolytic response. At higher Cd concentrations (i. e. 30 and 300 μM, which are considered as high pollution levels), protein oxidation state and protease activities are significantly modified in roots and leaves, although in different ways. Proteolysis responds earlier to Cd stress in leaves than in roots, probably because of differences in Cd compartmentation and/or sequestration, antioxidant response and metabolic sensitivity to Cd. The up-regulation of the 20S proteasome gene expression and proteolytic activity argues in favor of the involvement of the 20S proteasome in the degradation of oxidized proteins in plants, as already reported in mammals and yeasts.

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