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# Evidence for the Existence in *Arabidopsis thaliana* of the Proteasome Proteolytic Pathway

## ACTIVATION IN RESPONSE TO CADMIUM\*<sup>§</sup>

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Heavy metals are known to generate reactive oxygen species that lead to the oxidation and fragmentation of proteins, which become toxic when accumulated in the cell. In this study, we investigated the role of the proteasome during cadmium stress in the leaves of *Arabidopsis thaliana* plants. Using biochemical and proteomics approaches, we present the first evidence of an active proteasome pathway in plants. We identified and characterized the peptidases acting sequentially downstream from the proteasome in animal cells as follows: tripeptidyl-peptidase II, thimet oligopeptidase, and leucine aminopeptidase. We investigated the proteasome proteolytic pathway response in the leaves of 6-week-old *A. thaliana* plants grown hydroponically for 24, 48, and 144 h in the presence or absence of 50  $\mu\text{M}$  cadmium. The gene expression and proteolytic activity of the proteasome and the different proteases of the pathway were found to be up-regulated in response to cadmium. In an *in vitro* assay, oxidized bovine serum albumin and lysozyme were more readily degraded in the presence of 20 S proteasome and tripeptidyl-peptidase II than their nonoxidized form, suggesting that oxidized proteins are preferentially degraded by the *Arabidopsis* 20 S proteasome pathway. These results show that, in response to cadmium, the 20 S proteasome proteolytic pathway is up-regulated at both RNA and activity levels in *Arabidopsis* leaves and may play a role in degrading oxidized proteins generated by the stress.

Cadmium is a highly toxic and persistent environmental poison for plants, yeasts, and animals (1). Cadmium is released into the environment mainly through industrial wastes and is transferred to animals through the food chain (2). Cadmium interferes with many cellular functions mainly by complex formation with organic compounds such as proteins, lipids, and nucleotides leading to the inhibition of gene expression and

metabolic activities. Cadmium toxicity involves production of active oxygen species and free radicals, although the mechanism is still obscure (3). Cadmium is not a transition metal and does not produce hydroxyl radicals through Fenton or Haber-Weiss reactions, but it induces the production of superoxide anion, nitric oxide, and hydrogen peroxide, enhances lipid peroxidation and depletion of cellular glutathione, and finally generates oxidative stress (3, 4). Active oxygen species can lead to oxidation of side chains of amino acid residues and formation of protein-protein covalent cross-linkage, which can lead to protein inactivation or denaturation (5–7). If they are not rapidly degraded, oxidatively modified proteins can undergo direct fragmentation or can form large aggregates due to covalent cross-linking and increased surface hydrophobicity, which lead to cell death (5, 8).

In animal cells, the proteasome has been shown to recognize and degrade mildly oxidized proteins in the cytosol, nucleus, and endoplasmic reticulum, thus minimizing their cytotoxicity (5). From *in vitro* studies, it was shown that the 20 S proteasome recognizes and degrades oxidized proteins, probably through a “by default” degradation mechanism (9), although the 26 S proteasome does not (10–12). This may be explained by the fact that a mild oxidative stress rapidly inactivates both the ubiquitin-activating/conjugating system and 26 S proteasome activity in intact cells, but it does not affect 20 S proteasome activity (10, 12, 13).

In mammals, a set of proteases has been identified acting sequentially downstream of the proteasome to peptide degradation and releasing free amino acids into the cytosol. The peptides issued from the proteasome range from 3 to 25 amino acids and have different features according to their size. The smallest products (2–6 amino acids) are directly degraded by aminopeptidases, mainly leucine aminopeptidases (LAPs),<sup>3</sup> although larger peptides (>6 amino acids) are first cleaved by intermediate endopeptidases (14). The most important endopeptidases for these processes are the thimet oligopepti-

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<sup>3</sup> The abbreviations used are: LAP, leucine aminopeptidase; TPPII, tripeptidyl-peptidase II; TOP, thimet oligopeptidase; Mcc, 7-methoxycoumarin-3-carboxyl; AMC, 7-amino-4-methylcoumarin; Suc, succinimidyl; RT, reverse transcription; BSA, bovine serum albumin; LC-MS/MS, liquid chromatography/tandem mass spectrometry; pNA, p-nitroanilide; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DNPH, dinitrophenylhydrazine; CMK, chloromethyl ketone; AAF, Ala-Ala-Phe; Dnp, 2,4-dinitrophenyl; FW, fresh weight.

dase (TOP) (1) for peptides of 9–17 residues and tripeptidyl-peptidase II (TPPII) for longer products (15–25 amino acids) (15). The proteasome pathway (proteasome, TPPII, TOP, and aminopeptidases) constitutes the main protein degradation pathway in mammalian cells. It has been shown to play a central role in the degradation of many proteins in normal and disease states (16) and also in antigen processing for major histocompatibility complex class I presentation (17, 18). However, the specific involvement of the proteasome pathway in the degradation of oxidized protein has not been investigated so far.

In plants, the presence of the proteasome is now well established. It was first identified in pea (19) and then in all species investigated so far (20). Both the 20 S and 26 S proteasomes were characterized at molecular (21, 22) and biochemical levels (23) in *Arabidopsis thaliana*. As observed in animal and yeast cells, plant proteasome has been found to be up-regulated, at transcriptional or translational levels, in oxidative conditions such as carbon starvation (24) or cadmium stress (25–28), and its involvement in the response to oxidative stress has been postulated. In plants, the preferential involvement of the 20 S proteasome in the degradation of oxidized proteins was recently supported by *in vivo* experiments using *A. thaliana* mutants (29). The authors showed that the loss of function of some subunits of the 19 S regulatory particle of the 26 S proteasome resulted in a decrease of the 26 S accumulation at the benefit of the 20 S level, together with a higher tolerance to oxidative stress. The presence of TPPII, and many aminopeptidases, was also reported in plants (30–32), but no TOP-like protease has been identified to date. Thus, the following questions have been raised: (i) whether the proteasome pathway (including the 20 S proteasome and the downstream acting proteases) is similar in plants to that found in animals; (ii) whether this pathway is responsive to oxidative stress; and (iii) whether it is involved in the degradation of oxidized proteins.

To address these questions, proteomics and classical enzymatic approaches were used to investigate the presence of the following different peptidases of the proteasome pathway: proteasome, TPPII, TOP, and LAP in *Arabidopsis* leaves. The response of the proteasome pathway in the leaves of 6-week-old plants grown for 24, 48, and 144 h in the presence or absence of 50  $\mu\text{M}$  cadmium was also determined. Oxidative stress was estimated through protein oxidation level. The changes in RNA and protein accumulation and activity of the different proteases of the pathway were analyzed. Oxidized or nonoxidized bovine serum albumin, lysozyme, and casein were used to test the capacity of the 20 S proteasome and TPPII to degrade oxidized substrates.

## EXPERIMENTAL PROCEDURES

**Growth Conditions**—*A. thaliana* seeds (ecotype Columbia) were germinated in sand and then grown hydroponically in controlled conditions. The photoperiod was 14 h, with a photosynthetic photon flux density of 140–190  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The day/night temperatures were 23/18  $^{\circ}\text{C}$ , and the relative humidity was maintained close to 55%. The nutritive solution (pH 5.9) contained the following elements: 805  $\mu\text{M}$   $\text{Ca}(\text{NO}_3)_2$ , 2 mM  $\text{KNO}_3$ , 60  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$ , 695  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 1.1 mM  $\text{MgSO}_4$ , 20  $\mu\text{M}$   $\text{Na}_2\text{EDTA}$ , 20  $\mu\text{M}$   $\text{FeSO}_4$ , 74 nM

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , 3.6  $\mu\text{M}$   $\text{MnSO}_4$ , 3  $\mu\text{M}$   $\text{ZnSO}_4$ , 9.25  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , and 785 nM  $\text{CuSO}_4$ . The culture medium was renewed every 2 days and was continuously sparged with air. When seedlings were 6 weeks old, stress was induced by adding  $\text{Cd}(\text{NO}_3)_2$  to the mineral solution at a 50  $\mu\text{M}$  final concentration. No cadmium was added to control cultures. Cadmium-containing solutions were renewed every 2 days. At 0, 24, 48, and 144 h of cadmium exposure, leaves were harvested and quickly frozen in liquid  $\text{N}_2$ , ground to a powder in liquid  $\text{N}_2$ , and stored at  $-80^{\circ}\text{C}$  until analysis.

**Preparation of Clarified Extracts**—Five hundred mg of frozen leaf powder were homogenized in a mortar at 4  $^{\circ}\text{C}$  with four times their fresh weight of extraction medium (50 mM Tris-HCl (pH 7.5), 5 mM  $\beta$ -mercaptoethanol, and 0.3% (w/v) insoluble polyvinylpyrrolidone). The homogenate was centrifuged for 20 min at 16,000  $\times g$ , and the supernatant was used for protease purification and activity measurements.

**Chromatography Steps**—The soluble leaf protein extract (6 ml) was applied to a HiPrep 26/60 Sephacryl S-300 gel filtration column ( $M_r$  10,000–1,500,000; Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The column was calibrated with the following markers: thyroglobulin (670 kDa), bovine  $\gamma$ -globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin  $\text{B}_{12}$  (1.35 kDa). The fractions containing chymotrypsin-like AAF-AMC-like (TPPII), Mcc-PLGPK-Dnp-like (TOP) (1), and Leu-pNA-like activities were pooled and used for activity measurement in the presence or absence of various chemical agents to characterize the different proteases as described below.

**Determination of Protein-bound Carbonyls**—Carbonyl contents in soluble protein extracts, from 100 mg FW of each sample, were quantified by reaction with dinitrophenylhydrazine (DNPH) according to Ref. 33. Proteins were reacted with 200  $\mu\text{l}$  of 10 mM DNPH (in 2 M HCl) or 2 M HCl (control) for 1 h at 25  $^{\circ}\text{C}$ . For each determination, two replicates and their respective blanks were used. Proteins were precipitated with 20% (w/v) trichloroacetic acid, centrifuged, and washed three times with ethanol/ethyl acetate (1:1 (v/v)). The protein pellets were finally dissolved in 6 M guanidine hydrochloride, 20 mM potassium phosphate buffer (pH 2.3), and the absorption at 370 nm was measured. Carbonyl content was calculated using a molar absorption coefficient of 22,000  $\text{M}^{-1} \text{cm}^{-1}$ . To determine the yield of protein recovery after the carbonyl derivation step with DNPH, proteins were quantified before and after the derivation procedure by reading the absorption of the samples at 280 nm (33). Protein amounts were calculated from a BSA standard curve prepared in the same conditions. Protein recovery was  $88 \pm 4\%$ .

**Proteolytic Activity Measurements**—Global endopeptidase activity measurement was adapted from Brouquisse *et al.* (34). One hundred  $\mu\text{l}$  of clarified extract and 100  $\mu\text{l}$  of azocasein (5 mg  $\text{ml}^{-1}$  in 200 mM MES-KOH (pH 6.0)) were incubated for 3 h at 37  $^{\circ}\text{C}$ . The reaction was stopped by the addition of 100  $\mu\text{l}$  of 10% (v/v) trichloroacetic acid. After 10 min on ice, samples were centrifuged at 15,000  $\times g$  for 10 min; 250  $\mu\text{l}$  of supernatant were then added to 750  $\mu\text{l}$  of 1 M NaOH, and the absorbance was read at 440 nm. The extinction coefficient  $\epsilon_{1\%}$  azocasein in

## Proteasome Pathway Activation in Response to Cadmium

1 M NaOH = 37 liters  $\text{cm}^{-1} \text{g}^{-1}$  was used to calculate the azocasein degradation activity.

For chymotrypsin-like activity measurement, Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC; 2 mM stock in dimethyl sulfoxide) was used as a substrate. The assay mixture contained 100  $\mu\text{l}$  of 20 S proteasome fraction in 50 mM Tricine buffer (pH 8.1) and 100  $\mu\text{M}$  of Suc-LLVY-AMC. After incubation at 37 °C for 1 h, the reaction was stopped by the addition of 100  $\mu\text{l}$  of 10% (w/v) SDS and 700  $\mu\text{l}$  of Tris-HCl 100 mM (pH 9.0). The AMC radical released was measured fluorometrically ( $\lambda_{\text{excitation}} = 380 \text{ nm}$ ;  $\lambda_{\text{emission}} = 460 \text{ nm}$ ). Activity was calculated using AMC standard curve made in the same conditions.

For TPPII activity measurement, 100  $\mu\text{l}$  of sample fraction were mixed with 50 mM Tris-HCl buffer (pH 7.6), 5 mM  $\text{MgCl}_2$ , and 125  $\mu\text{M}$  Ala-Ala-Phe-AMC (AAF-AMC) as substrate. After 1 h of incubation period at 37 °C, the reaction was stopped with the addition of 800  $\mu\text{l}$  of 80 mM sodium acetate (pH 4.3). The AMC radical released was measured fluorometrically ( $\lambda_{\text{excitation}} = 380 \text{ nm}$ ;  $\lambda_{\text{emission}} = 460 \text{ nm}$ ).

TOP activity was measured by mixing a 100- $\mu\text{l}$  sample with 50 mM Tris-HCl buffer (pH 7.6), 5 mM  $\text{MgCl}_2$ , 0.1 mM dithiothreitol, 0.05% Brij 35% (v/v), and 20  $\mu\text{M}$  Mcc-Pro-Leu-Gly-Pro-D-Lys-OH-Dnp (Mcc-PLGPK-Dnp) as substrate. After 1 h at 37 °C, the reaction was stopped by adding of 800  $\mu\text{l}$  of 80 mM sodium acetate (pH 4.3). The product was measured fluorometrically ( $\lambda_{\text{excitation}} = 345 \text{ nm}$ ;  $\lambda_{\text{emission}} = 405 \text{ nm}$ ).

For LAP activity measurement, 300  $\mu\text{l}$  of fraction were mixed with 50 mM Tris-HCl buffer (pH 8) and 0.5 mM  $\text{MnCl}_2$ . The reaction was measured at 25 °C and started by the addition of 1 mM L-leucine-*p*-nitroanilide (Leu-*p*NA) as substrate. The *p*NA released was measured spectrophotometrically at 405 nm. The extinction coefficient ( $\epsilon$ ), *p*NA = 10,500 liters  $\text{cm}^{-1} \text{M}^{-1}$ , was used to calculate LAP-like activity.

**Protease Effectors**—Protease effector stock solutions were prepared in the following solvents: E64 (2 mM),  $\text{Na}_2\text{EDTA}$  (0.2 M), butabindide (1 mM), AAF-chloromethyl ketone (AAF-CMK), and benzyloxycarbonyl-AAF CMK (5 mM) in water; phenylmethylsulfonyl fluoride (0.2 M), 1,10-phenanthroline (0.2 M), and pepstatin (2 mM) in ethanol; lactacystine (1 mM), carboxy-3-phenylpropyl -AAF-polyclonal antibody (2 mM), and benzyloxycarbonyl-Leu-Leu-leucinal (MG132, 2 mM) and bestatin (2 mM) in dimethyl sulfoxide (DMSO). Effectors were first preincubated for 20 min at room temperature with protein extracts prior to substrate addition, and activities were measured as described above. Control assays were carried out with the corresponding solvent.

**Immunoprecipitation Experiments**—Polyclonal antibodies raised against the maize 20 S proteasome (24) and tomato LAP (35) were used for immunoprecipitation experiments. Serum IgG fractions were purified using 1 ml of HiTrap protein A column (Amersham Biosciences) as recommended by the manufacturer. Immunoprecipitation protocol was adapted from Ref. 24. Briefly, 100  $\mu\text{l}$  of either clarified extract or partially purified 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 5-fold (immunoglobulin-G binding) excess of protein A-agarose (Affi-Gel, Bio-

Rad) and then centrifuged for 5 min at 10,000  $\times g$ . The enzymatic activities (chymotrypsin-like or Leu-*p*NA-like) were measured in each supernatant fraction as described above.

**Two-dimensional Gel Electrophoresis**—Proteins were extracted according to Bestel-Corre *et al.* (36) and lyophilized. Proteins were dissolved in solubilization buffer for two-dimensional PAGE (37). Protein concentrations were determined using a modified Bradford assay using BSA as a standard. SDS-PAGE and two-dimensional PAGE were performed as described by Wang *et al.* (38). After electrophoresis, gels were stained with Coomassie Blue R-250 or electrophoretically transferred to nitrocellulose filters. Electro-transfer and protein blots procedures were performed as described by Gu *et al.* (35). Protein blots were incubated with a 1:500 dilution of a polyclonal serum made against the tomato LAP-A1 (35). Signals were analyzed and quantified using Quantity One software (Bio-Rad).

**Identification of Proteases of the Proteasome Pathway**—For in-gel digestion, 20  $\mu\text{g}$  of proteins from the Mono Q fractions were by SDS-PAGE. After coloration with Coomassie Blue R-250, the gel was cut into 1.5-mm slices. Each band was further cut and washed twice in 100  $\mu\text{l}$  of destaining solution (50 mM  $\text{NH}_4\text{HCO}_3/\text{CH}_3\text{CN}$  (50:50, v/v)) at room temperature for 30 min before dehydration with 100  $\mu\text{l}$  of pure  $\text{CH}_3\text{CN}$ . The solution was then removed, and the gel pieces were dried in a speed vacuum and rehydrated in 100  $\mu\text{l}$  of 7%  $\text{H}_2\text{O}_2$ , at room temperature for 15 min, in the dark. The oxidizing solution was removed, and the gel slices were rinsed in water and then dehydrated in  $\text{CH}_3\text{CN}$  as described above. After complete drying, the bands were rehydrated in 20  $\mu\text{l}$  of digestion buffer (150 mM Tris-HCl (pH 8.1), 10 mM  $\text{CaCl}_2$ , 100 mM urea/ $\text{CH}_3\text{CN}$  (95:5, v/v)), containing 150 ng sequencing grade modified trypsin (Promega, Madison, WI). After 15 min of incubation at 4 °C, 30  $\mu\text{l}$  of digestion buffer was added, and the digestion reaction was carried out at 37 °C for 5 h, under permanent shaking. The digestion solution was then collected, and peptides were extracted from the gel by diffusion in 50  $\mu\text{l}$  of 0.3 M urea, 90% (v/v)  $\text{CH}_3\text{CN}$  for 30 min with intermittent sonication. Digestion and extraction solutions were pooled and dried under speed vacuum. Peptide mixtures were redissolved in 25  $\mu\text{l}$  5% (v/v)  $\text{CH}_3\text{CN}$ , 0.2% (v/v) formic acid in water prior to LC-MS/MS analysis.

For nano-liquid chromatography (nano-LC)-electrospray ionization-MS/MS, chromatographic separation of digested proteins was accomplished by loading 0.15  $\mu\text{g}$  of peptide mixture onto a 15-cm fused silica C18 column (75  $\mu\text{m}$  inner diameter, 3  $\mu\text{m}$ , 100 Å, and 360  $\mu\text{m}$  outer diameter; Dionex). Peptide elution was achieved using the following linear gradients: (a) from 10 to 40% solvent B (90% (v/v)  $\text{CH}_3\text{CN}$ , 0.1% (v/v) formic acid in water) for 40 min and (b) from 40 to 90% solvent B for 5 min. The remaining percentage of the elution solvent was made of solvent A (water/ $\text{CH}_3\text{CN}$  (1:1, v/v)) containing 0.1% formic acid). Flow rate through the nano-LC column was 200–300 nl/min. For automatic LC-MS/MS analysis, the QTOF Ultima instrument was run in data-dependent acquisition mode with the following parameters: 1-s scan time and 0.1-s interscan delay for MS survey scans; 400–1400 and 50–2000 *m/z* mass ranges for the survey and the MS/MS scans, respectively; five

TABLE 1

Sets of PCR primer sequences used to amplify gene-specific regions and corresponding size of the amplified products

	Gene	AGI number	Forward primer (5' to 3')	Reverse primer (5' to 3')	Length	No. of cycles	
Proteasome 20 S subunits	$\alpha 7$	At2g27020	AAGAAGCGGTTATCGAGGTG	AAAGACTCCAATGGTTCATGC	317	23, 24, 25	
	$\beta 1$	At4g31300	TCTACGGGTTCTTCGACCAG	CGGGTCATTTCATTACAACAGAA	383	23, 24, 25	
	$\beta 2-1$	At3g27430	TGATCTACATCAAGGCTCTGTG	TTTCGAAAGGACGAAGTCTCTC	156	24, 25, 26	
	$\beta 2-2$	At5g40580	TGAATCTCGTGTGAGAGACAGA	TTTCAAACCGAATCATCCA	184	24, 25, 26	
	$\beta 5-1$	At1g13060	GTTTCTAATCGTTCCTCCCTTT	TTTTTGCAGAAAAAGAGAATCTTATAG	150	25, 26, 27	
	$\beta 5-2$	At3g26340	CATCACTTATGTTTGTTCATCAAT	TGTTATCTATATGCAACGTGATCATT	115	25, 26, 27	
	$\beta 6$	At3g60820	GGTGCTCAAGGTTCTGGTTC	CAAGGATTTCGAGTGATTCTCA	431	24, 25, 26	
	$\beta 7$	At1g56450	CTTGCTAGGCCAATTCTTCG	GATGTGGTTGCTTGGCTTCT	259	24, 25, 26	
	Other proteases	TPPII	At4g20850	CTAAATCAGTATCAGAACGCTTGG	CATGGCTAACCCCTTCTGGTATAG	391	21, 22, 23
		NEURO	At1g67690	GCTTTTGCCGCTTTTGACTCAG	GGTCAACACGTAGGCCCCGAG	307	30, 31, 32
		TOP	At5g10540	CGAACCATTTTCACATCTCCAC	AATCGGAGAATTTGGGTGGAGG	291	26, 27, 28
		TOPorg	At5g65620	CCATTTTCTCACCACAACCAG	GGGAAACTGAAGGAAAGGATTTG	314	26, 27, 28
		LAP1	At2g24200	GGAGTGGAGAGAAGCTGTGGAGG	CAGTCTCTCCCTCAGCTCTC	297	23, 24, 25
LAP2		At4g30920	GGAGTGGAGAGAAGTTATGGAG	GCAAAAACACAAC TAGTTTCACATC	314	21, 22, 23	
LAP3		At4g30910	GGAGTGGAGAGAAGTTATGGAG	CTGAGAATGTTAAATAAGAACTGGG	313	25, 26, 27	
PIP2		At2g14260	CGTCGGACTCACACTTGC	ACATTCGGTTTCGGTTAAC	357	25, 26, 27	
Constitutive genes		ACT2	At3g18780	GGTAACATTGTGCTCAGTGGTGG	AACGACCTTAATCTTCATGCTGC	108	22, 23, 24
		ACT8	At1g49240				

components; MS/MS to MS switch after 5 s; switchback threshold, 30 counts/s; include charge states 2, 3, and 4 with the corresponding optimized collision energy profiles. The acquired data were post-processed to generate peak lists (.pkl) with PeptideAuto, which is part of proteinLynx from Masslynx 4.0. Each peak list was submitted to searches using MASCOT 2.0. MASCOT search parameters used with MS/MS data were as follows: data base = *A. thaliana* (nuclear, mitochondrial, and chloroplastic genome), enzyme = trypsin/protein, one missed cleavage allowed, a peptide tolerance = 0.25 Da, MS/MS tolerance = 0.25 Da, peptide charge = 2+/3+/4+, and variable modifications such as oxidized methionine under sulfone and sulfoxide and cysteic acid. The algorithm can efficiently choose the best amino acid sequence, from all possible amino acid combinations, to interpret the MS/MS spectrum according to the same chemical modifications defined in Mascot.

**Extraction of Total RNA and Estimation of Relative Transcript Level by RT-PCR**—Total RNA was isolated from 100 mg of plant tissue using the TRIzol reagent protocol (Invitrogen). DNase treatment was performed to remove contaminant genomic DNA using the RQ1 DNase-RNase-free (Promega), according to the manufacturer's instructions. Reverse transcription was performed using 5  $\mu$ g of total RNA (first strand cDNA synthesis, Amersham Biosciences) as detailed in the manufacturer's instructions.

Semi-quantitative RT-PCR was performed using 0.5  $\mu$ l of a 10 $\times$  dilution of the RT reaction with 0.1  $\mu$ M of each oligonucleotide primer, using the TITANIUM<sup>TM</sup> Taq polymerase (Clontech). After an initial denaturation step of 3 min at 95  $^{\circ}$ C, PCR was performed as follow: 30 s denaturation (94  $^{\circ}$ C), 20 s hybridization (55, 60, or 62  $^{\circ}$ C according to the primers), and 45 s elongation (68  $^{\circ}$ C); the number of PCR cycles was defined for each gene studied to obtain a detectable signal without reaching saturation (Table 1). Amplification products (10  $\mu$ l) were loaded on a 1.3% (w/v) ethidium bromide-stained agarose gel. Oligonucleotide pairs used for amplification are listed in Table 1. Three independent experiments were analyzed. PCRs were performed in duplicate. Each set of reactions was stopped at three different numbers of cycles and analyzed on agarose gel. Signals were analyzed and quantified using Quantity One

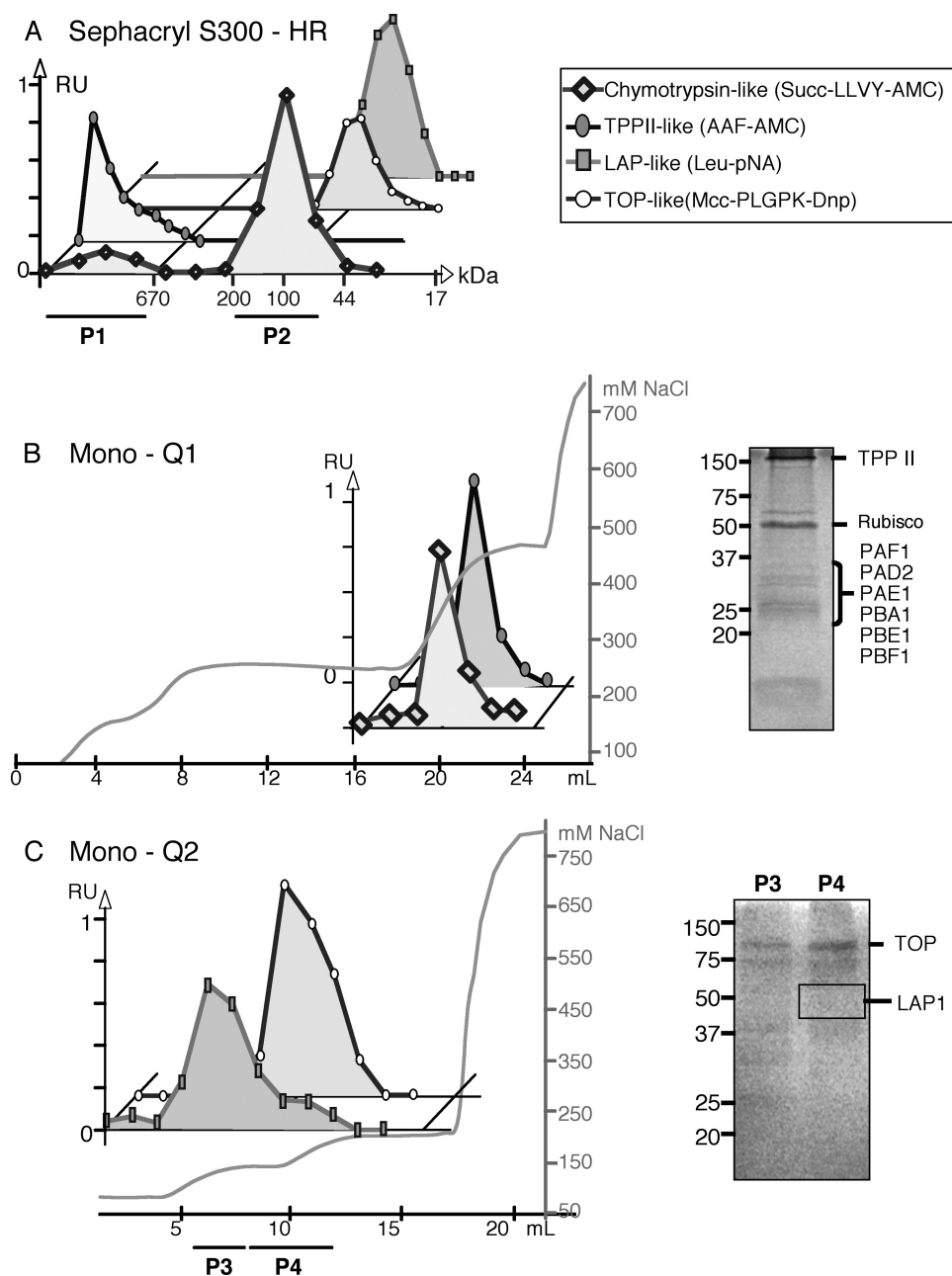
software (Bio-Rad). The expression level of each gene was normalized according to the ACT2/8 level and the control conditions.

**Preparation of Oxidized Protein**—To test the efficiency of the 20 S proteasome/TPPII couple in the degradation of oxidized proteins, oxidative treatment of 10–15 mg of either BSA, lysozyme, or  $\alpha$ -casein was performed *in vitro* according to Ref. 24. Proteins were incubated in 20 mM sodium phosphate buffer (pH 7.2) in the presence or absence of 200  $\mu$ M FeSO<sub>4</sub> and 50 mM ascorbate in a final volume of 1 ml, at 37  $^{\circ}$ C, for 3 h. Five  $\mu$ l of either oxidized or nonoxidized proteins was submitted to DNPH derivatization to check their oxidation status, according to Basset *et al.* (24). Ascorbate and FeSO<sub>4</sub> were eliminated using a 6-DG column (Bio-Rad). Primary amine residues were blocked by reductive methylation using the procedure of Ref. 39 with the following modifications: 0.2–0.5  $\mu$ mol of protein were incubated with an excess of 100-fold formaldehyde and sodium cyanoborohydride at 25  $^{\circ}$ C for 23 h. These two reagents were then removed using a 6-DG column (Bio-Rad). After protein measurement, the samples were stored at –80  $^{\circ}$ C.

**Degradation of Protein Substrates by the 20 S Proteasome/TPPII Mixture**—After a Sephacryl chromatography step performed with the leaf protein extract, the 20 S proteasome and TPPII containing fractions were pooled (P1 pool) and used to monitor the degradation of the proteins oxidized *in vitro*. Twenty  $\mu$ g of methylated protein substrates were incubated in 0.1 M sodium phosphate buffer (pH 7.2), 5% glycerol, 1 mM dithiothreitol, and 35  $\mu$ l of P1 pool (the protein concentration of P1 pool was 1 mg/ml), at 37  $^{\circ}$ C for 3, 4, and six h. Background changes of (i) the media, (ii) the protease mix, (iii) the substrates, and (iv) the protease inhibitors were monitored using a set of controls for each time point. Each measurement was made in duplicate.

The amount of newly formed  $\alpha$ -amino groups was measured with fluorescamine (Sigma). Ten  $\mu$ l of the proteolytic reaction assay were harvested, and proteolysis was stopped with 10  $\mu$ l of 10% trifluoroacetic acid. Then 100  $\mu$ l of sodium phosphate buffer (pH 6.8) and 50  $\mu$ l of fluorescamine (0.3 mg/ml in acetone) were added. The mixture was vortexed for 1 min, and water was added to a final volume of 1 ml. Fluorescence was

## Proteasome Pathway Activation in Response to Cadmium



**FIGURE 1. Partial purification and characterization of the proteases involved in the proteasome pathway from *A. thaliana* leaves.** *A*, enzymatic activity elution pattern during S-300 Sephacryl chromatography. *P1* refers to the fractions containing 20 S proteasome and TPPII activities; *P2* refers to the fractions containing TOP and LAP activities. *B*, anion-exchange chromatography (Mono Q) of the proteins present in the *P1* pool (from the Sephacryl S-300-HR) and SDS-polyacrylamide gel of the fraction containing the maximum activity of proteasome 20 S and TPPII used for protein identification by mass spectrometry analysis. *Rubisco*, ribulose-bisphosphate carboxylase/oxygenase. *C*, anion-exchange chromatography step (Mono Q) of the proteins present in the *P2* pool (from the Sephacryl S-300-HR) and SDS-polyacrylamide gels of the pools *P3* and *P4* used for protein identification by mass spectrometry analysis. Activities are expressed as relative units ranging from null to one. Salt concentration of elution buffer is expressed as millimolar NaCl. *RU*, relative units.

measured using an excitation wavelength of 370 nm and an emission of 480 nm.

**Other Assays**—Proteins were quantified (40) using the Bio-Rad microassay reagent. Bovine  $\gamma$ -globulin was used as the standard. Native-PAGE was performed with 6% (w/v) polyacrylamide gels. Gels were fixed, stained with Coomassie Blue, and destained using standard methods. Western blot analyses

were carried out as in Ref. 24 using polyclonal antibodies raised against either maize root 20 S proteasome or leaf tomato LAP.

## RESULTS

**Evidence for the Proteasome Pathway in *A. thaliana***—The presence of the different proteases involved in the proteasome pathway, including the 20 S proteasome, TPPII, TOP, and LAP, was investigated in the leaves of 6-week-old *A. thaliana* plants. A leaf protein extract was first submitted to a size-exclusion chromatography step, using a Sephacryl S-300 column. The pattern of the chromatogram obtained is presented in Fig. 1. Using specific substrates, the different peptidase activities were then assayed in the eluted fractions (Fig. 1A). The use of Suc-LLVY-AMC peptide has allowed the identification of two peaks of chymotrypsin-like activity (Fig. 1A). The first one, around 700 kDa, corresponds to the size of the 20 S proteasome, and its presence was confirmed by Western blot experiments using anti-plant 20 S proteasome antibodies (data not shown). The second major peak of chymotrypsin-like activity, which accounted for ~80% of the total activity, was eluted with proteins having a molecular mass of around 100 kDa (no cross-reaction was observed with 20 S proteasome antibodies; data not shown). Thus we conclude that, in contrast to mammals and yeast, the 20 S proteasome is not responsible for the major part of the chymotrypsin-like activity.

A TPPII-like activity, monitored using the AAF-AMC tripeptide as a substrate, was measured in the first peak corresponding to high molecular weight complexes (Fig. 1A). This is in agreement with a previous report showing that *Arabidopsis* TPPII is a large oligomeric 5–9-MDa complex (30). A TOP-like activity was measured using the specific fluorogenic substrate Mcc-PLGPK-Dnp. It was detected around the 80–100-kDa fractions, which are close to the 78-kDa size of mammalian TOP (41).

Leu-pNA was used as substrate for screening aminopeptidase activities with LAP or LAP-like activities. LAPs have been

**TABLE 2**

**Proteases of the proteasome pathway identified by mass spectrometry analysis**

This table gives the protein acronym of proteases identified after SDS-PAGE in Mono Q1 and Mono Q2 fractions of Fig. 1; the gene designation; theoretical isoelectric point (pI); Mascot score of in-gel digestion protocol (Score column); deduced molecular mass; protein sequence coverage; and number of peptides assigned using the in-gel digestion protocol. The protease family was assigned by the peptidase MEROPS database.

Proteins	Gene	pI	Score	Mass	Coverage	No. peptides
				Da	%	
<b>Proteases identified in Mono Q1 fractions</b>						
TPPII	At4g20850	5.92	584	152,273	7.1	9
20 S proteasome $\alpha$ -subunit D2 (PAD2)	At5g66140	8.73	84	27,307	9.2	2
20 S proteasome $\alpha$ -subunit F1 (PAF1)	At5g42790	4.99	73	30,457	10.1	2
20 S proteasome $\alpha$ -subunit E1 (PAE1)	At1g53850	4.70	75	25,931	14.4	3
20 S proteasome $\beta$ -subunit F1 (PBF1)	At3g60820	6.95	51	24,628	9.9	2
20 S proteasome $\beta$ -subunit E1 (PBE1)	At1g13060	5.77	75	25,264	12.2	2
20 S proteasome $\beta$ -subunit A1 (PBA1)	At4g31300	5.24	84	25,280	4.7	2
<b>Proteases identified in Mono Q2 fractions</b>						
Peptidase M3 family protein/thimet oligopeptidase family protein (Thimet, TOPorg)	At5g65620	5.90	1479	88701	36.0	27
Peptidase M3 family protein/thimet oligopeptidase family protein (Thimet, TOP)	At5g10540	5.45	736	78994	21.3	15
Leucine aminopeptidase (LAP1)	At2g24200	5.66	175	54475	14.4	4

described in mammals and plants, as hexameric complexes composed of six 50–55-kDa subunits (42). The *A. thaliana* genome encodes three LAP proteins; only LAP1 has a putative cytosolic localization (43, 44). However, after screening the size-exclusion chromatography fractions, only one peak of Leu-pNA hydrolyzing activity was detected in the 80–100-kDa protein fractions (Fig. 1A). These data show that Leu-pNA hydrolyzing *Arabidopsis* peptidases distinct from the hexameric LAPs may be involved in degrading the peptides generated by the proteasome.

Two complementary approaches were used to further identify and characterize the peptidases of the proteasome pathway. The first strategy was a proteomics approach consisting of an additional protein purification step followed by an SDS-PAGE fractionation and identification by mass spectrometry. As a second approach, protease activities were characterized according to their sensitivity to known inhibitors.

Fractions eluted from the gel filtration column exhibiting chymotrypsin- and TPPII-like activities were pooled together (Fig. 1A, P1 pool) and applied on an anion-exchange Mono Q column. It appeared that both chymotrypsin- and TPPII-like activities eluted in the same fractions (around 300 mM NaCl, Fig. 1B). Proteins present in the fraction containing the maximum activities were separated by SDS-PAGE (Fig. 1B). After Coomassie Blue staining, bands present in the gel were excised and trypsin-digested, and peptide mixtures were submitted to the identification of several subunits of the 20 S proteasome (Table 2; Fig. 1B). The chymotrypsin activity of P1 pool from the Sephacryl S-300 and from the Mono Q1 was inhibited by 98 and 99%, respectively, when measured either in the presence of 10  $\mu$ M lactacystin or after immunoprecipitation experiments with anti-20 S proteasome antibodies (Table 3). No inhibition was observed when chymotrypsin activity was measured in the presence of the TPPII inhibitor butabindide. These data clearly indicate that this activity was related to the 20 S proteasome.

TPPII was among the major proteins identified by SDS-PAGE and nano-LC-ESI-MS/MS (Fig. 1B; Table 2). Peptidase assays performed on either the Sephacryl S-300-P1 pool, or Mono Q1 fractions (Fig. 1), using two specific inhibitors of TPPII, butabindide and AAF-CMK, showed an inhibition close to 100% of this activity (Table 3). Moreover, benzyloxycarbonyl-AAF-CMK (a negative

**TABLE 3**

**Effect of different effectors on proteolytic activities**

Sephacryl S-300 fractions corresponding to pools 1 and 2 were used for inhibition measurement of the 20 S proteasome, TPPII, and TOP activities. Desalted clarified extracts were used for the immunoprecipitation of LAP activity. Activities were assayed as described under "Experimental Procedures." The inhibitor and activator effects are expressed as percentage of the control (100%). Data correspond to the means of at least eight independent measurements  $\pm$  S.D. The abbreviations used are as follows: Z, benzyloxycarbonyl; PMSF, phenylmethylsulfonyl fluoride; Cpp, carboxy-3-phenylpropyl; pAB, polyclonal antibody.

Effector	Effector concentration	% of the control
<b>20 S proteasome (P1 pool)</b>		
Control		100
Lactacystin	10 $\mu$ M	11 $\pm$ 2
Butabindide	20 $\mu$ M	103 $\pm$ 20
Anti-20 S antibody		1 $\pm$ 4
<b>Tripeptidyl peptidase II (P1 pool)</b>		
Control		100
Butabindide	20 $\mu$ M	1 $\pm$ 1
AAF-CMK	50 $\mu$ M	1 $\pm$ 1
Z-AAF-CMK	50 $\mu$ M	105 $\pm$ 9
MG132	20 $\mu$ M	95 $\pm$ 6
Bestatin	20 $\mu$ M	96 $\pm$ 6
PMSF	2 mM	1 $\pm$ 2
E64	20 $\mu$ M	105 $\pm$ 8
Pepstatin	20 $\mu$ M	113 $\pm$ 8
1,10-Phenanthroline	2 mM	94 $\pm$ 8
EDTA	2 mM	105 $\pm$ 8
<b>Thimet oligopeptidase (P2 pool)</b>		
Control		100
Cpp-AAF-pAB	20 $\mu$ M	21 $\pm$ 2
PMSF	2 mM	96 $\pm$ 10
E64	20 $\mu$ M	87 $\pm$ 1
1,10-Phenanthroline	2 mM	38 $\pm$ 1
EDTA	2 mM	59 $\pm$ 5
MnCl <sub>2</sub>	2 mM	441 $\pm$ 22
CoCl <sub>2</sub>	2 mM	220 $\pm$ 11
ZnCl <sub>2</sub>	2 mM	2 $\pm$ 2
<b>Leucine aminopeptidase (crude extract)</b>		
Control		100
Anti-LAP antibody		83 $\pm$ 2

control for the serine protease inhibitor AAF-CMK) did not inhibit this activity. TPPII activity also showed a high sensitivity to the serine protease inhibitor phenylmethylsulfonyl fluoride, although it was not affected by proteasome (MG132), Cys protease (E64), or metallopeptidase (EDTA and 1,10-phenanthroline) inhibitors (Table 3). Aminopeptidase (bestatin) and Asp protease (pepstatin) inhibitors did not have any effect on this activity either (Table 3). These results are in accordance with previous descriptions of the *Arabidopsis* TPPII protease (30) and confirm the assumption that the AAF-AMC-like activity measured in these pools was due to TPPII.

## Proteasome Pathway Activation in Response to Cadmium

**TABLE 4**

**Protein and carbonyl contents and global endopeptidase activities in the leaves of *Arabidopsis* plants exposed to 50  $\mu\text{M}$  Cd for 24, 48, and 144 h**

Data are the mean  $\pm$  S.D. of six (protein), two (carbonyl), and three (endopeptidase) independent experiments.

Cadmium concentration ( $\mu\text{M}$ )	24 h		48 h		144 h	
	0	50	0	50	0	50
Protein content (mg g <sup>-1</sup> FW)	19.9 $\pm$ 2.5	21.1 $\pm$ 2.8	18.8 $\pm$ 1.9	18.8 $\pm$ 1.8	20.0 $\pm$ 3.5	19.1 $\pm$ 2.6
Carbonyl content (nmol mg <sup>-1</sup> protein)	3.04 $\pm$ 0.14	2.73 $\pm$ 0.07	2.83 $\pm$ 0.23	3.56 $\pm$ 0.07 <sup>a</sup>	2.94 $\pm$ 0.38	5.57 $\pm$ 1.04 <sup>a</sup>
Endopeptidase activity (pg of azocasein min <sup>-1</sup> mg <sup>-1</sup> FW)	5.3 $\pm$ 0.3	5.4 $\pm$ 0.5	4.8 $\pm$ 0.5	6.0 $\pm$ 1.1	5.3 $\pm$ 0.4	8.7 $\pm$ 0.9 <sup>b</sup>

<sup>a</sup> Significant difference between Cd-treated and control plants is  $<0.05$  by Student's *t* test.

<sup>b</sup> Significant difference between Cd-treated and control plants is  $<0.01$  by Student's *t* test.

The TOP- and LAP-like activities corresponding to proteins ranging from 75 to 140 kDa were pooled (Fig. 1A, *P2 pool*) and submitted to an anion-exchange chromatography step (Fig. 1C, *Mono Q2*). TOP activity was eluted with 135 mM NaCl. The corresponding fractions were pooled (P4) and submitted to SDS-PAGE (Fig. 1C). Subsequent proteomics analysis allowed the identification of two metallopeptidases, TOP (At5g10540) and TOPorg (At5g65620) (Table 2), which are annotated in the TAIR data base as M3/thimet oligopeptidase family proteins. In the *Arabidopsis* genome, another gene (At1g67690) presenting sequence similarities to animal thimet oligopeptidase family is annotated M3 peptidase similar to the cytosolic neurolysin, and named TOP-like (TOPL) in this work. Among the three M3/thimet oligopeptidase family proteins identified in *A. thaliana*, two are classified as cytosolic peptidases (TOP and TOPL), and the third one (TOPorg), presenting a transit peptide, is thought to have a mitochondrial or chloroplastic localization. The absence of TOPL peptides in the proteomic analysis (Table 2) suggests that TOPL is probably less abundant than TOP in the cytosol. The relative abundance of TOP and TOPorg compared with TOPL was also correlated with the levels of these RNAs in *Arabidopsis* plants. Compared with TOP and TOPorg RNAs, five additional PCR cycles were necessary to detect a TOPL RNAs using RT-PCR gene-specific primers (supplemental Fig. S1). This is a likely explanation of why no peptide was found to match with the product of the TOPL gene when the proteins of the Mono Q2 (Fig. 1C) were analyzed by mass spectrometry.

The sensitivity of TOP-like activity to a set of protease inhibitors (Table 3) confirmed the expectation, based on sequence comparisons, that these proteases are members of the TOP family. TOP-like activity was inhibited by the metalloprotease inhibitors (EDTA and 1,10-phenanthroline) (Table 3) suggesting they belong to this family. Moreover, the use of the TOP selective inhibitor, carboxy-3-phenylpropyl-AAF-polyclonal antibody (14), in the peptidase assay led to an 80% inhibition of the activity. Taken together, these results strongly suggest that the protease activity was effectively due to TOP enzyme. In addition, we were able to demonstrate that TOP is activated by MnCl<sub>2</sub> (4.4-fold) and CoCl<sub>2</sub> (2.2-fold), but 98% is inhibited by ZnCl<sub>2</sub> (Table 3). Moreover, the inhibition of the TOP activity by EDTA was reversed and stimulated by the addition of MnCl<sub>2</sub>, but not by MgCl<sub>2</sub> or ZnCl<sub>2</sub>. This suggests that the *Arabidopsis* TOP could be a manganese-dependent metalloprotease.

As carried out for TOP, the MS characterization of LAP was investigated in the 80–100-kDa protein fractions exhibiting LAP-like activity (Fig. 1A, *P2 pool*). These fractions were first

loaded on a Mono Q column. Proteins exhibiting LAP-like activity were eluted from the Mono Q2 around 120 mM NaCl (Fig. 1C). The most active fractions were pooled (P3 pool) and subjected to SDS-PAGE fractionation. Mass spectrometry analysis allowed the identification of leucine aminopeptidase 1 (LAP1, At2g24200, see Table 2), corresponding to a 320–360-kDa hexameric enzyme (43, 45). AtLAP1 is cytosolic in location and is similar to the mammalian LAP that processes peptides from the proteasome. The co-localization of LAP activity and LAP1 peptides in the 80–100-kDa fractions was perplexing. Previous studies with the tomato LAP-A1 indicated that its hexameric structure was critical for LAP activity (46). Therefore, it was possible that the inactive LAP1 dimers were present in the 80–100-kDa fraction, and the Leu-*p*NA hydrolyzing activity (LAP-like activity) that was detected represented a novel aminopeptidase activity. Many different aminopeptidases are known to hydrolyze LAP substrates (32, 47). Given the possible complexity of Leu-*p*NA-hydrolyzing proteins in the leaf extracts, the use of aminopeptidase inhibitors with nonpurified protein fractions would not be useful. Therefore, LAP activity was determined in desalted leaf extracts in the presence or absence of anti-LAP antibodies. As reported in Table 3, LAP activity was found to represent 17% of the total Leu-*p*NA degrading activity in *Arabidopsis* leaves. This means that the major part of the Leu-*p*NA degrading activity is due to one or several nonhexameric LAPs. In *A. thaliana*, two other hexameric LAPs exist, LAP2 (At4g30920) and LAP3 (At4g30910), both predicted to be chloroplastic. On the basis of PCR analysis, LAP1 is the most abundantly expressed hexameric LAP in *Arabidopsis* leaves (supplemental Fig. S1), which may explain why only LAP1 was detected after MS analysis.

*Cadmium Treatment Increases Protein Oxidation and Up-regulates the 20 S Proteasome Pathway in the Leaves of A. thaliana*—As it has been hypothesized that the 20 S proteasome system in mammals could be involved in the degradation of oxidized proteins (8), the response of the proteasome pathway in response to cadmium stress was investigated. In addition, the capacity of the 20 S proteasome to degrade oxidized proteins was also determined.

To assess the impact of cadmium treatments on the proteasome pathway, *A. thaliana* plants were grown hydroponically and submitted to a 50  $\mu\text{M}$  cadmium treatment. The leaves of 24-, 48-, and 144-h cadmium-treated and control plants were analyzed for protein content and oxidation level, as well as for proteolytic activities. No change in total protein content was observed in cadmium-treated leaves compared with controls (Table 4). However, the increase in carbonyl groups, measured



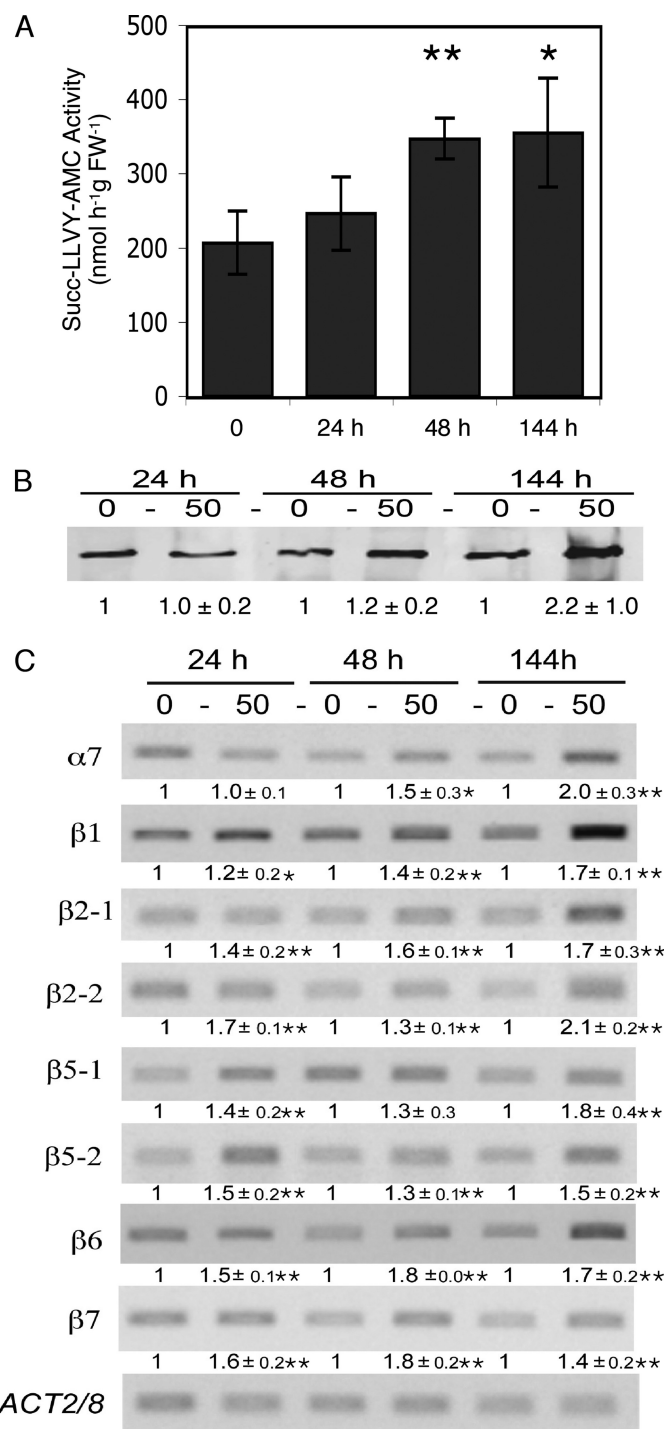
after derivatization with DNPH, indicates that the protein oxidation state increased after 48 and 144 h of treatment with cadmium (Table 4). The total endopeptidase activities in leaf protein extracts also increased after 144 h of 50  $\mu\text{M}$  cadmium addition (Table 4).

The 20 S proteasome activity was monitored during cadmium stress in the leaves of *Arabidopsis* plants harvested after 24, 48, or 144 h (6 days) of treatment. The chymotrypsin activity measured in the P1 pool after Sephacryl S-300 (Fig. 1A) was used to follow the 20 S proteasome activity. Our data indicated a 1.7-fold increase of the chymotrypsin activity of the 20 S proteasome after 48 and 144 h (Fig. 2A). Protein blot analysis also revealed an increase in 20 S proteasome content after 144 h of treatment (Fig. 2B).

To determine whether the increase of the 20 S proteasome activity in response to cadmium treatment was correlated with changes in proteasome subunit RNA levels, semi-quantitative RT-PCR experiments were performed. Specific primers were designed to monitor the RNAs of six proteasome subunits (Table 1). We chose three structural monogenic subunits as follows:  $\alpha 7$  (PAG1, At2g27020),  $\beta 6$  (PBF1, At3g60820), and  $\beta 7$  (PBG1, At1g56450) and the three catalytic subunits  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$ , respectively, encoded by either one gene,  $\beta 1$  (PBA1, At4g31300), or two genes,  $\beta 21$  (PBB1, At3g27430) and  $\beta 22$  (PBB2, At5g40580) and  $\beta 51$  (PBE1, At1g13060) and  $\beta 52$  (PBE2, At3g26340). The results, presented in Fig. 2C, showed an increase in transcript level for all the subunits analyzed from 24 h, except for  $\alpha 7$ , whose level increased only after 48 h. After 144 h, all the subunit transcripts presented a 1.4–2.1-fold increase. These data clearly indicate that RNAs encoding subunits of the 20 S proteasome were up-regulated in response to cadmium.

The expression and activity levels of TPPII, TOP, and LAP in response to cadmium treatment were also analyzed in *Arabidopsis* leaves. Specific primers were designed for each peptidase (Table 1), and the transcript analyses were performed using semi-quantitative RT-PCR (Fig. 3B). As for the 20 S proteasome, the TPPII activity from control and cadmium-treated leaves was measured in the P1 pool after the Sephacryl S-300 chromatographic step. From 24 h of treatment, TPPII presented a gradual increase in activity, reaching a 1.8-fold increase after 144 h (Fig. 3A). A similar increase was detected for its transcript level (At4g20850) (Fig. 3B).

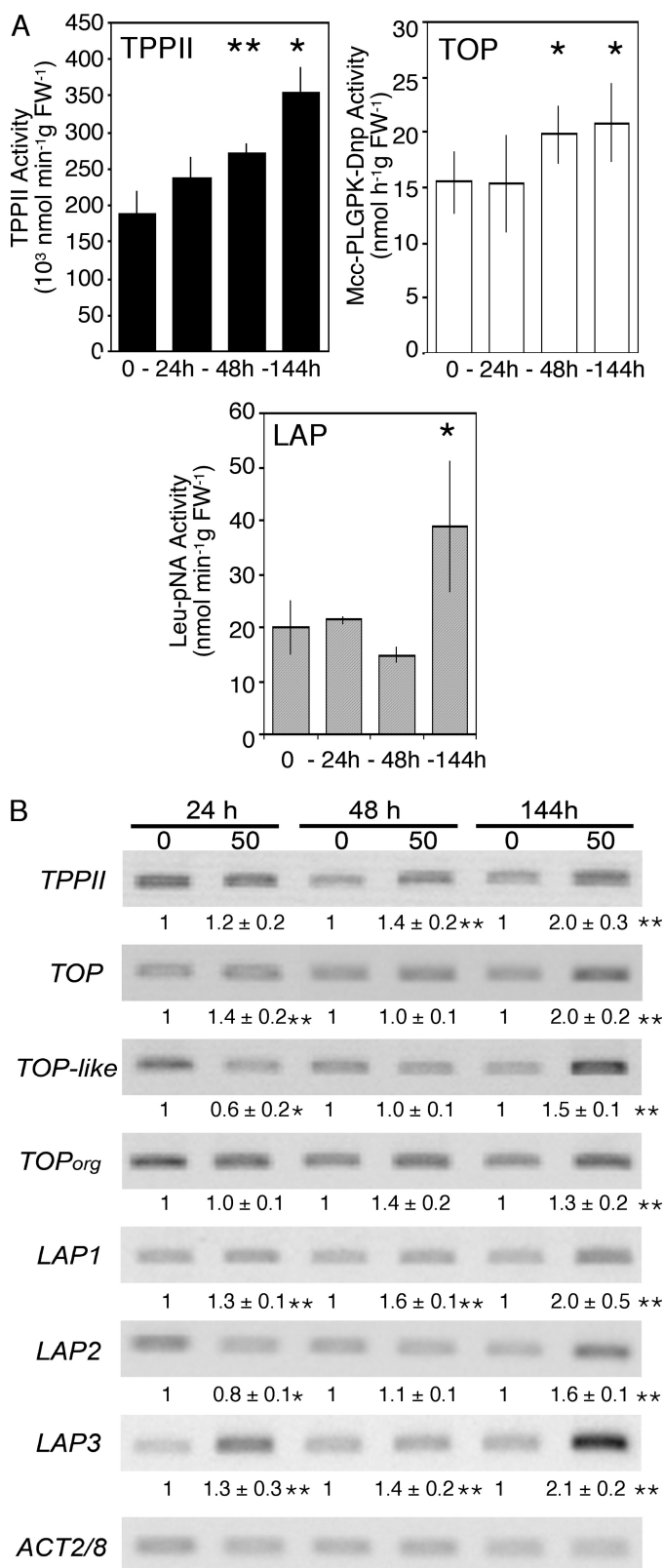
TOP activity from control and cadmium-treated leaves was measured in the P2 pool after the Sephacryl S-300 chromatographic step. It presented a moderate increase (1.3-fold) only after 48 h. This increase was maintained up to 144 h (Fig. 3A). In semi-quantitative RT-PCR experiments, the transcript level of the three *Arabidopsis* thimet oligopeptidases, TOP (At5g10540), TOPL (At1g67690), TOPorg (At5g65620), was monitored. The most abundant cytosolic thimet oligopeptidase isoform, TOP, was induced after 144 h (with 1.4- and 2.0-fold augmentations, respectively), whereas the minor isoform, TOPL, first decreased at 24 h and then increased to reach 1.5 times the control level after 144 h (Fig. 3B). The transcript level of the organelle specific isoform, TOPorg, was slightly affected by cadmium treatment (Fig. 3B). It should be noted that the final increase of TOP activity measured may thus be overestimated because it corresponds to the activity of a mix of TOP, TOPL, and TOPorg.



**FIGURE 2. Changes in 20 S proteasome activity, amount, and transcript levels in the leaves of *Arabidopsis* plants grown for 24, 48, and 144 h in the presence of 50  $\mu\text{M}$  cadmium.** 20 S proteasome chymotrypsin-like activity (A), amount (B), and subunit transcript levels measured by semi-quantitative RT-PCR (C). Western blot (B) and luminescent (C) signals were quantified using the Quantity One software (Bio-Rad). Values represent the relative intensity of each signal normalized to control (0 cadmium) in B and to ACT 2/8 signal in C. Data represent the mean of three (activity), six (Western blot) and three (transcript) biological replicates. Asterisks indicate significant differences between cadmium-treated and control plants (\*\*, <0.01; \*, <0.05, Student's *t* test).

The activity of the hexameric LAP was measured in the desalted clarified extracts of control and cadmium-treated plants after immunoprecipitation with anti-LAP antibodies. In these extracts, LAP antibodies were found to precipitate only

## Proteasome Pathway Activation in Response to Cadmium



**FIGURE 3. Changes in activity, amount, and transcript level of various proteases in the leaves of *Arabidopsis* plants grown for 24, 48, and 144 h in the presence of 50  $\mu\text{M}$  cadmium.** A, activity. TPP II and TOP + TOPL activities are expressed in  $\text{nmol h}^{-1} \text{g FW}^{-1}$ ; LAP activities are expressed in  $\text{nmol min}^{-1} \text{g FW}^{-1}$ . Data represent the mean  $\pm$  S.D. of three independent experiments. B, transcript levels measured by semi-quantitative RT-PCR. Luminescent signals were quantified using the Quantity One software (Bio-Rad). Values represent the relative intensity of each signal normalized to control

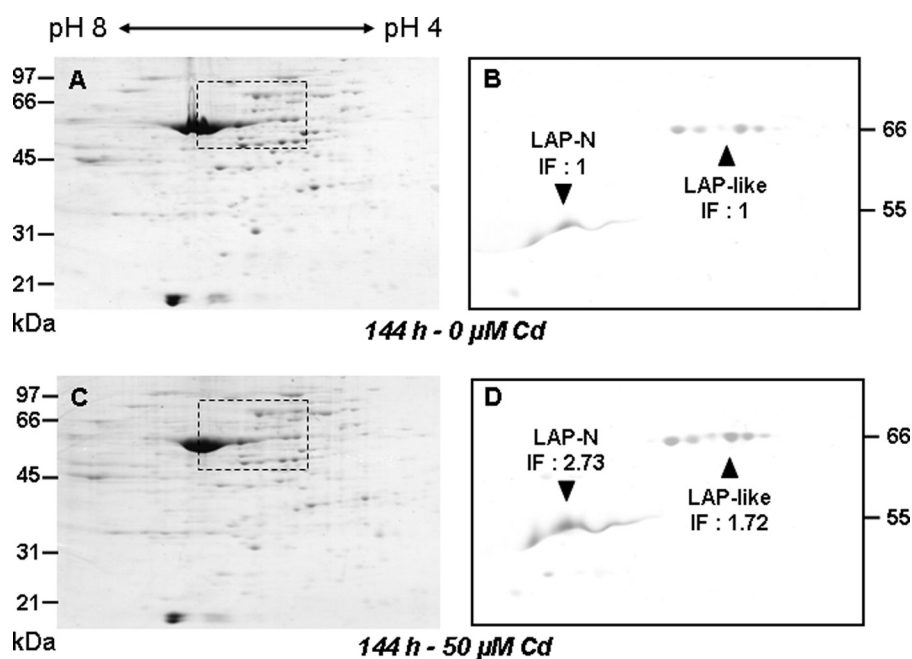
11–18% of the total Leu-pNA degrading activity, which means that hexameric LAPs are not the major pNA-degrading peptidases in cadmium-treated as well as in control *Arabidopsis* leaf extracts. LAP activity did not change during the first 48 h of treatment and increased by a factor of 1.8 only after 144 h (Fig. 3A). This increase in activity was correlated with an increase in LAP amount. Indeed, immunoblot analysis of LAP proteins after two-dimensional PAGE showed that LAP and 66-kDa LAP-like proteins increased 2.7- and 1.7-fold, respectively, in 144-h cadmium-treated leaves compared with the untreated control leaves (Fig. 4). Among the three LAP genes (*LAP1*-At2g24200, *LAP2*-At4g30920, and *LAP3*-At4g30910), transcript levels were found to increase after 24 h of treatment for *LAP1* and *LAP3* (Fig. 3B), but the three genes presented a similar increase in transcript level after 144 h (2.0-, 1.6-, and 2.1-fold for *LAP1*, *LAP2*, and *LAP3*, respectively).

It is noteworthy that all the different proteases of the 20 S proteasome pathway, including the 20 S proteasome, exhibited a similar increase in activity after 144 h (*i.e.* 1.3–1.8-fold increases). Transcript levels of the different proteases thought to belong to this pathway also increased in a proportional manner. These data indicate that the peptidases of the proteasome pathway are regulated at multiple levels, including transcript and protein level in response to cadmium, and suggest that the proteasome proteolytic pathway may be involved in the degradation of oxidized protein generated by the cadmium treatment.

**Degradation of Oxidized Proteins by 20 S Proteasome and TPP II**—Considering the increase in oxidized proteins in cadmium-treated *Arabidopsis* leaves (Table 4), and the induction of the different peptidases of the proteasome pathway (Figs. 2 and 3), we sought to determine whether oxidized proteins were preferential substrates for the 20 S proteasome pathway of *A. thaliana*. To test this hypothesis, BSA and lysozyme, two globular proteins whose structure can be significantly modified by oxidative treatment, were used as substrates.  $\alpha$ -Casein, a linear protein, served as a control. These proteins were submitted to oxidative treatments and used as substrates for degradation by the 20 S proteasome and TPP II. The three proteins were first subjected to a mild metal-catalyzed oxidative treatment (in the presence of 50 mM ascorbate and 200  $\mu\text{M}$   $\text{FeSO}_4$ ). Their oxidation state was checked using anti-DNP antibodies after derivatization of the carbonyl groups with DNPH (supplemental Fig. S2A). Oxidized and nonoxidized proteins were then submitted to a reductive methylation (using formaldehyde and sodium cyanoborohydride) to block primary amine groups. In these conditions, the amount of primary amines, quantified by fluorometric assays using fluorescamine (a primary amine group reagent), was reduced by a factor of 80–100 (supplemental Fig. S2B).

Methylated BSA, lysozyme, and  $\alpha$ -casein were used as substrates for degradation by the 20 S proteasome and TPP II (P1 pool issued from the Sephacryl S-300 step, Fig. 1A). The degradation products of oxidized and nonoxidized proteins were

(0 cadmium) and to ACT 2/8 signal. Data represent the means  $\pm$  S.D. of three independent biological replicates. Asterisks indicate significant differences between cadmium-treated and control plants (\*\*,  $<0.01$ , and \*,  $<0.05$ , Student's *t* test).



**FIGURE 4. Accumulation of LAP-A and 66-kDa LAP-like proteins in the leaves of *Arabidopsis* plants grown for 144 h in the presence of 50  $\mu\text{M}$  cadmium.** Total proteins (80  $\mu\text{g}$ ) were fractionated by two-dimensional PAGE and visualized by Coomassie Blue R-250 staining for protein loading control (A and C) or electroblotted and incubated with a 1:5000 (w/v) dilution of polyclonal LAP-A antiserum (B and D). LAP-A ( $\blacktriangledown$ ) and LAP-like ( $\blacktriangle$ ) proteins masses are shown (in kDa). The pH range of the isoelectric focusing gels and the protein size markers are mentioned. Immunoblots B and D correspond to the dashed rectangles in Coomassie-stained gels A and C, respectively. Western blot signals were quantified using the Quantity One software (Bio-Rad). The increase factor (IF) in protein amounts in cadmium-treated versus control plants is indicated below each protein name.

quantified using fluorescamine. The data in Fig. 5A show the ratio of oxidized versus nonoxidized degraded substrate for each protein. BSA and lysozyme, but not  $\alpha$ -casein, were better degraded under their oxidized form (1.5- and 1.3-fold, respectively). As expected, no enhanced degradation of oxidized  $\alpha$ -casein was observed consistent with the facts that  $\alpha$ -casein is a linear protein, whose structure is not further denatured by oxidation (5).

To assess the contributions of the proteasome and TPPII to oxidized protein degradation, the degradation of BSA was tested in the presence or absence of proteasome and TPPII inhibitors. In the presence of either 10  $\mu\text{M}$  lactacystin or 20  $\mu\text{M}$  butabindide, the degradation of oxidized BSA was inhibited by 46 and 33%, respectively (Fig. 5B). These data indicated that both the 20 S proteasome and TPPII were able to degrade oxidized BSA. With both inhibitors, BSA degradation was almost completely inhibited (88%) (Fig. 5B). Similar results were obtained using lysozyme as substrate (data not shown). Based on inhibitor efficiency (Table 3), these data show that, in our assay, the degradation of BSA and lysozyme was fully due to the activity of the 20 S proteasome and TPPII.

## DISCUSSION

**Proteasome Proteolytic Pathway Occurs in *A. thaliana***—The proteasome pathway, as described in the cytosol of mammalian cells, may be defined by the more or less sequential action of the proteasome, TPPII, TOP, and LAPs (15). In this study, we first report the occurrence and the identification of the different proteases involved in the proteasome proteolytic pathway in

the model plant *A. thaliana*. This characterization was done using classical enzymatic activity measurements with the help of specific inhibitors and effectors, by a proteomics approach, and by measuring gene expression at the transcript level. Besides the 20 S proteasome, which was previously identified in different plant species (19, 20), this work provides new insights into the functioning of peptidases previously identified in *Arabidopsis*-like TPPII (30) and LAP (48). In addition, we report the occurrence of proteases that have not been described in the plant kingdom until now, like TOP.

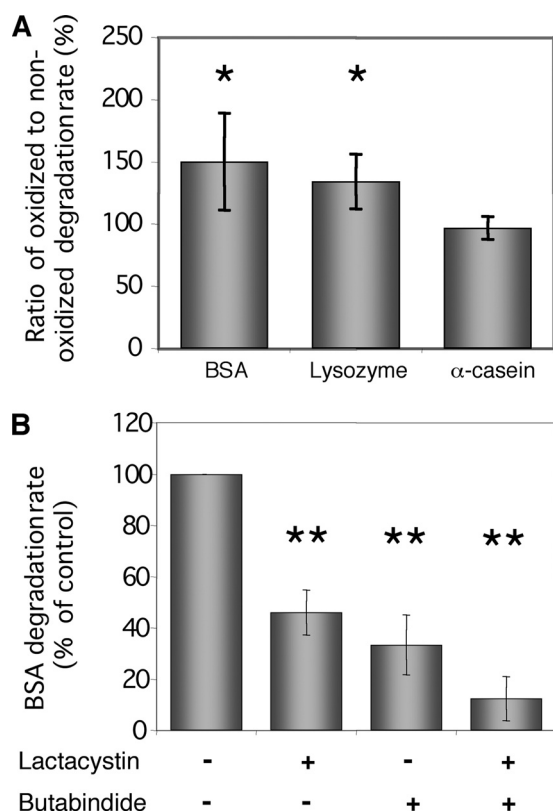
In animals, TPPII is the main exopeptidase capable of processing large proteasome products. Although not essential for the generation of antigenic peptides in antigen processing, it was supposed to be necessary for efficient protein turnover (49, 50). This protease was recently purified from *Arabidopsis* seedlings and characterized (30). In this study, we confirmed the presence of TPPII in the leaves of *Arabi-*

*dopsis* plants and demonstrated its role in the catabolism of oxidatively damaged proteins.

In mammals, TOP appears among the most important elements of the proteasome proteolytic pathway (14). This peptidase, which belongs to the peptidase M3 family, is described as a zinc-dependent metallopeptidase and preferentially cleaves peptides 5–17 amino acids in length (14, 51). In *A. thaliana*, genome analysis reveals the existence of a small multigenic family composed of three genes encoding proteins similar to the mammalian TOP family. The proteins encoded by At5g10540 (TOP) and At5g65620 (TOPorg) are more similar to the mouse TOP (EC 3.4.24.15) (28% amino acids sequence identities), whereas TOPL (At1g67690) is more similar to the mouse neurolysin (EC 3.4.24.16) (34% amino acid sequence identity), a closely related M3 oligopeptidase. TOPorg is expressed as a preprotein with a transit peptide, which predicted a chloroplastic location (Predotar algorithm). S-300 Sephacryl fractionation of leaf crude extracts, activity characterization, and mass spectrometry analyses confirmed that *Arabidopsis* TOP and TOPorg are expressed as monomers of  $\sim$ 80 kDa in healthy *Arabidopsis* leaves. The analysis of the influence of a set of compounds on TOP activity indicates that these proteases present similar inhibitor susceptibilities to mammalian TOPs.

In the proteasome pathway described in animal cells, aminopeptidases complete the total degradation of peptides into free amino acids. The mammalian LAP is involved in this process (14, 17). In plants, LAPs have been postulated to have functions in the salvage of C and N resources from the cotyledons of

## Proteasome Pathway Activation in Response to Cadmium



**FIGURE 5. Degradation of oxidized protein by 20 S proteasome/TPPII.** A, degradation rate of oxidized proteins are normalized to the degradation rate of nonoxidized proteins. The degradation rate of BSA, lysozyme, and  $\alpha$ -casein was determined by fluorescent assays using fluorescamine as described under "Experimental Procedures." Data represent the means  $\pm$  S.D. of three independent biological replicates. B, effect of 20 S proteasome (10  $\mu$ M lactacystin) and TPPII (20  $\mu$ M butabindide) inhibitors on the degradation rate of BSA. Asterisks indicate significant differences between treated and control assays (\*\*,  $<0.01$ , and \*,  $<0.05$ , Student's *t* test).

germinating seedlings, in the response to biotic and abiotic stresses, or for LAP-A to regulate jasmonic acid-mediated wound signaling (reviewed in Refs. 32, 44, 52). Our data show that although LAP1 subunits are detected in the Leu-*p*NA-hydrolyzing fraction, the hexameric LAP1 is not the dominant *p*NA-degrading peptidase in the cytosol and that other Leu-*p*NA-hydrolyzing enzymes are important in *Arabidopsis* leaves. Other candidate enzymes include three aminopeptidases of *Arabidopsis* encoded by M1-like peptidase genes (44), which are often referred to as LAPs in animals (31). Two of these M1-like peptidases have been characterized (53, 54), but peptides corresponding to these genes were not detected in the Leu-*p*NA fraction. Therefore, at present, there is no evidence supporting the role of the M1 family of peptidases in the proteasome degradative pathway. In the genome of *A. thaliana*, three genes encode leucine aminopeptidases, LAP1 (At2g24200), LAP2 (At4g30920), and LAP3 (At4g30910), which present 70–80% amino acid sequence identity with each other. Using gene-specific primers, we detected the transcripts of the three genes in the leaves of *Arabidopsis* (supplemental Fig. S1). Furthermore, LAP proteins were detected in healthy leaves after two-dimensional PAGE and immunoblot analysis with anti-LAP antibodies as reported previously (31).

**Proteasome Proteolytic Pathway Is Responsive to Cadmium Stress**—As reported in other plants species (25–27, 55–58), this study show that cadmium treatment causes an increase in oxidized proteins and endopeptidase activities in *Arabidopsis* leaves (Table 4). Recently, 20 S proteasome has been shown to be up-regulated in response to cadmium in sunflower, maize, and tomato leaves, as well as in *Arabidopsis* cells (25, 26, 28), suggesting that it could be involved in the degradation of oxidized proteins generated by cadmium-induced oxidative stress.

The data reported here demonstrate that the expression and activity of several proteasome pathway peptidases are up-regulated by cadmium stress. For example, 50  $\mu$ M cadmium treatment led to an increase of transcripts level of three structural and three catalytic proteasome subunits, TPPII, TOP, TOPL, and LAP1–3 (Figs. 2 and 3). It also triggered a final 30–90% increase in the activity of the main elements of the pathway, *i.e.* 20 S proteasome, TPPII, TOP, and LAP-like peptidase. The increases in proteasome structural and catalytic subunit transcripts, followed by increases in proteasome quantity and chymotrypsin-like activity, are in good agreement with studies reporting that, in mammalian cells, the overexpression of one subunit of the 20 S proteasome leads to an up-regulation of the other subunits, an increase in proteasome content, and an enhancement of proteasome activities (59–61). The analysis of the regulation of these proteasome pathway-related peptidases was based on the similarity of the proteasome pathway between mammalian and plant systems. However, it does not exclude *a priori* the potential involvement in this pathway of other peptidases of which expression and activity were not taken into account in this study (for instance, nonhexameric LAPs).

The relatively low induction factor of the proteasome pathway in response to cadmium may give some doubt about the significance of this response. However, it must be kept in mind that in plants up to 50% of the total proteins are replaced every week (62) and that 26 S/20 S proteasome-dependent proteolysis accounts for the major part of protein turnover (20). Thus, even moderate changes in this central pathway (a 1.3–1.6-fold increase in the proteolytic capacity) are highly significant for cellular metabolism.

The role of the 20 S and 26 S proteasome in the response to oxidative stress and degradation of oxidized proteins appears to be complex as revealed from studies in both yeast and plants. In yeast, the degradation of abnormal proteins resulting from cadmium treatment was shown to be mediated, in part, by proteasome and up-regulation of genes coding for the limiting components of the ubiquitin pathway (63). Indeed, overexpression of a polyubiquitin gene conferred resistance to moderate oxidative stress in respiring yeast cells (64). In contrast, a recent study carried out with *Arabidopsis* showed that mutations of one of the subunits of the 19 S regulatory particle of the 26 S proteasome resulted in an enhanced accumulation of the 20 S versus 26 S proteasome and a higher tolerance to oxidative stress (29). Moreover, in contrast to the 26 S proteasome, the 20 S proteasome appears more resistant to oxidative stress as it maintains its activity even in the presence of H<sub>2</sub>O<sub>2</sub> (13). In addition, Shringarpure *et al.* (65) demonstrated that, *in vivo*, oxidized proteins are degraded without ubiquitin conjugation. These data strongly support the involvement of the 20 S rather

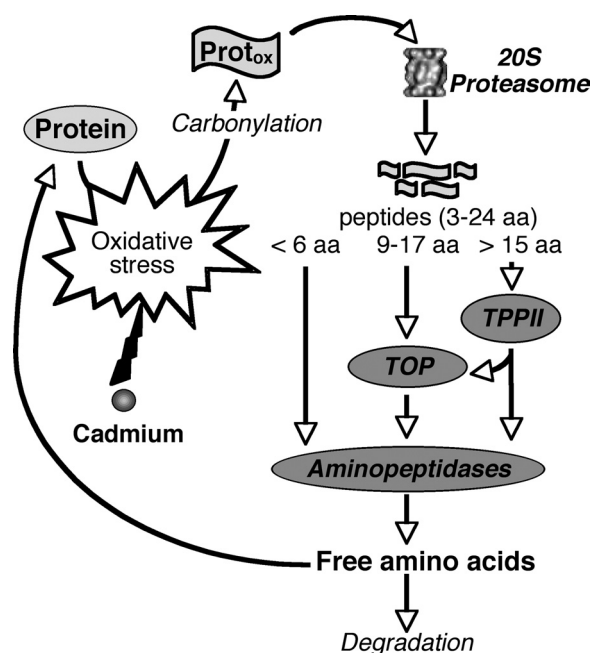


FIGURE 6. Putative function of the 20 S proteasome proteolytic pathway during cadmium and, more generally, oxidative stress. Results presented in this study strongly suggest that the 20 S proteasome, TPPII, TOP, and aminopeptidases participate in the degradation of oxidized proteins generated during stress to protect cells against toxicity. Free amino acids released from this pathway are used for the synthesis of new proteins, although oxidatively modified amino acids are degraded.

than the 26 S in the degradation of oxidized proteins, probably via a by default degradation mechanism (9).

This study indicated that the 20 S proteasome pathway is up-regulated at RNA and activity levels in response to cadmium in *Arabidopsis* leaves. This suggests that it is involved in the response to oxidative stress through the sequential degradation of oxidized proteins by the 20 S proteasome, TPPII, TOP, and finally LAPs. This suggestion is supported by the assay of oxidized versus nonoxidized protein degradation by the 20 S proteasome and TPPII (Fig. 5). The better degradation of oxidized BSA and lysozyme by the 20 S proteasome/TPPII couple, without any requirement for ATP, confirms the preferential degradation of oxidized proteins by the 20 S proteasome. Previous works reported that the degradation of oxidized proteins by the 20 S proteasome occurs via the recognition of hydrophobic amino acid residues that are exposed during oxidative rearrangement of secondary and tertiary protein structure (5, 66, 67).  $\alpha$ -Casein, which is a structure-less protein, was used as a control for this and was found not to be better degraded when oxidized (Fig. 5). Therefore, these data further support the hypothesis that, in *A. thaliana*, the 20 S proteasome pathway takes part in the degradation of oxidized proteins resulting from a stress, such as cadmium stress.

In summary, this work, together with previous knowledge acquired in mammalian and plant areas, led us to specify the function of the 20 S proteasome pathway during cadmium stress and more generally during oxidative stress. The general scheme of protein oxidation and degradation processes is summarized in Fig. 6. As a consequence of various oxidative stresses, oxygen radicals and activated oxygen species cause modifications to amino acids of proteins that result in the loss

of protein function or enzymatic activity. To avoid the toxic effects of oxidized protein accumulation, the 20 S proteasome and the proteases acting downstream (TPPII, TOP, and aminopeptidases) sequentially degrade denatured proteins into small peptides and amino acids. The free amino acids produced during this process are then either used for the synthesis of new proteins or degraded when they are present in oxidized form. Thus, the induction of the proteasome proteolytic pathway during cadmium stress could be part of the general response mechanism of plants to cope with daily and naturally occurring oxidative stresses.

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**Evidence for the Existence in *Arabidopsis thaliana* of the Proteasome Proteolytic Pathway: ACTIVATION IN RESPONSE TO CADMIUM**

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