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**Heat stress reveals high molecular mass proteasomes in *Arabidopsis thaliana*
suspension cells cultures**

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Abstract

Because of their sessile nature, plants have evolved complex and robust mechanisms to respond to adverse environments. Stress conditions trigger an increase in protein turnover and degradation. Proteasomes are essential to the cell for removing, in a highly regulated manner, partially denatured or oxidized proteins thus minimizing their cytotoxicity. We observed that suspension cells of *Arabidopsis thaliana* treated with high temperature (37 °C) directed the assembly of high molecular mass proteasomes. The removal of a 75% of the original ubiquitin conjugates and the maintenance of protein carbonyls at basal levels correlated with a specific proteasome profiles. The profiles obtained by the separation of different proteasomes populations by Blue-Native Polyacrylamide Gel Electrophoresis and western blot analysis suggest that synthesis, assembly, and heavy ubiquitination of 20S (CP) subunits are promoted by heat stress.

Keywords: *Arabidopsis thaliana* suspension cell culture, Proteasome, Heat stress, Blue-Native gel electrophoresis

Abbreviations:

BN/PAGE Blue Native Polyacrylamide Gel Electrophoresis
SDS/PAGE SDS Polyacrylamide Gel Electrophoresis

1. Introduction

Plants undergo various stressful environments across their lifetimes, but their sessile nature means they cannot escape from unfavorable conditions. Plants have developed unique strategies for stress mitigation or adaptation to their surroundings. Since stress environments activate an increase in protein turnover and degradation, one strategy to cope with it is the selective protein breakdown mediated by the proteasome in the nucleus, cytosol and endoplasmic, reticulum which decreased their toxicity (Smalle and Vierstra, 2004), (Thompson and Vierstra, 2005). 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 the ubiquitination 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 (Davies, 2001).

Proteasomes are protein degradative complexes involved in all processes of the living cell such as cell division, stress response, transcription, DNA repair, and signal transduction, among others (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998). In plants, proteasomes have been particularly involved in the differentiation of leaves, flowers, and xylem, in hormone response, as well as abiotic and biotic stress responses (Shibahara et al., 2002). Proteomic analysis of different organism reported that an estimated 80 to 90% of the cytosolic proteins are degraded via proteasomes (Glickman and Ciechanover, 2002). The minimal expression of a proteasome is the 20S core particle, or catalytic particle (CP) constituted by four stacked seven-membered rings of β_{1-7} (central) and α_{1-7} (distal) subunits in an arrangement α -

76 β - β - α , for a total of 28 subunits. In this hollow-cylinder structure, the interior domains
77 of the subunits β 1, β 2 and β 5 define catalytic domains that have trypsin, chymotrypsin,
78 and caspase-like activities respectively (Kish-Trier and Hill, 2013). Virtually any
79 protein in direct contact with the proteasome catalytic sites could be degraded
80 (Nussbaum et al., 1998). The first line of proteasome regulation, that limits the
81 inappropriate and non-selective protein degradation, are the N-terminus regions of
82 α subunits that constitute the proteasome gate (Groll et al., 2000). This basic 20S (CP)
83 proteasome version is responsible for cell removal of oxidatively modified proteins
84 (Davies, 2001). The function and structure of the 20S (CP) is highly conserved through
85 eukarya (Tanaka et al., 1988; Fort et al., 2015). 20S α subunits are also the binding sites
86 for different regulatory complexes. The 26S proteasome for example, is formed by the
87 basic 20S (CP) flanked by one or two 19S regulatory complexes (19S-20S or 19S-20S-
88 19S) docked on the distal-most surfaces of the α rings. For *Saccharomyces cerevisiae*,
89 *Schizosaccharomyces pombe* and human cells, the architecture of the 19S regulatory
90 particle has been reported at sub-nanometer level and the functions of most of the
91 subunits has been assigned (Beck et al., 2012; Schweitze et al., 2016; Sakata et al.,
92 2012; Bohn et al., 2010). As for the independent 20S (CP), the structure and function of
93 the 19S regulatory complex is conserved through evolution (Fort et al., 2015). The 19S
94 complex is in charge of recognizing ubiquitinated proteins, denature them by the action
95 of ATP-dependent “unfoldases”, opens the 20S (CP) gate by rearrangement of the α
96 subunits, and translocate the protein substrate to the catalytic sites for its degradation
97 (Peth et al., 2010). The 26S is the specialized proteasome version involved -along with a
98 ubiquitin activating (E1), the ubiquitin conjugating (E2) and the ubiquitin ligases (E3)
99 enzymes- in the Ubiquitin-Proteasome-System (UPS) (Hershko and Ciechanover,
100 1998). Approximately 5% of the *A. thaliana* proteome is dedicated to this system or its

101 regulation (Manzano et al., 2008). A different proteasome regulatory particle PA28
 102 (11S, REG), is a trimeric complex that degrades carbonylated proteins in an ATP
 103 independent manner (Hernebring et al., 2013). PA200 (in mammals) and the
 104 homologous Blm10 (in yeast), are alternative regulatory complexes which had been
 105 involved in the degradation of very specific non-ubiquitinated protein substrates (López
 106 et al., 2011; Blickwedehl et al., 2012). Ecm29 (in a human cell line) has been proposed
 107 to act as a structural stabilizing agent for the 26S proteasome, especially when the 20S
 108 (CP) maturation was impaired (Lehmann et al., 2010). Information on different
 109 proteasome regulators published so far has shown that the binding of the regulatory
 110 complexes has a deep influence in the selection of substrates, in the catalytic function of
 111 the 20S (CP) and in the peptides produced. In this scenario, the presence of “supra-20S”
 112 complexes allow the cell to respond to very specific metabolic stages. The active
 113 building of proteasomal complexes has a counterpart, the 26S proteasomes in yeast
 114 were disarmed into independent 19S and 20S (CP) particles during the stationary phase
 115 and this phenomenon correlated with cell viability. When ATP was available by
 116 medium refreshing, the 26S proteasomes were reconstituted (Bajorek et al., 2003).
 117 Dissociation and reassociation of the 26S proteasome have been reported during the
 118 adaptation of a human cell line to oxidative stress (Grune et al., 2011). Reversible 26S
 119 disassembly has been reported upon mitochondrial stress in yeast (Livnat-Levanon et
 120 al., 2014). Considering the assembly/disassembly capacities of the proteasomes and the
 121 repertoire of regulatory complexes, any cell in a specific environmental context, have
 122 the possibilities to “direct” the versions of the proteasome that respond better to the
 123 catalytic needs of the moment, and the proteasome versions selected would have an
 124 influence on cell fate. This information indicates that a very dynamic process of

125 assembly, selection of regulatory particles, and disassembly of proteasomes are
 126 continuously taking place in cells in close relationship with the environment.
 127 Great efforts have been made to characterize biochemically, genetically and by mixed
 128 “omics” techniques, the subunit composition and function of the proteasomes in plants.
 129 In *A. thaliana* particularly, their 20S (CP) and 26S proteasomes have been isolated and
 130 characterized (Polge et al., 2009; Yang et al., 2004; Book et al., 2010). Unique genes
 131 code for the 20S (CP) and the 19S subunits in yeast and mammalian cells, in *A. thaliana*
 132 instead, both complexes are encoded by gene pairs whose sequence differences, suggest
 133 protein products with altered functions (Yang et al., 2004), so potentially different 20S
 134 (CP), 26S particles and probably other proteasomes entities assembled with paralogous
 135 pairs coexist. By proteomic approach using an epitope-tagged 26S proteasomes as bait,
 136 more than 40 proteins interact with this complex. This in-depth mass spectrometric
 137 analysis strongly suggests the existence of a diverse array of proteasomes. Despite all
 138 the information on *A. thaliana* proteasomes, studies on the dynamics of the different
 139 proteasome versions in plants as has been reported for yeast (Bajorek et al., 2003;
 140 Livnat-Levanon et al., 2014) or mammalian cells (Grune et al., 2011; 34. Shibatani et
 141 al., 2006) are limited.

142 Blue Native Polyacrylamide Gel Electrophoresis (BN/PAGE) is a technique that
 143 allows the separation of native protein complexes based on their molecular mass
 144 differences (Wittig et al., 2006). BN/PAGE has been used for the complexomics
 145 analysis of different models (Wittig et al., 2006; Lasserre et al., 2006; Hashemi et al.,
 146 2016). This technique has been employed to successfully separate the different versions
 147 of proteasomes in whole cell lysates of a human embryonic cell line (HEK293)
 148 (Camacho-Carvajal et al., 2004) and to study the proteasome dynamics of rabbit
 149 reticulocytes (Shibatani et al., 2006). In the latter report, six native proteasome

150 populations (20S, 20S-PA28, PA28-20S-PA28, 19S-20S-PA28, 19S-20S and 19S-20S-
151 19S) were identified. By γ -interferon stimulation or the chemical inhibition of the
152 proteasome, an active interchange of proteasome regulatory “caps” was evidenced
153 (Shibatani et al., 2006). The applicability of BN/PAGE in combination with label-free
154 protein quantification and protein correlation profiling was employed to investigate the
155 20S proteasome from *Plasmodium falciparum*, even in the background of the whole
156 protein extract (Sessler et al., 2012). BN/PAGE was used for monitoring changes in the
157 quantity and subunit composition of the 20S (CP) when the $\alpha 3$ subunit was deleted in
158 yeast (Couttas et al., 2011).

159 In the present work, we adapted some of the above-mentioned protocols of
160 proteasome isolation and analysis by BN/PAGE to establish whether in *A. thaliana* cells
161 in suspension culture different proteasome versions coexist and if under drastic changes
162 in the culture conditions like heat stress, the basal proteasome populations were altered.
163 Our results showed that cells in suspension culture subjected to an increment of
164 temperature (from 25 to 37° C) experienced an enrichment of high molecular mass
165 proteasomes that in turn have an impact on the cell content of oxidized and
166 ubiquitinated proteins.

167

170 **2. Methods**

171

172 **2.1 Cell culture and heat stress treatment**

173

174 Suspension cell cultures were generated from hypocotyls dissected from *A. thaliana*
175 seedlings and were kindly provided by P. Guzmán and L. Aguilar (CINVESTAV,
176 Irapuato). Cells were maintained by weekly transfer in MS medium (Murashige and

177 Skoog, 1962) containing basal salt mixture, 3% sucrose and supplemented with 50 μg
 178 /L kinetin, 75 $\mu\text{g/L}$ 2,4-dichlorophenoxyacetic acid (2-4D) and 1X Gamborg's vitamin
 179 solution, pH 5.7. Cultures were incubated at 25 °C and 100 rpm under long day
 180 conditions of 16 h light/ 8 h dark, and 80 μM photons $\text{m}^{-2} \text{s}^{-1}$. For heat stress treatment,
 181 a one-week "mother culture" of exponentially growing cells was diluted (1:10) with
 182 fresh MS medium (plus supplements) and divided into independent 250 mL flasks
 183 containing 50 mL liquid medium. Cell cultures were incubated at 37 °C at 100 rpm for
 184 0.5, 1, 2 and 3 h (illuminated), then individual cultures were immediately filtered using
 185 regular paper towels to discard liquid medium. Cell packages (approximately 10 mL)
 186 were recovered with a spatula and immediately frozen in liquid nitrogen. Samples were
 187 kept at -70 °C until their processing for proteasome isolation.

188

189 **2.2 Proteasomes isolation**

190

191 Total cell lysate was obtained by adding to each frozen cell package, 25 mL of
 192 extraction buffer (Tris-HCl pH 7.5, 1 mM dithiothreitol, 2 mM adenosine triphosphate,
 193 0.25M sucrose, 1 mM MgCl_2 , 1% polyvinylpyrrolidone, Complete EDTA-free
 194 [Roche, used as recommended]), and "10 mL" of glass beads (4 mm). While thawing,
 195 cells were disrupted by five vortex cycles (5 min vortexing followed by 5 min
 196 incubation in ice). At this stage, aliquots from different cultures were taken for the
 197 determination of the total content of ubiquitin conjugates and protein carbonyls by
 198 western blot (see below). Total lysate was filtered on three layers of cheesecloth to
 199 retire glass beads and was centrifuged at 16 000 X g for 15 min at 4 °C. Pellet (P1,
 200 Fig. 1) was eliminated and supernatant (Sn1, Fig. 1) was centrifuged for 1h at 70 000 X
 201 g at 4 °C. Resultant pellet (P2, Fig. 1) was discarded and supernatant (Sn2, Fig. 1) was

centrifuged again, this time at 350 000 X g for 3.5 h at 4 °C. The supernatant (Sn3, Fig. 1) was discarded, the pellet (P3, Fig.1) which contained the proteasomes enriched fraction, was resuspended in buffer A (HEPES buffer pH 7.8, 75 mM NaCl, 375 mM MgCl₂, 40 mM DTT, glycerol 7.5% y 1.6 μM ATP). Aliquots were immediately separated by BN-PAGE or kept at -70 °C until their analysis.

2.3 BN/PAGE

Resolution of proteasomes according to their molecular mass was achieved by BN/PAGE (Wittig et al., 2006), and optimized for proteasome analysis (Camacho-Carvajal et al., 2004; Shibatani et al., 2006) with some modifications. Proteasomes enriched fraction in buffer A was directly loaded onto an 8 X 6 cm BN/PAGE mini gels (5 to 10% acrylamide gradient [acrylamide:bis-acrylamide 32:1] in 50 mM BisTris/HCl, pH 7.0, 500 mM α-aminocaproic acid, and overlaid by 4% stacking gel in the same buffer). Electrophoresis was carried out at 5 °C according to the program: 50 V for 1h, 150 V for 16h and 500 V for 1h. Cathode buffer: 50 mM Tricine, 15 mM BisTris-HCl pH 7.0 and 0.02% Coomassie G-250 (Cat. 1442C-1, Research Organics, Inc.), anode buffer: 50 mM Bis-Tris-HCl pH 7.0 in a Mini-PROTEAN System (Bio-Rad). Proteins in analytical BN/PAGE were visualized with Coomassie Brilliant Blue (section 2.5) or by silver stain [40] (Blum et al., 1986). For preparative purposes, BN/PAGE gels were fractionated and electroeluted (next section). The molecular mass of the protein complexes was estimated by the method of Wittig et al., 2010, using the endogenous HSP 60, Rubisco (both proteins identified by mass spectrometry in the fraction 4 and 7 respectively), and the independent 20S (CP) native complexes as molecular mass markers.

227

228 **2.4 Protein electroelution and concentration**

229

230 After electrophoresis running completion, a glass plate of the gel “sandwich” was
 231 removed. As the cathode buffer contained Coomassie G-250, four protein bands were
 232 stained during electrophoresis (Fig. 2b) and used as markers to cut the gels into eight
 233 horizontal fractions (Fig. 2c). For the analysis of the disassembled proteasomes
 234 components by western blot, each fraction was individually divided into smaller
 235 fragments (~ 2 X 2 mm) and heated at 95 °C for 10 min in the presence of 1 mL of 1X
 236 Laemmli sample buffer (Tris 50 mM pH 6.8, 2% SDS, 5% β-ME, 8% glycerol without
 237 bromophenol blue). After cooling, gel pieces and sample buffer were transferred to the
 238 sample traps of the electroelutor/concentrator system “Little Blue Tank” (ISCO, INC)
 239 containing 10 mL of Laemmli running buffer (25 mM Tris, 192 mM glycine, 0.1%
 240 SDS) at a 1:10 dilution. Laemmli running (1X) buffer was used in both electrode
 241 compartments. Protein electroelution was carried out at 3 W for 3 h at 5 °C. Samples
 242 recovered from the sample traps (~ 200 μL) were precipitated by methanol/chloroform,
 243 air dried, resuspended and heated at 95 °C for 10 min in 1X Laemmli sample buffer for
 244 their SDS/PAGE and western blot analysis. A similar technique was followed to recover
 245 native proteasome complexes from BN/PAGE, except that gel fractions were not heated
 246 or incubated in the presence of chaotropic agents. For this purpose,
 247 electroelutor/concentrator sample traps contained 10 mL of running buffer (1:10) and
 248 1X running buffer was used in electrode compartments (without SDS). Samples
 249 recovered were immediately loaded or preserved at -70 °C after glycerol addition (20%
 250 final) for additional BN/PAGE analysis.

251

252 **2.5 SDS/PAGE**

253

254 We analyzed the protein profile of each electroeluted sample by SDS/PAGE according to
 255 the protocol of Laemmli, 1970. Samples in sample buffer were loaded onto 4% stacking
 256 gels and resolved in 12% polyacrylamide-SDS gels. Runs were performed using the
 257 Tris/glycine/SDS running buffer (see previous section) at 200 V for 1h at 5 °C in a Mini-
 258 PROTEAN system (Bio-Rad). Gels were stained in a solution of Coomassie Brilliant
 259 Blue R-250 (0.1%), acetic acid (40%) and ethanol (40%). For silver staining of proteins
 260 on BN/PAGE, we used the improved method of Blum et al., 1986. [41]. We employed
 261 Pink pre-stained ladder, 15-175 kDa (Cat. MWP02, Nippon Genetics) as molecular
 262 weight markers.

263

264 **2.6 Western blot and slot blot analysis**

265

266 Proteins separated by BN/PAGE or SDS/PAGE were electrophoretically transferred to
 267 nitrocellulose membranes (0.45 µM HATF, Millipore) using transfer buffer (25 mM
 268 Tris, 192 mM glycine, 20% isopropanol) in a Mini-PROTEAN (Bio-Rad) transfer
 269 system at 360 mA for 1h at 6 °C. Proteins on BN/PAGE were denatured previous to
 270 their blotting by incubating the gel in a solution of 20mM Tris-HCl buffer (pH 7.4), 3%
 271 SDS for 10 min with agitation followed by heating in a microwave oven (medium level
 272 for 1 min). After an additional incubation for 10 min at room temperature, proteins were
 273 transferred as indicated. Proteins on membranes were fixed (25% isopropanol, 10%
 274 acetic acid) for 1h with agitation at room temperature. After distilled water wash,
 275 proteins were visualized with Ponceau S solution (0.2% Ponceau S in 5% acetic acid).
 276 For western blot assay, membranes were blocked 1h at 25 °C with 5% non-fat milk in

277 TBS-T buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 0.05% Tween-20) and incubated
 278 with primary or secondary antibodies in the solution of non-fat milk in TBS-T buffer.
 279 All intermediate washes were done with TBS-T. The following primary antibodies were
 280 used at the same 1:10 000 dilutions for 2 h at 25 °C: mouse-anti-proteasome 20S
 281 alpha+beta (Cat. ab22673, Abcam), rabbit-anti-proteasome 26S S2 (Rpn1) (Cat.
 282 ab98865, Abcam), rabbit-anti-proteasome Rpn6 (S9) (Cat. PW8370, Enzo), mouse-anti-
 283 Rpt2 (Cat. ab21882, Abcam), rabbit-anti-19S S5A/Rpn10 (Cat. ab56851, Abcam) and
 284 rabbit-anti-Ubiquitin antibody (Cat. sc-9133, Santa Cruz Biotechnology, Inc.). As
 285 secondary antibodies, we employed HRP-goat-anti-mouse IgG (H+L) (Cat. 62-6520,
 286 Zymed) or HRP-goat-anti-rabbit IgG (H+L) (Cat. 65-6120, Zymed) both at 1:10 000
 287 dilutions for 1 h at 25 °C. Western blots were developed with Super Signal West Femto
 288 (Cat. 34095, Thermo Scientific) and exposed to X-Ray films (Cat. 6040331, Kodak).
 289 Total carbonyl (Johansson et al., 2004) and ubiquitin conjugates (Tang et al., 2014)
 290 contents of heat stress and control samples, were estimated by slot blot analysis. For
 291 carbonyl content estimation, protein from total cell lysates (section 2.2) was precipitated
 292 with methanol/chloroform and resuspended in 1X Laemmli sample buffer. Five
 293 milligrams of each sample (determined by the method of Lowry, 1951 [44]) were
 294 derivatized with 2,4-dinitrophenylhydrazine (DNPH) and loaded on each well of the
 295 slot blot manifold (Cat. PR 648, Hoefer). Oxidatively modified proteins on
 296 nitrocellulose filters were determined by an immunochemical protocol (OxyBlot Protein
 297 Oxidation detection kit, from Chemicon International). Same slot blot technique was
 298 followed to determine ubiquitin conjugates, but samples were not derivatized.
 299 Quantification of western and slot blots was made by densitometry of the
 300 autoradiograms using NIH ImageJ 1.48 software. All data were standardized for growth
 301 in control conditions (wild type = 1).

302

303 **2.7 Protein quantification by a modified Lowry method**

304

305 We determined protein content in those samples that contained β -ME, by the Lowry
306 method according to the modification of Makkar *et al.*, 1980. Briefly, an aliquot of each
307 sample was vacuum dried to eliminate β -ME (from Laemmli sample buffer), which
308 interferes with the protein determination. Samples were resuspended with 0.1N NaOH
309 for its protein quantification by the classic Lowry method with Folin-Ciocalteu reagent
310 calibrated with crystalline bovine serum albumin.

311

312 **2.8 Mass spectrometry**

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314 For the mass spectrometric analysis, BN/PAGE slices were destained and chemically
315 modified prior to mass spectrometry analysis. After reduction (dithiothreitol) an
316 alkylation (iodoacetamide) samples were digested in-gel with trypsin (Promega,
317 Madison, WI, USA). Resultant peptides were desalted with Zip Tips C18 (Millipore-
318 Billerica, MA, USA) and applied to a LC-MS system (Liquid Chromatography-Mass
319 Spectrometry) composed by a nanoflow pump (EASY-nLC II, Thermo-Fisher Co. San
320 Jose, CA) and a LTQ-Orbitrap Velos (Thermo-Fisher Co., San Jose, CA) mass
321 spectrometer with a nano-electrospray ionization (ESI) source. The mass spectrometer
322 was calibrated with a Calmix solution containing N-butylamine, caffeine, Met-Arg-Phe-
323 Ala (MRFA), and Ultramark 1621. For LC, a 10%–80% gradient of solution B
324 (water/acetonitrile 0.1% formic acid) was used during 120 min through a home-made
325 capillary column (0.75 μ m in diameter $\times$ 10 cm in length; RP-C18) with a flux of 300
326 nL/min. Collision-Induced Dissociation (CID) and High-energy Collision Dissociation

(HCD) methods were used for peptide fragmentation, selecting only 2+, 3+ and 4+ charged ions. Single charged ions and those above 5+, as well as ions with undefined charges, were not considered. For data acquisition, a positive ion mode was set. Capture and performance of fragmentation data were done according to the total ion scanning and predetermined charge with 3.0 (m/z) isolation width, a collision energy of 35 arbitrary units, an activation Q of 0.250, an activation time of 10 milliseconds and a maximum injection time of 10 milliseconds per micro-scanning. The automatic capture of data was done using ion dynamic exclusion: (i) exclusion list of 400 ions; (ii) pre-exclusion time of 30 s; and (iii) exclusion time of 300 s. Data were searched against an available *A. thaliana* NCBI databases using Discoverer 1.4 software (Thermo-Fisher Co., San Jose, CA, USA).

2.9 Cell viability test

To determine cell viability, samples were collected from the stressed cell cultures at 30 min, 1, 2 and 3 h and from unexposed cells at 0 h and 3 h. For all cultures viability was also quantified after 3 h recovery at 25 °C. 100 µL of cell culture was mixed with one volume of 0.4 % trypan blue (Sigma-Aldrich) and were incubated for 3 min at 25 °C. Viable (unstained) and dead (stained) cells were counted in a Neubauer chamber under a light microscope. Cell viability was considered as the percentage of unstained cells out of the total of cells observed.

352 **3. Results**

353

354 **3.1 Proteasome isolation**

355

356 The isolation of proteasomes by a protocol of differential centrifugation has been
 357 published (Shibatani et al., 2006). This technique has proven effective in capturing all
 358 the possible proteasome versions present in a reticulocyte model. We adapted this
 359 protocol to isolate the different proteasome versions in suspension cells cultures of *A.*
 360 *thaliana*. The analysis by SDS/PAGE and western blot of representative aliquots
 361 (pellets and supernatants) collected along the isolation protocol revealed that the final
 362 pellet was effectively enriched in proteasomes (P3, Fig 1b). An enrichment factor
 363 (P3/Sn1) of 22.5 X was estimated by film densitometry of a western blot using an anti-
 364 20S proteasome.

365

366 **3.2 Separation of different proteasome versions of *A. thaliana* cells by BN/PAGE,** 367 **concentration by electroelution and protein blot analysis.**

368

369 Since potentially different proteasome versions were contained in the crude P3 fraction
 370 from *A. thaliana* cells (Fig. 1b), we loaded this sample directly onto the stacking well of
 371 a BN/PAGE for their separation, transfer and detection by western blot with an anti-20S
 372 antibody. A Coomassie-stained lateral strip from a BN/PAGE, revealed a profile of four
 373 major bands of a molecular mass (estimated by the method described by Wittig *et al.*
 374 2006) of 560, 750, 850 and 2600 kDa (Fig. 2b). Western blot from transferred native
 375 gels shown that only one of these bands with a molecular mass of ~ 750 kDa, had a
 376 strong reactivity with the anti-20S antibody (Fig. 2a). The migration of this band was

377 consistent with the native molecular mass of the independent 20S (CP) by BN/PAGE
378 (Shibatani et al., 2006; Camacho-Carvajal et al., 2004). Nevertheless, our western blot
379 analysis failed at showing proteasome complexes of a molecular mass higher than the
380 20S (CP) (Fig. 2a). One possibility to explain this result might be the low abundance of
381 superior proteasomes versions or the limitation of our detection system. To circumvent
382 this problem, we cut an entire mini BN/PAGE (8 X 6 cm) into eight horizontal fractions
383 that were independently electroeluted and analyzed (Fig. 2c). One of the major
384 advantages of electroelution in the system we used, in addition to its quantitative sample
385 recovery (Ohhashi et al., 1991; Sui et al., 1996), is that it concentrates the contained
386 proteins. For the protocol here described, we estimated a concentration factor of 35X.
387 The components of disassembled and electroeluted proteasomes in every gel piece
388 (section 2.4) were resolved by SDS/PAGE and analyzed by western blot using the anti-
389 20S antibody. Figure 2d shows a representative profile of the proteins recovered from a
390 whole BN/PAGE and immobilized on a nitrocellulose filter. Ponceau S stain evidenced
391 an abundant set of bands between 20 and 30 kDa (lane 5, Fig. 2d) electroeluted from the
392 fraction 5 that contained the band originally recognized by the anti-20S antibody when
393 an intact native gel was transferred (Fig. 2a). The analysis of an equivalent gel fraction
394 by mass spectrometry (Supplemental Table 1) indicated that this band corresponded to
395 the independent 20S (CP). Additional evidence that proteins in fraction 5 corresponded
396 to the 20S (CP) subunits, was given by the anti-20S antibody (Fig. 2e and f). A longer
397 film exposure to the same western blot membrane (Fig. 2f) produced a heavy smear in
398 the 40 to 100 kDa interval. Western blot analysis on a 20S (CP) purified from an
399 equivalent enriched proteasome fraction (P3, Fig. 1) by ion exchange chromatography
400 and size exclusion fractionation (Supplemental Fig. 1), suggests that the subunits of the
401 20S are heavily ubiquitinated even though the proteasome fraction was purified from *A.*

402 *thaliana* cells kept under the optimal culture conditions (Fig. 2e and f). By Ponceau S
 403 staining (or Coomassie staining on an equivalent gel) the characteristic 20S (CP) set of
 404 bands were hard to observe in those fractions that potentially contained proteasomes
 405 with a molecular mass higher than the independent 20S (CP) (lanes 1 to 4, Fig. 2d).
 406 Nevertheless, the anti-20S antibody tracked proteasomes up to lanes 2, 3 and 4 (Fig. 2e
 407 and f) that correspond to putative proteasome complexes of a nominal average
 408 molecular mass (estimated by BN/PAGE) of approximately 1600, 1100 and 850 kDa,
 409 respectively. The proteasomes profile described in Fig. 2 was considered as the basal for
 410 *A. thaliana* suspension cells under optimum culture conditions as defined in this paper,
 411 where the predominant population of proteasomes was constituted by the independent
 412 20S (CP) and the abundance of “heavier” proteasomes gradually decreased toward the
 413 top of the BN/PAGE (Fig. 2e and f).

414

415 **3.3 High molecular mass proteasomes in heat-stressed cells**

416

417 Same general methodology (proteasome isolation, separation of different proteasome
 418 versions by BN/PAGE, electroelution/concentration and western blot) was applied to *A.*
 419 *thaliana* cells exposed to heat stress to detect possible alterations in the basal
 420 proteasomes arrangement observed in control cells (Fig. 2). First, we needed to establish
 421 if the content of total 20S proteasomes changed at 37 °C exposition. Densitometry
 422 analysis of the western blot films showed a small increment in the anti-20S antibody
 423 signal from 11 to 17% between unexposed cells and those at 37 °C and less of an 8%
 424 among stressed cells (Supplementary Fig. 2a to c). Since 20S content among samples
 425 were considered equivalent, for comparative purposes the BN/PAGE of proteasome-
 426 enriched fraction (P3) prepared from all culture cells were loaded with the same protein

content (Fig. 3). After 30 min of heat treatment, a major difference was detected on fraction 1 (Lane 1, Fig. 3c). A faint signal produced by a proteasome population of a presumed molecular mass of ~ 2 600 kDa (based on its BN/PAGE mobility) was detected. The signal from the proteasomes versions contained in fractions 2 to 4 was equivalent to the unexposed cells (lanes 2 to 4 Fig. 3a and c). We also noticed the presence of signal bands between 50 to 100 kDa (lane 5 Fig. 3c) that probably correspond to ubiquitinated subunits of the 20S proteasome (Supplementary Fig. 1b). Parallel determinations were also carried out to establish the global levels of protein ubiquitination and carbonylation of total cell lysates (Fig. 4). Both parameters have been broadly used as markers of cellular stress (Lledías et al., 1999; Taylor et al., 2002; Bollineni et al., 2014). At 30 min of heat treatment we detected the removal of 10% of the original total ubiquitin conjugates content by slot blot analysis (Fig. 4a) and a slight ubiquitination signal clearance in the western blot image (lane 2, Supplementary Fig. 2d). The level of oxidatively modified proteins on the other hand, showed no changes at this time (Fig. 4b). The trypan blue cell viability assay showed no difference between the control and heat stressed cells. Trypan blue exclusion in stressed cells was equivalent to control culture even after 3 h recovery at 25 °C. A striking difference in the western blot proteasomes profile was detected at 1 and 2 h after the temperature increase, where an important enrichment of the higher order proteasome configurations was observed (lanes 1 to 4, Fig. 3d and e). In addition, a “new” anti-20S signal was detected in the fraction 6 from these heat-stressed cells cultures (lane 6 Fig. 3d and e) originated from a native protein complex of approximately 640 kDa. This molecular mass was significantly smaller than a functional 20S (CP). We speculated that in this fraction, because of the reactivity with the anti-20S (lane 6, Fig. 3 d to f), the estimated native molecular mass by BN/PAGE and the 20S peptides (α and β) obtained by mass

spectrometry (not shown), 20S assembly intermediate complexes known as half-proteasomes (13 -16S) could be localized (Schmidtke et al., 1997; Lehmann et al., 2002). The 50 -100 kDa smear of the 20S (CP) subunits was equivalent at both times (lane 5, Fig. 3d and e) suggesting a strong 20S (CP) subunits ubiquitination. The increment in high molecular mass proteasome populations correlated with the clearance of 60% (at 1h) and 75% (at 2h) of the basal ubiquitinated proteins levels (Fig. 4a and Supplementary Fig. 2d). The total amount of protein carbonyls was practically unaltered at these times (Fig. 4b). Cell viability test showed no changes with respect to control cultures when determined immediately after 37 °C treatment or the recovery for 3 h at 25 °C was allowed. The western blot analysis of cell suspension cultures at 3 h under heat stress, showed the higher enrichment of all the proteasome versions for all the times sampled, half-proteasomes included (Fig. 3 f). In addition, a noteworthy feature of this 3 h profile was the presence of 20S-immunoreactive bands between 60 and 70 kDa in all fractions (bracket in Fig. 3f, lanes 1 to 8). These bands are presumably produced by the ubiquitinated subunits of the 20S (CP) (Supplemental Fig. 1) that assembled the half-proteasomes, the free 20S CP and the higher molecular mass proteasome versions promoted by heat stress. There were also clear differences in the slot blot determination of total ubiquitin conjugates and carbonyl contents, a two-fold and a nine-fold increase respectively in comparison with the previous sampled hour (Fig. 4a and b). No changes in cell viability were observed relative to control cultures at 3h heating or after the recovery period.

473

3.3 19S regulatory particle subunits are part of the high molecular mass proteasomes.

476

477 The modular nature of proteasomes and their association/dissociation dynamics
478 directed by environmental clues has been reported (Bajorek et al., 2003; Grune et al.,
479 2011; Livnat-Levanon et al., 2014). High molecular mass proteasomes resolved by
480 BN/PAGE were result from the assembly of different regulators on the distal surface of
481 one or both the 20S α rings (Shibatani et al., 2006). To determine if the high molecular
482 mass proteasomes we observed contained 19S regulatory particle subunits, we probed
483 the eight fractions obtained from a control culture BN/PAGE with antibodies against
484 Rpn1 (19S base subunit), Rpt2 (19S base subunit), Rpn10 (19S lid subunit that keeps
485 together base and lid) and Rpn6 (lid subunit which holds together 20S and 19S
486 complexes). Fraction 5 that contained exclusively the independent 20S (CP) (lane 5,
487 figure 5b and c) showed minimum or null reactivity against all the anti-19S regulatory
488 subunits antibodies (lane 5, Fig. 5d to g). A strong signal with the immediate higher
489 molecular mass proteasome complex was obtained with the Rpn10 and Rpn1 antibodies
490 (Lane 4, Fig. 5d and e). Rpn10 signal showed a stepwise decrease toward the upper
491 region of the BN/PAGE (Lanes 1 to 5, Fig. 5d) while the Rpn1 signal kept constant in
492 three consecutive fractions and showed a decrease up to fraction 1 (Lanes 2 to 4, Fig.
493 5e). The use of the Rpt2 and Rpn6 antibodies shown a very different pattern, a stepwise
494 increase toward those fractions obtained from the top of the BN/PAGE. If we consider
495 that, all the subunits are found in the context of their respective 20S or 19S complex
496 (Livneh et al, 2016), our results suggest that some of the detected complexes probably
497 represent 26S maturation/assembly intermediates. Based on the BN/PAGE mobility of
498 the complex and the relative reactivity of the antibodies in each fraction, Rpn10 and
499 Rpn1 were associated with the 20S CP (figure 5d and e, lanes 3 and 4) before
500 association of Rpt2 and Rpn6 (Fig. 5f and g, lanes 3 and 4) as has been described
501 (Hendil et al., 2009). In this context BN/PAGE fractions (Lanes 3 and 4, Figure 5)

could be considered early steps in the way to consolidate the higher molecular mass proteasome complexes 20S/19S and 19S/20S/19S probably contained in fractions 2 and 1 respectively (Lane 2 and 1, Fig. 5).

4. Discussion

Different proteasomes versions have been shown by BN/PAGE analysis of samples from a human embryonic cell line (HEK293) (Camacho-Carvajal et al., 2004) and rabbit reticulocytes [34] (Shibatani et al., 2006). The separation of the different proteasomes versions by BN/PAGE revealed that stimulation with γ -interferon or MG132 (a proteasome inhibitor) directed a dynamic process of recruitment and exchange of proteasome regulatory complexes (Shibatani et al., 2006). We reasoned that the published protocols of proteasomes isolation and electrophoretic analysis could be applicable to *A. thaliana* suspension cells, and as a starting point, to detect whether different proteasome versions are present in plants. We also would be able to analyze the proteasome populations changes in response to an insult such as temperature increment. In our hands, the published protocol for proteasome isolation (Shibatani et al., 2006) with some modifications was effective for *A. thaliana* cells in suspension cultures (Fig. 1b). In our lab, the same technique was useful for the isolation of proteasomes (and their BN/PAGE analysis) from *A. thaliana* two-week seedlings, mature spinach and maize leaves, different succulent plants leaves and from yeast, mouse liver, zebra fish and human erythrocytes (Rivas and Lledías, unpublished). When the enriched proteasome fraction (P3) of *A. thaliana* cells was separated by BN/PAGE and transferred to membranes for western blot detection with an anti-20S antibody, only one band was detected (Fig. 2a). Higher molecular mass complexes were

not observed at this point. We discarded the possibility that in our adaptation of the isolation protocol, any of the buffers or additives employed or even the sample freezing, promoted the disassembly of complex proteasomes. The same enrichment protocol has been successfully used as a previous step to purify, by ionic exchange chromatography and size exclusion fractionation, the 26S proteasomes from *A. thaliana* suspension culture cells. An alternative possibility was that the abundance of higher order proteasomes was relatively scarce in this cell type and were beyond the limit of detection. The electroelution protocol was effective for concentrating the proteasomes in all BN/PAGE fractions and facilitate their visualization by western blot (Fig. 2 and 3). Four proteasome populations were revealed in suspension cells grown at optimum culture conditions (25 °C). The most abundant proteasome version corresponded to the independent 20S (CP) (Fig. 2 e and f). The identity of the 20S (CP) was verified by mass spectrometric analysis (Supplementary Table 1). We discarded the possibility that “heavier” proteasomes versions detected in unexposed or in heat stressed cells (Fig. 3) were product of an artifact caused by anomalous or not optimum electrophoretic separation of independent 20S (CP) particles, since fractions 1 to 6 (Fig. 3) electroeluted under native conditions and independently re-separated on fresh blue native gels, were detected at exactly the same original fractions. Even though samples were frozen at -70 °C after their electroelution, high molecular mass complexes, the independent 20S (CP) and lower complexes preserved their original electrophoretic mobility. We observed that in cells, even at optimum growth conditions, the subunits of the 20S (CP) were probably ubiquitinated (Supplemental Fig. 1 and smear in lane 5 Fig. 3b). The posttranscriptional modification of the 20S (CP) subunits by ubiquitination have been shown by proteomic techniques in *A. thaliana* (Book et al., 2010). High temperatures by themselves caused intracellular protein denaturation and substrates ubiquitination

(Lepock et al., 1988; Pinto et al., 1991). In *A. thaliana* suspension cell cultures, a moderate heat stress was detected when the temperature was raised at 37 °C and the production of reactive oxygen species (ROS) was enhanced (Volkov et al., 2006) that in turn may promote protein carbonylation. A classical marker of cellular oxidative stress is the increase of carbonyls in total protein samples (Levine et al., 1990; Wong et al., 2010; Bollineni et al., 2014). Oxidation partially denatures protein and hydrophobic patches exposure initializes the intricate action of the ubiquitin-proteasome system (UPS) (Pacifci et al., 1993; Murata et al., 2001). Protein ubiquitin conjugates are considered an early and sensitive cell stress marker (Shang and Taylor 2011). In our experiments, the levels of ubiquitin conjugates and oxidatively modified proteins suggest two phases in the *A. thaliana* cell response to the heat increment. During the first phase (30 min to 2 h at 37 °C) 75 % the basal ubiquitin conjugates were removed (Fig. 4a and Supplementary Fig. 2d) while the total protein carbonyl level remained relatively unaltered (Fig. 4b). These results indicate that an oxidative stress was not produced because the antioxidant machinery and the modified protein elimination mechanisms (ub-conjugates degradation among them) were effective. The western blot proteasome profiles (Fig. 3a to e) suggest that heat increment promoted the assembly of proteasome versions of a molecular mass higher than the free 20S (CP). In reticulocytes the differences in molecular mass of proteasomes complexes detected by BN/PAGE have been attributed to the interaction of the 20S (CP) with the 19S particle to constitute the 26S proteasome (Shibatani et al., 2006; Camacho-Carvajal et al., 2004) which in turn is responsible of ubiquitin conjugates elimination (Smalle and Vierstra, 2004; Voges et al., 1999) while the still independent 20S (CP) degrades oxidatively modified proteins (Ferrington et al., 2001; Grune et al., 1997). In *A. thaliana*, the abundance of these two proteasome entities is highly interrelated during cell growth and stress

577 tolerance (Kurepa et al., 2009). If the degradation of protein ubiquitin conjugates was
578 limited (by 26S synthesis impairment) the degradation of oxidized proteins by the
579 independent 20S (CP) was favored (Kurepa et al., 2008). We consider that the opposite
580 phenomenon as we observe during the first phase is also plausible. During the second
581 phase (3 h at 37 °C) both stress markers increased, doubled for ubiquitin conjugates and
582 a nine-fold increase was detected for protein oxidation (Fig. 4a and b). These results are
583 indicative that cellular antioxidant and damaged protein removal capacities were
584 surpassed, and an oxidative stress episode was initiated (Sies, 1997). The western blot
585 profile showed the higher enrichment for all the proteasomes versions observed (Fig. 3
586 f). The stress markers levels and the western blot results combined, suggest that high
587 molecular mass proteasomes still removed ubiquitin conjugates but with a relative
588 diminished efficiency (Fig. 4a); however, oxidized proteins greatly increased (Fig.4b).
589 We hypothesize that high temperature for 3 h, forced the cell machinery to assembly
590 high molecular mass proteasomes while decreasing the total amount of independent 20S
591 (CP), oxidatively modified proteins increased consequently. The 26S proteasome has
592 been shown inefficient at degrading oxidatively modified proteins (Davies, 2001) and in
593 addition, was inhibited by oxidative stress (Reinheckel et al., 1998). This observation
594 probably explains why despite “heavier” proteasomes were enriched at this time (Fig.
595 3f) a decrease of 50% in their capability of ubiquitinated proteins removal was detected
596 (Fig. 4a). An additional explanation for the impaired oxidized protein elimination is the
597 sensitivity of the trypsin and caspase-like 20S (CP) catalytic activities to oxidation
598 (Demasi et al., 2003). A heavy signal, attributable to 20S subunits ubiquitination was
599 observed for all the BN/PAGE fractions from suspension cells heat stressed for 3h (Fig.
600 3f and Supplemental Fig. 1). Under abiotic stress, the activity of specific *A. thaliana*
601 ubiquitin ligases (Pub22 and Pub23) increased, which destabilized the 19S regulator by

ubiquitination of its subunits (Cho et al., 2015). In human embryonic cells (HEK293) the ubiquitination of the subunit Rpn13 (a ubiquitinated proteins receptors located in the regulatory particle 19S) decreased its substrate binding capacities under proteotoxic stress conditions leading to ubiquitin conjugates build up (Besche et al., 20014). Inhibition of the 26S activity by substrate overload is plausible (Kurepa et al., 2009). All these processes are probably happening in cells in suspension cultures throughout 3 h of heat exposition. Severe heat stress causes an increase in ubiquitinated proteins (Ferguson et al., 1994) and the accumulation of protein carbonyls has been reported as a product of heat exposition in plants that produced an excess in ROS production (Hasanuzzaman et al., 20013). In our experiments, the elimination of ubiquitin conjugates and the avoidance of the accumulation of protein oxidation products during the first 2 h of heat exposition suggest that *A. thaliana* suspension cell cultures adapted the proteasome degradative machinery (and additional cellular mechanisms) to tolerate the temperature increment, a tolerance that seemed limited to the third hour where the cell stress markers increased (Fig 4a and b). Despite this fact, cell viability values remain unaltered for 3 h, a parameter that indicated that at least for this period, the cell capacity to cope with heat stress was not entirely compromised. The films in Fig. 3 (a, c to f) were exposed the same time to the chemiluminescence developing reaction to allow a direct comparison among the different proteasome populations present at different times under stress. The contrast between the proteasome complement of control cells with those of the stressed cultures (Fig. 3), even when a longer film exposure was practiced for control cells (Fig. 3b), strongly suggests that heat stress promoted the assembly of proteasome versions with a molecular mass higher than the independent 20S (CP). Our western blot analysis of total lysates showed that

there was not a significant net increase in the total content of proteasomes 20S (CP) subunits among the heat-treated cells (Supplemental Fig. 2). However, the profiles obtained by the separation of different proteasomes populations by BN/PAGE (Fig. 3) and 20S ubiquitination (Supplementary Fig. 1), suggest that synthesis, assembly and probably degradation of proteasomes subunits were promoted under heat stress. During this very dynamic process, proteasomes subunits could be synthesized and assembled as half-proteasomes through their activation as mature 20S (CP). Eukaryotic half-proteasomes are assembly complexes constituted by a seven-membered α ring and several β subunits proproteins (Schmidtke et al., 1997; Lehmann et al., 2002 and dedicated chaperones (Le Tallec et al., 2007). In line with our observation, mammalian half-proteasomes were localized just above the band of mature 20S (CP) by native gel electrophoresis (Schmidtke et al., 1997). Once 20S (CP) are completed, they are available as platforms to assembly higher molecular mass proteasomes versions, or they can remain independent, in close dependence with the protein turn over needs imposed by the environment. The western blot analysis of the BN/PAGE fractions 1 to 8 from control cells, showed that the 19S regulatory particle subunits Rpn10, Rpn1, Rpt2 and Rpn6 are associated to “supra” 20S proteasome assemblies (Fig. 5). The use of antibodies against the 19S regulatory particle subunits Rpn10, Rpn1 and Rpt2 produced a faint signal associated with the independent 20S and the putative semi-proteasome (Fig. 5, lanes 5 and 6 respectively). Signals from non-20S associated forms were detected in fractions 7 and 8 (Fig. 5d to g). We propose that some of the observed heat-enriched proteasome versions, corresponded to the 26S assembly and other alternative proteasome versions with ub-conjugates elimination capacities, while the isolated 20S (CP) kept oxidatively modified proteins at basal levels. We cannot discard the possibility that even other high molecular mass proteasome forms were also involved in

the ubiquitin conjugates and oxidized proteins elimination. Experimental evidenced have shown that during an oxidative stress event in yeast, Ecm29 increased its association with the 19S regulatory complex promoting 26S disassembly. Since independent 20S (CP) population increased oxidized modified proteins could be preferentially degraded (Wang et al., 2010). In murine fibroblasts, proteasomes with an array PA28-20S-PA28 induced during oxidative stress, degraded carbonylated proteins with a higher efficiency (Pickering et al., 2010).

The presence in the cell of *ad hoc* proteasomes could offer better possibilities for successfully coping with unfavorable growth conditions. Additional and higher resolution analyses are needed to identify the specific protein components in each fraction in the Ub-proteasome pathway context, nevertheless we consider that our approach of proteasome isolation by centrifugation, separation of discrete proteasomes populations based in their molecular mass differences by BN/PAGE, and the concentration of the samples by electroelution shown that an increment in culture temperature directed the assembly of “supra” 20S proteasome complexes. Our protocol (combined with mass spectrometry and western blot) could be considered a useful tool to characterize the regulators and the additional interacting proteins that contribute to the proteasomes function and dynamics.

670

671 **Contribution**

672

Daniel Aristizábal: performed the experiments and analyzed the data. Viridiana Rivas: performed some of the experiments. Fernando Lledías: conceived and designed the experiments, performed some of the experiments and wrote the manuscript. Gladys Cassab: wrote the manuscript.

677

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683

684 **Conflicts of interest**

685 The authors declare that they have no conflicts of interest.

686

687 **Ethical approval**

688 This article does not contain any studies with human participants or animals performed
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690

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FIGURES

Heat stress reveals high molecular mass proteasomes in *Arabidopsis thaliana* suspension cells cultures

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FIGURE LEGENDS

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Fig. 1. Proteasomes isolation scheme. A proteasome enriched fraction was obtained by differential centrifugation from total lysates of *A. thaliana* cell suspension cultures (a). Representative aliquots of all the pellets (P1 to P3) and supernatants (Sn1 to Sn3) were separated by SDS/PAGE and analyzed by western blot using an anti-20S antibody to determine the protocol efficiency (b). The proteasome enriched fractions (P3) resuspended in buffer A were directly loaded onto BN/PAGE gels to separate the proteasomes based on their molecular mass differences.

Fig. 2. Separation of the different proteasome versions of *A. thaliana* cells by BN/PAGE and analysis by western blot. Proteasome enriched fraction (P3) was separated by BN/PAGE (b). In-gel denatured proteins were transferred to a nitrocellulose membrane to identify 20S (CP) subunits with an anti-20S antibody (a). To detect higher molecular mass proteasomes, a complete BN/PAGE gel was cut into eight fractions (1 to 8, c). Proteins contained in each fraction were electroeluted/concentrated to be analyzed independently by SDS/PAGE and western blot using an anti-20S antibody. Transferred proteins were stained with Ponceau S (d) before 20S (CP) detection by western blot (e and f).

Fig. 3. Western blot proteasomes profiles of *A. thaliana* cells under heat stress. Proteasome enriched fractions (P3) from cells cultures at 37°C were separated by BN/PAGE. Eight fractions obtained from each BN/PAGE gel (lanes 1 to 8) were individually electroeluted/concentrated, precipitated and analyzed by western blot using an anti-20S antibody. Panel a correspond to unexposed cells, c to e show the profiles of the cells recovered at 30 min, 1, 2 and 3 h, respectively. Except for b, all films were exposed the same time to the chemiluminescent developing reaction. Above to each

film image, we positioned a silver stained BN/PAGE lane (**BN**) to show the actual protein content and profile of each P3 sample from where the eight fractions were obtained. The most abundant protein in all BN/PAGE, electroeluted from fraction 5 (lane 5, a to f) was the independent 20S (CP). The signals from fractions 1 to 4 (**a** to **f**) were produced by proteasome versions with a molecular mass higher than the 20S (CP).

Fig. 4. Ubiquitin conjugates and protein carbonyls of total lysates from *A. thaliana* cell cultures exposed to heat stress. 5 mg of total protein extract were loaded into slot blot wells and immobilized in nitrocellulose membranes for the determination of the total amount of ubiquitin conjugates present in cells incubated at 37 °C for 0.5, 1, 2 and 3 h with a monoclonal anti-Ubiquitin antibody (**a**). Same protocol was followed to determine the content of oxidatively modified proteins, except samples were derivatized with DNPH before their membrane immobilization (**b**). Detection was made with an anti-DNPH antibody. Densitometry films values were normalized to 1, considering the ubiquitin conjugates or total carbonyl contents from an unexposed control (0 h). Media and standard deviations of three independent experiments are represented in the graphs.

Fig. 5. 19S regulatory particle subunits were present in high molecular mass proteasomes. Denatured BN/PAGE fractions (1-8) from control *A thaliana* cells were transferred to nitrocellulose, stained with Ponceau red (**a**) and probed with anti-20S (**b** and **c**), anti-Rpn 10 (**d**), anti-Rpn 1 (**e**), anti-Rpt 2 (**f**) and anti-Rpn 6 (**g**) antibodies. In contrast with panel (**b**), the anti-20S profile in **c**, was obtained with five times higher protein loading.