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Research article

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

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A B S T R A C T

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.

## 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 80–90% 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 $\beta_{1\text{-}7}$ (central) and $\alpha_{1\text{-}7}$ (distal) subunits in an arrangement α-β-β-α, for a total of 28 subunits. In this hollow-cylinder structure, the interior domains of the subunits β1, β2 and β5 define catalytic domains that have trypsin, chymotrypsin, and caspase-like activities respectively (Kish-Trier and Hill, 2013). This basic 20S (CP) proteasome version is responsible for cell removal of oxidatively modified proteins (Davies, 2001). 20S α subunits are also the binding sites for different regulatory complexes. The 26S proteasome for example, is formed by the basic 20S (CP) flanked by one or two 19S regulatory complexes (19S–20S or 19S–20S–19S) docked on the distal-most surfaces of the α rings. For *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and human cells, the architecture of the 19S regulatory particle has been reported at subnanometer level (Beck et al., 2012; Schweitze et al., 2016; Sakata et al., 2012; Bohn et al., 2010). The 19S complex is in charge of recognizing ubiquitinated proteins, denature them by the action of ATP-dependent "unfoldases", opens the 20S (CP) gate by rearrangement of the α subunits, and translocate the protein substrate to the catalytic sites for its degradation (Peth et al., 2010). The 26S is the specialized proteasome version involved -along with a ubiquitin activating (E1), the ubiquitin

*Abbreviations:* BN/PAGE, Blue Native Polyacrylamide Gel Electrophoresis; SDS/PAGE, SDS Polyacrylamide Gel Electrophoresis

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conjugating (E2) and the ubiquitin ligases (E3) enzymes-in the Ubiquitin-Proteasome-System (UPS) (Hershko and Ciechanover, 1998).

Different proteasome regulatory particle like PA28 (Hernebring et al., 2013) PA200 (in mammals) Blm10 (in yeast), are alternative regulatory complexes which had been involved in the degradation of very specific non-ubiquitinated protein substrates (López et al., 2011; Blickwedehl et al., 2012). Ecm29 (in a human cell line) has been proposed to act as a structural stabilizing agent for the 26S proteasome, especially when the 20S (CP) maturation was impaired (Lehmann et al., 2010). Information on different proteasome regulators published so far has shown that the binding of the regulatory complexes has a deep influence in the selection of substrates, in the catalytic function of the 20S (CP) and in the peptides produced. In this scenario, the presence of "supra-20S" complexes allow the cell to respond to very specific metabolic stages. The active building of proteasomal complexes has a counterpart, the 26S proteasomes in yeast were disarmed into independent 19S and 20S (CP) particles during the stationary phase and this phenomenon correlated with cell viability. When ATP was available by medium refreshing, the 26S proteasomes were reconstituted (Bajorek et al., 2003). Dissociation and reassociation of the 26S proteasome have been reported during the adaptation of a human cell line to oxidative stress (Grune et al., 2011). Reversible 26S disassembly has been reported upon mitochondrial stress in yeast (Livnat-Levanon et al., 2014). Considering the assembly/disassembly capacities of the proteasomes and the repertoire of regulatory complexes, any cell in a specific environmental context, have the possibilities to "direct" the versions of the proteasome that respond better to the catalytic needs of the moment, and the proteasome versions selected would have an influence on cell fate. This information indicates that a very dynamic process of assembly, selection of regulatory particles, and disassembly of proteasomes are continuously taking place in cells in close relationship with the environment.

Great efforts have been made to characterize biochemically, genetically and by mixed "omics" techniques, the subunit composition and function of the proteasomes in plants. In *Arabidopsis thaliana* particularly, their 20S (CP) and 26S proteasomes have been isolated and characterized (Polge et al., 2009; Yang et al., 2004; Book et al., 2010). By proteomic approach using an epitope-tagged 26S proteasomes as bait, more than 40 proteins interact with this complex. Despite all the information on Arabidopsis proteasomes, studies on the dynamics of the different proteasome versions in plants as has been reported for yeast (Bajorek et al., 2003; Livnat-Levanon et al., 2014) or mammalian cells (Grune et al., 2011; Shibatani et al., 2006) are limited.

Blue Native Polyacrylamide Gel Electrophoresis (BN/PAGE) is a technique that allows the separation of native protein complexes based on their molecular mass differences (Wittig et al., 2006). BN/PAGE has been used for the complexomics analysis of different models (Wittig et al., 2006; Lasserre et al., 2006; Hashemi et al., 2016). This technique has been employed to successfully separate the different versions of proteasomes in whole cell lysates of a human embryonic cell line (HEK293) (Camacho-Carvajal et al., 2004) and to study the proteasome dynamics of rabbit reticulocytes (Shibatani et al., 2006). In the latter report, six native proteasome populations (20S, 20S-PA28, PA28-20S-PA28, 19S–20S-PA28, 19S–20S and 19S–20S–19S) were identified. By γ-interferon stimulation or the chemical inhibition of the proteasome, an active interchange of proteasome regulatory "caps" was evidenced (Shibatani et al., 2006). BN/PAGE was used for monitoring changes in the quantity and subunit composition of the 20S (CP) when the α3 subunit was deleted in yeast (Couttas et al., 2011).

In the present work, we adapted some of the above-mentioned protocols of proteasome isolation and analysis by BN/PAGE to establish whether in Arabidopsis cells different proteasome versions coexist and if under drastic changes in the culture conditions like heat stress, the basal proteasome populations were altered.

## 2. Methods

### 2.1. Cell culture and heat stress treatment

Suspension cell cultures were generated from hypocotyls dissected from Arabidopsis seedlings. Cells were maintained by weekly transfer in MS medium (Murashige and Skoog, 1962) containing basal salt mixture, 3% sucrose and supplemented with 50 μg/L kinetin, 75 μg/L 2,4-diclorofenoxiacetic acid (2-4D) and 1X Gamborg's vitamin solution, pH 5.7. Cultures were incubated at 25 °C and 100 rpm under long day conditions of 16 h light/8 h dark, and 80 μM photons $m^{-2}$ $s^{-1}$. For heat treatment, a one-week culture of exponentially growing cells was diluted (1:10) with fresh MS medium and divided into 250 mL flasks containing 50 mL liquid medium. Cultures were incubated at 37 °C at 100 rpm for 0.5, 1, 2 and 3 h (illuminated). Cell packages (10 mL) were recovered with a spatula after filter paper filtration and immediately frozen in liquid nitrogen and kept at −70 °C.

### 2.2. Proteasomes isolation

Total cell lysate was obtained by adding to each frozen cell package, 25 mL of extraction buffer (Tris-HCl pH 7.5, 1 mM dithiothreitol, 2 mM adenosine triphosphate, 0.25M sucrose, 1 mM $MgCl_2$, 1% polyvinylpolypyrrolidone, Complete EDTA-free [Roche]), and "10 mL" of glass beads (4 mm). While thawing, cells were disrupted by five vortex cycles (5 min vortexing/5 min incubation on ice). Aliquots were taken for the determination of the total content of ubiquitin conjugates and protein carbonyls by western blot. Total lysate was filtered on three layers of cheesecloth and centrifuged at 16 000 × *g* for 15 min at 4 °C. Pellet (P1, Fig. 1) was eliminated and supernatant (Sn1, Fig. 1) was centrifuged for 1 h at 70 000 × *g* at 4 °C. Pellet (P2, Fig. 1) was discarded and supernatant (Sn2, Fig. 1) was centrifuged again at 350 000 × *g* for 3.5 h at 4 °C. 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.

### 2.3. BN/PAGE

Resolution of proteasomes 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 fraction in buffer A was loaded onto an 8 × 6 cm BN/PAGE mini gels (5–10% 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 1 h, 150 V for 16 h and 500 V for 1 h. 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 (Bio-Rad). Proteins in analytical BN/PAGE were visualized with Coomassie Brilliant Blue or by silver stain (Blum et al., 1986). For preparative purposes, BN/PAGE gels were fractionated and electroeluted. The molecular mass of the protein complexes was estimated by the method of Wittig et al. (2010), using the endogenous HSP 60, Rubisco (identified by mass spectrometry in the fraction 4 and 7 respectively), and the independent 20S (CP) native complexes as molecular mass markers.

### 2.4. Protein electroelution and concentration

As the cathode buffer contained Coomassie G-250, four protein bands were stained during electrophoresis (Fig. 2b) and used as markers to cut the gels into eight horizontal fractions (Fig. 2c). For the analysis of the proteasomes by western blot, each fraction was divided into smaller fragments (~2 × 2 mm) and heated at 95 °C for 10 min in the

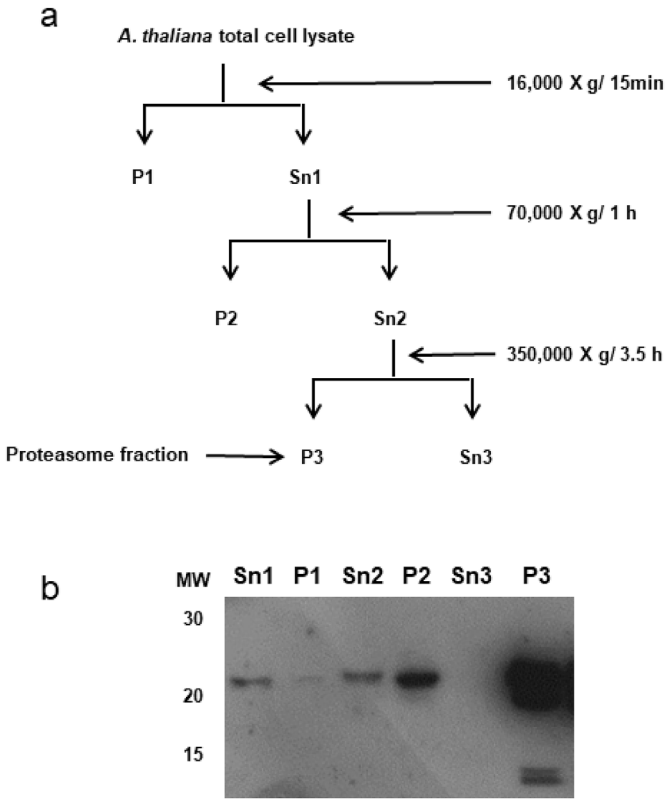


**Fig. 1. Proteasomes isolation scheme.** A proteasome enriched fraction was obtained by differential centrifugation from total lysates of *A. thaliana* cell suspension cultures (**a**). Representative aliquots of all the pellets (P1 to P3) and supernatants (Sn1 to Sn3) were separated by SDS/PAGE and 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.

presence of 1 mL of 1X Laemmli sample buffer (Tris 50 mM pH 6.8, 2% SDS, 5% β-ME, 8% glycerol without bromophenol blue). Gel pieces and sample buffer were transferred to the sample traps of the electroelutor/concentrator system "Little Blue Tank" (ISCO, INC) containing 10 mL of Laemmli running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at a 1:10 dilution. Laemmli running (1X) buffer was used in both electrode compartments. Protein electroelution was carried out at 3 W for 3 h at 5 °C. Samples from the sample traps (~200 μL) were precipitated by methanol/chloroform, resuspended and heated at 95 °C for 10 min in 1X Laemmli sample buffer for their SDS/PAGE and western blot analysis. We determined protein content in samples containing β-ME, by the Lowry method (calibrated with crystalline bovine serum albumin) according to the modification of Makkar et al. (1980). A similar technique was followed to recover native proteasome complexes from BN/PAGE, except that gel fractions were not heated or incubated in the presence of chaotropic agents. For this purpose, electroelutor/concentrator sample traps contained 10 mL of running buffer (1:10) and 1X running buffer was used in electrode compartments (without SDS). Samples recovered were immediately loaded or preserved at −70 °C after glycerol addition (20% final) for additional BN/PAGE analysis.

### 2.5. SDS/PAGE

We analyzed the protein profile of each electroeluted sample by SDS/PAGE according to Laemmli (1970). Samples were loaded onto 4% stacking gels and resolved in 12% polyacrylamide-SDS gels. Runs were performed using the Tris/glycine/SDS running buffer at 200 V for 1 h at 5 °C. Gels were stained in Coomassie Brilliant Blue R-250 (0.1%), acetic acid (40%) and ethanol (40%). For silver staining of proteins on BN/PAGE, we used the method of Blum et al. (1986). Pink pre-stained ladder, 15–175 kDa (Cat. MWP02, Nippon Genetics) were used as molecular weight markers.

### 2.6. Western blot and slot blot analysis

Proteins separated by BN/PAGE or SDS/PAGE were transferred to nitrocellulose using buffer (25 mM Tris, 192 mM glycine, 20% isopropanol) in a Mini-PROTEAN at 360 mA for 1 h at 6 °C. Proteins on BN/PAGE were denatured before their blotting by incubating the gel in a solution of 20 mM Tris-HCl buffer (pH 7.4), 3% SDS for 10 min with agitation followed by heating in a microwave oven (1 min). After an additional incubation (10 min, room temperature) proteins were transferred. Proteins were visualized with Ponceau S solution (0.2% Ponceau S in 5% acetic acid). For western blot assay, membranes were blocked 1 h at 25 °C with 5% non-fat milk in TBS-T buffer (20 mM Tris-HCl pH7.4, 150 mM NaCl, 0.05% Tween-20) and incubated with primary or secondary antibodies in the solution of non-fat milk in TBS-T buffer. All intermediate washes were done with TBS-T. The following primary antibodies were used at the same 1:10 000 dilutions for 2 h at 25 °C: mouse-anti-proteasome 20S alpha + beta (Cat. ab22673, Abcam), rabbit-anti-proteasome 26S S2 (Rpn1) (Cat. ab98865, Abcam), rabbit-anti-proteasome Rpn6 (S9) (Cat. PW8370, Enzo), mouse-anti-Rpt2 (Cat. ab21882, Abcam), rabbit-anti-19S S5A/Rpn10 (Cat. ab56851, Abcam) and rabbit-anti-Ubiquitin antibody (Cat. sc-9133, Santa Cruz Biotechnology, Inc.). As secondary antibodies, we employed HRP-goat-anti-mouse IgG (H + L) (Cat. 62–6520, Zymed) or HRP-goat-anti-rabbit IgG (H + L) (Cat. 65–6120, Zymed) both at 1:10 000 dilutions for 1 h at 25 °C. Western blots were developed with Super Signal West Femto (Thermo Scientific) and exposed to X-Ray films (Kodak). Total carbonyl (Johansson et al., 2004) and ubiquitin conjugates (Tang et al., 2014) contents of heat stress and control samples, were estimated by slot blot analysis. For carbonyl content estimation, protein from total cell lysates (section 2.2) was precipitated with methanol/chloroform and resuspended in 1X Laemmli sample buffer. Five milligrams of each sample (determined by the method of Lowry, 1951) were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and loaded on each well of the slot blot manifold (PR 648, Hoefer). Oxidatively modified proteins on nitrocellulose filters were determined by an immunochemical protocol (OxyBlot Protein Oxidation detection kit, Chemicon International). Same slot blot technique was followed to determine ubiquitin conjugates, but samples were not derivatized. Quantification of western and slot blots was made by densitometry of the autoradiograms using NIH ImageJ 1.48 software.

### 2.7. Mass spectrometry

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

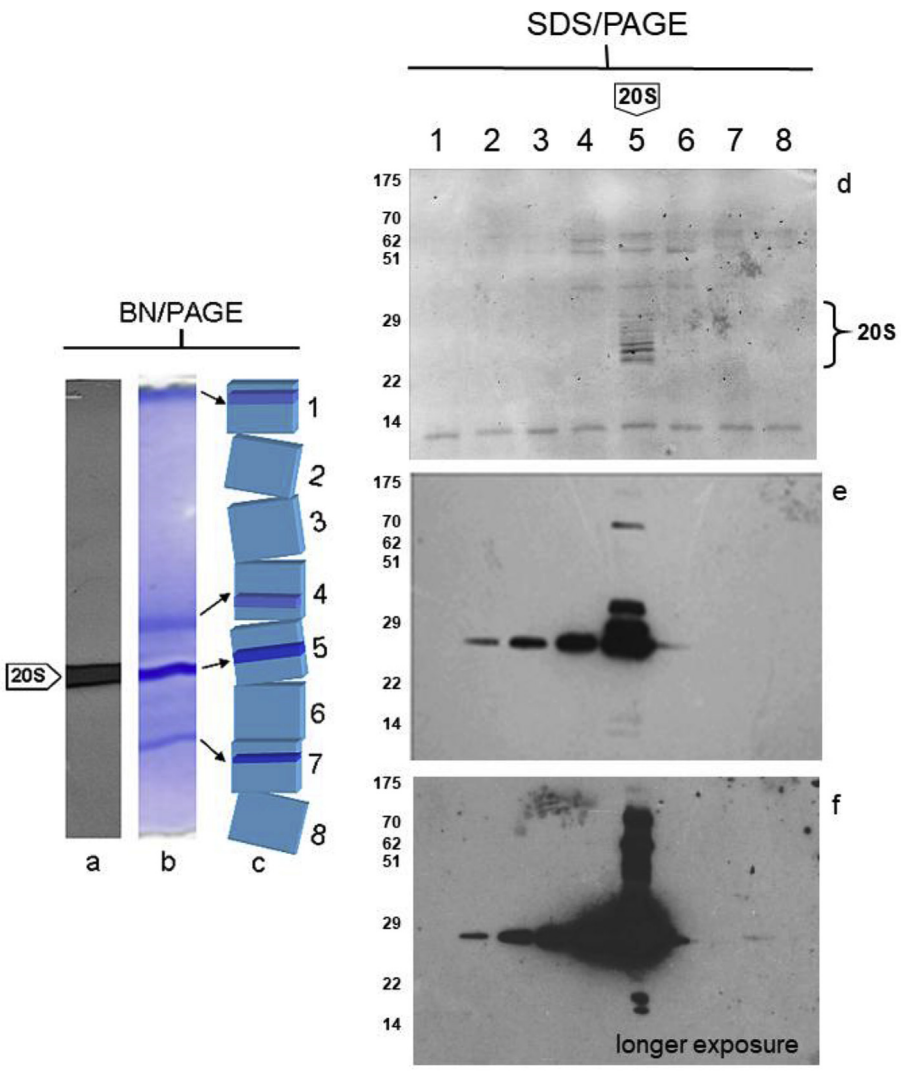


**Fig. 2. Separation of the different proteasome versions of Arabidopsis cells by BN/PAGE and analysis by western blot.** Proteasome enriched fraction (P3) was separated by BN/PAGE (**b**). In-gel denatured proteins were transferred to a nitrocellulose membrane to identify 20S (CP) subunits with an anti-20S antibody (**a**). To detect higher molecular mass proteasomes, a complete BN/PAGE gel was cut into eight fractions (1–8, **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**).

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 ms and a maximum injection time of 10 ms 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 Arabidopsis NCBI database using Discoverer 1.4 software (Thermo-Fisher Co., San Jose, CA, USA).

### 2.8. 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. Cell viability was considered as the percentage of unstained cells out of the total of cells observed.

## 3. Results

### 3.1. Proteasome isolation

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

### 3.2. Separation of different proteasome versions of Arabidopsis cells by BN/PAGE, concentration by electroelution and protein blot analysis

Since potentially different proteasome versions were contained in the P3 fraction from Arabidopsis cells (Fig. 1b), we loaded this sample directly onto the stacking well of a BN/PAGE for their separation, transfer and detection by western blot with an anti-20S antibody. A Coomassie-stained lateral strip from a BN/PAGE, revealed a profile of four major bands of a molecular mass (estimated as described by Wittig et al., 2006) of 560, 750, 850 and 2600 kDa (Fig. 2b). Western blot from native gels shown that only the band of 750 kDa had a strong reactivity

with the anti-20S antibody (Fig. 2a). The migration of this band was consistent with the native molecular mass of the independent 20S (CP) by BN/PAGE (Shibatani et al., 2006; Camacho-Carvajal et al., 2004).

Nevertheless, our western blot analysis failed at showing complexes of a molecular mass higher than the 20S (CP) (Fig. 2a). One possibility to explain this might be the low abundance of superior proteasomes versions or the limitation of our detection system. To circumvent this problem, we cut an entire mini BN/PAGE (8 × 6 cm) into eight horizontal fractions that were independently electroeluted and analyzed (Fig. 2c). One of the major advantages of electroelution in the system we used, in addition to its quantitative sample recovery (Ohhashi et al., 1991; Sui et al., 1996), is that it concentrates the contained proteins. For the protocol here described, we estimated a concentration factor of 35. Disassembled and electroeluted proteasomes in every gel piece (section 2.4) were resolved by SDS/PAGE and analyzed by western blot using the anti-20S. Fig. 2d shows a profile of the proteins recovered from a whole BN/PAGE and immobilized on nitrocellulose. Ponceau S evidenced an abundant set of bands between 20 and 30 kDa (lane 5, Fig. 2d) electroeluted from the fraction 5 that contained the band originally recognized by the anti-20S when an intact native gel was transferred (Fig. 2a). The analysis of an equivalent gel fraction by mass spectrometry (Supplemental Table 1) indicated that this band corresponded to the independent 20S (CP). Additional evidence that proteins in fraction 5 corresponded to the 20S (CP) subunits, was given by the anti-20S (Fig. 2e and f). Longer film exposure to the same western blot membrane (Fig. 2f) produced a heavy smear in the 40–100 kDa interval. Western blot on a 20S (CP) purified from an equivalent enriched proteasome fraction (P3, Fig. 1) by ion exchange chromatography and size exclusion fractionation (Supplemental Fig. 1), suggests that the subunits of the 20S are heavily ubiquitinated even though the fraction was purified from Arabidopsis cells were kept under optimal culture conditions (Fig. 2e and f). By Ponceau S staining (or Coomassie on an equivalent gel) the characteristic 20S (CP) set of bands were hard to observe in those fractions that potentially contained proteasomes with a molecular mass higher than the independent 20S (CP) (lanes 1 to 4, Fig. 2d). Nevertheless, the anti-20S antibody tracked proteasomes up to lanes 2, 3 and 4 (Fig. 2e and f) that correspond to putative proteasome complexes of a nominal molecular mass (estimated by BN/PAGE) of approximately 1600, 1100 and 850 kDa, respectively. The proteasome profile described in Fig. 2 was considered as the basal for Arabidopsis suspension cells under optimum culture conditions, where the predominant population of proteasomes was constituted by the independent 20S (CP) and the abundance of "heavier" proteasomes gradually decreased toward the top of the BN/PAGE (Fig. 2e and f).

### 3.3. High molecular mass proteasomes in heat-stressed cells

Same general methodology was applied to Arabidopsis cells exposed to heat stress to detect possible alterations in the basal proteasomes arrangement observed in control cells (Fig. 2). First, we needed to establish if the content of total 20S proteasomes changed at 37 °C exposition. Densitometry analysis of the western blot films showed a small increment in the anti-20S signal from 11 to 17% between unexposed cells and those at 37 °C and less of an 8% among stressed cells (Supplementary Figs. 2a–c). Since 20S content among samples were considered equivalent, the BN/PAGE of proteasome-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 of a presumed molecular mass of ~2600 kDa 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 and 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 cell lysates (Fig. 4). Both parameters have been 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 42% of the original total ubiquitin conjugates content by dot blot (Fig. 4a) and a slight ubiquitination signal clearance by western blot (lane 2, Supplementary Fig. 2d). The level of oxidatively modified proteins had a 60% significative decrease at this time (Fig. 4b). The trypan blue viability assay showed a 2% decrease between the control and stressed cells. This difference was maintained even after 3 h of 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 cultures (lane 6 Fig. 3d and e) originated from a protein complex of 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 (Supplementary Fig. 1). The increment in high molecular mass proteasome populations correlated with the clearance of 84% (1 h) and 87% (2 h) of the basal ubiquitinated proteins levels (Fig. 4a and Supplementary Fig. 2d). Total protein carbonyls were kept at control levels during the first hour while at 2 h 60% of the original content diminished (Fig. 4b). Cell viability decreased 7% 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 of cell suspension cultures at 3 h under heat stress, showed the higher enrichment of all the proteasome versions, half-proteasomes included (Fig. 3f). 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. There were also clear differences in the slot blot determination of total ubiquitin conjugates and carbonyl contents, a three-fold and a thirty-six-fold increase respectively, in comparison with the previous sampled hour (Fig. 4a and b). A 9–11% decrease in cell viability was observed relative to control cultures at 3 h at 37 °C or after the recovery period.

### 3.4. 19S regulatory particle subunits are part of the high molecular mass proteasomes

The modular nature of proteasomes and their association/dissociation dynamics directed by environmental cues has been reported (Bajorek et al., 2003; Grune et al., 2011; Livnat-Levanon et al., 2014). High molecular mass proteasomes resolved by BN/PAGE were result from the assembly of different regulators on the distal surface of one or both the 20S α rings (Shibatani et al., 2006). To determine if the high molecular mass proteasomes observed contained 19S regulatory particle subunits, we probed the eight fractions obtained from a control and heat stress cultures BN/PAGE with antibodies against Rpn1 (19S base subunit), Rpt2 (19S base subunit), Rpn10 (19S lid subunit) and Rpn6 (19S lid subunit). Control fraction 5, that contained exclusively the independent 20S (CP) (lane 5, Fig. 5a, c and e) showed minimum or null reactivity against all the anti-19S regulatory subunits antibodies (lane 5, Fig. 5f, h, j and l). A strong signal with the immediate higher molecular mass proteasome complex was obtained with the Rpn10 and Rpn1 antibodies (Lane 4, Fig. 5f and h). Rpn10 signal showed a stepwise decrease toward the upper region of the BN/PAGE (Lanes 1 to 4, Fig. 5f) while the Rpn1 signal kept constant in three consecutive

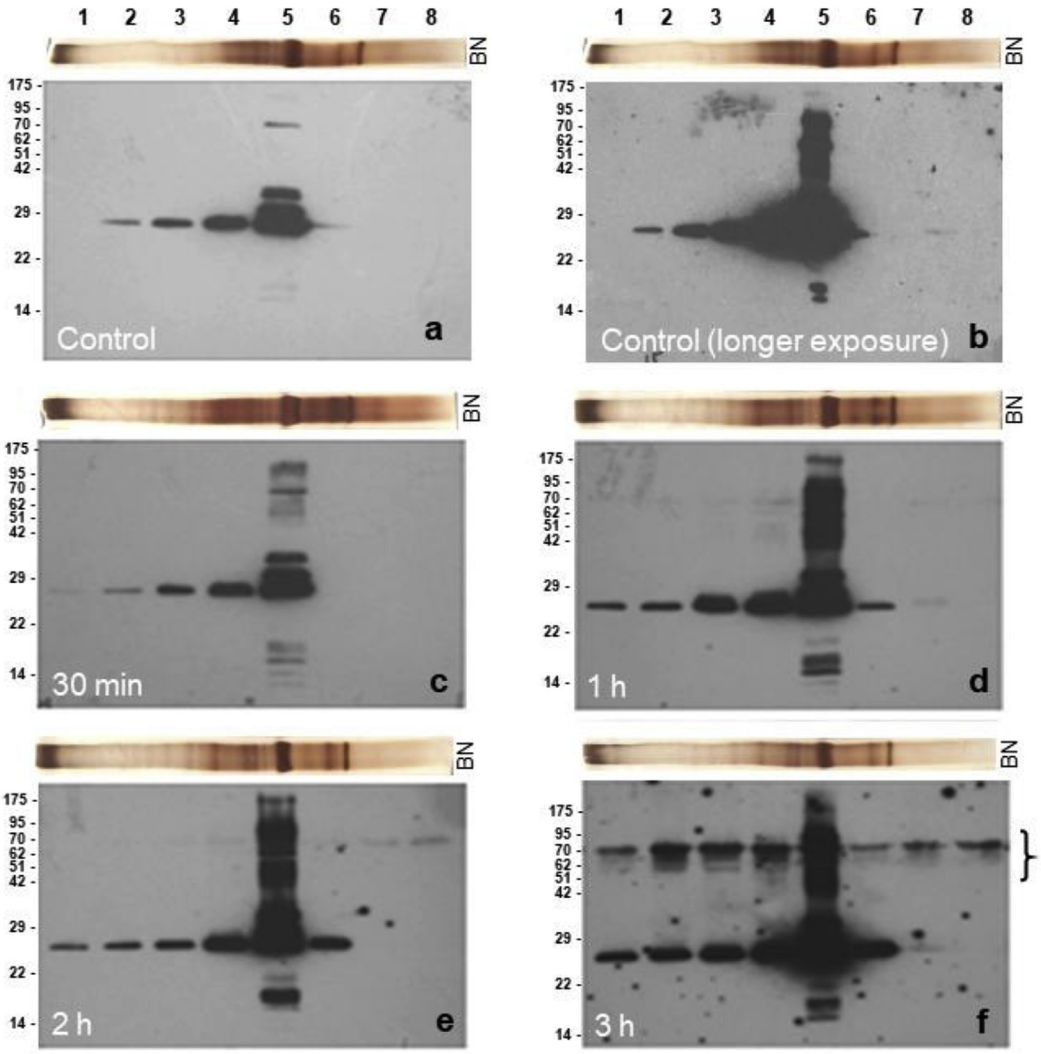


**Fig. 3. Western blot proteasomes profiles of *A. thaliana* cells under heat stress.** Proteasome enriched fractions (P3) from cells cultures at 37 °C were separated by BN/PAGE. Eight fractions obtained from each BN/PAGE gel (lanes 1 to 8) were individually electroeluted/concentrated, precipitated and 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).

fractions and showed a decrease up to fraction 1 (Lanes 1 to 4, Fig. 5h). The use of the Rpt2 and Rpn6 antibodies shown a different pattern, a stepwise increase toward those fractions obtained from the top of the BN/PAGE (Lanes 1 to 4, Fig. 5j and l). This control 19S subunits profile was altered in cells exposed for 1 h at 37 °C. Versus control culture, the anti-Rpn10 showed a higher reactivity with proteasome complexes in fraction 1 (Lane 1, Fig. 5g) and fractions 2 - 4 decreased (Fig. 5g). The use of anti-Rpt2 revealed a signal only from fraction 1 (Fig. 5k). Rpn1 and Rpn6 proteins had a fraction distribution equivalent to control culture, except for a significant increase in fractions 1, 7 and 8 for anti-Rpn1 (Fig. 5i) and in 6–8 fractions for Rpn6 (Fig. 5m). If we consider that, all the subunits are found in the context of their respective 20S or 19S complex (Livneh et al., 2016), our results suggest that some of the detected complexes probably represent 26S maturation/assembly intermediates. Based on the BN/PAGE mobility of the complex and the relative reactivity of the antibodies in each fraction, Rpn10 and Rpn1 were associated with the 20S CP (Fig. 5f and h, lanes 3 and 4) before association of Rpt2 and Rpn6 (Fig. 5j and l, lanes 3 and 4) as has been described (Hendil et al., 2009). In this context BN/PAGE fractions (Lanes 3 and 4, Fig. 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). The association scenario between 20S and its regulatory 19S subunits was shown altered under heat stress.

## 4. Discussion

Different proteasomes versions have been shown by BN/PAGE of samples from a human embryonic cell line (HEK293) (Camacho-Carvajal et al., 2004) and rabbit reticulocytes (Shibatani et al., 2006). The separation of the 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 Arabidopsis 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

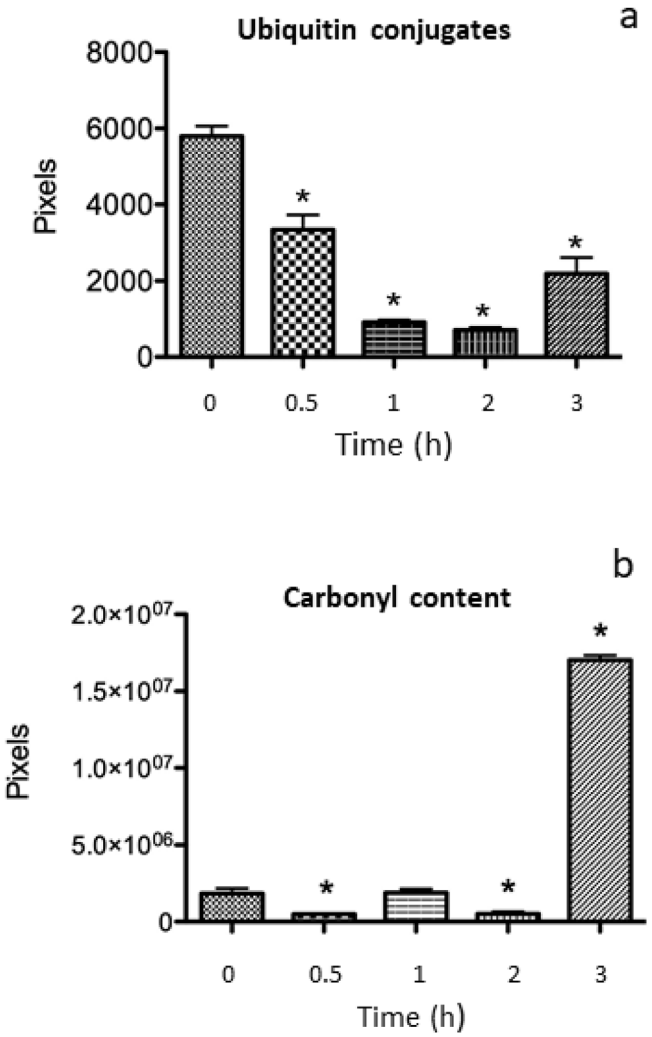


**Fig. 4. Ubiquitin conjugates and protein carbonyls of total lysates from Arabidopsis cell cultures exposed to heat stress.** Five mg of total protein extract were loaded into slot blot wells and immobilized on nitrocellulose membranes for determination of the total amount of ubiquitin conjugates present in cells incubated at 37 °C for 0, 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 immobilization and detection with an anti-DNPH antibody (**b**). Film densitometry was obtained by ImageJ (NIH). Data sets were analyzed by two-tailed paired Student *t*-test (Prisma Version 5.0b, GraphPad Software Inc.). Ubiquitin content significantly decreased after incubation at 37 °C; however, there is an increase of these conjugates after 3 h of treatment. P values for 0.5 h = 0.001, for 1 h = < 0.0001, 2 h = < 0.0001, and 3 h = 0.0003. Protein carbonyls of total cell lysates are significantly low after 0.5 and 2 h of heat shock; nevertheless, these contents did not differ between the control (0 h) and heat stressed cells for 1 h. After 3 h at 37 °C, carbonyl content significantly increased compared to the control. P values for 0.5 h = 0.0114, 2 h = 0.03, and 3 h = < 0.0001. (*) Depicts significant differences between the control (0 h) and the ubiquitin conjugates or protein carbonyl total lysates.

(Shibatani et al., 2006) with some modifications was effective for Arabidopsis cells in suspension cultures (Fig. 1b). In our lab, same technique was useful for the isolation of proteasomes (and their BN/PAGE analysis) from Arabidopsis 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 Arabidopsis cells was separated by BN/PAGE and transferred 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 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 Arabidopsis 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 (Figs. 2 and 3). Four proteasome populations were revealed in 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 spectrometry (Supplementary Table 1). We discarded the possibility that "heavier" proteasomes detected in unexposed or in heat stressed cells (Fig. 3) were product of an artifact 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 the same fractions. 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 modification of the 20S (CP) subunits by ubiquitination have been shown by proteomic techniques in Arabidopsis (Book et al., 2010). High temperatures by themselves caused intracellular protein denaturation and substrates ubiquitination (Lepock et al., 1988; Pinto et al., 1991). In Arabidopsis suspension cell cultures, a moderate heat stress was detected when 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 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) (Pacifici 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 Arabidopsis cell response to heat increment. During the first phase (30 min–2 h at 37 °C) 88% the basal ubiquitin conjugates were removed (Fig. 4a and Supplementary Fig. 2d) while the total protein carbonyl level decreased a 40% (Fig. 4b). These results indicate that an oxidative stress was not produced because the antioxidant machinery and the modified protein elimination mechanisms were effective. The western blot proteasome profiles (Fig. 3a-f) 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 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 Arabidopsis the abundance of these two proteasome entities is highly interrelated during cell growth and stress tolerance (Kurepa et al., 2009). If degradation of protein ubiquitin conjugates was limited (by 26S synthesis impairment) oxidized proteins degradation by the independent 20S (CP) was favored (Kurepa et al., 2008). We consider that the opposite phenomenon as we observe during the first phase, is also plausible. During the second phase (3 h at 37 °C) both stress markers increased, doubled for ubiquitin conjugates and a nine-fold increase was detected for protein oxidation (Fig. 4a and b). These results are indicative that cellular antioxidant and damaged protein removal

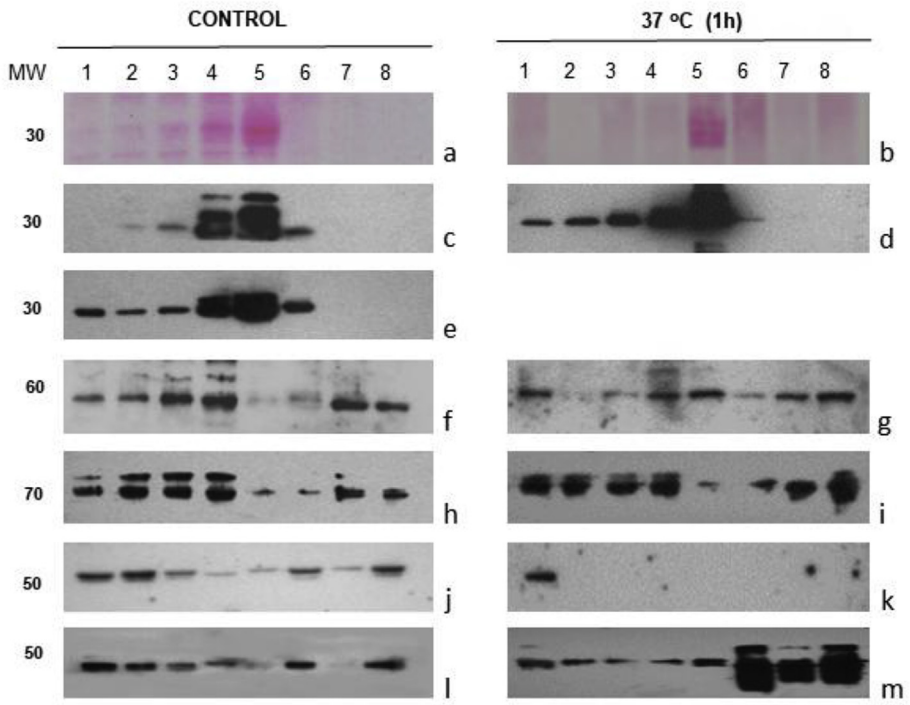


**Fig. 5. 19S regulatory particle subunits were present in high molecular mass proteasomes.** Denatured BN/PAGE fractions (1–8) from control and heat stressed (1 h at 37 °C) Arabidopsis cells were transferred to nitrocellulose, stained with Ponceau red (**a**, **b**) and probed with anti-20S (**c**–**e**), anti-Rpn 10 (**f**, **g**), anti-Rpn 1 (**h**, **i**), anti-Rpt 2 (**j**, **k**) and anti-Rpn 6 (**l**, **m**) antibodies. In contrast with panel (**c**), the anti-20S profile in **e**, was obtained with five times higher protein loading. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

capacities were surpassed, and an oxidative stress episode was initiated (Sies, 1997). The western blot profile showed the higher enrichment for all the proteasomes versions observed (Fig. 3 f). The stress markers levels and the western blot results combined, suggest that high molecular mass proteasomes still removed ubiquitin conjugates but with a relative diminished efficiency (Fig. 4a); however, oxidized proteins greatly increased (Fig. 4b). We hypothesize that high temperature for 3 h, forced the cell machinery to assembly high molecular mass proteasomes while decreasing the total amount of independent 20S (CP) and oxidatively modified proteins increased consequently. The 26S proteasome has been shown inefficient at degrading oxidatively modified proteins (Davies, 2001) and in addition, was inhibited by oxidative stress (Reinheckel et al., 1998). This observation probably explains why despite "heavier" proteasomes were enriched (Fig. 3f) a three-fold decrease in their capability of ubiquitinated proteins removal was detected (Fig. 4a). An additional explanation for the impaired oxidized protein elimination is the sensitivity of the trypsin and caspase-like 20S (CP) catalytic activities to oxidation (Demasi et al., 2003). A heavy signal, attributable to 20S subunits ubiquitination was observed for all the BN/PAGE fractions from suspension cells heat stressed for 3 h (Fig. 3f and Supplemental Fig. 1). Under abiotic stress, the activity of specific Arabidopsis ubiquitin ligases (Pub22 and Pub23) increased, which destabilized the 19S regulator by ubiquitination of its subunits (Cho et al., 2015). Inhibition of the 26S activity by substrate overload is plausible (Kurepa et al., 2009). 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 (Hasanuzzaman et al., 2013). 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 Arabidopsis suspension cell cultures adapted the proteasome degradative machinery 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 varied between 8 and 10% 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) allow a direct comparison among the different proteasome populations present under stress. The contrast between the proteasome complement of control cells with those of the stressed cultures (Fig. 3), 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 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 and heat stressed cells, showed that the 19S subunits Rpn10, Rpn1, Rpt2 and Rpn6 are associated to "supra" 20S proteasome assemblies (Fig. 5). This association profile was altered by the temperature increase. At 37 °C Rpn10 (a well-known receptor of Ub-conjugates) and Rpt2 (19S subunit that unfolds and inserts substrates into the 20S core protease) were preferentially detected in fraction 1 (Fig. 5g and k) where the 19S–20S–19S proteasome version is expected. We did not discard the possibility that heat-enriched proteasomes (detected in fractions 2–4) other than the 26S, have ub-conjugates elimination capacities, while the isolated 20S (CP) kept oxidatively modified proteins at basal levels.

The presence in the cell of *ad hoc* proteasomes offer better possibilities for successfully coping with unfavorable growth conditions. We consider that our approach of proteasome isolation, separation of

discrete proteasomes populations by BN/PAGE and the concentration of the samples by electroelution showed that an increment in culture temperature directed the assembly of “supra” 20S proteasome complexes. Our protocol could be considered a useful tool to characterize the regulators and the additional interacting proteins that contribute to the proteasomes function and dynamics.

## Contribution

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.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.034.

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