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The ubiquitin/proteasome pathway from *Lemna minor* subjected to heat shock

A.S. CAEIRO[1], P.C. RAMOS[2], A.R. TEIXEIRA[3] and R.B. FERREIRA[1,3]*

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal[1]
Departamento de Química e Bioquímica, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Faro, Portugal[2]
Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal[3]

Abstract

Exposure of *Lemna minor* L. to high temperatures leads to an initial decrease in the ubiquitin (Ub) monomer pool size and the accumulation of high molecular mass Ub-protein conjugates, possibly reflecting an increment in the supply of protein substrates to the Ub/proteasome pathway. Alternative explanations include, for example, changes in the transcription rates of one or more pathway components. To measure the effect of heat shock on the simultaneous rates of transcription of selected genes encoding five Ub pathway components (Ub, E1, E2, β subunit and ATPase subunit of the 26S proteasome), a semi-quantitative RT-PCR method was developed using cDNA normalized against the housekeeping gene encoding the 18S ribosomal RNA. Whilst Ub transcription is abruptly increased, there is a moderate increment in the transcription of E1 and the β subunit, a moderate reduction in the transcription of the ATPase subunit and a marked reduction in the case of E2, indicating a differential transcription pattern of the various components of the Ub/proteasome pathway in *L. minor* subjected to high temperatures. These observations suggest that the increment in the Ub/proteasome pathway intermediates is due to an augmented supply of substrates derived from the stress-induced damage imposed on the cellular proteins. The initial build up of intermediates occurs not only at the expense of the pre-existing pool of free Ub, but also as a result of the prompt increase in Ub expression.

Additional key words: ATPase subunit, duckweed, semi-quantitative RT-PCR, transcription.

Introduction

Plants are typically detrimentally affected upon exposure to high temperatures (Iba 2002). Thus, for example, in mulberry, high temperature stress markedly reduced the activities of Rubisco and sucrose phosphate synthase, decreased chlorophyll, total soluble protein content, photosystem 2 activity and foliar nitrogen and nucleic acids, and affected sugar metabolism through reduction in leaf starch content and sucrose-starch balance (Chaitanya *et al*. 2001). To cope with the stress, plants have to adapt their protein metabolism promptly, not only by synthesizing a group of stress-related proteins but also by removing damaged or unwanted proteins. Thus, heat shock proteins were synthesized in lentil seeds in response to heat treatment (Dell'Aquila 2000). In strawberry leaves and flowers, the content of most proteins decreased but a few new proteins appeared in response to heat stress (Ledesma *et al.* 2004). However, this study concluded that the results obtained differ between plant organs and between cultivars. Extracellular proteins were found to accumulate in intercellular spaces of barley primary leaves after heat shock (Tamás *et al*., 1997). In wheat leaves, the contents in Rubisco activase and Rubisco binding protein were found to increase under heat stress, in accordance with their role as chaperones

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Abbreviations: E1 - Ub-activating enzyme; E2 - Ub-conjugating enzyme; RT-PCR - reverse transcriptase polymerase chain reaction; Ub - ubiquitin.
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* Author for correspondence; fax: (+351) 214433644, e-mail: rbferreira@itqb.unl.pt

and the function of the binding protein as a heat shock protein (Demirevska-Kepova *et al.* 2005).

High temperatures increase the rate of postsynthetic protein denaturation due to the low free energy required to stabilize mature proteins (Goldberg 2003). Partially unfolded proteins can be highly toxic, because they act as nuclei for intracellular aggregation due to the tendency of normally buried hydrophobic domains to associate with one another. For this reason, cellular proteins are under continuous surveillance by the proteolytic systems (Goldberg 2003). The ubiquitin (Ub)/proteasome system, a tightly regulated and highly specific pathway for the degradation of individual proteins in eukaryotic cells, has been reported as a major proteolytic pathway responsible for the selective breakdown of abnormal proteins (Sullivan *et al.* 2003, Vierstra 2003). Ub-dependent proteolysis of proteins occurs in two sequential steps: tagging the protein substrates with Ub, followed by degradation by the 26S proteasome.

The highly conserved, 76-amino-acid protein Ub is covalently attached to specific protein targets *via* an ATP-dependent reaction cascade. Subsequent rounds of conjugation add polyUb chains to the target. The Ub-conjugating cascade involves the sequential action of three enzyme families, E1, E2 and E3 (Sullivan *et al.* 2003, Vierstra 2003). The exquisite selectivity of this pathway resides in the E3s, which are specific for different protein substrates, explaining the large number (> 1200) of E3 genes found in the *Arabidopsis* genome (Glickman and Ciechanover 2002).

The resulting polyubiquitylated protein conjugates are then recognized and degraded by the 26S proteasome, with the concomitant release of the reusable Ub monomers (Vierstra 2003). This ATP-dependent, self-compartmentalized protease is composed of a 20S core protease (CP), in which proteins are degraded to short peptides, and one or two 19S regulatory particles (RP), which confer ATP-dependence and substrate specificity (Voges *et al.* 1999).

The CP is a broad-spectrum, ATP- and Ub-independent peptidase created by the assembly of four, stacked heptameric rings of related α and β subunits surrounding a central cavity. Its two inner β rings form a central chamber containing the proteolytic sites, which face the central cavity. Access to this chamber is restricted by a narrow gated channel created by the α-subunit rings, which is normally maintained in the closed state. Even in its open state, controlled by the ATPases in the RP, it allows entry of only unfolded proteins (Benaroudj *et al.* 2003).

Each end of the CP is capped by an RP composed of 18 principal subunits. Apparently, the RP recognizes and binds to polyubiquitylated proteins, releases the attached Ubs as free monomers, unfolds the protein substrates, opens the α-subunit ring gate and directs the unfolded proteins into the CP lumen for degradation (Belknap and Garbarino 1996, Voges *et al.* 1999, Glickman 2000).

Several studies have been published on the effect of stress on the expression of individual Ub pathway components. However, to our knowledge, no study has been conducted on the various components of the pathway. In this work, the levels of the Ub pathway intermediates (*i.e.* free Ub and the high molecular mass Ub-protein conjugates) were analysed in *Lemna minor* L. cells subjected to heat shock. Semi-quantitative RT-PCR was then used to follow the changes in the levels of selected mRNAs encoding Ub, E1, E2, the β subunit of the CP, and an ATPase subunit of the RP.

Materials and methods

Plant growth and stress induction: Plants of *Lemna minor* L. were grown at temperature of 21 ºC and a 16-h photoperiod with irradiance of 200 μmol(photon) m^{-2} s^{-1} in a complete sterile medium, as described before (Ferreira and Teixeira 1992), supplemented with 10 mM sucrose. For stress induction the plants were incubated at 38 ºC for periods up to 6 h. Plant material was frozen in liquid nitrogen and stored at -80 ºC.

Isolation of total RNA: Total RNA was isolated from plants incubated at 38 ºC during 0, 0.5, 1, 2, 3, 4 and 6 h, using the *Invisorb* Spin Plant-RNA Mini Kit (*Invitek*, Berlin, Germany), according to the manufacturer's specifications. The RNA was DNase treated using the *TURBO DNA-free*™ kit (Ambion, Austin, TX, USA).

Generation of cDNA: Single-stranded complementary DNA (cDNA) was synthesized using the *Superscript*™ II RNase H reverse transcriptase (*Invitrogen*, Burlington, ON, Canada) according to the manufacturer's protocol. Approximately 250 ng of total RNA was used, in combination with 0.5 μg Oligo$(dT)_{12-18}$ (*Invitrogen*) and 100 ng of random hexamers pd$(N)_6$ (*Roche*, Penzberg, Germany) as primers, 500 μM of dNTPs (*Invitrogen*) and H_2O. The mixture was heated at 65 ºC for 5 min to denature secondary structures, then cooled rapidly in ice and incubated at 25 ºC during 10 min to allow annealing of random hexamers. The RNA was reverse transcribed in a final volume of 20 mm^3 using 200 U *Superscript*™ II RNase H reverse transcriptase, 40 U RNaseOUT™ (*Invitrogen*), 10 mM dithiothreitol (DTT), 4 mm^3 5× First-Strand buffer, at 42 ºC for 50 min, and then stopped by heating at 70 ºC for 15 min. The RT mix was incubated with 2 U RNase H at 37 ºC for 20 min.

Oligonucleotide primers (Table 1) were designed from conserved regions among known selected gene sequences, to amplify specific cDNA sequences encoding five different genes involved in the *L. minor* Ub/proteasome pathway. Polymerase chain reactions (PCR) were performed in a *Trio-Thermoblock*™ PCR machine (*Biometra*, Gottingen, Germany) using 1 mm^3 of the

cDNA, 50 ng of each oligonucleotide primer, 200 μM of dNTPs (*Invitrogen*), 1.25 U of Taq DNA polymerase (*Invitrogen*) and 5 mm^3 10× PCR buffer, in a 50 mm^3 volume. The PCR program initially started with a 95 ºC denaturing step for 3 min, followed by 3 min at the appropriate annealing temperature (a.t. 55 ºC for Ub, 60 ºC for E1, 55 ºC for E2, 60 ºC for the β subunit of the 26S proteasome, and 55 ºC for the ATPase subunit of the 26S proteasome) and 72 ºC for 3 min. It continued then with 36 cycles at 95 ºC for 90 s, a.t. for 90 s and 72 ºC for 90 s, and a final elongation step at 72 ºC for 5 min.

Agarose gel electrophoresis: The RT-PCR products were fractionated on 1.0 % (1.5 % for semi-quantitative analyses) (w/v) agarose gels in TAE buffer (40 mM Tris-acetate (pH 8.5), 2 mM $Na_2EDTA.2H_2O$). The gels were stained with ethidium bromide (0.5 ng cm^{-3}) and the UV illuminated gel images were digitally captured with a CCD-camera from Gel Doc™ 1000 Single Wavelength Mini-Transilluminator (BIO-RAD, Hercules, CA, USA) and acquired using Quantity One® version 4.01 program (BIO-RAD, Hercules, CA, USA).

Similarity search: RT-PCR products were purified using the high pure PCR product purification kit (*Roche*). These products were subsequently sequenced with *BigDyeTerminator v1.1* kit (*Applied Biosystems*, Foster City, CA, USA) and analyzed by multi-capillary electrophoresis with *ABI Prism 3700* DNA analyzer at Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal. DNA sequence similarity was searched at NCBI with the *BLAST 2.2.9* algorithm to confirm identity. New sequences were considered confirmed if they were > 80 % homologous against other plant sequences.

Semi-quantitative PCR: For semi-quantitative PCR (following the protocol provided by *Ambion*, with minor modifications), and for each cDNA species of interest, the range of cycles over which each species should be examined was initially determined. A PCR master mix was prepared and was split into 10 aliquots, which were then subjected to PCR amplification and removed at different cycle numbers, starting with cycle 24 and ending with cycle 38. The thermal cycle profile used included a denaturation step of 95 ºC for 3 min, followed by 3 min annealing, 72 ºC for 3 min, and then by 24 to 38 cycles of 95 ºC for 30 s, a.t. for 30 s, 72 ºC for 30 s and a final elongation at 72 ºC for 5 min. The PCR samples were fractionated on 1.0 % (m/v) agarose gels, visualized with ethidium bromide, quantified with *Scion Image Beta 4.0.2* program and plotted (log of the signal *vs*. cycle number). The ideal cycle number selected for subsequent experiments was one in the middle of the detectable linear range.

Subsequently, the cDNAs of interest were co-amplified with a "housekeeping" gene (the 18S ribosomal RNA; transcript of 315 bp), for the number of cycles determined before, using commercial primers from *QuantumRNA™ 18S* internal standards (*Ambion*). Previous to RT-PCR experiments, the ratio of 18S primers to competimers (needed to contain both the specific gene and the 18S internal control, and to give similar yields of products) was determined using different ratios of 18S primer:competimer (400 nM) in the PCR mixture.

PCR was utilized to quantify the relative amount of mRNA, using 1 mm^3 of the cDNA template, 50 ng of each gene specific primer pair, 400 nM of 18S primers:competimers (*Ambion*), 200 μM of dNTPs, 1.25 U of Taq DNA polymerase and 5 mm^3 10× PCR buffer, in a final volume of 50 mm^3. The thermal cycle profile was as follows: 95 ºC for 3 min, a.t. for 3 min, 72 ºC for 3 min, followed by 27 to 31 cycles of 95 ºC for 30 s, a.t. for 30 s and 72 ºC for 30 s, and a final elongation of the product for 5 min at 72 ºC. The RT-PCR products were separated and visualized on 1.5 % (m/v) agarose gels, and digitally recorded and quantified as previously described. Data were expressed in relation to the internal control, as a ratio of the specified gene signal divided by the 18S ribosomal signal, in arbitrary units.

Protein extraction: Proteins were extracted by homogenizing (2 cm^3 g^{-1} fresh mass) plant samples in 90 mM Tris-HCl buffer (pH 8.0), containing 45 % (v/v) ethylene glycol, 18 mM sodium disulphite, 10 mM *N*-ethyl-maleimide (NEM), 5 mM ethylene diaminetetraacetic acid (EDTA), and 1 mM phenyl methyl sulfonyl fluoride (PMSF). The homogenate was then squeezed through two layers of cheesecloth and centrifuged at 31 000 *g* for 12 min. The resulting supernatant was desalted into 25 mM Tris-HCl buffer (pH 7.4), containing 150 mM NaCl, 1 mM EDTA, and 10 mM NEM. Protein concentrations were estimated by the Bradford method, using bovine serum albumin (BSA) as the standard.

Electrophoresis and Western blotting: Protein samples (5 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). To perform the Western analysis, the separated proteins were subsequently transfered onto a polyvinylidene fluoride (PVDF) membrane (*Roche*). Immunoblotting was performed according to the standard procedures described previously (Ferreira and Shaw 1989). Enhanced chemiluminescence (ECL) was used as the detection system (*Pierce*, Erembodegem, Belgium). Ub-P conjugates and free ubiquitin were semi-quantified using GelPlot facility from *Scion Image for Windows* (version *Beta 4.0.2*, *Scion Corporation*).

Statistical analyses: RT-PCR values were averaged from 3 replicates and are reported as the mean ± SE. For the analysis of variance and for Post-hoc comparison of mean, Tukey honest significant difference (HSD) test and *Statistica® 6.0* software were used.

Results and discussion

Selection of the genes encoding Ub/proteasome pathway components: It is well known that many Ub pathway components are encoded by multigene families. For example, *Arabidopsis* contains two E1 genes, 37 E2 genes and ~1300 encoding E3 components (Vierstra 2003). However, in some cases, the encoding genes exhibit a high degree of homology, whereas in others only a small subset is responsive under stress conditions.

Different criteria were followed to design the primers. Amplification primers (Table 1) were designed on highly conserved regions, including protein motifs whenever possible. Because Ub, E1 and E2 are encoded by gene families (Smalle and Vierstra 2004), an attempt was made to select polyUb, E1 and E2 genes whose levels of transcripts have been reported in the literature to be affected by stress (Seufert and Jentsch 1990, Girod *et al.* 1993, Hatfield *et al.* 1997, Loser and Weltring 1998, Noventa-Jordao *et al.* 2000, Spees *et al.* 2002a,b).

Ubiquitin is encoded by a multigene family. Functional Ub is produced from two different types of Ub genes, namely polyUb genes an Ub extension genes (Schlesinger and Bond 1987, Callis and Vierstra 1989). PolyUb genes contain tandem head-to-tail repeats of 228 bp encoding the Ub protein. In the case of *Pisum sativum*, for example, four polyUb genes (*PUB1*, *PUB2*, *PUB3* and *PUB4*) were isolated from a genomic library and completely sequenced (Xia and Mahon 1998). Ub extension genes contain a single Ub repeat followed in frame by sequences encoding one of two ribosomal proteins. The initial translation products from both types of Ub genes are readily processed to intact, functional Ub monomers.

The Ub extension protein genes are not generally involved in stress responses (Hoffman *et al.* 1991, Rickey and Belknap 1991). On the contrary, polyUb genes have been shown to be transcriptionally activated by heat stress in a variety of plants, animals and fungi (Genschik *et al.* 1992, Myer and Schwartz 1996, Sun and Callis 1997, Loser and Weltring 1998, Rollfinke *et al.* 1998, Simon *et al.* 1999). However, heat stress induced the transcription of only specific members of the polyUb gene family in several species (Belknap and Garbarino 1996, Noventa-Jordao *et al.* 2000, Spees *et al.* 2002a).

The *Lemna* Ub sequence to be amplified was selected to target the Ub domain signature (PS00299), making the primers specific to polyUb transcripts, while excluding the Ub-fusion transcripts. In addition, the primers obtained originate multiple matching subsegments (products) in the polyUb mRNAs, reflecting the Ub repeats in the sequence. Thus, in the case of *Lemna*, three polyUb gene products were obtained (results not shown) with sizes estimated at 250, 500 and 750 bp. The multiple matching obtained experimentally for *Lemna* was compared with the theoretical multiple matching obtained for the four polyUb genes from *P. sativum* (GenBank accession numbers L81139, L81140, L81141 and L81142; Xia and Mahon 1998). To this end, the primers designed for *Lemna* polyUb genes were aligned with the *P. sativum* polyUb gene sequences (*BLAST* 2 sequences) and the range of amplified products predicted: three products (234, 462 and 690 bp) for *PUB2*, two products (234 and 462 bp) for *PUB3*, one product (234 bp) for *PUB4* and no product for *PUB1*. These observations indicate that the products obtained from *Lemna* probably represent a pool of polyUb transcripts synthesized from different polyUb genes.

Multiple E1 isoforms exist in organisms. Thus, E1s are encoded by one gene in *Saccharomyces cerevisiae*, by two genes in *Arabidopsis thaliana*, or by three genes in *Triticum aestivum*. The two *Arabidopsis E1* genes have been reported to be co-expressed in most, if not all, plant tissues and cells and not differentially expressed (Hatfield and Vierstra 1992, Hatfield *et al.* 1997). Since the number of E1 encoding genes in *Lemna* remains unknown, primers were designed that are potentially capable of amplifying the total pool of E1 transcripts. To this end, the region of the sequence to be amplified contained the prosite motif Ub-activating enzyme active site (PS00865).

Table 1. Sequence of primers and expected fragment size of RT-PCR products, used in the semi-quantitative RT-PCR analysis of mRNA levels of the genes expressed in *Lemna minor* in response to heat stress. The GenBank accession numbers for the cDNA partial sequences obtained in this work for *L. minor* genes are indicated.

Gene	Sequences of primers	Size [bp]	Number
Polyubiquitin	Sense: 5′-TAGACAATGTTAAGGCCAAGATTCAAGACAAGG-3′ Antisense: 5′- TGTCAATGGTATCAGAGCTCTCCACCTCAAGGG -3′	234	AY683447
Ubiquitin-activating enzyme (E1)	Sense: 5′-GGCAAGAATGTATGTTGACCAG-3′ Antisense: 5′-TGTGGGGAACGAGTGAGAGT-3′	180	AY683450
Ubiquitin-conjugating enzyme (E2)	Sense: 5′-CCAAGCGCATCCTCAAGGAGCTCAAGG-3’ Antisense: 5′-GGATAATCTGGAGGAAAATGAATGGTAACC-3	175	AY683451
Beta subunit of the 26S proteasome	Sense: 5′-CCATGGCTGGTGGGGCTGCTGAC-3′ Antisense: 5′-CCAGGCCCCGTTTCATCCCACCC-3′	199	AY683448
ATPase subunit of the 26S proteasome	Sense: 5′-GGTTCGACGCACGTGGCAACATCAAGG-3′ Antisense: 5′- CCTAATGTCGGCACCTGTGGAGTTTGG-3	236	AY683446

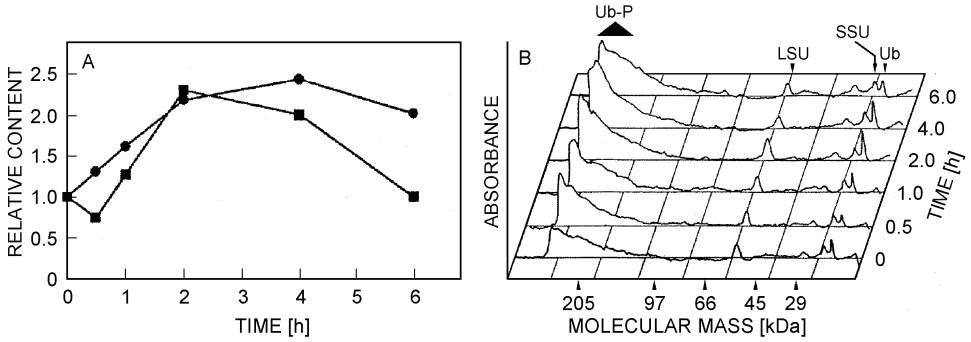

Fig. 1. Immunoblot quantification of the levels of free Ub (Ub) and high molecular mass Ub-protein conjugates (Ub-P) in *Lemna minor* exposed to 38 ºC for different periods of time (from 0 to 6 h). *A* - Relative content of free Ub (*squares*) and Ub-protein conjugates (*circles*). *B* - Profile plots generated for each lane in the immunoblot where the total *Lemna* protein was probed with anti-Ub antibodies; the data presented were averaged from several immunoblots. Molecular masses of standards in kDa; LSU and SSU - large and small subunits of ribulose bisphosphate carboxylase.

The various isoforms of E2s are all encoded by small multigene families containing from two to more than five members (Seufert and Jentsch 1990, Zhang *et al.* 2003). The primers to the *Lemna E2* gene sequence were designed to target the prosite motif of the Ub-conjugating enzyme family (PS50127).

In the case of the 26S proteasome, all genes encoding its subunits have been reported to be expressed simultaneously and up-regulated under stress conditions (Belknap and Garbarino 1996, Smalle and Vierstra 2004). RT-PCR analysis using suitable primers (Table 1) allowed the amplification of 236 bp and 199 bp fragments for the ATPase and β subunit of the 26S proteasome, respectively. The deduced amino acid sequence from the *L. minor* 26S proteasome ATPase subunit fragment contains a prosite motif AAA-protein family signature (PS00674).

Changes in the levels of Ub/proteasome pathway intermediates in *Lemna minor* in response to heat shock: The average relative (considering the values obtained for the control without heat stress as unit) contents of free Ub and high molecular mass Ub-protein conjugates were determined in plants of *L. minor* exposed to 38 ºC for 0, 0.5, 1, 2, 3, 4 and 6 h (Fig. 1*A*,*B*). These diagrams were prepared from several immunoblots that were probed with affinity chromatography purified, anti-Ub polyclonal antibodies. As noted before (Ferreira *et al.* 1995) these antibodies produced a measurable signal with the large (LSU) and small (SSU) subunits of Rubisco (Fig. 1).

As expected, heat shock induced a gradual build-up of large Ub conjugates in the intracellulars (Fig. 1). As concerns free Ub, there was a rapid reduction in its content following the onset of stress, possibly explained by a prompt demand for this protein to the ubiquitylation of heat-unfolded proteins. The subsequent boost in the *in vivo* content of free Ub might result from a large increase in its level of expression plus rate of release from the Ub conjugates, which exceeded during several hours its rate of conjugation. These results are supported by the previous observation that Ub is itself a heat shock protein (Bond and Schlesinger 1985).

It now seems well established that high temperatures, as other stresses in general, lead to decreased Ub monomer pool size and the accumulation of high molecular mass Ub-protein conjugates in plant tissues (Ferguson *et al.* 1990), possibly by increasing the supply of protein substrates to the Ub/proteasome pathway. In the particular case of heat shock, many proteins suffer partial unfolding and become toxic because of their tendency to form intracellular aggregates. For this reason, cellular proteins are under constant surveillance by the proteolytic systems, which continually monitor mature proteins for postsynthetic denaturation (Goldberg 2003). Indeed, the Ub/proteasome pathway is believed to function during heat stress by degrading the enhanced levels of damaged cellular proteins. However, alternative explanations for the observed decrease in free Ub/increase in Ub-conjugates during heat stress, such as, for example, a reduction in the expression and/or an increment in the proteolysis of one or more of the pathway components, cannot be ruled out.

Changes in the levels of selected mRNAs encoding Ub/proteasome pathway components in *Lemna minor* in response to heat shock: The information concerning the expression under stress conditions of the various Ub/proteasome pathway components as a whole is sparse. Most studies have focused on individual pathway components. Nevertheless, resistance to high temperatures in yeast has been shown to require both the synthesis of Ub monomers and other components of the Ub-conjugating system and the proteasome (Finley *et al.* 1987, Hilt and Wolf 1995).

Using suitable primers, designed from conserved regions among known gene sequences, a study was conducted to follow the changes in the levels of selected mRNAs encoding various Ub/proteasome pathway components (Ub, E1, E2, and β subunit from the CP and ATPase subunit from the RP of the proteasome) in *L. minor* exposed to high temperatures (38 ºC) for various

durations. After isolation of the total RNA fraction from each plant sample, RT-PCR was used to select and amplify specific cDNA sequences encoding the genes of interest. With the exception of Ub, for which three amplification products of the expected size were produced (see above), after conventional PCR performed under optimized conditions (data not shown) and for each primer pair tested, a single amplification product of the expected size was obtained. The amplified products or PCR amplicons were subsequently sequenced and the corresponding deduced amino acid sequences compared with those of other species (results not shown). These data indicate that there are very strong homologies when the amino acid sequences of the five proteins under study are compared with those of the other species, including man.

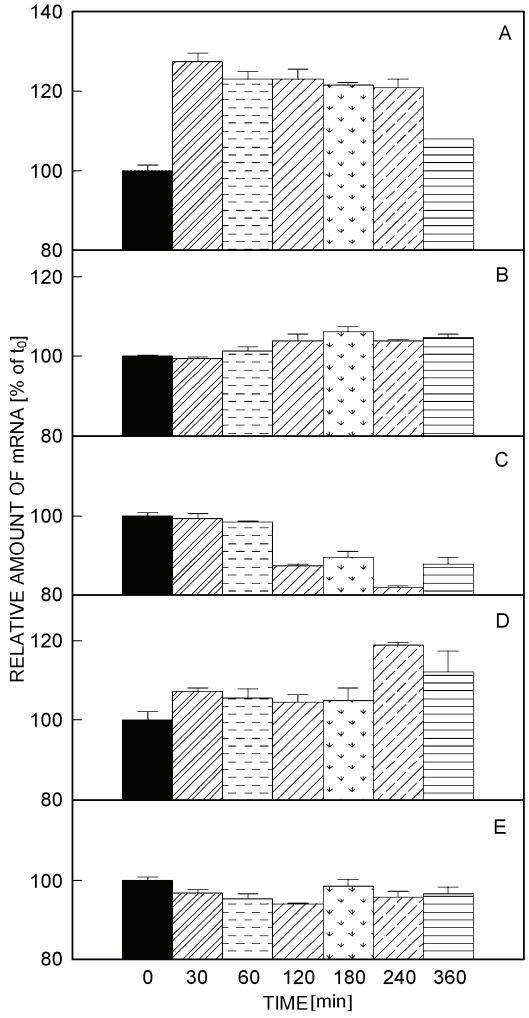

Fig. 2. Effect of heat stress on the relative amounts of the mRNAs encoding the five selected genes involved in the ubiquitin/proteasome pathway from *Lemna minor*. The plants were exposed to 38 ºC for 0, 0.5, 1, 2, 3, 4 and 6 h. *A* - ubiquitin; *B* - E1; *C* - E2; *D* - β subunit of the 26S proteasome; *E* - ATPase subunit of the 26S proteasome. Relative amounts of mRNA were measured by semi-quantitative RT-PCR. Means ± SE. Significant differences from the 0 h are marked as ** ($P < 0.01$) and *** ($P < 0.001$).

The primers specific for the polyUb, E1, E2, β subunit and ATPase genes were designed to span 234, 180, 175, 199 and 236 bp of the cDNA, respectively (Table 1). To follow the changes in the levels of the transcribed mRNAs in *L. minor* subjected to heat shock, a semi-quantitative RT-PCR method was developed using cDNA normalized against the housekeeping gene encoding the 18S ribosomal RNA (transcript of 315 bp). This method did not require radioactivity or expensive PCR instruments with real-time fluorescent detection (Meadus 2003). RT-PCR values were presented as a ratio of the specified gene's signal in the selected linear amplification cycle divided by the 18S ribosomal RNA signal.

The results of this experiment showed that there was an abrupt increase in the transcription of polyUb soon after the onset of stress, followed by a gradual decline thereafter (Fig. 2). However, the contents of polyUb mRNA were always above the control value. Moderate increases were observed for E1 and the β subunit of the proteasome. A moderate decrease was detected in the transcription of the ATPase subunit of the proteasome, and a marked reduction in the case of E2.

In the case of the 26S proteasome, all genes encoding its subunits have been reported to be expressed simultaneously and up-regulated under stress conditions (Belknap and Garbarino 1996, Smalle and Vierstra 2004). Ito *et al.* (1997) detected the simultaneous expression of the α and β subunits of the CP and an ATPase subunit of the RP during germination of *Spinacia oleracea* seeds. These observations contrast with the data presented in Fig. 2, in which the content of the β subunit mRNA increased and that of the ATPase subunit decreased when *Lemna* was exposed to heat shock. Shirley and Godman (Shirley and Goodman 1993) had already reported that a limited increase in mRNA encoding a proteasome subunit was induced by heat shock in *Arabidopsis*. In support of our results are the studies performed by Basset *et al.* (2002), who showed that both 20S and 26S forms coexisted in eukaryotic cells (Yang *et al.* 1995). Besides its functions as the proteolytic core of the 26S complex, the 20S proteasome may be involved in the degradation of oxidatively modified proteins (Basset *et al.* 2002), forming a selective mechanism to remove oxidatively damaged proteins from the cell (Grune *et al.* 1997). If this hypothesis is correct, it is not surprising to find differential expression patterns between CP and RP subunits, at least under selected conditions.

With the exception of the 26S proteasome subunits, the other results (Fig. 2) are in reasonable agreement with the scarce information available on this subject in the literature. Heat stress has been shown to result in transcriptional activation of polyUb genes in a variety of plant species (Belknap and Garbarino 1996). However, in several plant species, such as potato, maize and sunflower, only specific members of the polyUb gene

family are transcriptionally activated by heat stress. Furthermore, polyUb genes that are either nonresponsive or down-regulated by heat shock have also been identified in *Arabidopsis* and parsley (Belknap and Garbarino 1996). Although the genes of certain E2s are transcriptionally activated by heat shock in animals (Goldberg 2003) and yeast (Seufert and Jentsch 1990), their plant homologs are not induced by high temperatures in either *Arabidopsis* (Sullivan *et al.* 1994) or *Nicotiana sylvestris* (Genschik *et al*. 1994).

In summary, the data illustrated in Fig. 2 indicate a differential expression of the various components of the Ub/proteasome pathway in *Lemna minor* exposed to high temperatures.

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