Plant Cell Physiol. 47(9): 1262–1273 (2006)
doi:10.1093/pcp/pcj096, available online at www.pcp.oxfordjournals.org
© The Author 2006. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.
All rights reserved. For permissions, please email: journals.permissions@oxfordjournals.org

Exposure of *Lemna minor* to Arsenite: Expression Levels of the Components and Intermediates of the Ubiquitin/Proteasome Pathway

Cláudia Santos [1,*], Margarida Gaspar [2], Ana Caeiro [1], Cristina Branco-Price [1,3], Artur Teixeira [2] and Ricardo Boavida Ferreira [1,2]

[1] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal*
[2] *Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal*
[3] *Department of Botany and Plant Sciences, University of California, Riverside, CA 92621, USA*

In animal cells, arsenite has been reported to cause sulfhydryl depletion, generate reactive oxygen species and increase the level of large ubiquitin–protein conjugates. Plant viability tests and DNA laddering experiments have shown that *Lemna minor* remains viable after exposure to 50 μM $NaAsO_2$ for periods of at least 6 h. However, protein metabolism is affected in two major ways: the synthesis of an array of stress proteins, which confer thermotolerance; and an increase in the amount of large ubiquitin–protein conjugates, particularly evident after 2–3 h of stress, indicative of a role for the ubiquitin/proteasome pathway. This outcome is primarily attributed to an increased availability of protein substrates during arsenite treatment for three main reasons: an increase in protein carbonyl content after 1–2 h of stress; moderate increments in the transcript levels of the sequences coding for the ubiquitin pathway components chosen as markers (polyubiquitin, E1 and E2, and the β subunit and the ATPase subunits of the 26S proteasome); the observed increase in ubiquitin conjugates does not depend on de novo protein synthesis. This study is the first report on the involvement of the ubiquitin/proteasome pathway in response to arsenite in plants. In addition, it addresses the simultaneous expression of selected genes encoding the various components of the pathway. The results suggest that in plants, unlike in animals, the response to a relatively low level of arsenite does not induce apoptotic cell death. As a whole, the response to arsenite apparently involves a conjugation of salvage and proteolytic machineries, including heat shock protein synthesis and the ubiquitin/proteasome pathway.

Keywords: Arsenite stress — *Lemna minor* — protein oxidation — thermotolerance — ubiquitin/proteasome.

Abbreviations: CP, core protease; DNP, 2,4-dinitrophenylhydrazone; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; Hsp, heat shock protein; ICP-OES, inductively coupled plasma-optical emission spectroscopy; PC, Phytochelatin; ROS, reactive oxygen species; RP, regulatory particle; RT–PCR, reverse transcription–PCR; Ub, Ubiquitin.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under the accession numbers AY683450 for the ubiquitin-activating enzyme (E1) transcript, AY683451 for the ubiquitin-conjugating enzyme (E2) transcript, AY683447 for the polyubiquitin transcript, AY683446 for the ATPase subunit of the 26S proteasome transcript and AY683448 for the beta subunit of the 26S proteasome transcript.

Introduction

Arsenic (As) is ubiquitous in the environment and is derived from both natural and anthropogenic sources. It is a non-essential element for plants, and inorganic As species are generally highly phytotoxic. Arsenate [As (V)] is the predominant As species in aerobic soils, whereas arsenite [As (III)] dominates under anaerobic conditions (Wang et al. 2002). Organisms take up arsenate via phosphate transporters and arsenite by aquaglyceroporins (Rosen 2002). Endogenous thiols, such as glutathione (GSH), act as reducing agents for arsenate, probably playing an important role in the metabolic conversion of As (V) to As (III) (Mandal and Suzuki 2002).

As (V) acts as a phosphate analog and can disorganize phosphate metabolism, leading to the disruption of the energy flow in cells (Ullrich-Eberius et al. 1989). For example, it uncouples oxidative phosphorylation. As (V) may also replace the phosphorus in DNA, and this appears to inhibit the DNA repair mechanism. On the other hand, As (III), regarded as the more toxic form of the element, reacts with sulfhydryl groups of enzymes and tissue proteins, leading to inhibition of cellular function and death (Meharg and Hartley-Whitaker 2002). Indeed, the affinity of arsenite for sulfur has been invoked to account for the toxicity of As (III) compounds (Mandal and Suzuki 2002). As (III) forms strong bonds with functional groups, such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine residues (Rosen 2002). The citric acid cycle enzyme pyruvate dehydrogenase, containing lipoic acid as a cofactor, is strongly affected by arsenite. In the presence of As (III), the enzyme replaces the two hydrogen atoms from the thiol groups and attaches with a sulfur molecule forming a dihydrolipoylarsenite chelate

*Corresponding author: E-mail, csantos@itqb.unl.pt; Fax, +351-21-4433644.

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

complex, which prevents the reoxidation of the dihydrolipoyl group that is necessary for continued enzyme activity (Schmoger et al. 2000, Mandal and Suzuki 2002). Other enzymes known to be inhibited by As (III) include glutathione reductase, pyruvate oxidase, *S*-amino acid oxidase, choline oxidase and transaminase.

As (V) resistance has been identified in a range of plant species, which is generally achieved through a decreased uptake of As (V) due to the suppression of the high-affinity phosphate uptake system (Meharg and Macnair 1991, Meharg and Macnair 1992, Meharg and Hartley-Whitaker 2002). Once inside plant cells, As (V) is detoxified through a prompt reduction to As (III), which is subsequently complexed with thiols, particularly phytochelatins (PCs; thiol-rich peptides derived from GSH) (Pickering et al. 2000, Schmoger et al. 2000, Hartley-Whitaker et al. 2001b).

Exposure of plants to As (V) has been shown to originate an increase in reactive oxygen species (ROS) (Hartley-Whitaker et al. 2001a). Theoretically, this effect is likely to boost the ubiquitin (Ub)/proteasome pathway through an increment in available protein substrates. In fact, it is becoming clear that proteins in cells are under permanent surveillance by the proteolytic systems, which continually monitor mature proteins for post-synthetic denaturation or chemical damage (Goldberg 2003).

The Ub/proteasome system, a tightly regulated and highly specific pathway for targeted proteolysis, has been reported as a major pathway responsible not only for the selective degradation of damaged and misfolded proteins but also for the regulated turnover of critical cell regulators and partial proteolysis of propeptides (Sullivan et al. 2003, Vierstra 2003, Smalle and Vierstra 2004, Miller and Gordon 2005). In this pathway, the highly conserved, 76 amino acid globular protein Ub serves as a reusable tag for selective protein breakdown. Proteolysis occurs in two sequential steps: (i) ubiquitylation of the target protein, in which multiple Ub moieties become covalently attached to specific protein targets via an ATP-dependent reaction cascade. This Ub-conjugating system involves the sequential action of three enzyme families, E1, E2 and E3 (Sullivan et al. 2003, Vierstra 2003, Smalle and Vierstra 2004). The exquisite selectivity of this pathway resides in the E3s, which are specific for different protein substrates (Glickman and Ciechanover 2002). This control of specificity is reflected in the very large number of E3 genes found in eukaryotes.

(ii) The resulting polyubiquitylated protein conjugates are subsequently recognized and degraded by the ATP-dependent, self-compartmentalized 26S proteasome with the concomitant release of the Ub monomers for reuse (Voges et al. 1999, Vierstra 2003). The 26S proteasome is composed of a 20S core protease (CP), in which proteins are digested to short peptides, and one or two 19S regulatory particles (RPs), which confer ATP dependence and substrate specificity (Voges et al. 1999). Apparently, the RP recognizes and binds to polyubiquitylated proteins, releases the attached Ubs as free monomers, unfolds the protein substrates and directs the unfolded proteins into the CP lumen for degradation (Belknap and Garbarino 1996, Voges et al. 1999, Glickman 2000). In this way, Ub conjugation, the proteasome architecture and their linkage to ATP hydrolysis ensure that only unwanted proteins are selectively degraded (Goldberg 2003).

In this work, the response of *Lemna minor* cells to arsenite stress (50 μM $NaAsO_2$) was analyzed with regard to arsenite uptake, cell viability, stress protein synthesis, protein carbonyl content and changes in the free Ub and Ub–protein conjugates. The observed large increment in polyubiquitylated protein conjugates observed, indicative of an important involvement of the Ub/proteasome pathway, prompted us to follow simultaneously the changes in transcript abundance of selected genes encoding the major components of the pathway (polyUb, E1, E2, the β subunit of the CP and the ATPase subunit of the RP) by relative quantitative reverse transcription– PCR (RT–PCR).

Results

Response of Lemna minor to arsenite stress

Most studies on the effects of arsenite on biological systems used either environmentally relevant concentrations (0.5–10 μM) or very high concentrations of arsenite (1–2 mM). To analyze the effect on *L. minor* L. cells, a 50 μM arsenite concentration was selected for a number of reasons (data not shown): (i) it is a non-lethal treatment, for periods of at least 8 h (see below); (ii) heat shock protein (Hsp) synthesis can be detected with arsenite concentrations as low as 25 μM and increases for higher arsenite concentrations; with 50 μM arsenite, Hsps are detected after 1 h of stress; and (iii) a 6 h incubation in the presence of 50 μM arsenite confers thermotolerance to *Lemna*. Indeed, the plants die after a 2 h exposure to 42°C, but survive the lethal treatment if previously subjected to 38°C for 6 h or to 50 μM arsenite for 6 h.

Lemna minor plants were incubated in complete growth medium containing a relatively low level of arsenite (50 μM $NaAsO_2$), for periods up to 6 h. The results presented in Fig. 1A show the time course analysis of arsenite uptake by the plants (expressed in μg As g^{-1} DW) as determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES). The arsenite uptake rate by the plants is approximately constant during the first hour of stress and then gradually decreases, following a polynomial model ($y = -0.0497x^2 + 6.1956x + 13.0610$; $r^2 = 0.9906$).

Plant viability tests conclusively demonstrated that the exposure of *Lemna* plants to 50 μM $NaAsO_2$ is non-lethal

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

for periods of at least 8 h. In addition, since arsenite is known to induce apoptosis in animal cells, the integrity of the genomic DNA extracted from the stressed plants (Fig. 1B) was confirmed. Total DNA was extracted from *Lemna* fronds, fractionated by electrophoresis in an agarose gel and stained with ethidium bromide. No detectable bands were visible below 2,000 bp (results not shown). The total *Lemna* DNA was further analysed by Southern blotting using a method similar to that described previously (Hoeberichts et al. 2001). The absence of any DNA laddering is very obvious in Fig. 1B, indicating the absence of apoptotic cells. Indeed, DNA laddering is considered a hallmark of apoptotic death and represents a time point well beyond the 'point of no return' at the moment of death (Jones 2001, Woltering 2004). Not even a necrosis process was detected, as evidenced by the absence of DNA degradation (Fig. 1B).

Supplying *Lemna* plants with a suitable radioactive protein precursor, such as L-[^{35}S]methionine, which is readily absorbed by this aquatic plant during exposure to arsenite stress (or any other stress), allows the in vivo labeling of the de novo synthesized stress proteins, which may be subsequently detected by fluorography. The result of such an experiment is illustrated in lane arsenite of the fluorogram presented in Fig. 1C. The synthesis of the arsenite stress proteins depends on the metalloid concentration: it is detected for arsenite concentrations as low as 25 μM and increases for higher arsenite concentrations (data not shown). For the 50 μM concentration, the stress proteins are detected 1 h after addition of the arsenite.

For comparison purposes, the fluorogram depicted in lane 38°C of Fig. 1C represents the ^{35}S-labeled proteins synthesized by *Lemna* cells incubated at 38°C during 6 h (heat shock). Table 1 lists the major and minor Hsps and arsenite stress proteins. Of the 24 polypeptides identified as Hsps and the 24 polypeptides identified as arsenite stress proteins, 19 are common to both stresses. Although no protein identification has been performed, the large proportion of the stress proteins which are apparently common to both conditions, probably explains the acquisition of thermotolerance by plants exposed for 6 h to 50 μM arsenite.

Under the conditions used to grow the aquatic, higher plant *L. minor*, a 2 h exposure at 42°C was selected as the lethal treatment, i.e. the minimum temperature that kills 100% of the plants. This treatment obviously varies with the conditions utilized. Thus, for example, in the absence of light, the lethal treatment is 2 h at 43°C. The term acquisition of thermotolerance was therefore employed to mean any condition in which a suitable accumulation of Hsp takes place to ensure survival at the otherwise lethal treatment. This may be accomplished by a 4–8 h exposure at 38°C or 6–8 h incubation in the presence of 50 μM arsenite.

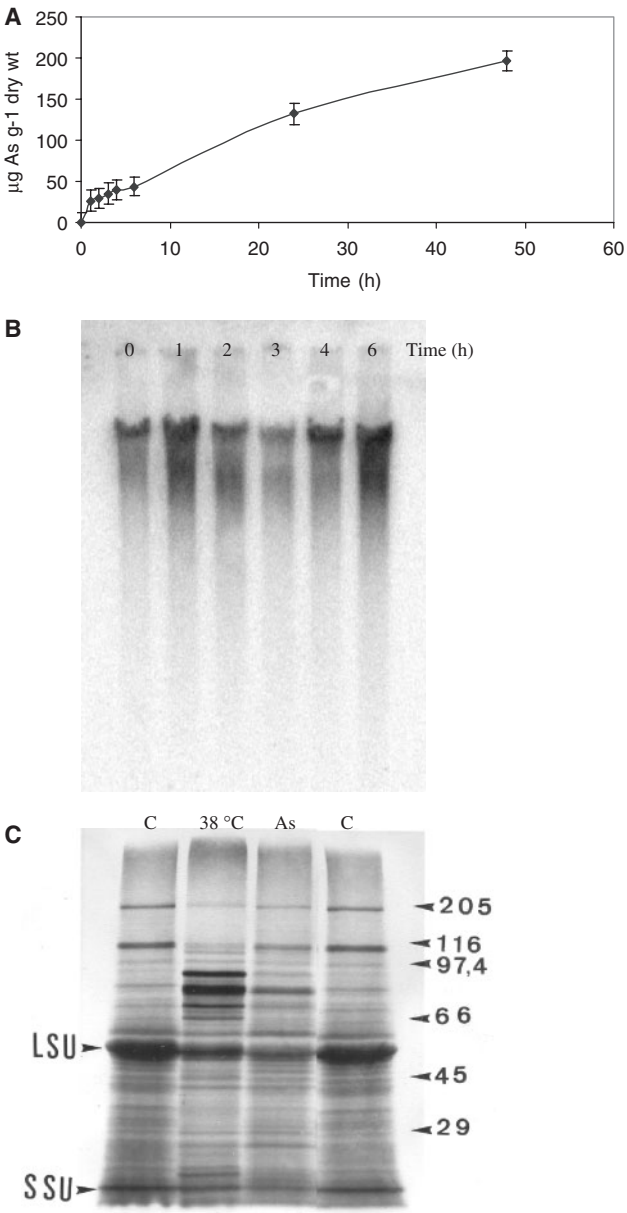

Fig. 1 Response of *L. minor* to arsenite stress. Plants of *L. minor* were exposed for various lengths of time to 50 μM $NaAsO_2$. (A) Time course analysis of arsenite absorption by the plants as determined by inductively coupled plasma-atomic emission spectroscopy (ICP-OES). (B) Southern blotting of genomic DNA from plants to detect DNA laddering. The plants were exposed to arsenite for the times (hours) indicated on the top of the blot. (C) Fluorography of the total soluble proteins extracted from *L. minor* plants incubated under control (C) conditions (22°C) or subjected to heat shock (38°C, 6 h) or arsenite stress (50 μM As, 6 h). The proteins were labeled in vivo by supplying L-[^{35}S]methionine to the plants. Molecular masses of standards are indicated in kDa. LSU and SSU, large and small subunits, respectively, of the photosynthetic enzyme ribulose bisphosphate carboxylase. The images are representative of results obtained for three independent experiments.

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

Table 1 Stress proteins synthesized in *L. minor* in response to arsenite or heat shock

Arsenite stress proteins (kDa)	Heat shock proteins (kDa)
133	–
–	119
104	**104**
–	99
89	**89**
88	**88**
87	**87**
85	85
82	**82**
80	**80**
78	**78**
76	**76**
–	74
72	72
67	**67**
64	64
–	62
60	**60**
58	58
47	–
44	44
38	38
34	–
30	–
28	28
26	**26**
–	20
19	**19**
18	–

Plants of *L. minor* were exposed for 6 h to 50 μM $NaAsO_2$ or to 38°C in the presence of L-[^{35}S]methionine. The stress proteins, detected by fluorography as shown in Fig. 1C, were identified by their molecular mass (kDa). The prominent ones are highlighted in bold.

Once acquired, thermotolerance seems to be a long adaptative response to high temperatures, as suggested by the data presented in Fig. 2. As expected, prolonged exposures of the plants at 38°C (Fig. 2A) or to arsenite (Fig. 2D) do not produce any significant change in the pattern of polypeptide synthesis. However, a significant but steady synthesis of stress proteins is readily detected when the plants are either exposed for up to 4 h at 42°C after being subjected for 8 h at 38°C (Fig. 2B) or exposed for up to 4 h at 38°C (Fig. 2E) or 42°C (Fig. 2F) after being incubated for 8 h in the presence of arsenite. Conversely, the pattern of protein synthesis is not altered when the plants previously exposed for 8 h at 38°C are incubated in the presence of arsenite (Fig. 2C).

Effect of arsenite stress on the level of ubiquitin–protein conjugates

Lemna minor plants were exposed to 50 μM arsenite for periods up to 6 h. At intervals, samples were collected and the total protein fraction was extracted, fractionated by SDS–PAGE, blotted onto a membrane and probed with affinity-purified, anti-Ub polyclonal antibodies. The levels of free Ub and of the high molecular mass Ub–protein conjugates were semi-quantified by image analysis. The results obtained, presented in Fig. 3A and B, show that there is a significant accumulation of the high molecular mass Ub–protein conjugates 2–3 h after the onset of the stress. This effect may be due to increased availability in protein substrates, to enhanced activity of the Ub-conjugating system (due to increased expression, post-translational activation or even a decreased rate of proteolysis) and/or to a lower level of 26S proteasome proteolytic activity (conversely due to increased proteolysis or biochemical/post-translational deactivation).

In an attempt to understand the arsenite-induced increment in the concentration of large Ub–protein conjugates, the changes in protein carbonyl content were followed during the incubation of *Lemna* in 50 μM $NaAsO_2$. The resulting oxyblot, illustrated in Fig. 4, indicates that there is an increased protein carbonyl content, particularly evident during the first 2 h of stress and in the case of the large subunit of ribulose bisphosphate carboxylase.

Changes in the levels of transcripts encoding the ubiquitin/proteasome pathway components in Lemna exposed to arsenite

After observing an increase in the Ub–protein conjugates during arsenite treatment, our attention focused on the regulation of the Ub/proteasome pathway components, and our primary approach was to evaluate changes in the transcript levels in response to the stress. Conserved regions in selected genes encoding polyUb, E1 and E2, and the β subunit and the ATPase subunits of the 26S proteasome were used to construct suitable primers. The criteria used to select genes and design primers are presented in Table 2 and briefly discussed in Materials and Methods. Subsequent RT–PCR allowed the synthesis of *Lemna* products of the expected size, which were identified by sequencing. Relative quantitative RT–PCR was then used to detect the changes in transcript levels corresponding to the five genes under study when *L. minor* is subjected to arsenite stress. The results obtained indicate that there are moderate but significant increases in the steady-state abundance of transcripts for the five genes under study, with a peak after 2 h of stress for polyUb, E1, E2 and the β subunit of the 26S proteasome (Fig. 5). This observation may reflect increased transcription or decreased transcript turnover rates. With regard to the ATPase subunit of the 26S proteasome,

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

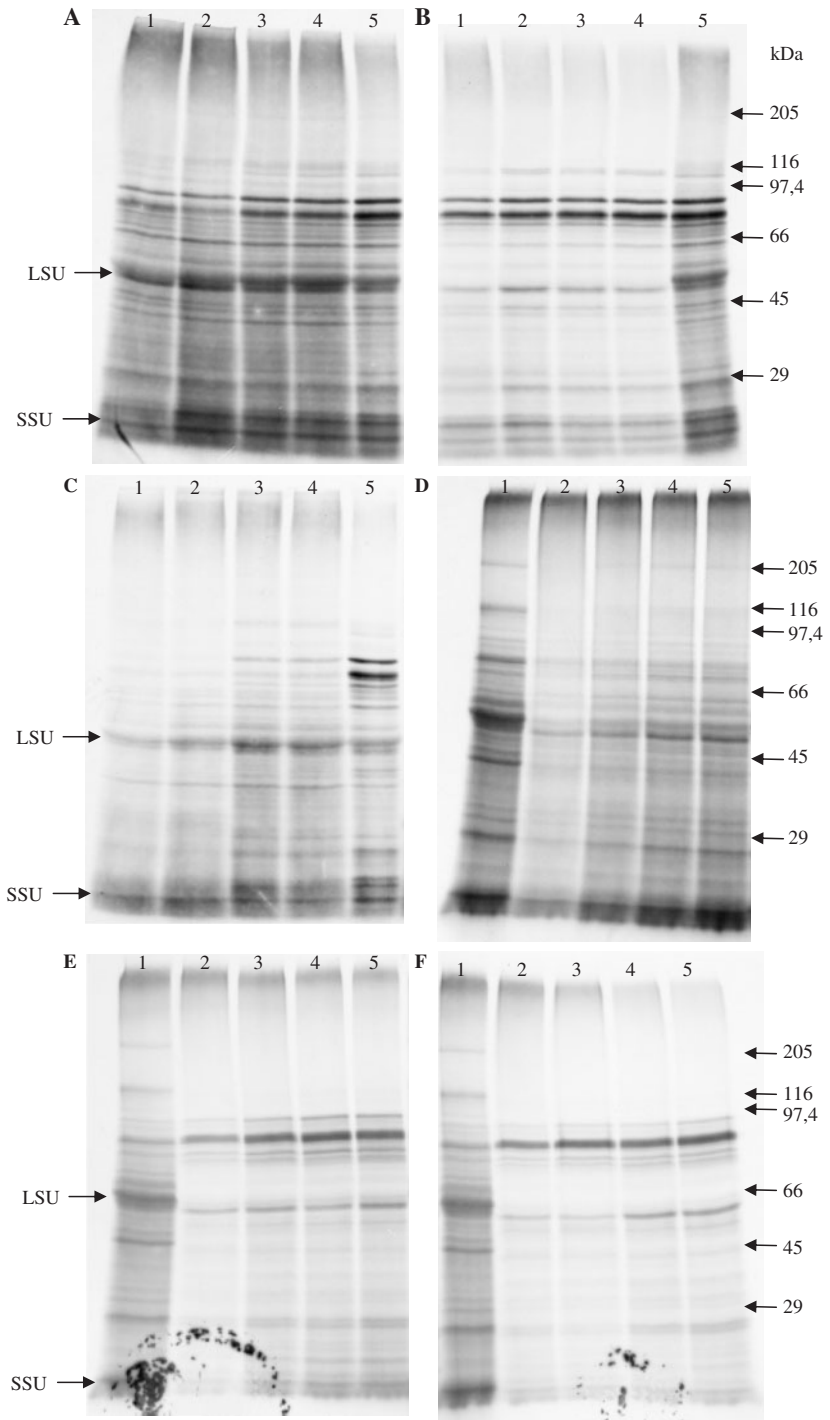

Fig. 2 Fluorography of ^{35}S-labeled proteins synthesized in thermotolerant *L. minor*. (A) Plants incubated at 38°C for 1 h (lane 1), 2 h (lane 2), 3 h (lane 3) or 4 h (lane 4) after 8 h at 38°C; lane 5, control (8 h at 38°C). (B) Plants incubated at 42°C for 1 h (lane 1), 2 h (lane 2), 3 h (lane 3) or 4 h (lane 4) after 8 h at 38°C; lane 5, control (8 h at 38°C). (C) Plants exposed to arsenite for 1 h (lane 1), 2 h (lane 2), 3 h (lane 3) or 4 h (lane 4) after 8 h at 38°C; lane 5, control (8 h at 38°C). (D) Plants exposed to arsenite for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4) or 4 h (lane 5) after 8 h in the presence of arsenite; lane 1, control (8 h in the presence of arsenite). (E) Plants incubated at 38°C for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4) or 4 h (lane 5) after 8 h in the presence of arsenite; lane 1, control (8 h in the presence of arsenite). (F) Plants incubated at 42°C for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4) or 4 h (lane 5) after 8 h in the presence of arsenite; lane 1, control (8 h in the presence of arsenite). Molecular masses of standards are indicated in kDa. LSU and SSU, large and small subunits of ribulose bisphosphate carboxylase, respectively.

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

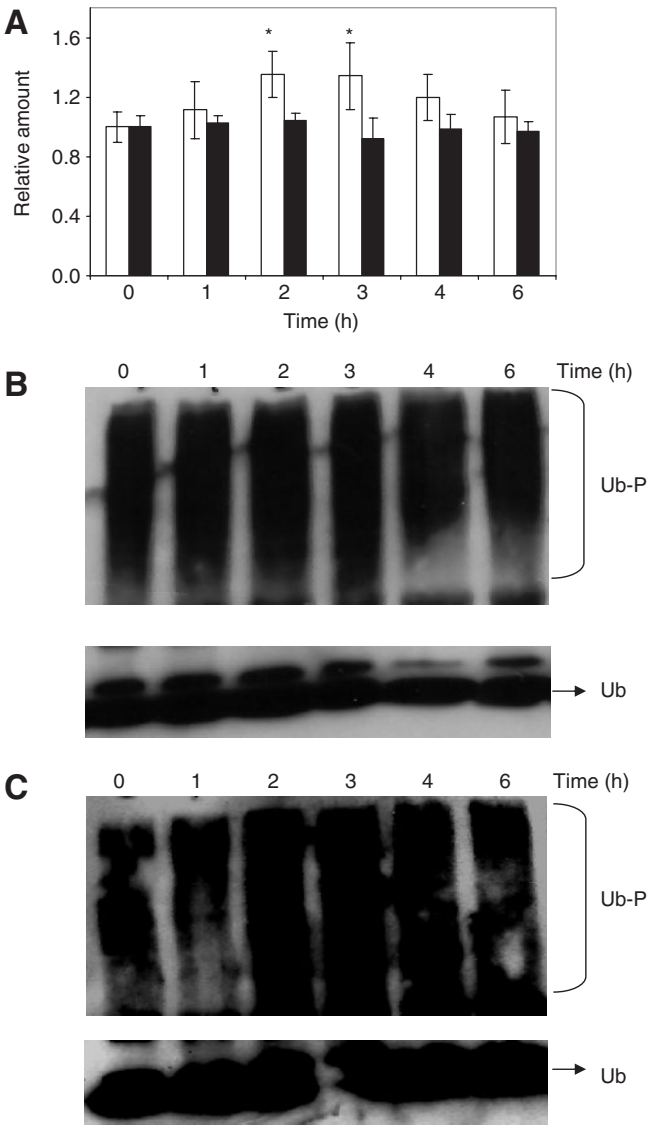

Fig. 3 Involvement of the Ub/proteasome pathway in the response of *L. minor* to arsenite. (A) Plants of *L. minor* were exposed to 50 μM $NaAsO_2$ for different periods of time. At intervals, samples were collected and the total protein fraction extracted, fractionated by SDS–PAGE, blotted onto a membrane and probed with anti-Ub antibodies. The levels of free Ub (filled squares) and of the high molecular mass Ub–protein conjugates (open squares) were determined by image semi-quantitative analysis with the Scion Image program. Data are shown as the percentage of pixels (arbitrary unit) measured relative to the control (100%). The asterisk indicates a significant difference compared with the control value (time 0) at $P<0.05$ for the Ub–protein levels. No significant differences at $P<0.05$ were observed for the free Ub levels. The results are the averages of three independent experiments and are represented as the mean ± SD. (B) and (C) Representative immunoblots showing the high molecular mass Ub–protein conjugates detected in *Lemna* exposed to arsenite stress without or with previous cycloheximide treatment, respectively.

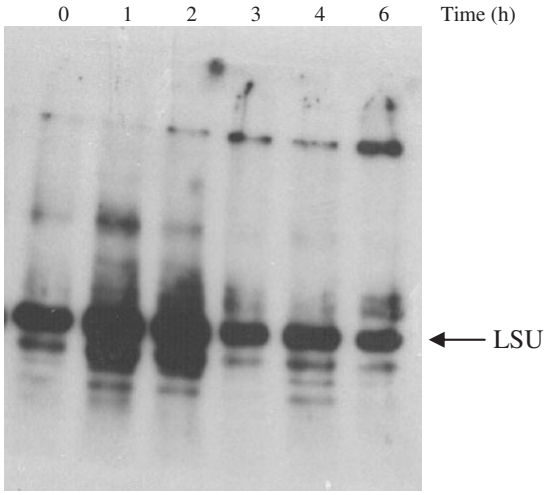

Fig. 4 Immunological detection of protein carbonyl groups in *L. minor* subjected to arsenite stress. Plants of *L. minor* were exposed to 50 μM $NaAsO_2$ for the periods of time (hours) indicated on top of the blot. After extracting the total protein fraction, the protein carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone (DNP). The total protein was subsequently fractionated by SDS–PAGE, blotted onto a membrane and probed with anti-DNP antibodies. LSU, large subunit of ribulose bisphosphate carboxylase, as previously identified by immunoblotting using anti-ribulose bisphosphate carboxylase antibodies. The image is representative of results obtained for three independent experiments.

a gradual but significant increase in transcript level is observed throughout the period investigated (0–6 h). The simultaneous increase in Ub–protein conjugates and in transcript levels suggested a regulation of the Ub-conjugating system dependent on levels of stable transcript. To verify this hypothesis, *L. minor* plants were incubated for periods up to 6 h in the simultaneous presence of 50 μM $NaAsO_2$ and 200 μg ml^{-1} of the cytosolic protein synthesis inhibitor cycloheximide. The pattern of variation of the large Ub–protein conjugates (Fig 3C) was identical to the one depicted in Fig. 3B, indicating that the arsenite-dependent increase in Ub conjugates does not depend on de novo protein synthesis.

Discussion

Exposure of the aquatic higher plant *L. minor* to 50 μM $NaAsO_2$ results in a typical uptake profile of the metalloid (Fig. 1A), as previously reported for arsenite and other metals in plants (Bondada et al. 2004). Following an initial linear trend, the uptake pattern subsequently levels off, with the continuous influx of arsenite into cellular compartments due to an active detoxification capacity and the slowing down in the uptake probably reflecting the failure of

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

Table 2 Sequence of primers used in the relative quantitative RT–PCR analysis of transcripts levels of the selected genes expressed in *L. minor* in response to arsenite

Product	Primers	Sequences used to design the primers	Annealing temperature (°C)	Accession No.	Criteria used in selecting the product and the region of the sequence to be amplified
Ubiquitin-activating enzyme (E1)	5′-GGCAAGAA TGTATGTT GACCAG-3′	E1(UBA2) from *Arabidopsis thaliana* (GenBank accession No. U80808)	60	AY683450	To contain the Prosite motif Ub-activating enzyme active site (PS00865).
	5′-TGTGGGG AACGAGTG AGAGT-3′				The product amplified is probably derived from more than one E1-encoding gene; in *Arabidopsis*, for example, E1-encoding genes are co-expressed and not differentially expressed (Hatfield et al. 1997)
Ubiquitin-conjugating enzyme (E2)	5′-CCAAGCGC ATCCTCAAG GAGCTCAA GG-3′	E2 (UBC4) from *Pisum sativum* (GenBank accession No. L29077)	55	AY683451	To contain the Prosite motif Ub-conjugating enzymes family (PS50127).
	5′-GGATAATCT GGAGGAAAA TGAATGGT AACC-3′				High identity with *Arabidopsis* UBC8-12, the most influential E2-encoding gene family (Smalle and Vierstra 2004)
Polyubiquitin	5′-TAGACAATG TTAAGGCAA GATTCAAGAC AAGG-3′	Polyubiquitin (PUB4) from *Pisum sativum* (GenBank accession No. L81142)	55	AY683447	To contain the Prosite motif Ub domain signature (PS00299) and to include polyUb transcripts, excluding Ub fusion transcripts
	5′-CCTTGAGGT GGAGAGCTC TGATACCAT TGACA-3′				These primers originate multiple matching subsegments (products) reflecting the Ub repeats. Each product, may represent a pool of polyUb transcripts synthesized from the different polyUb genes. These genes were described to be induced by: osmotic stress (Spees et al. 2002); heat shock (Belknap and Garbarino 1996; Sun and Callis 1997); drugs and hydrogen peroxide (Noventa-Jordao et al. 2000)
ATPase subunit of the 26S proteasome	5′-GGTTCGACG CACGTGGCAA CATCAAGG-3′	26S proteasome ATPase subunit from *Spinacia oleracea* (GenBank accession No. D86121	55	AY683446	To contain the Prosite motif AAA-protein family signature (PS00674) in the ATPase product.
	5′-CCTAATGTC GGCACCTGTG GAGTTTGG-3′				All genes encoding 26S proteasome subunits are referred to be expressed simultaneously and up-regulated under stress (Belknap and Garbarino 1996; Smalle and Vierstra 2004)
β subunit of the 26S proteasome	5′-CCATGGCTG GTGGGGCTG CTGAC-3′	26S proteasome β subunit from *Spinacia oleracea* (GenBank accession No. D78172)	60	AY683448	
	5′-CCAGGCCC CGTTTCATC CCACCC-3′				All genes encoding 26S proteasome subunits are referred to be expressed simultaneously and up-regulated under stress (Belknap and Garbarino 1996; Smalle and Vierstra 2004)

The sequences used to design the primers, the annealing temperatures and the GenBank accession numbers for the cDNA partial sequences obtained from *L. minor* are indicated, as well as the criteria followed to select the region to be amplified.

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

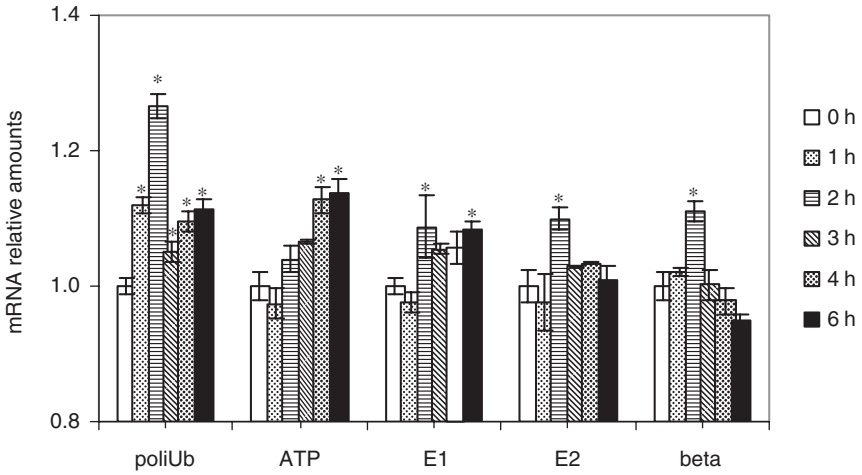

Fig. 5 Changes in the levels of transcripts encoding several Ub/proteasome pathway components in *L. minor* subjected to arsenite stress. The relative amounts of transcript, taken as a percentage of the time zero value, were determined by relative quantitative RT–PCR using suitable primers (see Table 2) and 18S rRNA as the internal control (see Materials and Methods). Five different transcripts were analysed (polyUb, E1, E2, and the β subunit and the ATPase subunits of the 26S proteasome) at 0, 1, 2, 3, 4 and 6 h of arsenite stress. The asterisk indicates a significant difference compared with the control value (time 0) at $P < 0.001$ for the transcript relative levels. The results are the averages of three independent experiments and are represented as the mean ± SD.

that detoxification mechanism (Bondada et al. 2004). The analysis of Fig. 1A reveals no saturation in *Lemna* uptake of 50 μM $NaAsO_2$ for periods up to 48 h.

This study demonstrates that the exposure of *Lemna* cells to low level arsenite (50 μM) does not induce DNA laddering (Fig. 1B). In contrast, in several animal cell lines arsenite has been shown to induce programmed cell death; even low levels of arsenite (25–50 μM) lead to apoptotic death (Tsou et al. 2003, Shi et al. 2004, Woltering 2004). In addition, it has been recently reported that in mammalian cells, high concentrations of arsenite are involved in the production of ROS (Chen et al. 1998, Liu et al. 2001, Shi et al. 2004), oxidative DNA damage (Lynn et al. 2000) and the induction of DNA strand breaks (Wang et al. 2001). Only very low levels of arsenite (up to 5 μM) were linked to cell proliferation (Valko et al. 2005). The DNA laddering experiment illustrated in Fig. 1B shows that *Lemna* genomic DNA remains intact after 6 h of stress, supporting the observation that the incubation of *Lemna* with arsenite for at least 6 h is non-lethal.

Several studies reported the arsenite induction of thermotolerance in animal (100 μM arsenite) and microbial cells (750 μM arsenite) (Li and Dewey 1992, Sanchez et al. 1992, Kampinga et al. 1995). In plants, there are several references on the acquisition of thermotolerance, involving a number of treatments other than arsenite exposure: mild heating regimes (Larkindale and Knight 2002); pre-treatments with salicylic acid, abscisic acid, calcium chloride, hydrogen peroxide, 1-aminocyclopropane-1-carboxylic acid (Larkindale and Knight 2002, Larkindale and Huang 2004); and the symbiotic interactions between plants and fungi (Redman et al. 2002). However, to date, there is no direct evidence of arsenite-induced thermotolerance in plants. *Lemna minor* plants survive a 2 h exposure to the otherwise lethal temperature of 42°C, if previously treated for 6 h with 50 μM arsenite (or, alternatively, 38°C). This is probably due to the induction of stress proteins by arsenite, forming a pattern that is essentially similar to that induced by heat shock (Fig. 1C). Overall, the data presented in Fig. 2 support the hypothesis that the proteins with identical estimated molecular masses (see Table 1) synthesized under heat shock or arsenite stress conditions are indeed identical. In fact, exposure to arsenicals either in vitro or in vivo in a variety of animal model systems has been shown to cause the induction of a number of the major stress protein families, such as Hsps. Among them are members with a low molecular mass, such as metallothionein and Ub, as well as ones with molecular masses of 27, 32, 60, 70, 90 and 110 kDa (Del Razo et al. 2001). In plants there is evidence that the response to oxidative stress results in the accumulation of some Hsps and that thermotolerance can be induced by compounds that generate oxidative bursts (Dat et al. 1998). The accumulation of Hsps may therefore indicate the oxidative nature of arsenite stress in plants.

Oxidized proteins are known to be better substrates for proteases. Protein carbonyl content, considered as a biomarker of oxidative stress, is presently the most general indicator and by far the most commonly used marker of protein oxidation (Dalle-Donne et al. 2003). The analysis of *Lemna* protein carbonyl content obtained when this plant is exposed to 50 μM arsenite for periods up to 6 h

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

(Fig. 4) reveals an increase in protein carbonyls, particularly evident after 1–2 h of stress. Indeed, the number of bands detected increases during arsenite treatment and this effect is particularly evident for the large subunit of the photosynthetic enzyme ribulose bisphosphate carboxylase. The signal corresponding to the very high molecular mass fraction seen on top of the blot for stress periods longer than 2 h may be due to the presence of oxidized proteins in large molecular mass Ub–protein conjugates. Alternatively, a number of proteins have been reported to undergo a polymerization process before proteolysis. For example, a model has been proposed for plant ribulose bisphosphate carboxylase degradation involving enzyme oxidation, polymerization and interaction with chloroplast membranes (Ferreira et al. 2000). In addition, polymerized ribulose bisphosphate carboxylase is readily detected on top of gels and blots in a number of stress conditions in a manner identical to that depicted in Fig. 4 (Ferreira et al. 1996).

For the reasons explained above, arsenite stress is likely to boost the Ub/proteasome pathway in plants, as has been previously shown in animal and yeast cells. Indeed, a number of studies performed with animal cells have reported the accumulation of large molecular mass Ub–protein conjugates in response to either low (0.5–10 μM) or high concentrations (2 mM) of arsenite (Mimnaugh et al. 1997, Chen et al. 2002, Kirkpatrick et al. 2003, Bredfeldt et al. 2004). These observations denote that arsenite directly affects the Ub/proteasome pathway in animal cells. We report a similar effect in *Lemna* cells exposed to arsenite stress: a gradual, transient and significant increase in the cellular concentration of large Ub conjugates, particularly evident after 2–3 h of stress (Fig. 3). Theoretically, this increment may result from an increased availability in protein substrates (assuming that the Ub-conjugating capacity is present in excess), from an increase in Ub-conjugating activity and/or a reduction in the activity of the Ub-degrading machinery, or from a combination of them. This is the first report on the involvement of the Ub/proteasome pathway in response to arsenite stress in plants.

Arsenite stress has also been reported to affect the activity of the Ub pathway components in animal cells. Doses of 10 μM arsenite decreased the cellular activity of the 20S proteasome by 40% in precision-cut rabbit renal cortical slices and by 15% in human embryonic kidney cells, but did not cause any notable difference in Ub-conjugating activity in the same cells (Kirkpatrick et al. 2003). However, earlier work reported by Klemperer and Pickart (1989) on intact rabbit reticulocytes and in a reticulocyte lysate (fraction II) showed that very high concentrations of arsenite (2 mM) inhibited the activity of Ub-conjugating enzymes in vitro by interacting with critical cysteine residues. In yeast, an ubiquitin ligase SCF plays a central role in the response to cadmium and arsenic which activate a transcription factor important for the cellular response (Yen et al. 2005).

Relative quantitative RT–PCR analysis of *Lemna* exposed to 50 μM arsenite for up to 6 h, using 18S rRNA as the internal control and suitable primers (Table 2), revealed that there are moderate increases in the amounts of some of the transcripts encoding Ub/proteasome pathway components, with peaks after 2 h of stress, particularly evident in the case of polyUb itself (Fig. 5). This observation is not surprising considering that Ub is itself an Hsp (Del Razo et al. 2001). Some of the selected genes described in this study have already been reported to be induced by stress and then considered as markers (Belknap and Garbarino 1996, Sun and Callis 1997, Noventa-Jordao et al. 2000, Spees et al. 2002, Smalle and Vierstra 2004). The exception seems to be the ATPase subunit of the 26S proteasome, for which a gradual but continuous increment in the amount of the corresponding transcript is observed throughout the 6 h period studied.

A comparision between Figs. 3A and 5 suggests that the increment in total (i.e. free plus conjugated) Ub (Fig. 3A) is due to enhanced translation of polyUb genes, as evidenced by the enhanced levels of Ub transcripts (Fig. 5). The general and moderate increase in the levels of transcripts encoding the other Ub pathway components observed during arsenite stress (Fig. 5) may contribute to the increased involvement of the Ub pathway during arsenite stress. Alternatively, if the Ub-conjugating activity is present in excess, this increment in transcript levels may compensate for the possible stress-enhanced turnover rates of the pathway components. Further experiments, involving the incubation of *Lemna* in the simultaneous presence of arsenite and protein synthesis inhibitors, showed that the arsenite-dependent increase in Ub conjugates does not require de novo protein synthesis. Therefore, the available evidence suggests that in *Lemna*, the arsenite-dependent increase in large Ub–protein conjugates is primarily attributed to an increment in the availability of protein substrates.

As a whole, the data presented here show that *L. minor* exposed to 50 μM arsenite remains viable for periods of at least 6 h but exhibits two major alterations in its protein metabolism: (i) the synthesis of an array of stress proteins; the large proportion of the stress proteins (19 out of 24) which are apparently common, as far as molecular mass is concerned, between arsenite stress and heat shock probably explains the acquisition of thermotolerance by the plants exposed to arsenite; (ii) an increase in the amount of large molecular mass Ub–protein conjugates, indicative of a role for the Ub/proteasome pathway in the response of *Lemna* to arsenite stress, an effect primarily attributed to an increased availability of protein substrates.

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

The results presented in this work indicate that in plants the response to low level arsenite does not induce apoptotic cell death but suggest a conjugation of salvage and proteolytic machineries including Hsp synthesis and the Ub/proteasome pathway.

Materials and Methods

Plant material, growth conditions and stress induction

Lemna minor L. was grown autotrophically at 22°C, with 16 h light, in a complete sterile culture medium as described earlier (Ferreira and Teixeira 1992), supplemented with 10 mM sucrose. Arsenite stress during 1, 2, 3, 4 and 6 h was performed by including 50 μM $NaAsO_2$ in the growth medium.

Arsenite determination

After stress treatments, *Lemna* fronds were washed with water, dried to a constant weight, ashed at 500°C and the ashes dissolved in 3 M HCl (2 ml), after which the samples were filtered and the volumes adjusted to 25 ml with Milli-Q water (Millipore, Billerica, MA, USA). Arsenite concentrations were subsequently determined by ICP-OES at 193.759 nm (Jobin-Yvon JY 24, Longjumeau, France) using calibration curves in 3 M HCl over a concentration range of 0.5–5 μg ml^{-1} (correlation coefficient of 0.9999). To minimize mineral contamination, all plasticware and glassware used were soaked in 50% (v/v) HCl before use.

Total protein extraction

Samples of *L. minor* were frozen in liquid nitrogen, then ground to a fine powder and the protein extracted essentially as described previously (Jabben et al. 1989), in 90 mM Tris–HCl buffer, pH 8.0, containing 45% (v/v) ethyleneglycol, 18 mM sodium metabisulfite, 10 mM *N*-ethylmaleimide, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) (2.0 ml g^{-1} FW). The homogenate was squeezed through two layers of cheesecloth and clarified by centrifugation (48,000×*g*, 12 min at 4°C). The supernatant was collected and immediately boiled for 3 min in the presence of 2% (w/v) SDS and 0.1 M 2-mercaptoethanol (Ferreira 1986).

Electrophoresis and immunoblotting

All protein samples were subjected to SDS–PAGE in 10% (w/v) acrylamide slab gels as described before (Ferreira and Davies 1986), except that *m*-cresol purple was used as the tracking dye and 0.1 M sodium acetate was included in the anode buffer solution (Christy et al. 1989).

The separated polypeptides were blotted onto a PVDF membrane at 70 V for 1 h at 4°C. After protein transfer, the membranes were air-dried and autoclaved for 30 min (Swerdlow et al. 1986). The blots were then processed as described previously (Ferreira and Shaw 1989) to visualize free Ub and the Ub–protein conjugates, except that 0.05% (v/v) Tween-20 was included in the antibody-containing solutions and the detection was performed with the chemiluminescent substrate SuperSignal® West Femto (Pierce Biotechnology, Rockford, IL, USA). The polyclonal anti-Ub antibodies were produced in rabbits injected with FPLC-purified, SDS-treated bovine Ub and the IgG fraction subsequently isolated by immunoaffinity chromatography on a Ub–Sepharose column. Ub–protein conjugates and free Ub were semi-quantified using the GelPlot facility from Scion Image for Windows (version Beta 4.0.2, Scion Corporation, Frederick, MD, USA). To each lane, profile plots were generated, and the area of the peaks calculated.

Oxyblotting

To detect the presence of carbonyl groups introduced in proteins as a result of stress, an Oxyblot® protein oxidation detection kit (Intergen, Burlington, MA, USA) was used. Plants submitted to stress were homogenized in 100 mM Tris–HCl buffer, pH 7.5, containing 0.1% (v/v) Triton X-100, 1.5% (v/v) 2-mercaptoethanol and protease inhibitor cocktail [2.0 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 1.0 mM EDTA, 130 μM bestatin, 14 μM *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), 1.0 μM leupeptin and 0.3 μM aprotinin] (2.0 ml g^{-1} FW). The homogenate was squeezed through two layers of cheesecloth and clarified by centrifugation (50,000×*g*, 30 min at 4°C) (Talent et al. 1998). The supernatant was collected and immediately used for the derivatization of the carbonyl groups to 2,4-dinitrophenylhydrazone (DNP) with 2,4-dinitrophenylhydrazine, according to the manufacturer's instructions. The reaction was stopped by the addition of the neutralization solution. SDS–PAGE sample buffer containing 2% (w/v) SDS and 0.1 M 2-mercaptoethanol was subsequently added. The samples were analysed by immunoblotting and processed as described above, using rabbit anti-DNP antibody as the primary antibody.

DNA laddering

The procedure used for DNA laddering was based on that described by Hoeberichts and co-workers with modifications (Hoeberichts et al. 2001). Genomic DNA was extracted from *L. minor* tissue using a DNeasy plant maxi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Samples of 25 μg each were run on a 1% (w/v) agarose gel in Tris/borate. The DNA was denatured for 30 min in NaOH (0.5 M)/NaCl (1 M), neutralized with Tris (1.5 M, pH 7.5)/NaCl (3 M) and blotted onto a nylon membrane overnight with 10× NaCl (1.5 M)/sodium citrate (0.15 M) (SSC). The membrane, after UV cross-linking, was pre-hybridized for 2 h with 5× SSC, 0.1% (w/v) SDS and 5× Denhart's solution at the hybridization temperature. The DNA on the blot was hybridized overnight at 65°C with an [α-^{32}P]CTP random oligonucleotide primer-labeled probe composed of *Hin*dIII-digested genomic *Lemna* DNA in the same buffer. Final washes were done at the hybridization temperature for 15 min with a solution containing 0.1% (w/v) SDS and 2× SSC and twice for 15 min with 0.1× SSC and 0.1% (w/v) SDS. The blot was exposed to phosphorus screens that were scanned using the STORM 860 scanner phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA).

In vivo labeling and fluorography

Lemna fronds were incubated at 25°C in 5 ml of growth medium containing 148 kBq of L-[^{35}S]methionine (specific activity 37 TBq $mmol^{-1}$). After 30 min, the arsenite stress conditions were applied. After the labeling period, the tissues were thoroughly washed with water containing 1 mM L-[^{32}S]methionine, weighed, frozen in liquid nitrogen, then ground to a fine powder and the protein extracted (3.0 ml g^{-1} FW) essentially as described earlier (Nover and Scharf 1984), in 50 mM Tris–HCl buffer, pH 7.8, containing 25 mM KCl, 10 mM $MgCl_2$, 1 mM PMSF, 1% (v/v) Triton X-100, 0.5% (v/v) 2-mercaptoethanol, 1 mM NaF and 250 mM sucrose. The homogenate was squeezed through two layers of cheesecloth and clarified by centrifugation (48,000×*g*, 12 min at 4°C). The supernatant was collected and immediately

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

boiled for 3 min in the presence of 2% (w/v) SDS and 0.1 M 2-mercaptoethanol (Nover and Scharf 1984).

The polypeptides were separated in SDS–polyacrylamide gels as described above, and fixed in 7% (v/v) acetic acid for 30 min. Fluorography was performed essentially as described earlier (Bonner and Laskey 1974). The gels were immersed in 20 vols of dimethylsulfoxide (DMSO) twice for 30 min, then in 4 vols of 22.2% (w/v) 2,5-diphenyloxazole in DMSO overnight, and finally in water for 1 h. The gels were dried under vacuum at 80°C for 2 h. Detection of the ^{35}S-label was carried out using Hyperfilm-MP (Amersham Pharmacia Biotech, Buckinghamshire, UK) in cassettes held at –70°C (Bonner and Laskey 1974).

Isolation of partial cDNA

Partial cDNA, encoding putative proteins, was isolated by the RT–PCR technique. Total RNA was extracted from *Lemna* plants using an Invisorb® Spin Plant-RNA Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. The RNA was treated with DNase using the TURBO DNA-free™ kit (Ambion, Austin, TX, USA) to eliminate DNA contamination. First-strand cDNA was prepared from 250 ng of total RNA with Superscript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) using 100 ng of random primers (Roche, Diagnostics, Mannheim, Germany). A 1 µl aliquot of cDNA was used for PCR. Amplification primers were designed on highly conserved regions, including proteins motifs whenever possible (Table 2). In addition, a selection was made for those genes encoding polyUb, E1 and E2, typically encoded by gene families (Smalle and Vierstra 2004) whose levels of transcripts have been reported in the literature to be affected by stress; these genes were chosen as markers in this study (see references in Table 2). 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 (see references in Table 2). DNA amplification reactions were conducted with the *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) under the following conditions: one cycle of 3 min (denaturation at 95°C, annealing temperature according to Table 2, extension at 72°C), followed by 35 cycles of 90 s at the same temperatures, and a final 5 min extension at 72°C. The partial cDNA synthesized was purified with the High Pure PCR Product Purification Kit (Roche, Diagnostics, Mannheim, Germany) and then sequenced by capillary electrophoresis (ABI Prism 310, Applied Biosystems, Foster City, CA, USA). Its identity was verified by comparative analysis in the sequence database at the NCBI. The sequences were submitted to the database and the corresponding accession numbers are presented in Table 2.

Relative quantitative RT–PCR

A 1 µl aliquot of cDNA preparation was used as template for the PCRs. The PCRs were performed as described above, except that after the first cycle of 3 min the subsequent cycles were of 30 s and the number of cycles was defined previously (in the linearity range of the reaction for each pair of primers).

As an internal control, in the multiplex reaction, the 18S rRNA was amplified using the kit Quantum RNA Universal 18S Internal Standards primers (Ambion, Austin, TX, USA). RT–PCR products were separated by 1% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. The images of the gels were captured with Gel Doc 1000 (BioRad, Hercules, CA, USA) and quantification was done with the GelPlot facility from Scion Image for Windows (version Beta 4.0.2, Scion Corporation, Frederick, MD, USA) as described above.

Statistical analysis

The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were analysed by one-way analysis of variance (ANOVA) with Tukey's HSD (honest significant difference) multiple comparison test ($\alpha = 0.05$) using STATISTICA for Windows (StatSoft, Inc., Tulsa, OK, USA).

Acknowledgments

This work was financially supported by the Fundação para a Ciência e a Tecnologia, Lisboa, Portugal, under grant no. PRAXIS XXI/BD/5699/95. The facilities provided by ESAS, Santarém, Portugal, for the ICP-OES analysis experiments are gratefully acknowledged.

References

Belknap, W.R. and Garbarino, J.E. (1996) The role of ubiquitin in plant senescence and stress responses. *Trends Plant Sci.* 1: 331–335.

Bondada, B.R., Tu, S. and Ma, L.Q. (2004) Absorption of foliar-applied arsenic by the arsenic hyperaccumulating fern (*Pteris vittata* L.). *Sci. Total Environ.* 332: 61–70.

Bonner, W.M. and Laskey, R.A. (1974) A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46: 83–88.

Bredfeldt, T.G., Kopplin, M.J. and Gandolfi, A.J. (2004) Effects of arsenite on UROtsa cells: low-level arsenite causes accumulation of ubiquitinated proteins that is enhanced by reduction in cellular glutathione levels. *Toxicol. Appl. Pharmacol.* 198: 412–418.

Chen, F., Zhang, Z., Bower, J., Lu, Y., Leonard, S.S., Ding, M., Castranova, V., Piwnica-Worms, H. and Shi, X. (2002) Arsenite-induced Cdc25C degradation is through the KEN-box and ubiquitin–proteasome pathway. *Proc. Natl Acad. Sci. USA* 99: 1990–1995.

Chen, Y.C., Lin-Shiau, S.Y. and Lin, J.K. (1998) Involvement of reactive oxygen species and caspase 3 activation in arsenite-induced apoptosis. *J. Cell Physiol.* 177: 324–333.

Christy, K.G., La Tart, D.B. and Osterhoudt, H.W. (1989) Modifications for SDS–PAGE of proteins. *Biotechniques* 7: 692–693.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. and Colombo, R. (2003) Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* 329: 23–38.

Dat, J.F., Foyer, C.H. and Scott, I.M. (1998) Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. *Plant Physiol.* 118: 1455–1461.

Del Razo, L.M., Quintanilla-Vega, B., Brambila-Colombres, E., Calderon-Aranda, E.S., Manno, M. and Albores, A. (2001) Stress proteins induced by arsenic. *Toxicol. Appl. Pharmacol.* 177: 132–148.

Ferreira, R.B. and Davies, D.D. (1986) Is protein degradation correlated with either the charge or size of *Lemna* proteins?. *Planta* 169: 278–288.

Ferreira, R.B., Esquível, M.G. and Teixeira, A.R. (2000) Catabolism of ribulose bisphosphate carboxylase from higher plants. *Curr. Top. Phytochem.* 3: 129–165.

Ferreira, R.M., Franco, E. and Teixeira, A.R. (1996) Covalent dimerization of ribulose bisphosphate carboxylase subunits by UV radiation. *Biochem. J.* 318: 227–234.

Ferreira, R.B. and Shaw, N.M. (1989) Effect of osmotic stress on protein turnover in *Lemma minor* fronds. *Planta* 179: 456–465.

Ferreira, R.M. and Teixeira, A.R. (1992) Sulfur starvation in *Lemna* leads to degradation of ribulose-bisphosphate carboxylase without plant death. *J. Biol. Chem.* 267: 7253–7257.

Glickman, M.H. (2000) Getting in and out of the proteasome. *Semin. Cell Dev. Biol.* 11: 149–158.

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022

Glickman, M.H. and Ciechanover, A. (2002) The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev*. 82: 373–428.

Goldberg, A.L. (2003) Protein degradation and protection against misfolded or damaged proteins. *Nature* 426: 895–899.

Hartley-Whitaker, J., Ainsworth, G. and Meharg, A.A. (2001a) Copper and arsenate-induced oxidative stress in *Holcus lanatus* L. clones with differential sensitivity. *Plant Cell Environ*. 24: 713–722.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W.T., Schat, H. and Meharg, A.A. (2001b) Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiol*. 126: 299–306.

Hartfield, P.M., Gosink, M.M., Carpenter, T.B. and Vierstra, R.D. (1997) The ubiquitin-activating enzyme (E1) gene family in arabidopsis thaliana. *Plant J*. 11: 213–226.

Hoeberichts, F.A., Orzaez, D., van der Plas, L.H. and Woltering, E.J. (2001) Changes in gene expression during programmed cell death in tomato cell suspensions. *Plant Mol. Biol*. 45: 641–654.

Jabben, M., Shanklin, I. and Vierstra, R.D. (1989) Ubiquitin–phytochrome conjugates. *J. Biol. Chem*. 264: 4998–5005.

Jones, A.M. (2001) Programmed cell death in development and defense. *Plant Physiol*. 125: 94–97.

Kampinga, H.H., Brunsting, J.F., Stege, G.J.J., Burgman, P.W.J.J. and Konings, A.W.T. (1995) Thermal protein denaturation and protein aggregation in cells made thermotolerant by various chemicals: role of heat shock proteins. *Exp. Cell Res*. 219: 536–546.

Kirkpatrick, D.S., Dale, K.V., Catania, J.M. and Gandolfi, A.J. (2003) Low-level arsenite causes accumulation of ubiquitinated proteins in rabbit renal cortical slices and HEK293 cells. *Toxicol. Appl. Pharmacol*. 186: 101–109.

Klemperer, N.S. and Pickart, C.M. (1989) Arsenite inhibits two steps in the ubiquitin-dependent proteolytic pathway. *J. Biol. Chem*. 264: 19245–19252.

Larkindale, J. and Huang, B. (2004) Thermotolerance and antioxidant systems in *Agrostis stolonifera*: involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene. *J. Plant Physiol*. 161: 405–413.

Larkindale, J. and Knight, M.R. (2002) Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiol*. 128: 682–695.

Li, J.J. and Dewey, W.C. (1992) Relationship between thermal tolerance and protein degradation in temperature-sensitive mouse cells. *J. Cell Physiol*. 151: 310–317.

Liu, S.X., Athar, M., Lippai, I., Waldren, C. and Hei, T.K. (2001) Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc. Natl Acad. Sci. USA* 98: 1643–1648.

Lynn, S., Gurr, J.R., Lai, H.T. and Jan, K.Y. (2000) NADH oxidase activation is involved in arsenite-induced oxidative DNA damage in human vascular smooth muscle cells. *Circ. Res*. 86: 514–519.

Mandal, B.K. and Suzuki, K.T. (2002) Arsenic round the world: a review. *Talanta* 58: 201–235.

Meharg, A.A. and Hartley-Whitaker, J. (2002) Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. *New Phytol*. 154: 29–43.

Meharg, A.A. and Macnair, M.R. (1991) The mechanisms of arsenate tolerance in *Deschampsia cespitosa* (L.) Beauv and *Agrostis capillaris* L. *New Phytol*. 119: 291–297.

Meharg, A.A. and Macnair, M.R. (1992) Suppression of the high-affinity phosphate uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. *J. Exp. Bot*. 43: 519–524.

Miller, J. and Gordon, C. (2005) The regulation of proteasome degradation by multi-ubiquitin chain binding proteins. *FEBS Lett*. 579: 3224–3230.

Mimnaugh, E.G., Chen, H.Y., Davie, J.R., Celis, J.E. and Neckers, L. (1997) Rapid deubiquitination of nucleosomal histones in human tumor cells caused by proteasome inhibitors and stress response inducers: effects on replication, transcription, translation, and the cellular stress response. *Biochemistry* 36: 14418–14429.

Noventa-Jordao, M.A., do Nascimento, A.M., Goldman, M.H., Terenzi, H.F. and Goldman, G.H. (2000) Molecular characterization of ubiquitin genes from *Aspergillus nidulans*: mRNA expression on different stress and growth conditions. *Biochim. Biophys. Acta* 1490: 237–244.

Nover, L. and Scharf, K.D. (1984) Synthesis, modification and structural binding of heat-shock proteins in tomato cell cultures. *Eur. J. Biochem*. 139: 303–313.

Pickering, I.J., Prince, R.C., George, M.J., Smith, R.D., George, G.N. and Salt, D.E. (2000) Reduction and coordination of arsenic in Indian mustard. *Plant Physiol*. 122: 1171–1178.

Redman, R.S., Sheehan, K.B., Stout, R.G., Rodriguez, R.J. and Henson, J.M. (2002) Thermotolerance generated by plant/fungal symbiosis. *Science* 298: 1581.

Rosen, B.P. (2002) Biochemistry of arsenic detoxification. *FEBS Lett* 529: 86–92.

Sanchez, Y., Taulien, J., Borkovich, K.A. and Lindquist, S. (1992) Hsp104 is required for tolerance to many forms of stress. *EMBO J*. 11: 2357–2364.

Schmoger, M.E., Oven, M. and Grill, E. (2000) Detoxification of arsenic by phytochelatins in plants. *Plant Physiol*. 122: 793–801.

Shi, H., Hudson, L.G. and Liu, K.J. (2004) Oxidative stress and apoptosis in metal ion-induced carcinogenesis. *Free Radic. Biol. Med* 37: 582–593.

Smalle, J. and Vierstra, R.D. (2004) The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol*. 55: 555–590.

Spees, J.L., Chang, S.A., Snyder, M.J. and Chang, E.S. (2002) Osmotic induction of stress-responsive gene expression in the lobster *Homarus americanus*. *Biol. Bull*. 203: 331–337.

Sullivan, J.A., Shirasu, K. and Deng, X.W. (2003) The diverse roles of ubiquitin and the 26S proteasome in the life of plants. *Nat. Rev. Genet*. 4: 948–958.

Sun, C.W. and Callis, J. (1997) Independent modulation of *Arabidopsis thaliana* polyubiquitin mRNAs in different organs and in response to environmental changes. *Plant J*. 11: 1017–1027.

Swerdlow, P.S., Finley, D. and Varshavsky, A. (1986) Enhancement of immunoblot sensitivity by heating of hydrated filters. *Anal. Biochem*. 156: 147–153.

Talent, J.M., Kong, Y. and Gracy, R.W. (1998) A double stain for total and oxidized proteins from two-dimensional fingerprints. *Anal. Biochem*. 263: 31–38.

Tsou, T.-C., Tsai, F.-Y., Wu, M.C. and Chang, L.W. (2003) The protective role of NF-[kappa]B and AP-1 in arsenite-induced apoptosis in aortic endothelial cells. *Toxicol. Appl. Pharmacol*. 191: 177–187.

Ullrich-Eberius, C.I., Sanz, A. and Novacky, A.J. (1989) Evaluation of arsenate and vanadate-associated changes of electrical membrane potential and phosphate transport in *Lemna gibba* GI. *J. Exp. Bot*. 40: 119–128.

Valko, M., Morris, H. and Cronin, M.T. (2005) Metals, toxicity and oxidative stress. *Curr. Med. Chem*. 12: 1161–1208.

Vierstra, R.D. (2003) The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends Plant Sci*. 8: 135–142.

Voges, D., Zwickl, P. and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem*. 68: 1015–1068.

Wang, J., Zhao, F.-J., Meharg, A.A., Raab, A., Feldmann, J. and McGrath, S.P. (2002) Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant Physiol*. 130: 1552–1561.

Wang, T.-S., Hsu, T.-Y., Chung, C.-H., Wang, A.S.S., Bau, D.-T. and Jan, K.-Y. (2001) Arsenite induces oxidative DNA adducts and DNA–protein cross-links in mammalian cells. *Free Radic. Biol. Med*. 31: 321–330.

Woltering, E.J. (2004) Death proteases come alive. *Trends Plant Sci*. 9: 469–472.

Yen, J.L., Su, N.Y. and Kaiser, P. (2005) The yeast ubiquitin ligase SCFMet30 regulates heavy metal response. *Mol. Biol. Cell*. 16: 1872–1882.

(Received July 3, 2006; Accepted July 25, 2006)

Downloaded from https://academic.oup.com/pcp/article/47/9/1262/2329635 by guest on 21 August 2022