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Proteome Profiling of *Populus euphratica* Oliv. Upon Heat Stress

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• *Background and Aims Populus euphratica* is a light-demanding species ecologically characterized as a pioneer. It grows in shelter belts along riversides, being part of the natural desert forest ecosystems in China and Middle Eastern countries. It is able to survive extreme temperatures, drought and salt stress, marking itself out as an important plant species to study the mechanisms responsible for survival of woody plants under heat stress.
• *Methods* Heat effects were evaluated through electrolyte leakage on leaf discs, and LT_{50} was determined to occur above 50 °C. Protein accumulation profiles of leaves from young plants submitted to 42/37 °C for 3 d in a phytotron were determined through 2D-PAGE, and a total of 45 % of up- and downregulated proteins were detected. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)/TOF analysis, combined with searches in different databases, enabled the identification of 82 % of the selected spots.
• *Key Results* Short-term upregulated proteins are related to membrane destabilization and cytoskeleton restructuring, sulfur assimilation, thiamine and hydrophobic amino acid biosynthesis, and protein stability. Long-term upregulated proteins are involved in redox homeostasis and photosynthesis. Late downregulated proteins are involved mainly in carbon metabolism.
• *Conclusions* Moderate heat response involves proteins related to lipid biogenesis, cytoskeleton structure, sulfate assimilation, thiamine and hydrophobic amino acid biosynthesis, and nuclear transport. Photostasis is achieved through carbon metabolism adjustment, a decrease of photosystem II (PSII) abundance and an increase of PSI contribution to photosynthetic linear electron flow. Thioredoxin *h* may have a special role in this process in *P. euphratica* upon moderate heat exposure.

Key words: *Populus euphratica*, moderate heat stress, mass spectrometry, proteome profiling, carbon metabolism.

INTRODUCTION

Changes in environmental temperature induce stress in most crop plants, especially in those not adapted to face extreme temperatures. Most crops cultivated worldwide are exposed to severe heat stress during their life cycle, suffering a reduction in yield and quality of fruits (Maestri *et al*., 2002). Accelerated global warming is a growing concern as it represents an increase in the average temperature with which worldwide crops have to cope. Efforts to improve crop performance upon exposure to high temperatures have been focused on plant transformation with genes offering enhanced thermal tolerance (Grover *et al*., 2000; Sharkey, 2000; Iba, 2002; Wang *et al*., 2003). However, heat effects are still not as well understood as other abiotic stresses, such as cold or high-intensity light. In recent years, however, knowledge of the molecular mechanisms behind plants' responses to heat stress has grown substantially. Nowadays, it is clear that heat induces signalling cascades, in prokaryotic and eukaryotic cells, which trigger the transcription of a specific set of genes through the activation of several transcription factors. High temperature in eukaryotes induces nuclear import and binding of heat shock factor 1 (HSF1) trimers to heat shock promoter elements. HSFs promote transcription of these genes against depletion of constitutive genes (Mishra *et al*., 2002; Bharti *et al*., 2004; Port *et al*., 2004). The result is the increase of proteins involved in proteolysis and chaperone activity (Mathew *et al*., 1998). Changes in the redox state of the chloroplast electron transport chain or in pools of photosynthesis-coupled redox-active compounds (thioredoxin, glutathione) exert regulation over both plastid- and nuclear-encoded chloroplast-expressed proteins (Dat *et al*., 2000). Hydrogen peroxide, mainly resulting from the activity of NADPH oxidases, is rapidly accumulated upon high temperature, among other stresses (Laloi *et al*., 2004), and may act as an important signalling molecule (Foyer *et al*., 2005). A group of proteins, part of the plant antioxidant system, are rapidly activated in response to oxidative stress generated by heat, including superoxide dismutases, catalases and peroxidases. Modulation of the heat stress response is also dependent on cellular control of degradation and maintenance of quality of proteomes by the ubiquitin–proteasome system (Mathew and Morimoto, 1998; Mathew *et al*., 1998). This system, which is involved on the regulation of transcription factors

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(Ingvardsen *et al*., 2001), removes denaturated and misfolded proteins through proteolysis of soluble cytosolic and nuclear proteins.

Plant species of particular interest for the study of thermal stress tolerance or resistance are those highly adapted to survive in extreme environments (Robertson *et al*., 1994; Lund *et al*., 1998; Skylas *et al*., 2002; Wang *et al*., 2003; Wullschleger and Difazio, 2003) since they may retain regulatory mechanisms enabling their survival. The dissection of such mechanisms may reveal a set of genes, and their products, that may contribute to genetic improvement for thermal stress tolerance in other plants, such as economically important cultivars (Bohnert *et al*., 2000, 2001; Hanson and Tabita, 2003). This is the case with *Populus euphratica*, a light-demanding species ecologically characterized as a pioneer that grows during the summertime in shelter belts along riversides (Shiji *et al*., 1996), as part of the natural desert forest ecosystems in China and in Middle Eastern countries (Youlin *et al*., 2001). Euphrates poplar is highly adapted to salt stress, extreme temperatures and drought (Ma *et al*., 1997; Gu *et al*., 1999, 2004*b*). Hence, *P. euphratica* may be considered an important plant species to study the events responsible for woody plants survival under heat stress.

Most components of the heat stress response mechanism in plants can be identified through high-throughput transcriptomic and proteomic analysis (Thiellement *et al*., 2002; Hanson and Tabita, 2003; Gu *et al*., 2004*a*). Proteomic studies are currently performed through a combination of 2D-PAGE gels and mass spectrometry analysis. Protein separation in 2D gels may be followed by tryptic *in gel* digestion and mass spectrometry analysis of the resulting peptides. The original proteins can be identified with mass spectrometry and database searching through the use of either peptide mass fingerprinting or tandem mass spectrometry information, using a bottom-up strategy. These protein identification approaches are dependent on databases of already known peptide or nucleotide sequences. As genomic sequencing projects are relatively recent, only a few genomes are available in public databases (Liska and Shevchenko, 2003). *Populus* is a genus that already has a broad collection of expressed sequence tags (ESTs) available to public use and has a completely sequenced genome from *Populus trichocharpa.* The aim of our study was to analyse leaf proteomes of *P. euphratica* and identify up- and downregulated proteins through the use of different databases, aiming to contribute to the knowledge of the molecular mechanisms underlying Euphrates poplar tolerance/resistance to high temperatures.

MATERIALS AND METHODS

Plant propagation and hydroponic culture

Plants of *Populus euphratica* Oliv. maintained under greenhouse conditions were used, after entering dormancy, to prepare grafts. Rooting was performed by immersion of grafts in 500 μg L^{-1} 1-naphthaleneacetic acid (NAA) solution for 48 h under greenhouse conditions. Rooted grafts were transferred to cuvettes of 5 × 5 × 8 cm, filled with river sand, and covered with plastic to maintain high humidity. After 3 weeks in the greenhouse (rooting ratio of 80 %), grafts were transferred to hydroponic culture in a phytotron, to mimic their natural habitat. Hydroponic culture was necessary to maintain the growth rate of *P. euphratica* plantlets in the absence of natural sunlight. The nutrient solution, containing 8·3 mM Ca^{2+}, 3·44 mM K^{+}, 1 mM Mg^{2+}, 8·3 mM NO^{3-}, 1·84 mM H_2PO^{4-}, 1 mM SO_4^{2-}, 37·8 μM Fe·EDTA, 0·32 μM Cu^{2+}, 0·8 μM Zn^{2+}, 0·4 μM Mn^{2+}, 50 μM H_3BO_3 and 0·082 μM MoO_4^{2-}, was set to pH 5·5–5·8. Temperature and relative humidity (RH) in the Fitoclima 700 EDTU phytotron (Aralab, S. Domingos de Rana, Portugal) were set as 30 °C and 80 % for 16 h of light (350 μE m^{-2} s^{-1}) and as 20 °C and 95 % for 8 h of darkness, corresponding approximately to the season photoperiod. Forty-eight plants were selected for sampling during heat treatments.

Cell membrane thermostability

Fifteen mature leaves were taken from young plants growing under greenhouse conditions and washed with distilled water to remove contaminants. Leaf discs of 3 mm diameter were prepared starting from total leaf width (average of 4 mm, including midrib) and kept in culture vials, each containing 15 discs prepared from five leaves. Leaf disc-containing vials were closed and submerged in a water bath (Thermomix BU coupled to Frigomix U, B. Braun, Melsungen AG, Germany) at each of the selected experimental temperatures (25, 40, 45, 50 and 55 °C). Each experimental temperature effect on leaf discs was assessed with nine replicates from three independent experiments. After 30 min, vials were withdrawn from the water bath and leaf discs were immediately immersed in sterile de-ionized water and then incubated for 2 h at room temperature under agitation at 30 r.p.m. on an Agitorb 300E (Aralab, S. Domingos de Rana, Portugal). Electrolyte leakage, from control and heat treatments, was immediately measured at +18 °C with a CDM 83 conductivity meter (Radiometer, Copenhagen, Denmark), using potassium hydroxide at 1 M for calibration. Maximum conductivity was measured after autoclaving for 15 min at 1·1 Pa and 121 °C, and after cooling at room temperature overnight under agitation. Significant membrane damage was considered to occur at the temperature at which the calculated percentage damage would exceed 50 % (lethal temperature, LT_{50}). Electrolyte leakage (EL), directly proportional to cell membrane damage, was calculated using the equation $EL(\%) = [(C_x - C_i)/C_m] \times 100$, where C is water conductivity under control conditions (i), experimental high temperature (x) and at maximum conductivity (m; after autoclaving).

Heat treatment design

For the heat experiment, it was necessary to mimic greenhouse conditions inside a controlled environment

TABLE 1. *Experimental design programmed in the phytotron regarding photoperiod, temperature and relative humidity for sample collection*

Parameters	Control				Day 1				Day 2				Day 3	
S_d (h)	5	6	5	8	5	6	5	8	5	6	5	8	5	6
T_b (°C)	20	30	30	20	20	42	42	37	37	42	42	37	37	42
T_e (°C)	30	30	20	20	42	42	37	37	42	42	37	37	42	42
RH_b (%)	95	80	80	95	95	80	80	95	95	80	80	95	95	80
RH_e (%)	80	80	95	95	80	80	95	95	80	80	95	95	80	80

Daily cycles in the phytotron were set as four segments per day. S_d(h), segment duration in h; for each segment, the temperature and relative humidity are presented. T_b (°C), temperature at the beginning of the segment in°C; T_e (°C), temperature at the end of the segment in°C; RH_b (%), percentage relative humidity at the beginning of the segment; RH_e (%), percentage relative humidity at the end of the segment.

cabinet or phytotron (Fitoclima 700 EDTU). Control conditions were set close to the season climatic parameters, allowing small deviations to the adaptation status of *P. euphratica* growth under greenhouse conditions. Daily cycles of temperature and RH were monitored and plotted to enable proper design of the experiment. Table 1 presents the established phytotron conditions for the heat experiment regarding RH, temperature and photoperiod. RH was set to 80 % during the light period. Since *P. euphratica* culture was carried out under hydroponic conditions, a high RH was chosen to allow higher thermal conductance between air and the plant body and to ensure proximity between these two values. Control samples were collected on the day before starting the heat treatment, after submitting *P. euphratica* plants to 30 °C for 6 h, under phytotron full light intensity (350 µmol photons m^{-2} s^{-1}). Heat treatment started with a temperature increase from 20 to 42 °C for 5 h on the first day, corresponding to an increase of 4·2 °C h^{-1}. This gradual increase in temperature during the heat treatment generates a lower inhibition of the photosynthetic machinery, compared with a rapid temperature increase, and allows an adaptation process (Law and Crafts-Brander, 1999). In this way, heat shock was induced and maintained for the remaining treatment. However, since transition from day to night implies a natural reduction in environmental temperature, a slight decrease in temperature (5 °C) on the transition from light to dark was introduced. Hence, the maximum temperature in the light period, set to 42 °C, was decreased to 37 °C for 5 h (–1 °C h^{-1}) and maintained for the following 8 h of darkness. The total experiment lasted for 4 d and sampling was always performed at the end of the 6 h segment for control (30 °C) and heat stress (42 °C) conditions on day 1 (6 h), day 2 (30 h) and day 3 (54 h). Samples consisted of ten fully mature leaves taken from each plant of a total of 12 plants used for each experimental condition. Continuous light intensity was set for the whole light period in the culture chamber (except for 30 min of transition at the beginning of the first segment) in order to minimize light-induced oscillations of protein accumulation profiles. All the samples were taken at exactly the same time point each day so that protein accumulation profiles dependent on circadian control would not mask heat-induced accumulation profiles, producing misleading differences detected on protein profiles.

Protein precipitation

Mature leaves were used for protein precipitation. Pooled samples, representing 120 leaves—ten leaves from each of 12 plants—were ground in liquid nitrogen and approx. 1·5 g of powder was suspended in ethanol at four times the sample volume. After 1 h at −20 °C, the same volume of cold acetone was added and proteins were allowed to precipitate overnight, at −20 °C. Proteins were collected through centrifugation at 26 000 *g* (–10 °C; 15 min), followed by the addition of nine times the sample volume of a solution of ethanol : acetone : triple distilled water 4 : 4 : 1 (v/v/v). Proteins were then re-suspended and reprecipitated for 6 h at −20 °C. Following centrifugation at 26 000 *g* (–10 °C, 40 min), two more washing steps were performed as before and the final pellet was dried overnight at room temperature.

Protein solubilization and IEF

Proteins were solubilized by adding 3·5 mL of lysis buffer [7 M urea, 1·9 M thiourea, 1 % (v/v) of Pharmalyte 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden), 2 % (w/v) CHAPS and 0·4 % (w/v) dithiothreitol (DTT)] to the dry pellet. Proteins were allowed to solubilize for 24 h at room temperature (20 °C) and protein quantification was performed with Bradford standard assay. IPG strips of 18 cm, linear pH 4–7 (Amersham Pharmacia Biotech, Uppsala, Sweden), were rehydrated overnight in lysis buffer containing 200 µg of protein for each gel. IPG strips were transferred to a Multiphor II isoelectric focusing (IEF) unit and IEF was performed at 20 °C, 5 mA and 5 W to a total of 16 000 Vh. IPG strips were shaken for 15 min in equilibration buffer [2 % (w/v) SDS, 10 % (v/v) glycerol, 50 mM Tris–HCl pH 6·8 and 5 % (v/v) 2-mercaptoethanol] and stored at −70 °C until use.

2D-PAGE

IPG strips were thawed and re-equilibrated for 15 min using fresh equilibration buffer, and immediately loaded onto 20 × 18·5 × 0·1 cm, 12·5 % polyacrylamide gels (acrylamide to bisacrylamide ratio of 200:1) without SDS. Electrophoresis was performed in recirculating running buffer overnight at 20 °C, under constant current settings.

Staining, scanning and image analysis

Two-dimensional polyacrylamide gels were fixed and stained with Sypro Ruby. Gel images were obtained with a cooled CCD camera system and analysed with BioImage 2-D Analyzer software (Version 6·1). Protein expression was determined through the sum of all the pixel values within the boundary of each spot expressed as a

percentage (integrated optical density percentage, IOD%) of the sum of IOD values for all the detected spots. All the detected spots were submitted to matching. After automated detection, spots were edited manually, matched, and the IOD% values were exported and analysed with Microsoft Excel.

Spot excision and protein digestion

Spots of interest were manually excised from 2D gels with a scalpel, washed with 70 μL of deionized water followed by acetonitrile 100 % (90 μL, 15 min; 30 μL, 2 min). The spots were dehydrated in a vacuum centrifuge and rehydrated with a solution of 67 ng of trypsin in 50 mM NH_4HCO_3, at 4 °C. After 20 min, 30 μL of 50 mM NH_4HCO_3 were added and digestion proceeded at 37 °C overnight, followed by storage at −20 °C until use.

Sample preparation and mass spectrometry

Desalting was performed on custom-made reverse-phase microcolumns, prepared with R2 resin (Perseptive Biosystems Inc., Framingham, MA, USA) as described elsewhere (Gobom *et al*., 1999). Peptide solution, obtained from digestion of each spot, was loaded onto the microcolumn, followed by washing with 10 μL of 1 % trifluoroacetic acid (TFA). Bound peptides were eluted with 0·8 μL of matrix solution [α-cyano-4-hydrocynnamic acid (5 g L^{-1}) in 70 % acetonitrile/0·1 % TFA] directly onto the matrix-assisted laser desorption ionization (MALDI) target plate. Peptide mass spectra were acquired in positive reflector mode on a 4700 Proteomics Analyzer MALDI-time of flight (TOF)/TOF (Applied Biosystems, Foster City, CA, USA) using 20 kV of acceleration voltage. Each spectrum was obtained with a total of 1000–1200 laser shots and was externally calibrated using peptides derived by tryptic digestion of lactoglobulin. Further processing and interpretation of the MS spectra was performed with *m/z* software (Genomic Solutions Inc., Ann Harbor, MI, USA). Tandem mass spectra were acquired using the same instrument in MS/MS positive mode. Further processing and interpretation of the MS/MS spectra were performed using Data Explorer (version 4·4, Applied Biosystems). All MS/MS data from each individual spot were merged into a single file before search.

Database search

Peptide mass maps and sequences obtained by tandem mass spectrometry were searched against all *Viridiplantae* entries of NCBInr and against a poplar EST database (Shevchenko *et al*., 1997) from Umea Plant Science Centre (Umea, Sweden) using the Mascot search engine 2·0 (Matrix Science Ltd, London, UK) (Perkins, 1999). Proteins identified in both ways were always manually checked to exclude false-positive hits. Search parameters were carbonyl propionamidation (cystein) as fixed modifications, methionine oxidation as variable modifications, peptide mass tolerance of 70 ppm at the most and a general fragment mass tolerance of 0·25 Da (up to 0·8 Da when necessary). According to the search engine, a score of 65 represents a significant identification ($P > 0·05$) when the database is restricted to the *Viridiplantae* taxonomy (NCBInr 20041113).

RESULTS

Euphrates poplar has a high cell membrane thermostability

Under greenhouse conditions, *P. euphratica* plants are sensitive to daylight intensity. Stress signals include, in these cases, stem darkening, changing from light pink to magenta from basis to apex, and leaves darkening to dark green. The internodes stop elongation and the apical meristem loses morphogenic activity. Exposure of *P. euphratica* plants to 42 °C for 54 h did not affect their survival. Heat-induced changes on stem coloration were reversed after recovery from heat stress. Apical dominance was re-aquired and initial flushing of axillary buds was interrupted. Cell membrane thermostability was estimated in order to choose a non-lethal, nor sublethal, temperature for the heat treatment. Cell membrane thermostability can be estimated through electrolyte leakage following exposure to physical stress factors, such as heat or cold, as has been used in soybean (*Glycine max*), sorghum (*Sorghum bicolor*) and melon (*Cucumis melo*). It has also been used in cool season crops such as wheat (*Triticum aestivum*) and Kentucky bluegrass (*Poa pratensis*) (see Ismail and Hall, 1999 and references therein) and to assess osmotic stress tolerance in transgenic plants (Abe *et al*., 2003). The evaluation of cellular membrane thermostability through analysis of membrane electrolyte leakage revealed a small increase of cell membrane ion permeability up to 45 °C, presenting thereafter a higher increase up to 55 °C (Fig. 1). The lethal temperature (LT_{50}) was determined to be between 50 and 55 °C, since membrane damage at these temperatures due only to heat was estimated to be 31 and 63 %, respectively. A temperature of 42 °C was selected as the maximum temperature to which to expose *P. euphratica* plants, since this should be non-lethal.

Moderate heat exposure induces growth arrest in P. euphratica

Euphrates poplar presents active growth under high temperature in its natural habitat, as long as it has access to the water table. It is possible to find this species as part of Chinese and Middle Eastern desert communities, distributed along riversides and following the track of the underground water. Therefore, it is not surprising that, even with a high salt concentration in the soil, or with atmospheric temperatures as high as 50 °C, it does not show a significant drought stress in its natural habitat (Brosche *et al*., 2005). When working with *P. euphratica*, we must use an experimental design where water availability and high light quality requirements are provided for this species, in order to represent true natural conditions. The system described in this work enabled

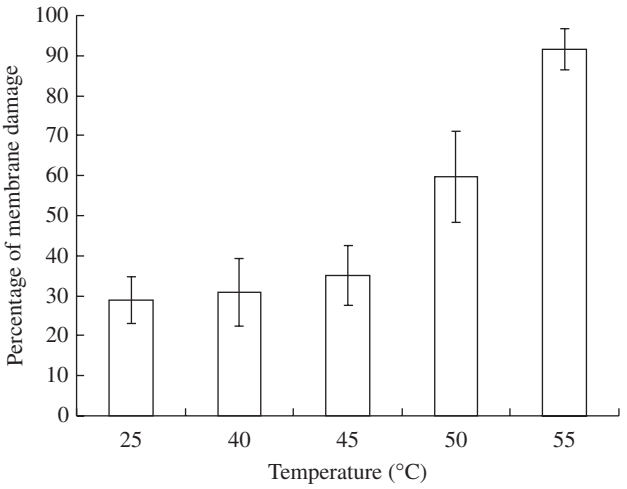


FIG. 1. Percentage of membrane damage of leaf cells from plants of *P. euphratica* submitted to temperatures up to 55 °C. Columns represent the average percentage membrane damage, calculated from the results of three independent experiments. The *y*-axis represents the total percentage of electrolyte leakage and the *x*-axis represents the temperature, in °C, to which leaf discs were exposed.

those requirements to be guaranteed, compensating for low-quality light through direct nutrient supply to the root system. Macroscopic evaluation of the effects of temperature increase on *P. euphratica* plants was performed during the heat experiment. A slight curling of the younger leaves (generally the third and fourth node below the apex) and a change in stem coloration (from light pink to light green) were observed after 30 h of exposure to 42 °C. After 24 h of recovery from heat stress, *P. euphratica* axillary buds begun to flush. Although this initial flush was sustained for 4–6 d, it never gave rise to new shoots because apical meristem started developing again and regained dominance during that period. Plants recovering for 8–10 d again showed their light pink stem coloration; the young leaves were no longer curled and no other stress symptoms were observed. Observations of *P. euphratica* plants during the heat treatment and recovery reported here confirmed our prediction from electrolyte leakage results.

Protein identification by homology in a woody plant species

Proteomic and genomic studies in trees have been difficult to accomplish due to several problems related to genome sizes and recalcitrance to *in vitro* manipulation (Canovas *et al*., 2004), but the completion of genome sequencing of *P. trichocharpa* provided public availability of a precious information resource, as it is now possible to obtain more accurate data on gene and protein sequences on woody plant species. Therefore, protein identification by homology through cross-species search (within woody plant species) may achieve a higher level of accuracy. The number of identifications achieved for *P. euphratica* proteins in the work reported here was particularly high, considering that this study was performed in a woody plant species without an annotated genome. The combination of two different approaches (protein identification based on cross-species sequence homology among *Viridiplantae* and protein identification based on an EST collection of a closely related species, *P. trichocarpa*) contributed to these results, together with good quality spectra and database researching. In the case of citrate synthase identification by homology search, some of the most intense peaks matched SwissProt/TrEMBL accession no. O24259. This was considered sufficient to accept this entry as the closest homologue, although a statistically non-significant MASCOT algorithm score has been obtained (Table 2). The sequence tag MASCOT scores were obtained using the most powerful search tool (Sequence Query on http://www.matrixscience.com/search_form_ select.html), through the use of a combination of molecular weight and sequence data information (Mann and Wilm, 1994). Using this combination of data, it became possible to achieve the best homology match because several peptide sequences from the same protein increase the information available and allow higher accuracy of matches compared with the use of single sequence data.

Image analysis of leaf proteomes allowed detection and quantification of 1355 spots, of which 653 showed IOD% changes of more than ±0·5-fold when compared with control values. Overall upregulation was observed for 19·9 % of the spots, while 16·4 % showed downregulation. Moreover, 2·1 % of the spots showed an accumulation profile classified as random. Therefore, 55 % of the 1355 spots have been classified as constant, showing changes of IOD% in the range of less than ±0·5-fold of control values. The up- or downregulated spots were grouped according to their accumulation profile as a function of time (Table 3). No statistical test or algorithm was applied to define these groups or to select the spots in each of them. Mass spectrometry analysis was performed for approx. 10 % of the spots of each profile group, selected based on their quality, to allow coverage of all groups. It was possible to identify protein spots for only nine of the 13 delimited groups, and proteins of four groups could not be identified (Table 3). MALDI-MS and MALDI-MS/MS analysis (Table 2) allowed significant homology matches to 51 out of 62 spots (82·3 % success rate) to be obtained. Thus, it became possible to achieve the best homology match as several peptide sequences for the same protein increase the information and allow higher accuracy of matches compared with the use of single sequence data. All proteins reported here were submitted to the UniProt via SPIN tool and were accepted after UniProt revision (accession numbers in Table 2). Twenty-one identifications were determined using a private *Populus* EST database, from which 17 identifications were obtained due to matches to homologous proteins from other plant species (Habermann *et al*., 2004) and four identifications were based only on EST database information. Eighteen of the 51 spots analysed matched ribulose-1,5-biphosphate carboxylase-oxygenase (Rubisco) entries and most of them showed upregulation (data not shown).

TABLE 2. *Protein spots from 2D gels identified through peptide mass fingerprinting and tandem mass spectrometry after tryptic digestion*

Spot no.	IOD % change (-fold)			Peptide mass fingerprint (score, e-value); peptide sequences (score/e–value)	Homologue protein	Species	SwissProt/TrEMBL accession no. (UniProt accession no.)
	6 h	30 h	54 h				
Amino acid metabolism							
310	+1·6	0·0	–0·2	VSLAGHDEYIVR ($64/4{\cdot}8 \times 10^{-5}$)	Putative ketol-acid reductoisomerase	*Oryza sativa* (japonica cultivar group)	REFSEQ: NM_192395
				FYEKEGLPAFPMoxGK ($65/4{\cdot}4 \times 10^{-5}$)	Ketol-acid reductoisomerase, chloroplast precursor	*Spinacea oleracea*	Q01292 (P84534)
				FYEKEGLPAFPMoxGK ($63/1 \times 10^{-4}$)			BLAST of EST: Q01292
335	0·0	–0·1	–0·6	TEFGPSQPFKGAK ($48/9{\cdot}1 \times 10^{-3}$)	Adenosylhomocysteinase*		P68172 (P84534)
338	+1·1	–0·1	–0·7	AEFGPSQPFKGAK ($50/2{\cdot}7 \times 10^{-3}$)	Adenosylhomocysteinase	*Catharanthus roseus*	P35007 (P84532)
				AEFGPSQPFKGAK ($50/5{\cdot}9 \times 10^{-3}$)			BLAST of EST: P35007
352	–0·3	–0·4	–0·7	AEFGPSQPFKGAK (40/0·021)	Adenosylhomocysteinase	*Catharanthus roseus*	P35007 (P84532)
				AEFGPSQPFKGAK (40/0·046)			BLAST of EST: P35007 (P84532)
941	–0·0	–0·2	–0·6	8 (61/0·13) LIVAIFPSFGER (64) TPNSYILQQFENPANPK ($102/6{\cdot}1 \times 10^{-10}$)	*O*-Acetylserine (thiol) lyase	*Populus alba* × *P. tremula*	Q6V3A7 (P84538)
1526	0·0	+1·3	–0·6	7 (60/0·16) YLFAGVVDGR ($82/1{\cdot}3 \times 10^{-5}$); GVTGFGFDLVR + *G*→*S* [+30·01] ($65/8 \times 10^{-4}$)	Arginine decarboxylase Methionine synthase	*Capsella bursa-pastoris* *Catharanthus roseus*	O81178 Q42699
Carbon metabolism							
183	–0·2	+1·1	–0·6	10 ($76/3{\cdot}8 \times 10^{-3}$) GLIYPPLSNIR (31/0·042) SIQVIVVTDGER (24/0·47) 11 ($77/2 \times 10^{-3}$)	NADP-dependent malic enzyme	*Populus trichocarpa*	P34105 (P84539)
							BLAST of EST: P34105
214	–0·2	–0·3	–0·6	8 (66/0·045)	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase*	*Oryza sativa*	REFSEQ: NM_191088
222	–0·7	–0·3	–0·2	ALPTYTPESPADATR ($60/2{\cdot}3 \times 10^{-3}$)	Transketolase	*Solanum tuberosum*	Q43848 (P84540)
				8 ($73/4{\cdot}5 \times 10^{-3}$)	BLAST of EST: transketolase	*Craterostigma plantagineum*	BLAST of EST: Q42676
286	–0·1	0·0	–0·7	6 ($70/8{\cdot}9 \times 10^{-3}$)	BLAST of EST: dihydrolipoamide dehydrogenase*	*Bruguiera gymnorrhiza*	BLAST of EST: Q93WQ1
327	–0·2	+1·5	–0·3	8 ($83/8{\cdot}6 \times 10^{-4}$)	Enolase	*Brassica rapa*	Q6W7E8
				AAVPSGASTGVYEALELR ($74/9{\cdot}1 \times 10^{-6}$) FRAPVEPY (17/5·5)	Enolase 2	*Zea mays*	P42895 (P84541)
519	–0·4	–0·2	–0·6	6 (54/0·68) YYTVLFGVSR (46/0·049) ALGLPLERPK (38/0·32)	Citrate (si)-synthase, mitochondrial	*Populus balsamifera*	O24259 (P84543)

558	–0·7	–0·6	–0·4	7 (48/2·7) ALPTYTPESPADATR (41/0·018) 9 (72/0·006) ALPTYTPESPADATR (41/0·03)	Transketolase, chloroplast BLAST of EST: transketolase	*Craterostigma plantagineum* *Solanum tuberosum* *Craterostigma plantagineum*	Q42676 Q43848 (P84540) BLAST of EST: Q42676
553	+1·2	+1·5	–0·4	AVSLVLPQLK ($62/5{\cdot}9 \times 10^{-4}$)	Glyceraldehyde-3-phosphate dehydrogenase B*	*Pisum sativum*	P12859 (P84544)
1040	–0·1	+1·7	–0·1	VGKFPLLANSR (45/0·04)	Putative dihydrolipoamide dehydrogenase precursor*	*Oryza sativa*	REFSEQ: NM_183836 (P84545)
Fatty acid synthesis							
431	+2·1	–0·3	–0·1	ITAYLPSGGPFVR ($78/2{\cdot}3 \times 10^{-6}$) LLEEAPSPALTPELR ($52/8 \times 10^{-4}$) ILVANRGEIAVR ($37/6{\cdot}5 \times 10^{-3}$)	Biotin carboxylase	*Brassica napus*	Q93Y50 (P84546)
Sulfate assimilation							
449	+1·8	0·0	–0·4	KADAVFAFQLR (40/0·008)	ATP sulfurylase*	*Brassica oleracea* var. *botrytis*	Q9SBL0 (P84547)
Vitamin biosynthesis							
916	+3·2	0·0	–0·9	5 (48/2·7) LFNAVAAEDLIVKGGR ($83/4{\cdot}8 \times 10^{-7}$) LFNAVAAEDLIVK ($69/1{\cdot}5 \times 10^{-5}$) FQPIKESIVSR ($49/1{\cdot}5 \times 10^{-3}$) EIVPGMIVTGMEVAEIDGAPR 2 oxidation (M) (26/0·77)	Thiazole synthase, chloroplast	*Alnus glutinosa*	Q38709 BLAST of EST: Q38709 (P84548)
Cytoskeleton							
486	+1·7	+1·1	–0·4	10 ($81/1{\cdot}3 \times 10^{-3}$) AIFVDLEPTVIDEVR ($90/6{\cdot}6 \times 10^{-8}$) SLDIERPTYTNLNR ($49/1{\cdot}8 \times 10^{-3}$) FDGAINVDVTEFQTNLVPYPR (36/0·03) 10 ($86/2{\cdot}5 \times 10^{-4}$)	Tubulin α-1 chain BLAST of EST: tubulin α-3/α-5 chain	*Anemia phyllitidis* *Oryza sativa* *Arabidopsis thaliana*	P33623 P28752 (P84549) BLAST of EST: REFSEQ: NM_121982
Signal transduction							
997	+1·6	–0·3	+1·1	ALPNQQTVDYPSFK N-acetyl (protein) [+42·01] ($64/1{\cdot}4 \times 10^{-4}$) SNYNFEKPFLYLAR ($53/4{\cdot}6 \times 10^{-4}$) SNYNFEKPFLYLAR ($53/1{\cdot}7 \times 10^{-3}$)	GTP-binding nuclear protein RAN/TC4	*Vicia faba*	P38548 (P84557) BLAST of EST: P38548
Transcription/translation							
1342	–0·6	–0·4	–0·3	LGAEISSLTLEEAR ($61/2{\cdot}8 \times 10^{-4}$)	BLAST of EST: 50S ribosomal protein L12-1*	*Arabidopsis thaliana*	BLAST of EST: REFSEQ: NM_113699 (P84558)
4341	–0·5	–0·4	–0·3	QIIEANLALR ($47/1{\cdot}4 \times 10^{-3}$) QIIEANLALRR ($42/6{\cdot}3 \times 10^{-3}$) GFGFVTFGNEK ($82/2{\cdot}7 \times 10^{-6}$)	ATP synthase ε chain BLAST of EST: glycine-rich RNA-binding protein	*Androya decaryi* *Euphorbia esula*	Q8MF84 (P84559) BLAST of EST: O48567 (P84560)
Photosynthesis							
724	–0·2	–0·3	–0·6	GFGILDVGYR ($55/2{\cdot}9 \times 10^{-4}$) GTGITEEFEEVPVQSR (27/0·33)	PSII stability/assembly factor, chloroplast	*Arabidopsis thaliana*	REFSEQ: NM_122218 (P84561)
1418	+2·8	+2·6	+4·4	EAPVGFTPPELDPSTPSPIFG -GSTGGLLR ($105/5{\cdot}3 \times 10^{-9}$)	BLAST of EST: PSI-D (PSI 20 kDa subunit)*	*Cucumis sativus*	BLAST of EST: P32869 (P84563)

TABLE 2. *continued.*

Spot no.	IOD % change (-fold) 6 h	30 h	54 h	Peptide mass fingerprint (score, e-value); peptide sequences (score/e–value)	Homologue protein	Species	SwissProt/TrEMBL accession no. (UniProt accession no.)
Redox homeostasis							
3885	+2·5	+2·6	+2·0	MIAPIFAELAK (52/1·1 × 10^{-3})	Thioredoxin *h*	*Populus tremula* × *P. tremuloides*	Q8S3L3 (P84564)
				TVGADKDGLPTLVAK (53/5·1 × 10^{-4})			
				MIAPIFAELAK (52/2·4 × 10^{-3})			BLAST of EST: Q8S3L3
				TVGADKDGLPTLVAK (53/1·3 × 10^{-3})			
Protein metabolism							
74	–0·3	–0·4	–0·5	21 (151)	OSJNBa0039C07·4 protein	*Oryza sativa*	Q7F9I1
				GSGFVAVEIPFTPR (56/4·2 × 10^{-4})	Chloroplast ATP-dependent Clp protease ATP-binding subunit ClpA homologue CD4B	*Lycopersicon esculentum*	P31542 (P84565)
				VLELSLEEAR (18/2·2)			
				VLENLGADPSNIR (70/1·6 × 10^{-5})			
				FLPDKAIDLIDEAGSR (119/2·1 × 10^{-10})			
				VLELSLEEAR (22/2·2)	Chloroplast ATP-dependent Clp protease ATP-binding subunit ClpA homologue CD4A	*Lycopersicon esculentum*	P31541
				FLPDKAIDLIDEAGSR (89/2·1 × 10^{-10})			
				FQPVKVPEPSVDETIQILK (65/1·5 × 10^{-4})			
76	–0·2	–0·1	–0·5	25 (180)	ATP-dependent Clp protease ATP-binding subunit/ClpC	*Arabidopsis thaliana*	REFSEQRELEASE: NM_124471
				VLENLGADPSNIR (63/9·7 × 10^{-5})	Chloroplast ATP-dependent Clp protease ATP-binding subunit ClpA homologue CD4B	*Lycorpersicon esculentum*	P31542 (P84565)
				GSGFVAVEIPFTPR (55/6·3 × 10^{-4})			
				VLELSLEEAR (41/0·015)			
159	+1·5	+1·2	–0·2	VFISDDFDGELFPR (93/7·7 × 10^{-8})	Putative heat shock protein (strong similarity to HSP90)	*Arabidopsis thaliana*	REFSEQ: NM_179601 (P84577)
				FLSVTEPSLLGDGGDLEIR G→A [+14·02] (76/4·2 × 10^{-6})			
				GVVDSDDLPLNVSR (65/3·8 × 10^{-5})			
242	–0·5	–0·6	–0·6	12 (91/1·2 × 10^{-4})	FtsH-like protein Pftf	*Nicotiana tabacum*	Q9ZP50 (P84578)
				FLEYLDKDR (26/6)			
				VRVQLPGLSQELLQK (18/31)			
				TPGFSGADLANLLNEAAILAGR (93/5·4 × 10^{-4})			
				TPGFSGADLANLLNEAAILAGRR (19)			
				8 (81/6 × 10^{-4})			BLAST of EST: Q9ZP50
				TPGFSGADLANLLNEAAILAGR (93/4 × 10^{-8})			
				TPGFSGADLANLLNEAAILAGRR (19/13)			
939	–0·6	–0·1	–0·4	IVDTFPGQSIDFFGALR (37/0·037)	Rubisco activase	*Cucumis sativus*	Q01587 (P84562)
				LVDTFPGQSIDFFGALR (37/0·066)			BLAST of EST: Q01587
				EGPPTFEQPAMTIEK (25/1)			

1138	+1·5	+1·3	–0·2	VAEAEEKTAGGLLLTETTK T→A [–30·01] ($105/3{\cdot}5 \times 10^{-9}$) EKPSIGTVIAVGPGSLDEEGKITP E→V [–29·97] ($80/1{\cdot}2 \times 10^{-6}$) YTSIKPLGDR ($55/1{\cdot}6 \times 10^{-4}$)	Chloroplast protein Cpn 10	*Arabidopsis thaliana*	O65282 (P84579)
1558	+1·6	+2·3	+1·8	VVAAGANPVLITR ($47/6{\cdot}9 \times 10^{-4}$) DLVNVLEDAIR (27/1) VVAAGANPVLITR ($73/5{\cdot}3 \times 10^{-5}$)	Chaperonin 60 β subunit (Rubisco subunit binding-protein, β subunit)	*Solanum tuberosum* BLAST of EST: P93570	P93570 (P84581)
ATP synthesis							
1461	+3·9	+6·9	+6·3	6 (62/0·055) TQFQEIISSTK ($51/2{\cdot}8 \times 10^{-3}$) KFLVELR (22/0·71)	BLAST of EST: plastid ATP synthase CF1 α chain	*Nicotiana tabacum*	BLAST of EST: P00823 (P84582)
3570	+1·6	+1·6	–0·3	9 (60/0·15) GQPVLNTGSPITVPVGR (17/3·2) VVDLLAPYQR (33/0·09) QISELGIYPAVDPLDSTSR ($50/3{\cdot}1 \times 10^{-3}$)	Mitochondrial F1 ATP synthase β subunit BLAST of EST: mitochondrial F1 ATP synthase β subunit	*Arabidopsis thaliana* *Arabidopsis thaliana*	P83483 BLAST of EST: P83483
4341	–0·5	–0·4	–0·3	QIIEANLALR ($47/1{\cdot}4 \times 10^{-3}$) QIIEANLALRR ($42/6{\cdot}3 \times 10^{-3}$) GFGFVTFGNEK ($82/2{\cdot}7 \times 10^{-6}$)	Chloroplast ATP synthase ε chain Glycine-rich RNA-binding protein	*Androya decaryi* *Euphorbia esula*	Q8MF84 (P84581) BLAST of EST: O48567
Proteins of unknown function							
1453	+1·1	+1·9	+1·2	IINDFTNLVNQVEPLK ($105/2{\cdot}9 \times 10^{-9}$) HIVFVR ($44/2{\cdot}1 \times 10^{-3}$) MIMDYYLF ($41/5{\cdot}9 \times 10^{-3}$)	BLAST of EST: stable protein 1 Wound-responsive mRNAs	*Populus tremula* *P. trichocarpa* × *P. deltoides*	BLAST of EST: Q9AR79 (P84580) Q42482

The ESTs-based identifications were always accompanied by a BLAST search (Zhang and Madden, 1997) against publicly available databases to obtain information based on homologous protein function. All the identifications were manually evaluated and found to be reliable, and all the sequences obtained by tandem mass spectra have been submitted to UniProt via SPIN tool, and their accession numbers are also presented in the table.

*Indicates the updated homology matches through new mascot searches in NCBInr giving strong evidence for the presented protein identity.

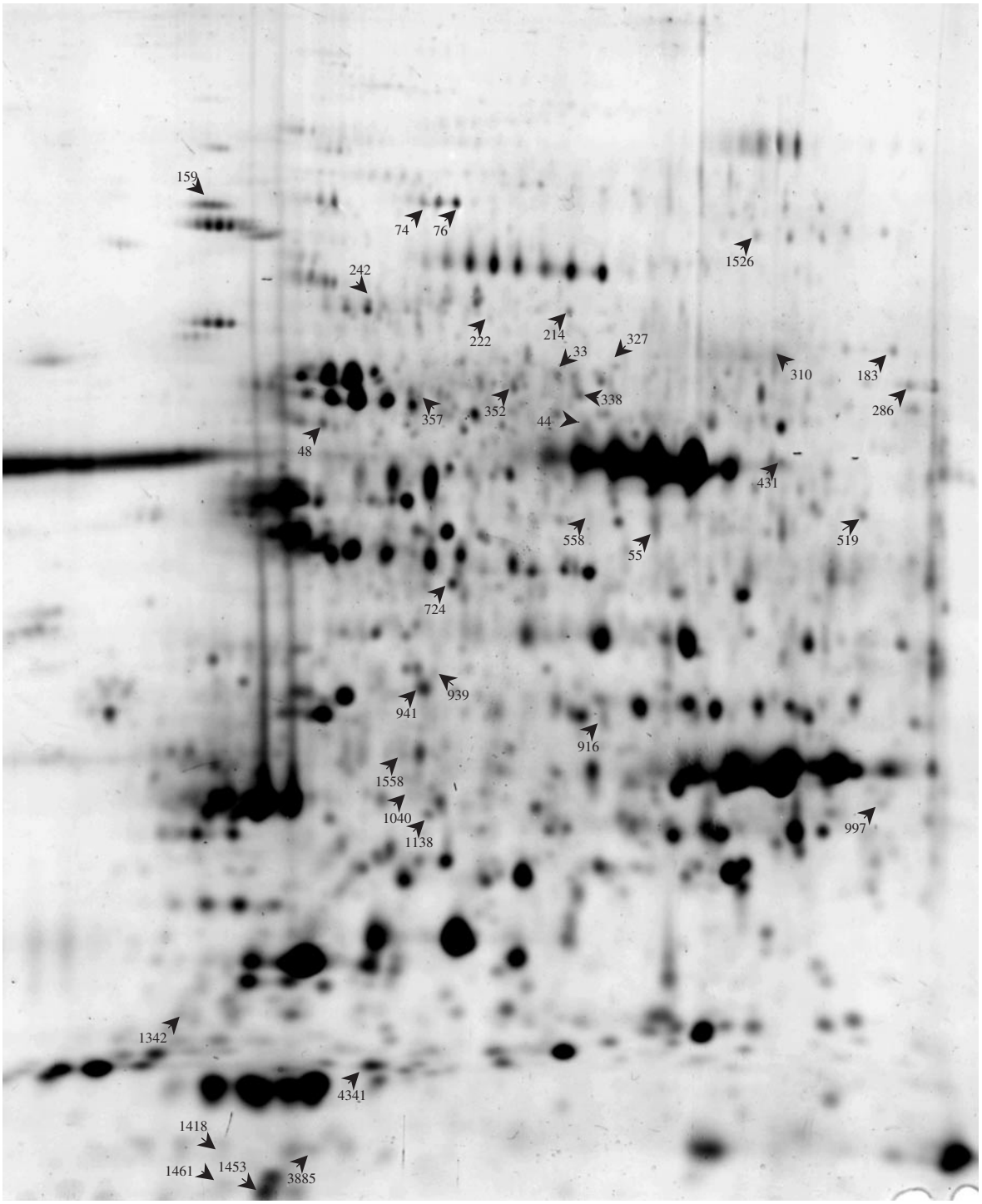
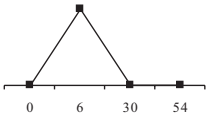
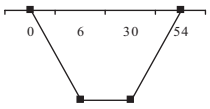
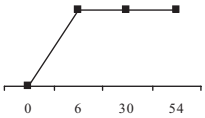
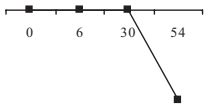
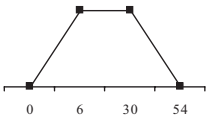
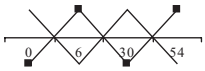
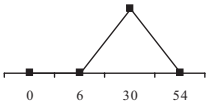
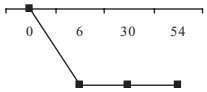
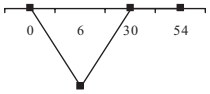
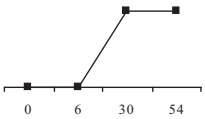
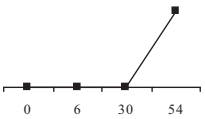
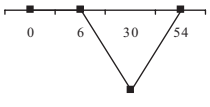
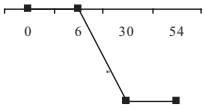


FIG. 2 Position of identified spots from 2D 12·5 % polyacrylamide gels. From left to right, pI 4–7 linear range. Arrowheads indicate the spots identified by mass spectrometry, together with the respective spot number presented in Table 2.

Proteins showing short-term upregulation (U6, U30 and U54)

Ketol-acid reductoisomerase, biotin carboxylase, α-tubulin, GTP-binding nuclear protein RAN, heat stress protein 90 (HSP90), chaperonin 10 (CPN10) and ATP sulfurylase (ATPS) were upregulated early upon heat stress in *P. euphratica*, returning later to control values (group U6, Table 3). The fact that α-tubulin was upregulated early in heat-stressed *P. euphratica* suggests that the short-term heat stress response leads to cytoskeleton remodelling. Since the first committed step

TABLE 3. *Classification of spots according to their IOD% changes of ±0.5 in comparison with control values*

Protein accumulation pattern	Identified proteins	Protein accumulation pattern	Identified proteins
 **U6 – 75 spots**	• Ketol-acid reductoisomerase • Biotin carboxylase • ATP sulfurylase • Tubulin alpha-chain • GTP-binding nuclear protein RAN • Putative HSP90 • Chloroplast protein Cpn10	 **D6_30 – 13 spots**	• Chloroplast transketolase
 **U6m – 72 spots**	• PSI-D • Thioredoxin h • Cpn60 β-subunit • Plastid ATP synthase CF1 alpha-chain	 **D54 – 131 spots**	• Adenosylhomocysteinase • O-acetylserine (thyol) lyase • Methionine synthase • NADP-dependent malic enzyme • Putative 2-3-biphosphoglycerate-independent phosphoglycerate mutase • Dihydrolipoamide dehydrogenase • Mitochondrial citrate (si)-synthase • Thiazole synthase • Chloroplast PSII stability/assembly factor
 **U6_30 – 47 spots**	• Mitochondrial F1 ATP synthase beta-subunit	 **UDR – 30 spots**	• Thiazole synthase
 **U30 – 75 spots**	• Enolase • Glyceraldehyde-3-phosphate dehydrogenase B • SP1 • Putative dihydrolipoamide dehydrogenase precursor	 **D6m – 19 spots**	• FtsH-like protein
 **D6 – 49 spots**	• Transketolase • 50S ribosomal protein L12-1 • ATP synthase ε-chain • Glycine-rich RNA-binding protein • Rubisco activase		
 **U30m – 23 spots**	 **U54 – 36 spots**	 **D30 – 21 spots**	 **D30m – 21 spots**

Graphical representation: the x-axis represents experimental time points in h and the y-axis represents changes in spot intensity incomparison with control, with no attributed scale. Thus, the control value is represented by zero and the arbitrary variations of +1 or −1 signify up- or downregulation, respectively. Group nomenclature: up/down only at 6 h (U6/D6); up/down only at 30 h (U30/D30); up/down only at 54 h (U54/D54); up/down at 6 h and maintained thereafter (U6m/D6m); up/down at 30 h and maintained thereafter (U30m/D30m); up/down only at 6 h and 30 h (U6_30/D6_30); up/down at 6 h with sucessive inversions in the following time points (UDR, where R stands for random).

of fatty acid synthesis is catalysed by biotin carboxylase, as part of the acetyl-CoA carboxylase multicomponent enzyme, the early upregulation of biotin carboxylase in *P. euphratica* suggests an increase in fatty acid synthesis in the first hours of heat stress exposure, most probably to face membrane instability. Membrane fluidity changes, which influence tensile forces, and cytoskeleton organization (Sangwan *et al*., 2002) have been reported previously

in response to environmental stimuli (Volkmann and Baluska, 1999). Cytosolic Ca^{2+} oscillations upon cold exposure lead to Ca^{2+} binding to tubulin molecules, which destabilize microtubules (MTs) (Mazars *et al*., 1997). MTs disassemble upon low temperatures and modulate the sensitivity of cold-sensitive calcium channels (Mazars *et al*., 1997), for which membrane fluidity changes have been reported to be essential (Sangwan *et al*., 2002). As could be predicted by electrolyte leakage analysis in *P. euphratica*, cell membrane stability would not be greatly affected by exposure to 42 °C, which is supported by the return of biotin carboxylase abundance levels to the control range during this experiment. GTP-binding nuclear protein RAN was upregulated after 6 h of heat stress, later returning to control values. This suggests strong transient nucleocytoplasmic interactions as GTPase RAN proteins are involved in protein import into the nucleus and RNA export from the nucleus, in chromatin condensation and in cell cycle control (Kahana and Cleveland, 1999; Stochaj and Rother, 1999; Yang, 2002; Yamazaki *et al*., 2004). ATPS was upregulated early in heat-stressed *P. euphratica* and is involved in sulfate pathway in plants. It catalyses the activation of sulfate through binding to AMP and forming 5′-adenylylsulfate (APS). In *P. euphratica* under high temperature, the upregulation of ATPS suggests an increase of APS synthesis and, indirectly, also of cysteine synthesis. As the final product of the sulfate reduction pathway, cysteine acts as a sulfur donor to methionine (Droux, 2004) and it is part of glutathione (GSH), which removes toxic metabolites from the cell while maintaining the reduced form of sulphydryl groups. Environmental conditions that induce GSH synthesis require higher sulfur assimilation into cysteine. In *Arabidopsis thaliana* under heavy metal stress, the high cysteine biosynthesis rate has been related to the synthesis of GSH and phytochelatins, as part of the plant detoxification mechanism (Dominguez-Solis *et al*., 2001). In the same way, ketol-acid reductoisomerase was upregulated after 6 h of heat stress, being involved in the biosynthesis pathway of the amino acids valine, leucine and isoleucine, which are hydrophobic amino acids. Ketol-acid reductoisomerase is under the control of the *rpoH* homologue of *Escherichia coli* and was found to be upregulated upon heat stress in *Agrobacterium tumefaciens* (Rosen *et al*., 2002). The *rpoH* gene codes for δ^{32}, one of the transcription factors controlling the heat shock regulon in *E. coli*, which is responsible for transcription of all major assimilation HSPs. In *Avicennia marina*, which is a mangrove with high salinity tolerance, the stress responsiveness of the ketol-acid reductoisomerase-encoding gene has been reported for the first time (Tanaka *et al*., 2002). Increased hydrophobic amino acid synthesis may be related to *de novo* protein synthesis in a more oxidative environment, which may favour isoforms with a higher percentage of these amino acids.

Enolase and glyceraldehyde-3-phosphate dehydrogenase (GDPDH), which are integral enzymes of the glycolytic pathway, stable protein 1 (SP1) and a putative dihydrolipoamide dehydrogenase (DLDH) precursor have shown short-term upregulation in *P. euphratica* (group U30, Table 2). Enolase transcripts were reported to be induced upon different abiotic stresses, such as water stress in *Lycopersicon esculentum* (Van der Straeten *et al*., 1991), and in *Zea mays* (Riccardi *et al*., 1998), in response to heat shock, salt stress and abscisic acid (ABA) treatment in ice plant (Forsthoefel *et al*., 1995) and during fruit ripening in tomato (Van der Straeten *et al*., 1991). Enolase and GDPDH upregulation suggest early acceleration of the glycolytic pathway upon exposure to high temperature, with a consequent increase in pyruvate production, followed by a decrease. This seems to be supported by the late downregulation of putative 2-3-bisphosphoglycerate-independent phosphoglycerate mutase, which is the enzyme responsible for the conversion of 2-phosphoglycerate into 3-phosphoglycerate in the glycolytic pathway, using its own phosphoryl group to carry out that conversion. Bridging the accumulation profile of enolase, GDPDH and 2-3-biphosphoglycerate-independent phosphoglycerate mutase is the accumulation profile of a putative DLDH precursor. Its accumulation levels increased in *P. euphratica* after 30 h of heat stress but then decreased after 54 h. As part of the pyruvate dehydrogenase (PDH) complex, as its E3 subunit, DLDH acts in the final step of pyruvate conversion into acetyl-CoA, where it re-oxidizes the dihydrolipoamide moiety using NAD^+. Its increase, followed by a decrease in abundance, suggests a transient increase in thiamine biosynthesis and a further reduction in its availability. This is also in agreement with the late downregulation profile determined for thiazole synthase, which is involved in thiamine biosynthesis in mitochondria and chloroplasts. Thiamine is a cofactor for two enzymatic complexes, PDH and α-ketoglutarate dehydrogenase in the tricarboxylic acid (TCA) cycle (Belanger *et al*., 1995), and PDH may have been upregulated early upon heat exposure, as described before.

SP1 is transiently upregulated in *P. euphratica* submitted to heat stress (group U30, Table 2). SP1 is a hydrophilic, homo-oligomeric protein composed of 12 subunits, each of 12·4 kDa, and is highly thermostable under extreme conditions (Wang *et al*., 2002). The SP1 protein characteristics of, simultaneously, an LEA protein and an sHSP, suggest that SP1 may be part of a new protein family due to its unique thermostability and distinct function in stress protection (Dgany *et al*., 2004). In fact, there is already a patent application for its use in industrial processes that require thermal stabilization of other proteins under extreme conditions (patent WO2004022697). SP1 is constitutively expressed in aspen plants and accumulates upon salt, cold, heat and desiccation stress, and during stress recovery (Wang *et al*., 2002; Renaut *et al*., 2004), and it acts as a chaperone to stabilize other proteins. Previous reports state that SP1 accumulation is particularly significant after heat stress is removed and when plants enter the recovery period (Wang *et al*., 2002). The common accumulation profile between SP1 and the glycolytic pathway enzymes found in this work suggests some sort of interaction with proteins related to carbon flow pathways or with other proteins that, indirectly, intervene in those pathways.

Proteins showing long-term upregulation (U6 m, U30 m and U6_30)

Photosystem I subunit D (PSI-D), thioredoxin *h*, chaperonin 60 β-subunit (CPN60-β) and ATP synthase CF1 α-chain were upregulated in the long term in heat-stressed *P. euphratica*.

CPN60-β was found to maintain high levels of accumulation, in contrast to HSP90 or CPN10 proteins. The major role of CPN60-β is in assisting in protein folding and preventing unfolding (Horwich *et al*., 2001), which is very similar to the function of other stress proteins, such as HSP90. CPN60 is composed of two subunits, α and β, it exists in chloroplasts and mitochondria, and it is involved in protein folding, together with CPN10, in an ATP-dependent manner. CPN60 seems to be an obligatory molecular chaperone in the folding/assembly pathway of Rubisco in higher plants. The folding reaction assisted by CPN60 develops through a binding–release cycle with CPN10, which alternates with a binding–release cycle with the unfolded protein substrate (Lund, 2005). Another chaperone molecule, HSP90, showed early upregulation, suggesting that, together with co-chaperones, HSP90 intervenes in the folding of newly synthesized proteins as well as stabilizing and refolding denatured proteins upon stress. The majority of HSP90 target proteins, among the several hundred already identified in eukaryotic cells (Sreedhar *et al*., 2004), are molecules involved in signal transduction.

Prolonged accumulation of thioredoxin *h* upon heat stress in *P. euphratica* may be related to its role in the oxidative stress response. Thioredoxin *h* is part of the thioredoxin antioxidant system in plants, acting after the oxidative stress generated in the electron transport chain through heat exposure, which induces redox changes in different compartments (Laloi *et al*., 2004). Thioredoxin *h* is able specifically to reduce small proteins containing intramolecular disulfide bonds (Besse and Buchanan, 1997). It has been shown to accumulate in salt-stressed *Hordeum vulgare* roots (Ueda *et al*., 2002) and, in *Populus tremula* × *P. tremuloides*, it is able to reduce peroxiredoxin Q, among other molecules which GSH is not able to reduce completely (Rouhier *et al*., 2004). Thioredoxin is able to regulate biosynthesis of the hydrophobic amino acids leucine, valine and isoleucine in *Chlamydomonas reinhardtii* (Lemaire *et al*., 2004). Valine and isoleucine share ketol-acid reductoisomerase in their biosynthesis, and this protein was, in fact, the single amino acid biosynthesis-related enzyme detected as upregulated in *P. euphratica* upon moderate heat exposure.

Long-term upregulation of the PSI-D subunit upon heat exposure of *P. euphratica* suggests the search for maintenance of structural stability of PSI. The PSI-D N-terminus allows stable binding of PSI subunit C and subunit E to the PSI complex, also contributing to ferredoxin docking (Andersen *et al*., 1992). Low amounts of PSI-D in *A. thaliana* were related to a decrease of all the other subunits of PSI, suggesting that the absence of PSI-D leads to incorrect PSI assembly and to its degradation (Haldrup *et al*., 2003). PSI-D accumulation, in the work here presented, suggests that PSI enhanced stability and minimized degradation. Similarly, chloroplastidial F_1 α- (CF_1-α) and mitochondrial F_1 β-subunits were continuously upregulated in *P. euphratica* upon 42 °C exposure, although the CF_1 α-subunit showed a higher extent of upregulation. It has been shown that it is the interaction between α, β and γ ATP synthase subunits that makes up the necessary force to stabilize CF_1 under thermal denaturation (Wang *et al*., 1993). Wang *et al*. (1993) propose that the CF_1 α-subunit has an organizing function in the assembly of the multisubunit enzyme ATP synthase upon thermal denaturation, as it contains non-catalytic sites where ATP can bind and stabilize ATP synthase CF_1. The CF_1 α–βcomplex is the ATP synthase minimum catalytic core (Avital *et al*., 1991), suggesting that upregulation of CF_1 α- and β- subunits in *P. euphratica* is related to their central role in the enzyme complex. It has been proposed previously that ATP synthase ε-subunit may have a minor influence on ATP synthase thermal stability, as suggested by some authors (Wang *et al*., 1993), in contrast to α- and β-subunits.

Proteins showing short-term downregulation (D6, D30 and D54)

Proteins which were downregulated early at 42 °C in *P. euphratica* included transketolase, 50S ribosomal L12-1 protein, ATP synthase ε-subunit, glycine-rich RNA-binding protein and Rubisco activase. Downregulation of the 50S ribosomal L12-1 protein suggests that, at moderately high temperature, protein synthesis might have been affected early on, although a later increase in levels suggests a recovery of protein synthesis. The 50S ribosomal L12 protein seems to be the binding site for several factors involved in protein synthesis, with an essential role in accurate translation (Munchbach *et al*., 1999). A similar regulatory mechanism has been reported in *E. coli*, where ribosomes might be sensors for the heat shock response (VanBogelen *et al*., 1990). Glycine-rich RNA-binding proteins, which bind to RNA, may help in protecting these molecules at non-optimal temperature. A recent study reports the temperature-regulated expression of two distinct genes that code for two glycine-rich RNA-binding proteins in *Dunaliella salina* (Zchut *et al*., 2003). Rubisco activase, which is highly sensitive to high temperature, was detected as being downregulated immediately after the beginning of heat exposure, later returning to control range values. As part of the AAA^+ family, its function comprises the regeneration of carbamylated active sites in Rubisco, maintaining Rubisco activity through ATP hydrolysis. The ability of Rubisco activase to promote activation, or to maintain the Rubisco active state in *in vitro* conditions, decreased above 30 °C in cotton and tobacco leaves (Crafts-Brandner and Salvucci, 2000). As the ATPase activity of Rubisco activase is not directly involved in activase association with the Rubisco enzyme, and as it increases with

temperature up to 42 °C, the ability of activase to promote Rubisco activation at moderately high temperature was reported as being related to instability of the quaternary structure of activase under those conditions. In the same way, transketolase was also detected as being downregulated after 6 h of heat exposure. A homologue of a chloroplastid transketolase isoform was also identified as being downregulated, but this returned to control range values after only 54 h of heat exposure. Transketolases are key enzymes in the reductive and oxidative pentose phosphate pathways, as they are responsible for the synthesis of sugar phosphate intermediates, and their downregulation in *P. euphratica* shown in this work indicates a transient reduction of carbon flux in those pathways.

Late downregulated proteins included proteins related to sulfate assimilation. Although early upregulation of ATPS suggests enhanced sulfate assimilation early upon high temperature exposure in *P. euphratica*, an inhibition may have occurred in later stages, as suggested by the OAS-TL and methionine synthase accumulation profiles. OAS-TL catalyses the last step of the sulfate reduction pathway, converting *O*-acetylserine into cysteine through incorporation of the sulfur atom. Methionine, which results from homocysteine conversion catalysed by methionine synthase, is the immediate precursor of *S*-adenosyl-methionine, which plays a crucial role in the biosynthesis of ethylene and polyamines, being these compounds involved in the plant abiotic stress response (Capell *et al*., 2004).

Proteins showing long-term downregulation (D6 m and D30 m)

In this category only FtsH-like protein was identified. FtsH proteins are chaperone metalloproteases of the AAA^+ family. Downregulation of *P. euphratica* FtsH during the whole heat experiment suggests a possible reduction in proteolysis of specific proteins that may become necessary during the heat exposure such as, for instance, homologues of *E. coli* σ factor, which control heat stress response in those bacteria. FtsH proteases of *E. coli* are partially responsible for the degradation of heat stress transcription factor δ^{32} (Shotland *et al*., 1997; Fischer *et al*., 2002), which accumulates upon FtsH depletion or upon exposure to high temperature. It has been suggested that *E. coli* FtsH may also have a non-proteolytic role since its absence causes abnormal orientation of some proteins of the plasma membrane (Langer, 2000). *In silico* analysis of the peptide sequences determined for *P. euphratica* using Clustal W (Thompson *et al*., 1994) allows confirmation that there is no signal peptide and, therefore, the FtsH-homologue protein found in *P. euphratica* is not a precursor but one of its isozymes. A recent report suggests that, unlike what was expected previously, the relative importance of different chloroplast FtsH isozymes is determined by their abundance, and not necessarily by different specific functions or specialized expression under certain conditions (Sinvany-Villalobo *et al*., 2004). This brings about the observation of a downregulation of the identified FtsH homologue in this work that, although not possible to locate in the chloroplast, may be related to heat stress response triggering.

DISCUSSION

In this work, we have analysed leaf proteomes of *P. euphratica* upon moderate heat stress. The main effects of the mimicked abiotic stress were detected as changes in the abundance of proteins involved in photosynthesis and carbon metabolism, suggesting a tight connection between those processes on reaching photostasis in *P. euphratica*. As enzymes related to lipid biogenesis, cytoskeleton structure, sulfate assimilation, thiamine and hydrophobic amino acid biosynthesis, and nuclear transport have been detected as being upregulated early in the short term in our experiment, signalling upon short-term moderate heat exposure in *P. euphratica* may involve structures and processes where these enzymes intervene. Simultaneously, there should have been an immediate inhibition of the communication between the pentose phosphate pathway and glycolysis, accomplished through transient downregulation of transketolase and Rubisco activase. A change in the cellular redox status was expected in *P. euphratica* leaves upon moderate heat, and redox homeostasis-related enzymes were expected to be upregulated. However, thioredoxin *h* was the only antioxidant enzyme found. Thioredoxins *h* comprise a large and diverse group of protein disulfide reductases, but the function of each thioredoxin *h* isoform is still unclear although the role of these enzymes in chloroplast redox homeostasis is well known (Foyer *et al*., 2005; Gelhaye *et al*., 2005). It is noteworthy that thioredoxin *h* has been detected as early long-term upregulated and that most proteins identified in this work have already been suggested as probable thioredoxin *h* chloroplast targets (Gelhaye *et al*., 2005). Recently, Lemaire *et al*. (2004) reported the identification of a thioredoxin-linked nuclear transport factor, RAN, in *C. reinhardtii*, the first ever found in a photosynthetic organism. Thioredoxin *h* may have a particular role in signalling related to photostasis in response to heat stress in *P. euphratica*, as in the redox regulation of the activity of several chloroplast enzymes.

Photostasis of P. euphratica

Changes in environmental temperature are primarily reflected in photosynthesis, which triggers a response aimed at reaching the best possible performance in the new situation. For this, a balance is sought between absorbed light energy, carbon assimilation and consumption in metabolic sinks. Overall plant growth is temperature dependent, while primary steps of photosynthesis are temperature independent. Photosynthetic non-biochemical processes are much quicker (10^{-15}–10^{-12} s) than biochemical reactions catalysed by plant enzymes (10^{-3}–10^{0} s) (Ensminger *et al*., 2006). Upon a thermal shift, photosynthetic biochemical processes, affected to a

much larger extent, induce a negative feedback control on non-biochemical photosynthetic processes (Fey *et al*., 2005), probably using singlet oxygen species generated on thylakoid membranes. This photostasis depends on an efficient sensory system to perceive cellular energetic status, for which the primary component is the plastoquinone (PQ) pool. Under moderate heat exposure, there is an increase of thylakoid proton conductance; linear electron flow is also affected and may be partially replaced by cyclic electron flow around PSI. According to Joliot *et al*. (2002), it is controlled by PSI assembly in supercomplexes, which depends on the ATP concentration. These authors suggest that an increase in ATP concentration, promoted by cyclic electron flow, would increase PSI availability to integrate linear electron flow and would decrease cyclic electron flow itself. The way in which the redox state of the PQ pool regulates plant acclimatory responses to light and temperature shifts is still not understood, although an organized signalling cascade has been suggested (Fey *et al*., 2005). PSII is highly heat susceptible, whereas PSI is relatively heat stable; thus, cyclic electron flow around PSI may help in reducing PSII activity and production of reactive oxygen species (ROS) under moderate heat stress. The late downregulation of PSII stability/assembly factor and the early long-term upregulation of PSI-D detected in *P. euphratica* suggest a strong involvement of PSI in thylakoid membrane electron flow. State transition is one of the strategies that plants use to cope with an excess of energy in photosystems. Photosynthetic linear electron flow is enhanced upon moderate heat exposure unless CO_2 availability suffers a reduction. This enhancement was not the case in the experiment reported here in *P. euphratica*, raising the possibility of the existence of a transient cyclic electron flow. A possible downregulation of linear electron flow could be related, not to PSII damage under moderate heat stress, but to photostasis balance through adjustment of carbon metabolism. Plant cells can maintain photostasis under cold exposure through several mechanisms, the increase of electron sink capacity being one of these mechanisms, through upregulation of CO_2 assimilation and carbon metabolism (Ensminger *et al*., 2006). The results reported here show that some carbon metabolism-related enzymes of *P. euphratica* are upregulated only after plants are maintained under moderate heat exposure (37 °C) during the night. Photostasis after 30 h of heat exposure may have induced emission of retrograde signals and changed expression of nuclear-encoded plastid proteins, to match the functional status of plastids under light conditions. In *P. euphratica*, the identified carbon metabolism-related enzymes have decreased in abundance after a second cycle of dark period exposure to moderate heat stress, returning to control range values; this fact probably represents shifts in photostasis. Equilibrium recovery between carbon sources and sinks may have been reached by *P. euphratica* plants at that time. NADP-dependent malic enzyme has been related to lower stomatal conductance when overexpressed in tobacco (Laporte *et al*., 2002). Its late downregulation in *P. euphratica* may be related to a decrease in carbon metabolism, with possible consequences for stomatal conductance. This late decrease in carbon metabolism is reflected in the late downregulated enzymes to an abundance level below that of the control, thus suggesting that carbon metabolism develops at a lower rate after 54 h of moderate heat exposure, in comparison with control conditions. Photostasis may have been reached through a reduction in steady-state molecular oxygen evolution, with a consequent decrease of CO_2 assimilation, possibly limited by the number of stable PSII complexes in thylakoid membranes. Reduction of Krebs cycle and glycolysis, thiamine synthesis, acetyl coenzyme-A and methionine synthesis, malate conversion into pyruvate and sulfur assimilation in *P. euphratica* plants after 54 h of exposure to moderate heat suggests a general reduction in overall metabolism which may represent an achievement of equilibrium and, thus, an adaptation to the imposed conditions. This slower growth rate or plant development arrest, even if transient, observed in the *P. euphratica* plants used in the experiment reported here, may reflect this general reduction on the overall plant metabolism.

Finally, it would be interesting to confirm if the photostasis maintenance strategy of *P. euphratica*, under prolonged exposure to moderate heat stress, includes a partial replacement of linear electron flow by cyclic electron flow, as reported for other plants under heat stress, drought stress or for cyanobacteria upon salt exposure (Hibino *et al*., 1996; Sudhir *et al*., 2005). Confirmation of this photosynthetic shift would enable a better understanding of chloroplast metabolic regulation, through energetic imbalance, with plant cell redox regulation of carbon metabolism under moderate high temperature.

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LITERATURE CITED

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2003. Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* **15**: 63–78.

Andersen B, Scheller HV, Moller BL. 1992. The PSI-E subunit of photosystem I binds ferredoxin:$NADP^+$ oxidoreductase. *FEBS Letters* **311**: 169–73.

Avital S, Gromet-Elhanan Z. 1991. Extraction and purification of the beta subunit and an active alpha beta-core complex from the spinach chloroplast CFoF1-ATP synthase. *Journal of Biological Chemistry* **266**: 7067–7072.

Belanger FC, Leustek T, Chu B, Kriz AL. 1995. Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. *Plant Molecular Biology* **29**: 809–21.

Besse I, Buchanan BB. 1997. Thioredoxin-linked plant and animal processes: the new generation. *Botanical Bulletin of Academia Sinica* **38**: 1–11.

Bharti K, von Koskull-Doring P, Bharti S, Kumar P, Tintschl-Korbitzer A, Treuter E, Nover L. 2004. Tomato heat stress transcription factor HsfB1 represents a novel type of general transcription coactivator with a histone-like motif interacting with the plant CREB binding protein ortholog HAC1. *Plant Cell* **16**: 1521–1535.

Bohnert HJ, Cushman JC. 2000. The ice plant cometh: lessons in abiotic stress tolerance. *Journal of Plant Growth Regulation* **19**: 334–346.

Bohnert HJ, Ayoubi P, Borchert C, Bressan RA, Burnap RL, Cushman JC, *et al.* 2001. A genomics approach towards salt stress tolerance. *Plant Physiology and Biochemistry* **39**: 295–311.

Brosche M, Vinocur B, Alatalo ER, Lamminmaki A, Teichmann T, Ottow EA, *et al.* 2005. Gene expression and metabolite profiling of *Populus euphratica* growing in the Negev desert. *Genome Biology* **6** R101.

Canovas FM, Dumas-Gaudot E, Recorbet G, Jorrin J, Mock HP, Rossignol, M. 2004. Plant proteome analysis. *Proteomics* **4**: 285–298.

Capell T, Bassie L, Christou, P. 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proceedings of the National Academy of Sciences of the USA* **101**: 9909–9914.

Crafts-Brandner SJ, Salvucci ME. 2000. Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. *Proceedings of the National Academy of Sciences of the USA* **97**: 13430–13435.

Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F. 2000. Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences* **57**: 779–795.

Dgany O, Gonzalez A, Sofer O, Wang W, Zolotnitsky G, Wolf A, *et al.* 2004. The structural basis of the thermostability of SP1, a novel plant (*Populus tremula*) boiling stable protein. *Journal of Biological Chemistry* **279**: 51516–51523.

Dominguez-Solis J, Gutierrez-Alcala G, Vega J, Romero L, Gotor C. 2001. The cytosolic o-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. *Journal of Biological Chemistry* **276**: 31474–31474.

Droux M. 2004. Sulfur assimilation and the role of sulfur in plant metabolism: a survey. *Photosynthesis Research* **79**: 331–348.

Ensminger I, Busch F, Huner N. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. *Physiologia Plantarum* **126**: 28–44.

Fey V, Wagner R, Brautigam K, Pfannschmidt T. 2005. Photosynthetic redox control of nuclear gene expression. *Journal of Experimental Botany* **56**: 1491–1498.

Fischer B, Rummel G, Aldridge P, Jenal U. 2002. The FtsH protease is involved in development, stress response and heat shock control in *Caulobacter crescentus*. *Molecular Microbiology* **44**: 461–478.

Forsthoefel NR, Cushman MA, Cushman JC. 1995. Posttranscriptional and posttranslational control of enolase expression in the facultative crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Plant Physiology* **108**: 1185–1195.

Foyer C, Noctor G. 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell and Environment* **28**: 1056–1071.

Gelhaye E, Rouhier N, Navrot N, Jacquot JP. 2005. The plant thioredoxin system. *Cell and Molecular Life Sciences* **62**: 24–35.

Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P. 1999. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *Journal of Mass Spectrometry* **34**: 105–116.

Grover A, Agarwal M, Katiyar-Agarwal S, Sahi C, Agarwal S. 2000. Production of high temperature tolerant transgenic plants through manipulation of membrane lipids. *Current Science* **79**: 557–559.

Gu RS, Jiang XN, Guo ZC. 1999. Structure characteristics associated with salt tolerance of *Populus euphratica*. *Acta Botanica Sinica* **41**: 576–579.

Gu, RS, Fonseca S, Puskas LG, Hackler L, Zvara A, Dudits D, Pais MS. 2004*a*. Transcript identification and profiling during salt stress and recovery of *Populus euphratica*. *Tree Physiology* **24**: 265–276.

Gu RS, Liu QL, Pei D, Jiang XN. 2004*b*. Understanding saline and osmotic tolerance of *Populus euphratica* suspended cells. *Plant Cell Tissue and Organ Culture* **78**: 261–265.

Habermann B, Oegema J, Sunyaev S, Shevchenko A. 2004. The power and the limitations of cross-species protein identification by mass spectrometry-driven sequence similarity searches. *Molecular and Cellular Proteomics* **3**: 238–249.

Haldrup A, Lunde C, Scheller HV. 2003. *Arabidopsis thaliana* plants lacking the PSI-D subunit of photosystem I suffer severe photoinhibition, have unstable photosystem I complexes, and altered redox homeostasis in the chloroplast stroma. *Journal of Biological Chemistry* **278**: 33276–33283.

Hanson TE, Tabita FR. 2003. Insights into the stress response and sulfur metabolism revealed by proteome analysis of a *Chlorobium tepidum* mutant lacking the rubisco-like protein. *Photosynthesis Research* **78**: 231–248.

Hibino T, Lee B, Rai A, Ishikawa H, Kojima H, Tawada M, *et al*. 1996. Salt enhances photosystem I content and cyclic electron flow via NAD(P)H dehydrogenase in the halotolerant cyanobacterium *Aphanothece halophytica*. *Australian Journal of Plant Physiology* **23**: 321–330.

Horwich AL, Fenton WA, Rapoport TA. 2001. Protein folding taking shape. Workshop on molecular chaperones. *EMBO Reports* **2**: 1068–1073.

Iba K. 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Annual Review of Plant Biology* **53**: 225–245.

Ingvardsen C, Veierskov B. 2001. Ubiquitin- and proteasome-dependent proteolysis in plants. *Physiologia Plantarum* **112**: 451–459.

Ismail A, Hall, A. 1999. Reproductive-stage heat tolerance, leaf membrane thermostability and plant morphology in cowpea. *Crop Science* **39**: 1762–1768.

Joliot P, Joliot A. 2002. Cyclic electron transfer in plant leaf. *Proceedings of National Academy of Sciences of the USA* **99**: 10209–10214.

Kahana JA, Cleveland DW. 1999. Beyond nuclear transport. RAN-GTP as a determinant of spindle assembly. *Journal of Cell Biology* **146**: 1205–1210.

Laloi C, Apel K, Danon A. 2004. Reactive oxygen signalling: the latest news. *Current Opinion in Plant Biology* **7**: 323–328.

Langer T. 2000. AAA proteases: cellular machines for degrading membrane proteins. *Trends in Biochemical Science* **25**: 247–251.

Laporte MM, Shen B, Tarczynski MC. 2002. Engineering for drought avoidance: expression of maize NADP-malic enzyme in tobacco results in altered stomatal function. *Journal of Experimental Botany* **53**: 699–705.

Law RD, Crafts-Brandner SJ. 1999. Inhibition and acclimation of photosynthesis to heat stress is closely correlated with activation of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Plant Physiology* **120**: 173–182.

Lemaire SD, Guillon B, Le Marechal P, Keryer E, Miginiac-Maslow M, Decottignies P. 2004. New thioredoxin targets in the unicellular photosynthetic eukaryote C*hlamydomonas reinhardtii*. *Proceedings of National Academy of Sciences of the USA* **101**: 7475–7480.

Liska AJ, Shevchenko A. 2003. Expanding the organismal scope of proteomics: cross-species protein identification by mass spectrometry and its implications. *Proteomics* **3**: 19–28.

Lund AA, Blum PH, Bhattramakki D, Elthon TE. 1998. Heat-stress response of maize mitochondria. *Plant Physiology* **116**: 1097–1110.

Lund P. 2005. The chaperonin cycle and protein folding. *BioEssays* **16**: 229–231.

Ma HC, Fung L, Wang SS, Altman A, Hutterman A. 1997. Photosynthetic response of *Populus euphratica* to salt stress. *Forest Ecology and Management* **93**: 55–61.

Maestri E, Klueva N, Perrotta C, Gulli M, Nguyen HT, Marmiroli N. 2002. Molecular genetics of heat tolerance and heat shock proteins in cereals. *Plant Molecular Biology* **48**: 667–681.

Mann M, Wilm M. 1994. Error tolerant identification of peptides in sequence databases by peptide sequence tags. *Analytical Chemistry* **66**: 4390–4399.

Mathew A, Morimoto RI. 1998. Role of the heat-shock response in the life and death of proteins. *Stress of Life* **851**: 99–111.

Mathew A, Mathur SK, Morimoto RI. 1998. Heat shock response and protein degradation: regulation of HSF2 by the ubiquitin–proteasome pathway. *Molecular and Cellular Biology* **18**: 5091–5098.

Mazars C, Thion L, Thuleau P, Graziana A, Knight MR, Moreau M, Ranjeva R. 1997. Organization of cytoskeleton controls the changes in cytosolic calcium of cold-shocked *Nicotiana plumbaginifolia* protoplasts. *Cell Calcium* **22**: 413–420.

Mishra SK, Tripp J, Winkelhaus S, Tschiersch B, Theres K, Nover L, Scharf KD. 2002. In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. *Genes and Development* **16**: 1555–1567.

Munchbach M, Dainese P, Staudenmann W, Narberhaus F, James P. 1999. Proteome analysis of heat shock protein expression in *Bradyrhizobium japonicum*. *European Journal of Biochemistry* **264**: 39–48.

Perkins DP, Creasy DM, Cottrell JS. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**: 3551–3567.

Port M, Tripp J, Zielinski D, Weber C, Heerklotz D, Winkelhaus S, *et al.* 2004. Role of Hsp17·4-cii as coregulator and cytoplasmic retention factor of tomato heat stress transcription factor HsfA2(1). *Plant Physiology* **135**: 1457–1470.

Renaut J, Lutts S, Hoffmann L, Hausman JF. 2004. Responses of poplar to chilling temperatures: proteomic and physiological aspects. *Plant Biology* **6**: 81–90.

Riccardi F, Gazeau P, de Vienne D, Zivy M. 1998. Protein changes in response to progressive water deficit in maize. Quantitative variation and polypeptide identification. *Plant Physiology* **117**: 1253–1263.

Robertson AJ, Ishikawa M, Gusta LV, Mackenzie SL. 1994. Abscisic acid-induced heat tolerance in bromus-inermis leyss cell-suspension cultures—heat-stable, abscisic acid-responsive polypeptides in combination with sucrose confer enhanced thermostability. *Plant Physiology* **105**: 181–190.

Rosen R, Buttner K, Becher D, Nakahigashi K, Yura T, Hecker M, Ron EZ. 2002. Heat shock proteome of *Agrobacterium tumefaciens*: evidence for new control systems. *Journal of Bacteriology* **184**: 1772–1778.

Rouhier N, Gelhaye E, Gualberto JM, Jordy MN, De Fay E, Hirasawa M, *et al.* 2004. Poplar peroxiredoxin Q. A thioredoxin-linked chloroplast antioxidant functional in pathogen defense. *Plant Physiology* **134**: 1027–1038.

Sangwan V, Orvar BL, Beyerly J, Hirt H, Dhindsa RS. 2002. Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *Plant Journal* **31**: 629–638.

Sharkey TD. 2000. Perspectives: plant biology. Some like it hot. *Science* **287**: 435, 437.

Shevchenko A, Wilm M, Mann M. 1997. Peptide sequencing by mass spectrometry for homology searches and cloning of genes. *Journal of Protein Chemistry* **16**: 481–490.

Shiji W, Bingao C, Huqun L. 1996. *Euphrates poplar forest*. Beijing: China Environmental Science Press.

Shotland Y, Koby S, Teff D, Mansur N, Oren DA, Tatematsu K, *et al.* 1997. Proteolysis of the phage lambda CII regulatory protein by FtsH (HflB) of *Escherichia coli*. *Molecular Microbiology* **24**: 1303–1310.

Sinvany-Villalobo G, Davydov O, Ben-Ari G, Zaltsman A, Raskind A, Adam Z. 2004. Expression in multigene families. Analysis of chloroplast and mitochondrial proteases. *Plant Physiology* **135**: 1336–1345.

Skylas DJ, Cordwell SJ, Hains PG, Larsen MR, Basseal DJ, Walsh BJ, *et al.* 2002. Heat shock of wheat during grain filling: proteins associated with heat-tolerance. *Journal of Cereal Science* **35**: 175–188.

Sreedhar AS, Kalmar E, Csermely P, Shen YF. 2004. Hsp90 isoforms: functions, expression and clinical importance. *FEBS Letters* **562**: 11–15.

Stochaj U, Rother K. 1999. Nucleocytoplasmic trafficking of proteins: with or without RAN? *BioEssays* **21**: 579–589.

Sudhir P, Pogoryelov D, Kovacs L, Garab G, Murthy S. 2005. The effects of salt stress on photosynthetic electron transport and thylakoid membrane proteins in the cyanobacterium *Spirulina platensis*. *Journal of Biochemistry and Molecular Biology* **38**: 481–485.

Tanaka S, Ikeda K, Ono M, Miyasaka H. 2002. Isolation of several anti-stress genes from a mangrove plant *Avicennia marina*. *World Journal of Microbiology & Biotechnology* **18**: 801–804.

Thiellement H, Zivy M, Plomion C. 2002. Combining proteomic and genetic studies in plants. *Journal of Chromatography B* **782**: 137–149.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Ueda A, Shi WM, Nakamura T, Takabe T. 2002. Analysis of salt-inducible genes in barley roots by differential display. *Journal of Plant Research* **115**: 119–130.

VanBogelen RA, Neidhardt FC. 1990. Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the USA* **87**: 5589–5593.

Van der Straeten D, Rodrigues-Pousada RA, Goodman HM, Van Montagu M. 1991. Plant enolase: gene structure, expression, and evolution. *Plant Cell* **3**: 719–735.

Volkmann D, Baluska F. 1999. Actin cytoskeleton in plants: from transport networks to signaling networks. *Microscopy Research and Technique* **47**: 135–154.

Wang WX, Pelah D, Alergand T, Shoseyov O, Altman A. 2002. Characterization of SP1, a stress-responsive, boiling-soluble, homo-oligomeric protein from aspen. *Plant Physiology* **130**: 865–875.

Wang WX, Vinocur B, Altman A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* **218**: 1–14.

Wang ZY, Freire E, McCarty RE. 1993. Influence of nucleotide binding site occupancy on the thermal stability of the F1 portion of the chloroplast ATP synthase. *Journal of Biological Chemistry* **268**: 20785–20790.

Wullschleger SD, Difazio SP. 2003. Emerging use of gene expression microarrays in plant physiology. *Comparative and Functional Genomics* **4**: 216–224.

Yamazaki D, Motohashi K, Kasama T, Hara Y, Hisabori T. 2004. Target proteins of the cytosolic thioredoxins in *Arabidopsis thaliana*. *Plant and Cell Physiology* **45**: 18–27.

Yang Z. 2002. Small GTPases: versatile signaling switches in plants. *Plant Cell* **14**: S375–S388.

Youlin Y, Squires V, Qi L. 2001. *Global alarm: dust and sandstorms from the world's drylands*. Bangkok: United Nations.

Zchut S, Weiss M, Pick U. 2003. Temperature-regulated expression of a glycine-rich RNA-binding protein in the halotolerant alga *Dunaliella salina*. *Journal of Plant Physiology* **160**: 1375–1384.

Zhang J, Madden TL. 1997. Powerblast: a new network blast application for interactive or automated sequence analysis and annotation. *Genome Research* **7**: 649–656.