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Heat, Salinity, and Acidity, Commonly Upregulate A1aB1b Proglycinin in Soybean Embryonic Axes

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1. Introduction

All organisms respond to elevated temperatures and to chemical and physiological stress by increasing the synthesis of heat shock proteins. By definition, heat shock proteins (HSPs) are a group of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures (De Maio, 1999). Brief exposure of cells to sub-lethal high temperature toughens the cells and provides protection from subsequent and even more severe temperature. In 1962, Ritossa reported that heat induced a characteristic pattern of puffing in the chromosomes of *Drosophila*. This discovery eventually led to the identification of the heat-shock proteins (HSPs) whose expression these puffs represented. Increased synthesis of specific proteins in *Drosophila* cells following heat shock was first reported in 1974 by Tissières *et al*. The regulation of heat shock gene expression in eukaryotes is largely mediated by the conserved heat shock transcription factors (HSFs). HSFs are present in a latent state under normal conditions. They are activated upon heat stress by induction of oligomerization and high-affinity binding to DNA and by exposure of domains for transcriptional activity (Wu, 1995). Experimental manipulation by molecular engineering of the heat response aims to select plant species resistant to hot weather, mainly in arid soils.

Stress factors other than heat also alter the cell physiology and induce stress responses comparable to that induced by thermal shock. Cellular energy depletion, extreme concentrations of ions, presence of uncommon osmolytes, rare gases, pollutants including many toxic substances, high or low temperatures, and drought, among others, all are stressing factors (Feder and Hofmann, 1999).

Beginning in the mid-1980's, researchers recognized that most stress-induced proteins, including HSPs, function as molecular chaperons. The chaperones are proteins that assist other proteins to fold, refold, travel to their place of residence (cytosol, organelle,

membrane, extracellular space), and translocate across membranes. Molecular chaperones participate in a variety of physiological processes and are widespread in organisms, tissues, and cells. Chaperone failure will have an impact on one or more cellular functions, which may lead to disease or even to death (Macario and Macario, 2007).

The principal heat-shock proteins with chaperone activity belong to five conserved families: HSP60, HSP70, HSP90, HSP100, and the small HSPs (HSP10, HSP20-30, and HSP40) (Schlesinger, 1990, 1994; Li and Srivastava, 2004). Heat-shock proteins are components of the stress response of eukaryotic and prokaryotic cells subjected to a variety of adverse conditions.

In plants, production of high levels of heat shock proteins can also be triggered by their exposure to different kinds of environmental stress conditions other than heat, including nitrogen deprivation, salinity in general and sodicity in particular, pollutants (heavy metals and pesticides), cold, and dryness, among others (Wang *et al*., 2003; Mahajan and Tuteja, 2005). Hence, HSPs are also known as stress proteins (Santoro, 2000). Hundreds of studies on plants of nutritious or economical importance subjected to a diversity of stress conditions, aimed to select resistant plant varieties, have been reported. *Glycine max* (LaFayette and Travis, 1990; Hsieh *et al*., 1992), *Arabidopsis thaliana* (Queitsch *et al*., 2000; Banti *et al*., 2008; Tonsor *et al*., 2008), *Oryza sativa* (Pareek, *et al*, 1995), *Nicotiana tabacum* (Usami *et al*., 1995), *Triticum aestivum* (Hendershot *et al*., 1992; Blumenthal, *et al*., 1998; Efeoglu and Terzioglu, 2007; Sancho *et al*., 2008), *Zea mays* (Cooper and Ho, 1983; Lund *et al*., 1998, Nieto-Sotelo *et al*., 2002), *Prosopis chilensis* (Ortíz *el al*., 1995; Ortíz and Cardemil. 2001), *Pisum sativum* (Lee *et al*., 1995), *Lycopersicon esculentum* (Polenta *et al*., 2007), *Sorghum bicolor* (Ougham and Stoddart 1986), *Cicer arietinum* (Bibi *et al*., 2009) and *Phaseolus vulgaris* (Nagesh-Babu and Devaraj, 2008) are within the many plants whose stress responses have been investigated.

Soybean subjected to thermal shock for brief periods respond by increasing the expression of certain proteins. The response is variable depending on several factors, mainly temperature, length of the shock, age of the crop at the time the thermal shock is applied, and source of the sample (embryonic axes, cotyledon, leaves, roots, etc.). Depending on these factors, several HSPs have been identified belonging to the five HSP families (Key *et al*., 1981; Mansfield and Key, 1987; Lin *et al*., 1984; Hsieh *et al*., 1992).

Stress response of soybean to high salinity has also been investigated; high salt concentrations have negative effects on growth, nodulation, seed quality and quantity, thus reducing the yield of soybean. To cope with salt stress, soybean has developed several resistance mechanisms, including: maintenance of ion homeostasis; adjustment in response to osmotic stress, restoration of osmotic balance, and other metabolic and structural adaptations (Phang *et al*., 2008).

As far as we know, the study of acidity and alkalinity as stressing factors on soybean has not been addressed. In this communication we report the experimentally-induced stress response of embryonic axes of soy to thermal shock (40ºC, 42ºC), salinity (200 mM NaCl), and acidity (pH 5.5) in a comparative manner. Hot soils and soils with high salt concentration, as well as acid- or alkaline- soils, certainly constitute inappropriate substrates for the satisfactory growth of soybean and other plant species.

2. Materials and methods

Chemicals

Except otherwise indicated, chemical reagents were from Sigma/Aldrich Chemical Co. (Branch in Mexico).

Soybean seeds

The variety Huasteca-100 of soybean (*Glycine max*) was used in this study; this variety was selected out of three other soybean varieties (Crystalline, UFV-1 and FT-0191) because of its good quality (Arce-Paredes *et al*, 2009) and because it is a prospect for high cultivation in certain regions in México (it is expected to cultivate up to 80 million square meters of land with soy Huasteca-100 by the year 2011). Soybean Huasteca-100 was contributed by Ing. Rafael Reza Alaman from Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), campo experimental at Iguala, Guerrero, México, and it was a recent crop.

Soybean minicrops

Healthy soybean seeds were selected, decontaminated for 10 min in 1% hypochlorite in water, rinsed once with distilled water (DW), let to hydrate for 4 h with DW, and planted on moisten cotton beds at 28ºC. Moistening solutions depended on the experiment and were: 10 mM phosphate buffer, pH 7.0; 200 mM NaCl in 100 mM phosphate buffer, or 10 mM acetate buffer, pH 5.5.

Heat shock

Soybean seeds were grown on a cotton bed moistened with 10 mM phosphate buffer, pH 7.0, at 28ºC for 48 h in the dark before they were subjected to thermal shock for 2 h. At this time, germinated embryonic axes measured about 1.0 cm in length. Three lots were prepared and subjected to no heat-shock (lot 1, control), 40ºC (lot 2), or 42ºC (lot 3). Lots were then reincubated at 28ºC for 4 h. At this time, cultures were harvested and examined for changes in length, mass, and protein content profiles of embryo axes.

Saline shock

Lots of 50 soybean seeds were grown at 28ºC on cotton beds moistened with 200 mM NaCl in 10 mM phosphate buffer, pH 7.0. From previous results from heat-shocked soybean seeds, crops were harvested at 54 h of cultivation, time at which embryonic axes were excised, measured, weighted, and subjected to protein extraction.

pH shock

Lots of soybean seeds were grown at 28ºC on cotton beds moistened with 10 mM acetate buffer, pH. 5.5. Based on previous results, crops were harvested at 54 h of cultivation and embryonic axes were collected, measured, weighted and subjected to protein extraction.

Extract preparation

At each harvesting time, embryonic axes were excised from germinated soybean seeds, measured, and weighted. Extracts were prepared from equal amounts (1.0 gram) of embryonic axes collected from each stress-treated soybean lot. Embryonic axes were cut into small (2-3 mm) pieces and then they were ground in a mortar with 5.0 ml of 25 mM Tris, 192 mM Glycine, pH 8.3 solution in the presence of 1 mM ethyl maleimide and 1 mM phenylmethylsulfonyl fluoride (PMSF). After filtering trough Whatman No. 2 filter paper, extracts were centrifuged at 10,000 x g (Sorvall RC-5B, DuPont Inst., USA) for 15 min at 4°C and soluble extracts were sterilized through 0.2 µm filters (Millipore), separated into 0.5 ml aliquots, and stored frozen at -20°C until used.

Protein contents

The amount of protein in each extract was determined by a micro-adaptation to the Lowry's method (Lowry *et al*., 1951). Assay was carried out in ELISA plates containing 200 µl of

alkaline reagent per well. The alkaline reagent consisted of 0.2% sodium-potassium tartrate, 0.1% cupric sulfate and 2% sodium carbonate in 0.1N sodium hydroxide. Ten microliters of serially diluted samples or protein standard (BSA, 1 mg per ml) were added per well. The plates were thoroughly shaken and then left undisturbed for 15 min. Then, 10 µl of Folin-Ciocalteau's reagent were added per well. Well-contents were individually homogenized by pipetting and plates were left undisturbed for 30 min at room temperature. Finally, absorbance at 600 nm was measured in an ELISA-reader (Labsystems multiscan Plus, Finland).

Protein precipitation

One ml of soluble extracted protein was mixed with 8 ml of 100% ice-cold acetone and 1 ml of 100% TCA. The mixture was kept at -20ºC for 1 h and then centrifuged at 18,000 x g (Sorvall, RC-5B, DuPont Instr., USA) for 15 min at 4ºC. Supernatant was discarded and the precipitate was washed with 1 ml of ice-cold acetone and recovered by centrifugation as above. Finally, the precipitate was dissolved in lysis buffer as described below.

Protein solubilisation

Proteins were dissolved in 2-D lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) and incubated at room temperature for 30 min on a shaker. Protein solutions were centrifuged for 30 min at 16,000 x g (Sorvall, RC-5B, DuPont Instr., USA) at room temperature, supernatants were collected and their protein content were measured using the micro-Lowry method already described.

CyDye labeling and Two dimensional differential in gel electrophoresis (2D-DIGE)

To label proteins, 30 µg of each protein samples were incubated with 0.7 µl of CyDye solutions (Cy2, Cy3 or Cy5) diluted 1:5 in dimethyl formamide (DMF) from 1 nmol/µl stock, (GE Healthcare, Piscataway, NJ) at 4°C for 30 min. Labeling was stopped by adding 0.7 µl of 10 mM L-Lysine and incubating at 4°C for 15 min. Then, labeled samples were mixed together, and equal volume of 2X 2-D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiotreitol (DTT), 2% pharmalytes and a trace amount of bromophenol blue) and 100 µl of destreak solution (GE Healthcare) were added. Total sample volumes were adjusted to 260 µl by adding Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and a trace amount of bromophenol blue). Samples were incubated at room temperature for 10 min on a shaker and centrifuged for 10 min at 16,000 x g (Sorvall, RC-5B, DuPont Instr., USA). Finally, supernatants were loaded onto 13 cm IPG strip holder (GE Healthcare).

Isoelectrofocusing (IEF) and SDS-PAGE

Thirteen cm IPG strips (pH 3-10) were put on the loaded samples and 1 ml of mineral oil was added on top of the strip. Isoelectrofocusing experiments (IEF) were run following the protocol provided by the manufacturer (GE Healthcare). Upon completion of IEF, strips were equilibrated in buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), a trace amount of bromophenol blue and 10 mg/ml DTT) for 15 minutes and then in buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml DTT) for 10 min with gentle agitation. IPG strips were then rinsed once in the SDS-gel running buffer, transferred to a 10.5% SDS-gel prepared using low fluorescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). Electrophoresis was performed at room temperature until dye fronts run out of the gels.

Image scan and data analysis

Upon completion of electrophoresis, gels were scanned using Typhoon TRIO (Amersham BioSciences) following the manufacturer's protocol. The scanned images were then processed by Image Quant software (version 5.0, Amersham BioScience). The quantitative analysis of protein spots was performed using DeCyder software (version 6.5).

Preparative gel

Unlabeled proteins (600-700 μg) were run in analytical gels and stained with Deep Purple total protein stain (GE Health care). Then, gels were scanned and images were processed by DeCyder software to generate a pick list.

Protein identification by Mass Spectrometry

Spot picking and Trypsin digestion

Spots of interest were picked up by Ettan Spot Picker (Amersham BioSciences) based on the in-gel analysis and spot picking design by DeCyder software. The gel spots were washed a few times then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). Digested tryptic peptides were desalted by Zip-tip C18 spin columns (Millipore) and peptides were eluted from the Zip-tip columns with 0.5 μl of matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate), and spotted on a MALDI plate (model ABI 01-192-6-AB).

Mass Spectrometry Analysis

MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Database search

Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the database of National Center for Biotechnology Information non-redundant (NCBInr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamido methylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant.

3. Results

Protein profiles of soybean embryonic axes grown in different stress conditions obtained by two dimensional differential in gel electrophoresis (2D-DIGE)

Proteins extracted from the embryonic axes of soybean subjected to nil (control lot 1), HS40°C (lot 2), HS42°C (lot 3), acid shock (AS) at pH 5.5 (lot 4), or saline shock (SS) at 200 mM NaCl (lot 5) were fractionated by two-dimensional differential gel electrophoresis (2D-DIGE) as mentioned in Materials and Methods. In each case, matching of protein spots across the gels allowed us to calculate the relative volume of each spot (RV) as the ratio:

volume of a particular spot/ volume of all spots detected in the gel. Those protein spots that showed an increase or a decrease in their RV of at least 1.5 times in relation to control sample (lot 1) were qualified as stress proteins (SPs) or HSPs. Under this criterion, we detected 35 HSPs in lot 2 (HS40°C), 15 HSPs in lot 3 (HS42°C), 31 ASPs in lot 4 (pH 5.5), and 29 SSPs in lot 5 (NaCl 200 mM). In addition, 11 protein spots showed a diminution in lot HS42°C. In general, most stressing conditions induced changes in the same proteins. In other words, same proteins were affected by the several stressing factors, with some exceptions.

2D-DIGE maps

Figure 1 shows the 2D-DIGE map of proteins extracted for embryonic axis of soybean grown under normal conditions (Control lot 1, in green), and the protein map for embryonic axes of soybean subjected to HS of 40°C for 2 h (lot 2, in red). Over 2000 protein spots were detected in all lots. Thermal shock (HS 40°C/2 h) induced the over-expression of 35 proteins, the largest number of up-regulated proteins in the experiment. Those proteins over expressed more than 1.5 fold in relation to the control lot appear encircled and orderly numbered from top to bottom.

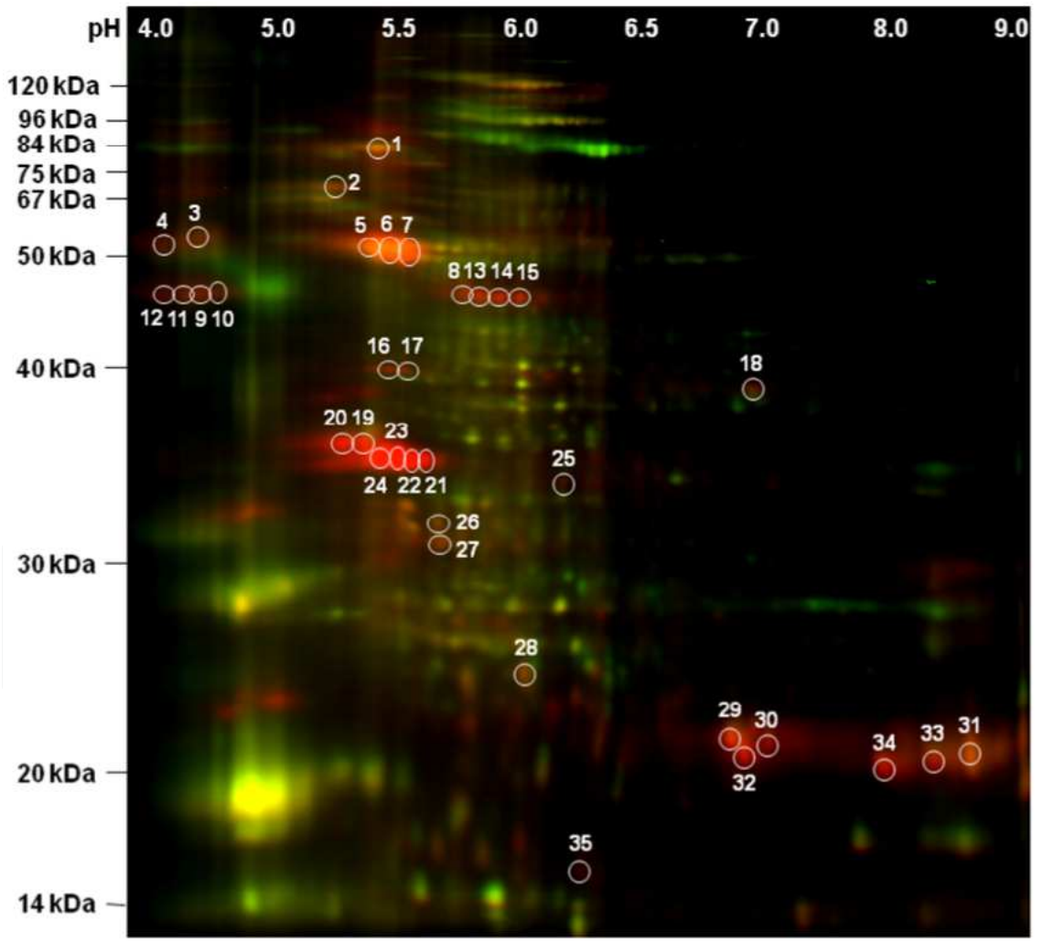


Fig. 1. 2D-DIGE expression map of the proteins extracted from embryonic axes of intact soybean (green) and soybean subjected to thermal shock at 40°C for 2 h (red). Thirty-five proteins (encircled and numbered) were over- expressed in relation to the control lot sample.

Figure 2 shows the 2D-DIGE map of proteins extracted from the embryonic axes of soybean subjected to thermal shock at 42 °C for 2 h (lot 3). In the image, proteins that were over-expressed (15) are encircled and numbered, proteins that were under expressed (11) are only encircled but not numbered, and proteins whose expression was not modified are not pointed at. All protein spots retained their number given in relation to spot proteins in lot 2.

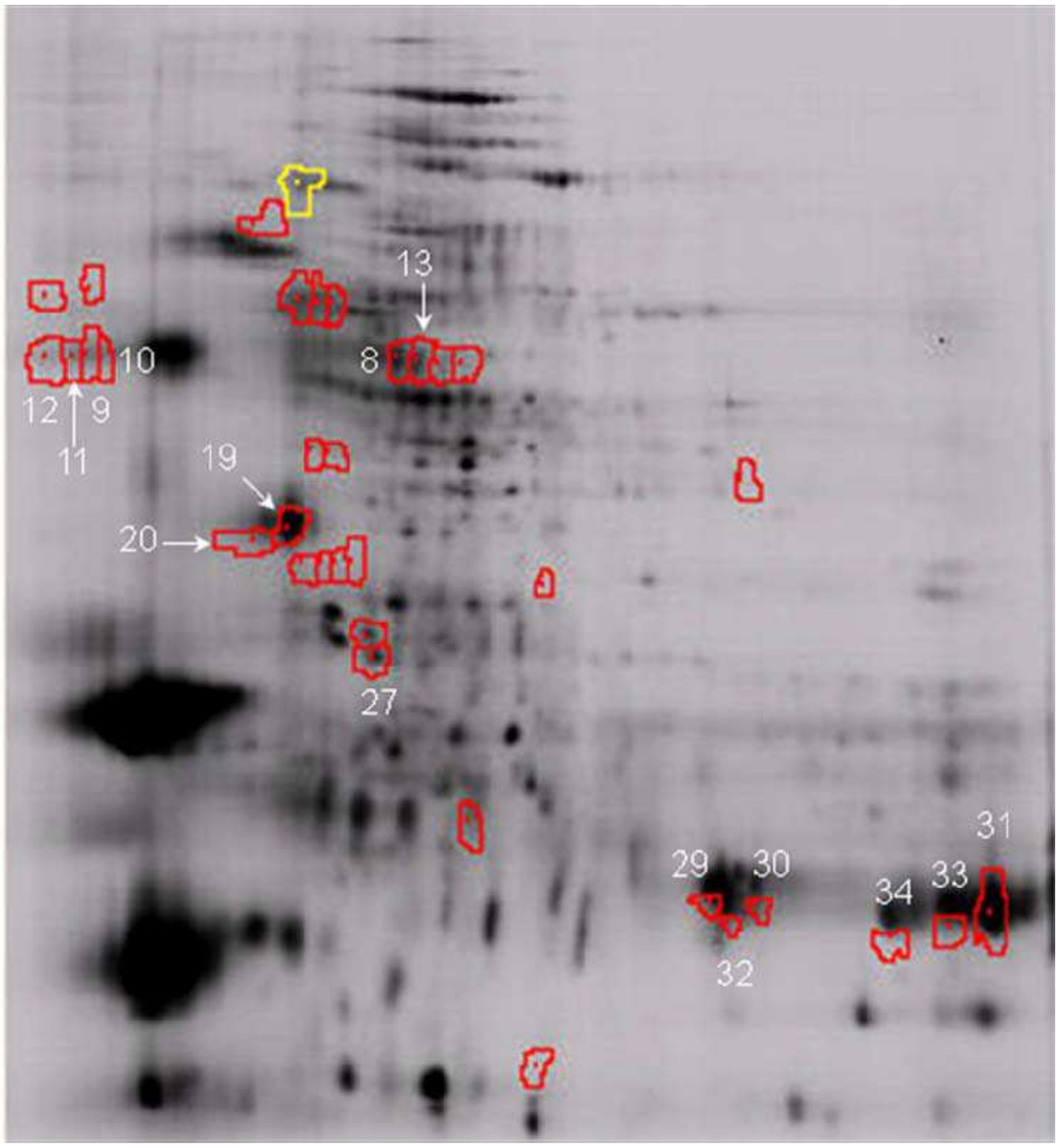


Fig. 2. 2D-DIGE map of the proteins extracted from the embryonic axes of soybean subjected at 42°C for 2 h. Fifteen proteins were over-expressed (encircled and numbered), 11 proteins were under-expressed (encircled but not numbered) and 9 proteins were not modified (not pointed at). Numbers given in reference to the protein map of soybean subjected to 40°C/2 h (lot 2). Arrows identify single spots within a tight group of spots. Yellow circle marks a specific spot not particularly relevant for this study.

2D-DIGE map of the proteins extracted from embryonic axes of soybean subjected to pH 5.5 is shown in Figure 3. Compared with the map for soybean subjected to thermal shock at 40°C (lot 2), 31 proteins were over-expressed (encircled and numbered spots). Protein spots are numbered in relation to spot proteins in lot 2.

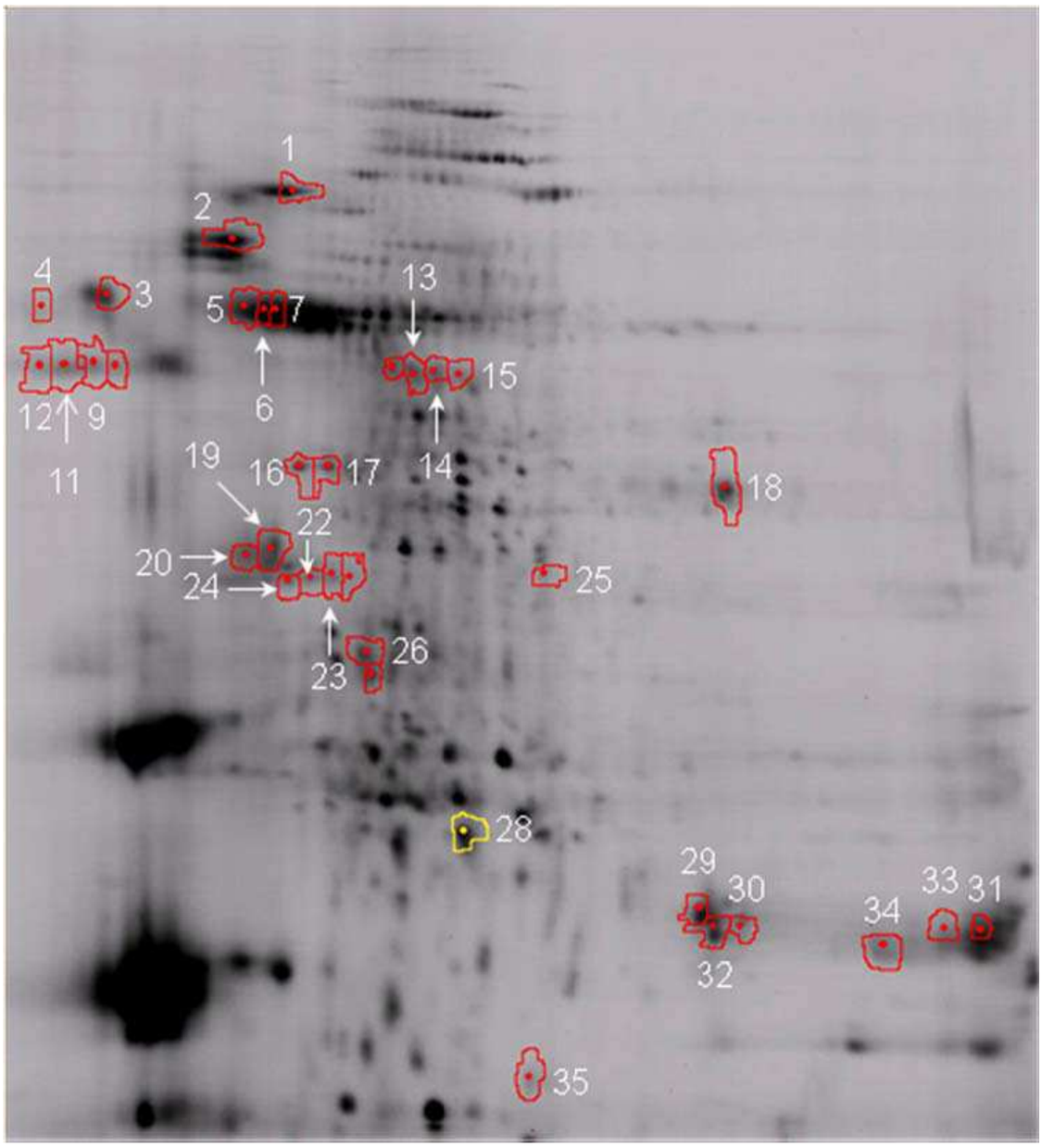


Fig. 3. 2D-DIGE image of proteins extracted from the embryonic axes of soybean grown at pH 5.5. Thirty-one proteins were over-expressed (encircled and numbered). Arrows identify single spots within a tight group of spots. Yellow circle marks a specific spot not particularly relevant for this study.

2D-DIGE spot map of proteins extracted from embryonic axes of soybean grown in the presence of 200 mM NaCl is shown in Figure 4. Twenty-nine proteins behaved as HSPs by increasing their expression beyond 1.5 fold (spots encircled and numbered) in relation to control sample.

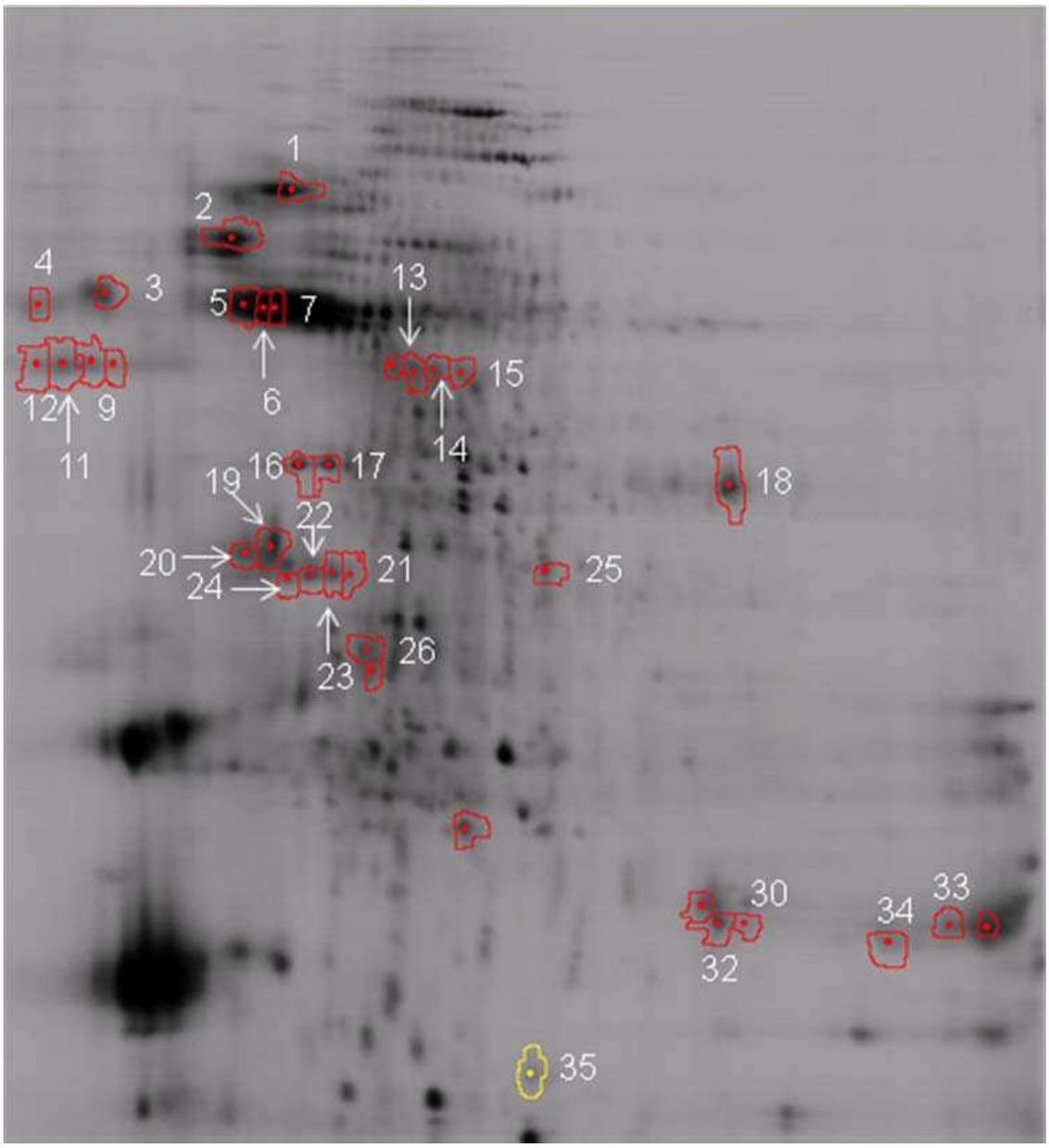


Fig. 4. 2D-DIGE spot map of proteins of embryonic axes of soybean cultured in the presence of 200 mM NaCl. Twenty-nine proteins were over expressed (encircled and numbered). Arrows identify single spots within a tight group of spots. Yellow circle marks a specific spot not particularly relevant for this study.

The relative volume ratio (stressing factor/control) of the proteins whose expression was modified in response to the several stressing factors tested is shown in Table 1. The strongest stressing, non-lethal factor was thermal shock of 40°C for 2 h. This stressing factor

Protein ID	HS40 °C/Control Volume Ratio	HS42 °C/Control Volume Ratio	pH5.5/Control Volume Ratio	200mM/Control Volume Ratio
1	2,79	-1,63	1,63	2,36
2	2,65	-1,99	2,58	3,00
3	2,98	-5,84	2,33	2,33
4	2,17	-1,84	1,90	3,53
5	5,21	-2,03	2,54	5,63
6	5,99	-1,97	2,51	4,76
7	8,07	-2,20	2,73	5,06
8	6,99	3,59	1,04	1,22
9	4,68	1,68	2,18	2,55
10	4,28	1,80	1,39	1,34
11	4,75	1,82	3,77	4,63
12	4,00	1,73	3,40	3,71
13	5,34	2,89	2,10	1,79
14	4,95	1,08	2,18	2,17
15	5,50	-1,35	1,63	1,85
16	7,24	1,31	3,48	6,00
17	3,41	-1,07	1,66	2,61
18	1,50	-4,68	2,31	2,89
19	5,56	3,18	1,67	2,14
20	16,11	2,18	2,05	3,25
21	29,55	-1,59	1,34	2,10
22	**87,07**	1,01	**15,48**	**24,32**
23	50.00	-1,26	2,51	4,48
24	**78,67**	-1,06	**10,07**	**12,63**
25	1,90	-2,47	2,18	4,05
26	2,07	1,44	1,51	1,64
27	2,73	1,94	1,48	1,21
28	1,97	-1,50	1,87	1,05
29	7,55	3,30	1,93	1,23
30	10,40	4,52	3,32	2,32
31	3,70	4,33	1,88	1,46
32	15,86	4,43	3,57	2,10
33	6,78	3,17	1,72	1,56
34	13,02	2,83	2,83	1,98
35	3,42	1,01	3,01	2,94
Up-regulated	35	15	31	29
Down-regulated	0	11	0	0
Non affected	0	9	4	6

Table 1. Relative volume ratios (stress factor/ control) of 2D-DIGE spot proteins of soybean subjected to diverse stressing conditions.

induced the over-expression of 35 proteins that in the 2D-DIGE map are identified from top to bottom with a progressive identification (ID) number (spots ID-1 to ID-35). A similar effect was observed when pH 5.5 and 200 mM NaCl were the stressing factors: 31 proteins were over-expressed at pH 5.5 and 29 proteins were over-expressed at 200 mM NaCl. The most deleterious effect on the germination of soybean was observed when the stressing factor was thermal shock of 42°C for 2 h. Under this condition, of the 35 proteins identified, 11 were down-regulated, 15 proteins were over-expressed and 9 proteins were unaffected.
The most interesting observation that comes out from the results shown in Table 1 is that 2 protein spots, ID-22 and ID-24, maximally increased their expression under all of the stressing situations tested, excepting the heat shock at 42°C for 2 h, which was the most lethal stressing factor in this study.

Volume graphs

The volume graphs for spot proteins ID-22 and ID-24 appear in Figures 5 and 6, respectively. The images, obtained with the DeCyder Differential Analysis Software version 6.5, show the spot number and position in the gel, the calculated relative volume, the peak height, and the area of each spot. Protein ID-22, increased 87.07 times at 40°C for 2 h in relation to the same protein in the control lot (lot-1) (Figure 5), while protein ID-24 increased 78.67 times at 40°C for 2 h over the same spot in the control lot (Figure 6).

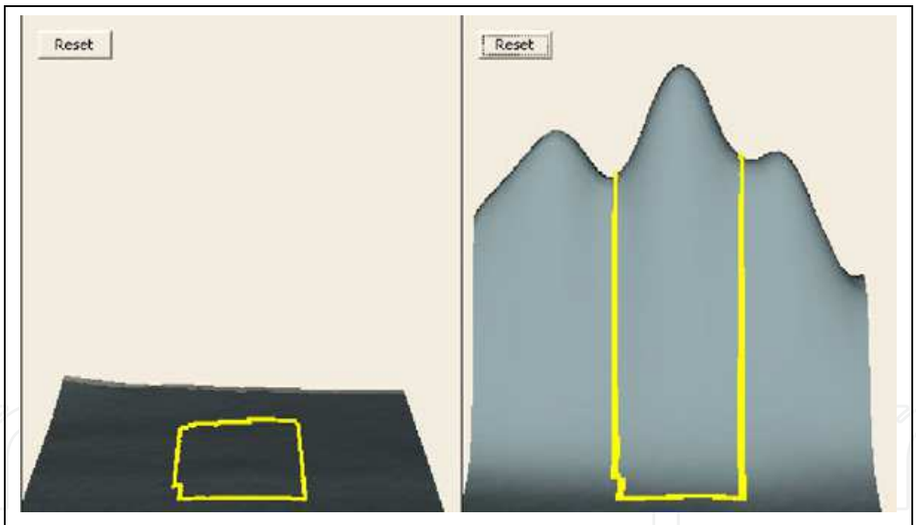
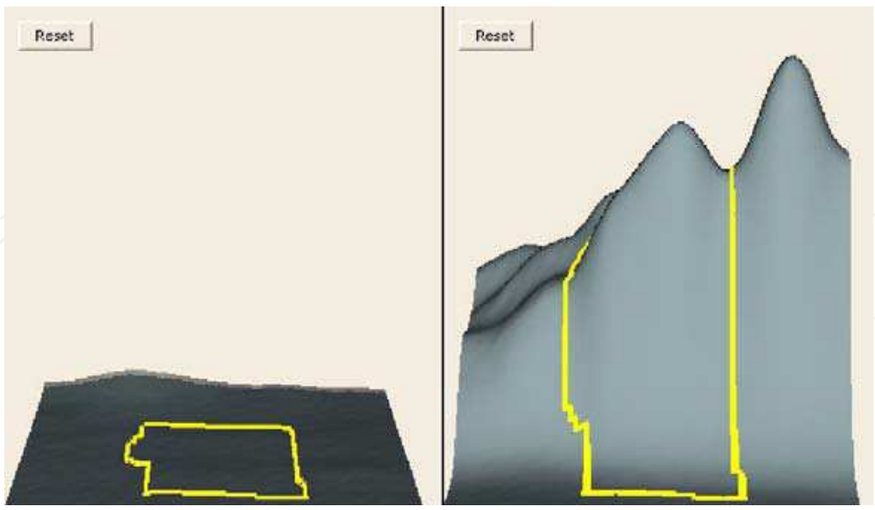


Fig. 5. Relative volume graph of 2D-DIGE spot ID-22 from soybean subjected to thermal shock of 40°C for 2 h (right panel) compared to the relative volume of the same spot in the control, intact lot (left panel).



Spot No. 1371	Spot No. 1371
Position 344,647	Position 344,647
Volume: 6.076e+004	Volume: 4.774e+006
Peak height: 84	Peak height: 11542
Area: 901	Area 901

Fig. 6. Relative volume graph of 2D-DIGE spot ID-24 from soybean subjected to thermal shock of 40°C for 2 h (right panel) compared to the relative volume of the same spot in the control, intact lot (left panel)

Protein identification by peptide mass fingerprint from two-dimensional gels

To identify the ID-22 and ID-24 spot proteins, we proceeded to analyze them by MALDI-TOF MS and TOF/TOF tandem MS/MS as described in Material and Methods.

The mass spectrum (MS) obtained for tryptic peptides eluted from 2-D gel spot ID 22 is shown in Figure 7. After baseline correction, background subtraction and peak deisotoping, 10 high-scored ions were submitted to Mascot. Four of the submitted ions matched to theoretical tryptic peptides from chain C, crystal structure of soybean proglycinin A1aB1b homotrimer (Accession No. gi|15988119). The sequences of these peptides are given together with the mass of monoisotopic, single charged ions. The peptide with the highest ion score (IS 42, CI 96.051 %) appears within a rectangle. The mass spectrum MS/MS for this ion is shown in Figure 8.

Figure 9 shows the mass spectrum obtained for tryptic peptides eluted from 2-D gel spot ID-24. The 10 high-scored ions were submitted to Mascot server. Four of the submitted ions matched to theoretical tryptic peptides from chain C, crystal structure of Soybean proglycinin A1aB1b homotrimer (gi|15988119). The sequences of these peptides are given together with the mass of monoisotopic, single charged ions. The peptide with the highest ion score (42, CI 96.051 %) appears within a rectangle. The mass spectrum MS/MS for this ion is shown in Figure 10.

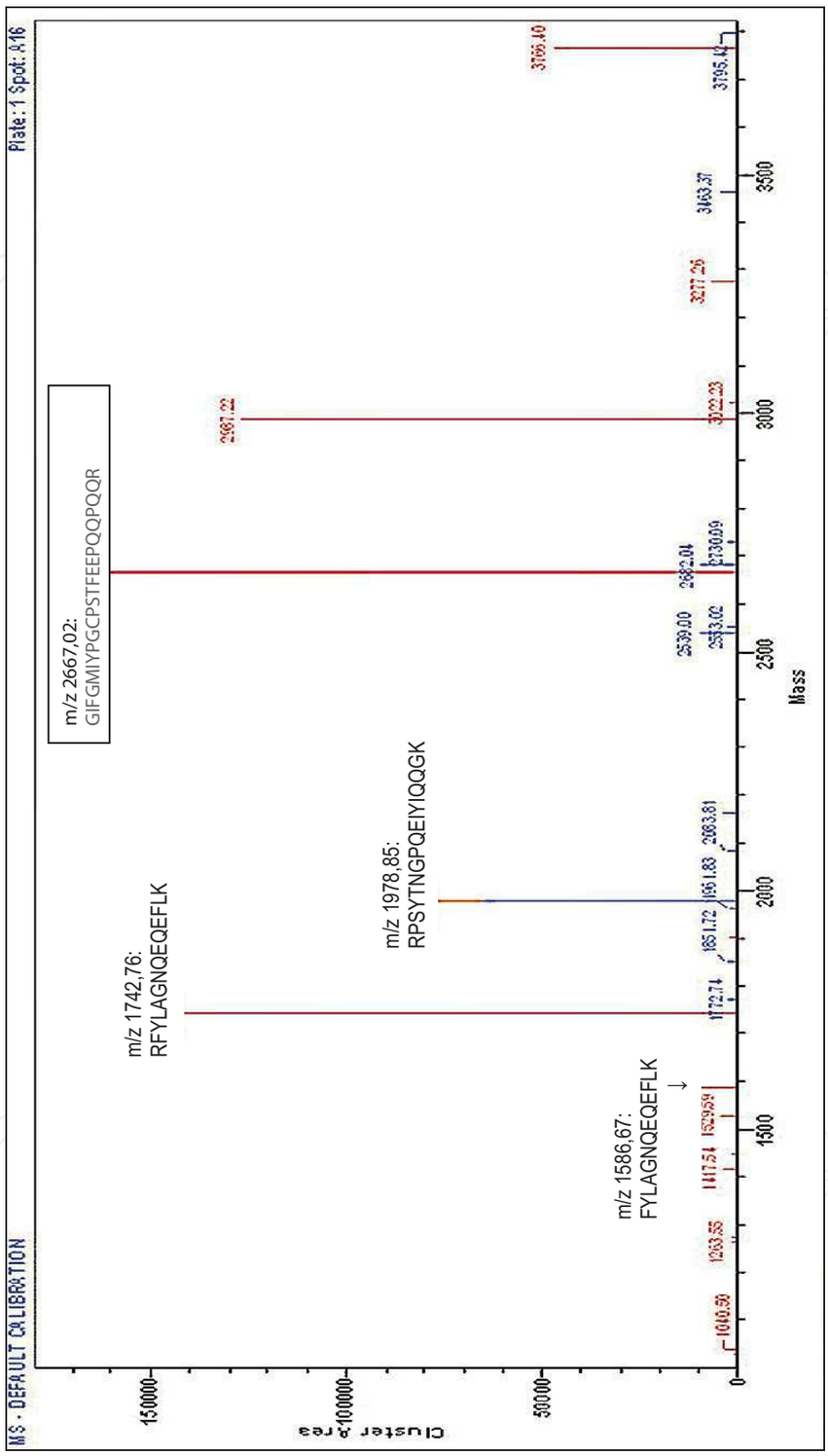


Fig. 7. Mass spectrum (MS) of tryptic peptides eluted from 2D-DIGE spot 22. The m/z value and sequence for each peptide is shown in the graph. The peptide with the highest ion score (42, CI 96.051 %) appears within a rectangle.

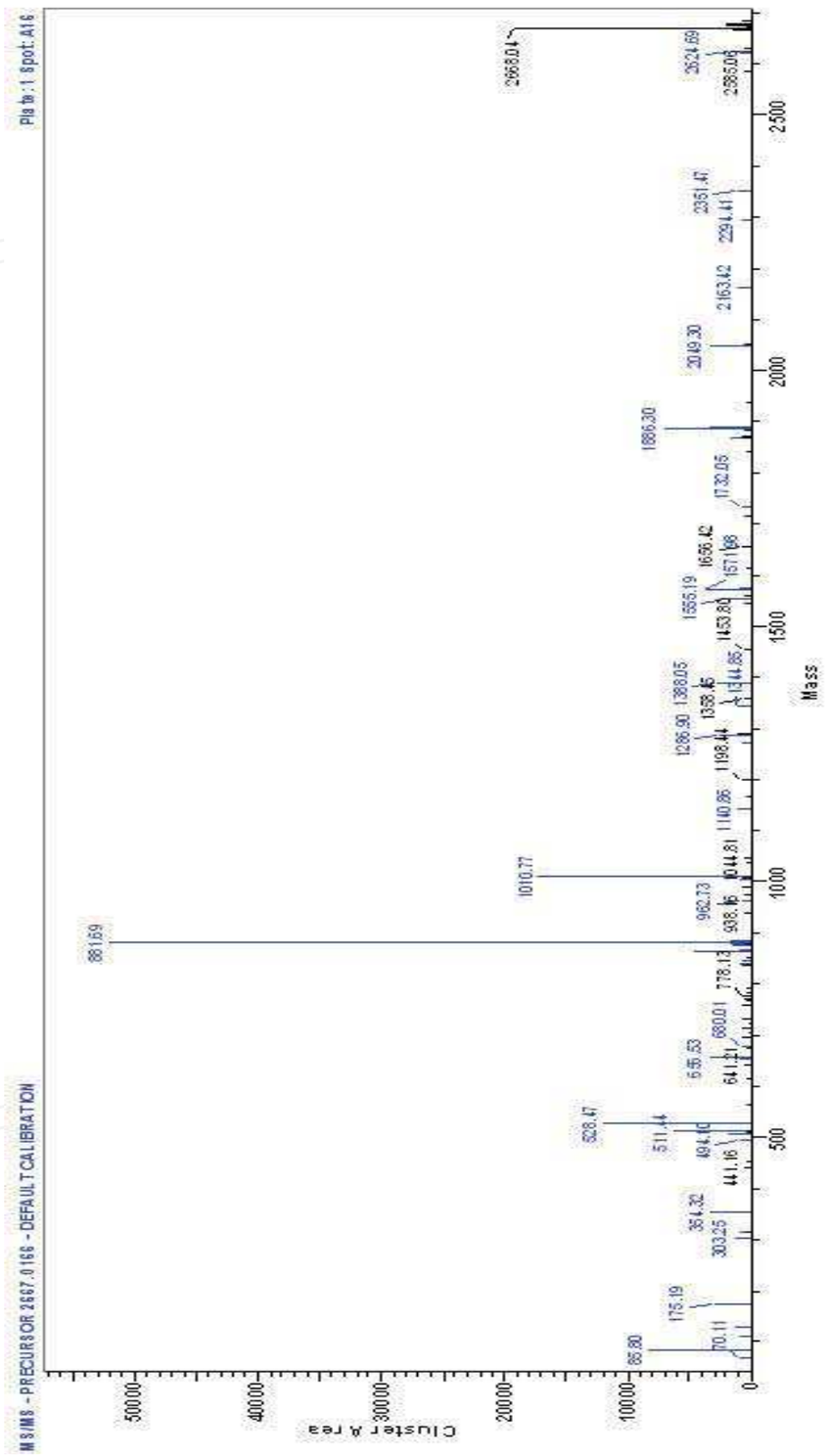


Fig. 8. MS/MS spectrum of peptide m/z 2667.02 (GIFGMIYPGCPSTFEEPQQPQQR) from protein spot ID 22. Sequences of these peptide ions fit within the sequence of peptide m/z 2667.02

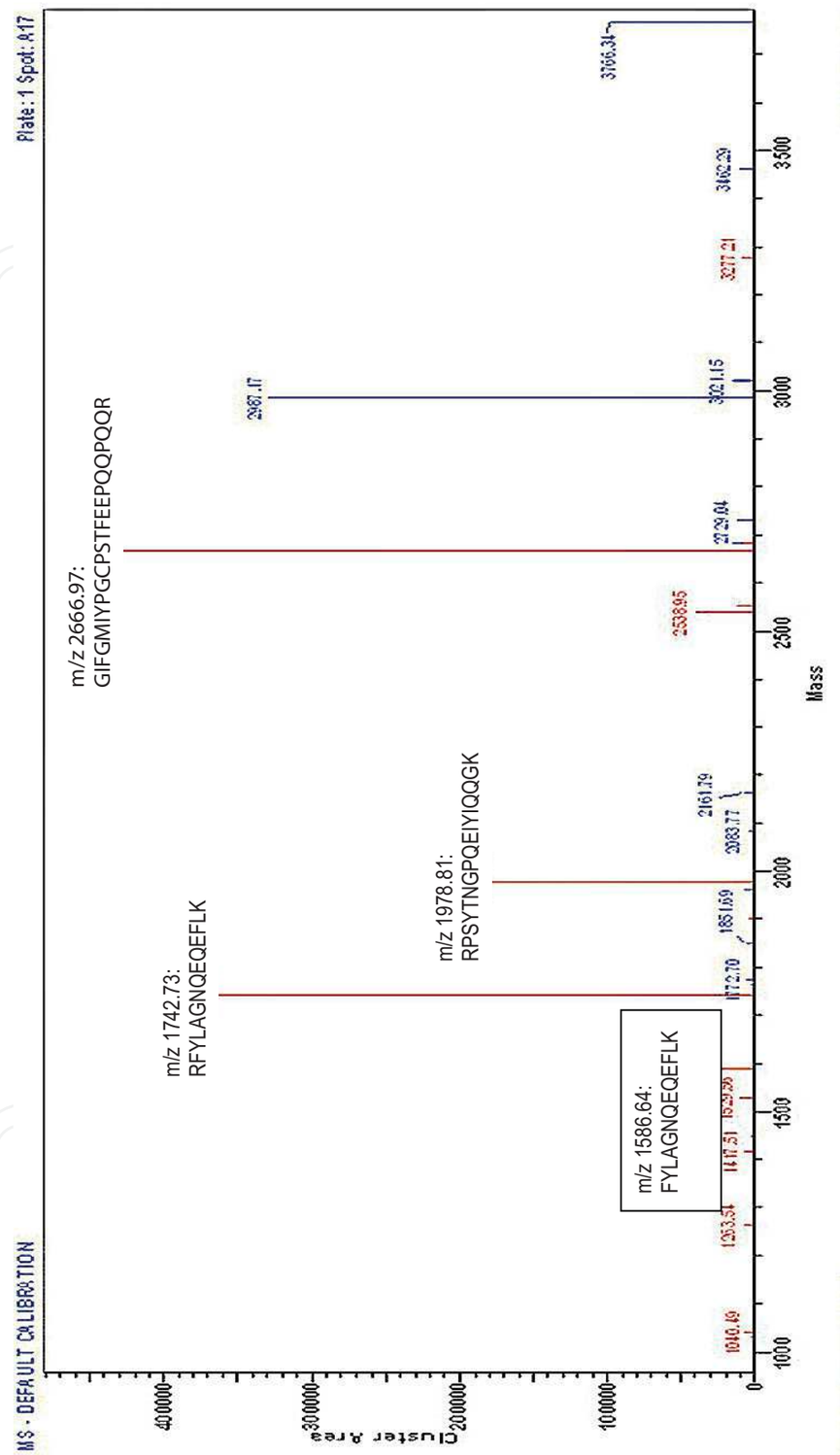


Fig. 9. Mass spectrum (MS) of tryptic peptides eluted from 2D-DIGE spot 24. The m/z value and sequence for each peptide is shown in the graph. The peptide with the highest ion score (42, CI 96.051 %) appears within the rectangle.

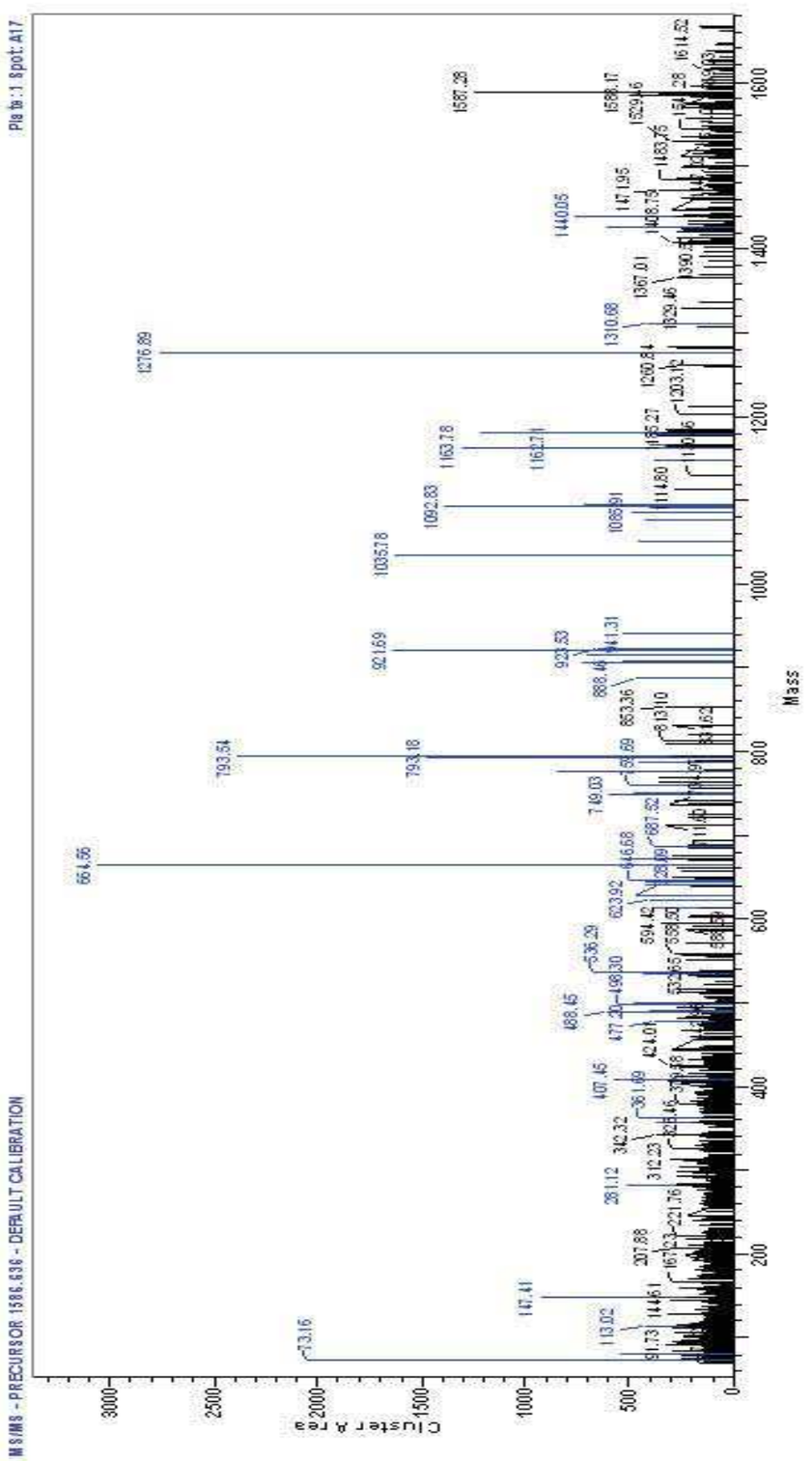


Fig. 10. MS/MS spectrum of peptide m/z 1586.64 (FYLAGNQEQEFLK) from protein spot ID 24. Sequences of these peptide ions fit within the sequence of peptide m/z 1586.64.

The physicochemical parameters relative to the molecular identification of proteins in spots ID22 and ID24 are shown in Table 2.

Spot ID	Accession No.	Identity	Molecular weight (Da)	Isoelectric point	Pep. Count	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion C.I. %
22	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	53590,6	5,78	12	198	100	89	100
24	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	53590,6	5,78	10	199	100	124	100

Table 2. Chemical characterization of proteins in 2D-DIGE spots 22 and 24 from the embryonic axes of soybean subjected to thermal shock at 40°C for 2 h.

The increment in the volume of spots 22 and 24 (proglycinin A1aB1b isomers) is shown in Table 3. The two protein isomers showed the highest expression under each stressing conditions tested (the exception was HS42°C/2 h).

Spot ID	Accession No.	Identity	HS/40°C/2h	HS/42°C/2h	pH 5.5	200 mM NaCl
22	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	87,07*	1,01	15,48	24,32
24	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	78,67	-1,0	10,07	12,63

*Upregulation or downregulation of proteins in relation to control lot (fold times).

Table 3. Increment in the expression of spot proteins ID-22 and ID-24 in the embryonic axes of soybean subjected to several stressing conditions.

Peptide localization.

Localization of peptides m/z 2667.02 (from 2D-DIGE spot ID 22) and m/z 1586.64 (from 2D-DIGE spot 24) within the Chain C, crystal structure of soybean proglycinin A1aB1b homotrimer (Accession No. gi|15988119) (Adachi et al. 2001), is shown below. Peptide m/z 2667.02 goes from a. a. 79 to 101 (bold, underlined sequence) and peptide m/z 1586.64 goes from a.a. 163 to 175 (underlined sequence).

1 FSSREQPQQN ECQIQKLNALKPDNRIESEG GLIETWNPNN KPFQCAGVAL SRCTLNRNAL
61 RRPSYTNGPQ EIYIQQGK **GI FGMIYPGCPS TFEEPQQPQQ** RGQSSRPQDR HQKIYNFREG
121 DLIAVPTGVA WWMYNNEDTP VVAVSIIDTN SLENQLDQMP RR FYLAGNQEQEFLK YQQEQ
181 GGHQSQKGKH QQEEENEGGS ILSGFTLEFL EHAFSVDKQI AKNLQGENEG EDKGAIVTVK
241 GGLSVIKPPT DEQQQRPQEE EEEEEDEKPQ CKGKDKHCQR PRGSQSKSRR NGIDETICTM
301 RLRHNIGQTS SPDIYNPQAG SVTTATSLDF PALSWLRLSA EFGSLRKNAM FVPHYNLNAN
361 SIIYALNGRA LIQVVNCNGE RVFDGELQEG RVLIVPQNFV VAARSQSDNF EYVSFKTNDT
421 PMIGTLAGAN SLLNALPEEV IQHTFNLKSQ QARQIKNNNP FKFLVPPQES QKRAVA

4. Discussion

Soybean (*Glycine max*), as a crop of upmost nutritional and industrial importance, has been the subject of an enormous amount of research; the effect of adverse environmental conditions on soybean germination, aiming to develop resistant varieties, has been one of the most investigated aspects. As a result, many proteins have been found to be modified in their expression, and also in their function.

Heat, was probably the first stressing factor studied in plants. Key *et al.* in 1981 showed that when the growth temperature of soybean seedlings was shifted from 28°C to 40°C the pattern of protein synthesis changed rapidly; normal protein synthesis decreased and a new set of proteins was induced. These "new" proteins were identified as heat shock proteins or HSPs. A strong positive correlation between HSPs and the acquisition of thermal tolerance was noticed thereafter (Lin *et al*., 1984). From the time when these initial publications appeared, the study of heat-induced HSPs has been continuous, aiming to identify particular molecular species. The most recent investigations have made use of the proteomic technology; most HSPs have been identified as stabilizing (refolding) proteins, storage proteins or respiratory-related proteins (Ren *et al*., 2009).

Although HSPs are meant to prevent, and even reverse, the molecular alterations induced by heat (Boston *et al*., 1996), they might be inefficient in cell protection when the temperature intensity is beyond the tolerance threshold (Schöffl *et al*., 1999). Direct effects of heat include denaturation and aggregation of proteins, changes in the structure and function of cell membranes, including lipid fluidization, enzyme inactivation in mitochondria and chloroplasts, inhibition in the synthesis of proteins and many other alterations (Howarth, 2005). The diminution in the expression levels of 11 proteins in the lot shocked at 42ºC in the present study (Table 1) indicated that this stressing condition overwhelmed the tolerance threshold in the soybean seeds.

The effect of salinity has also been studied in soybean. Protein changes similar to those noted in soybean subjected to thermal shock have been described in soybean subjected to

high salinity. High salinity may lead to rapid accumulation of reactive oxygen species (ROS) in the plant tissues (Zhu, 2001). In turn, ROS may produce degradation of photosynthetic pigments, lipid peroxidation, membrane permeability alteration, protein denaturation, and DNA mutations (Mittler, 2002). Salinity leads to reduction in photosynthesis, leaf area and biomass (Kao *et al*., 2006). To cope with salt stress, soybean has developed several tolerance mechanisms, including maintenance of ion homeostasis; restoration of osmolite balance, and the synthesis of antioxidants (phenols) and enzymes such as superoxide dismutase and peroxidase (Gossett *et al.,* 1994; Phang *et al.*, 2008), among others. In addition, several metabolic proteins in soybean have been found to be down-regulated by high salinity (glyceraldehydes-3-phosphate dehydrogenase and fructokinase-2, among them) while others have been found to be up-regulated (stem 31 kDa glycoprotein precursor, for instance) (Sobhanian *et al.,* 2010). In our study, germination of soybean in the presence of 200 mM NaCl led to a significant increase in the expression of 29 out of the 35 proteins over expressed in soybean shocked at 40ºC. Connection between high salinity, extreme heat, dryness, and oxidative stress, as stressing factors in plants has been recognized (Zhu, 2001).

To our knowledge, acidity as a stressing factor has not been studied, at least not in soybean; therefore, our results are pioneers in this subject. As pointed out in the Results section, acidity at pH 5.5, induced the overexpression of 31 proteins, two of them being the same proteins over-expressed in response to the other stressing factors tested (HS40°C and 200 mM NaCl).

The importance and novelty of the present study is that the stress response of soybean was investigated in a comparative manner, in soybean-minicrops subjected to four different stressing conditions, namely: heating at 40°C (HS40°C/2h), heating at 42°C (HS42°C/2 h), acidic environment (pH 5.5), and high salt concentration (200 mM NaCl).

The finding that 35 proteins out of the ≈2000 proteins detected in soybean grown under normal conditions (10 mM phosphate, pH 7.0, 28°C) showed modification in their expression, most of them at increase, was very informative. These 35 proteins behaved as heat-shock proteins under the principle that they increased (the majority) or decreased their expression in ≥ 1.5 times in comparison with the control lot. Within those proteins that increased their expression there were two spot-proteins, ID-22 and ID-24 (by 2D-DIGE), that maximally increased under all of the stressing situations applied; the exception was HS42°C/2 h that resulted the most deleterious factor as this factor negatively affected not only the expression of proteins but also the germination of the grain. As proteins ID-22 and ID-24 maximally over expressed under the stressing factors HS40°C/2 h, pH 5.5, and 200 mM NaCl, we thought of these two proteins as vital elements for the growing of soybean exposed to all kinds of adverse environments. Therefore, these proteins deserved further analysis and on this base we decided to analyze the nature of these proteins by MALDI TOF and MALDI TOF/TOF. The analysis of peptides allowed us to identify the two proteins as isomers of the glycinin in its immature form, pro-glycinin. Seed proteins are usually synthesized as precursors having a leader pre-peptide sequence that contains the signal for transport, processing and targeting from the site of synthesis to the storage organelles. The signal sequence is involved in directing the nascent chain from the rough side of the endoplasmic reticulum (ER) to the ER lumen. After cotranslational cleavage of the signal peptide, the polypeptides are subjected to glycosylation and/or to folding through enzyme-catalyzed disulfide formation. Then, mature polypeptides are directed to storage protein bodies with the help of chaperones. Close to 90 % of the proteins in soybean exist as storage proteins, which mostly consist of β-conglycinin (7S) and glycinin (11S). Glycinin, the protein found maximally up-regulated under all stressing situations tested in this study, is a 300-380 kDa hexamer (Peng *et al,* 1984; Utsumi, 1992); each

subunit is composed of acidic (~35 kDa) and basic (~20 kDa) polypeptides linked together by a disulfide bond. Five subunits have been identified and classified within group I: A1aB1b (53.6 kDa), A2B1a (52.4 kDa) and A1bB2 (52.2 kDa), or within group II: A5A4B3 (61.2 kDa) and A3B4 (55.4 kDa) (Maruyama *et al*, 2006). Glycinin subunits are polymorphic (*i.e*., there are some amino acid replacements in the same kind of subunit among different soybean cultivars). In addition, glycinin exhibits molecular heterogeneity, because the molecule is a hexamer with different subunit composition. Glycinin hexamers dissociate to their constituent polypeptide subunits, depending upon pH, ionic strength, and heating temperature (Adachi *et al*., 2001, 2003). The reason for the impressive over-expression of glycinin when soybean is subjected to heat, acidity or salinity (this study) is not yet understood. However, because this protein shows chaperone activity (Choi *et al*., 2004), it is possible that acting as a finely tuned sensor of stress, glycinin A1aB1b is efficiently up-regulated to cope with the adverse effects of environmental stressants in general, including heat, salinity and acidity (see text), waterlogging (Alam *et al*, 2010), Chilling (Cheng *et al*, 2010, and radioactivity (Danchenko *et al*, 2009). Reports on other proteins with hexameric structure and chaperone activity, reinforce this idea (Sauer *et al*., 2004; Lee *et al*, 2010).

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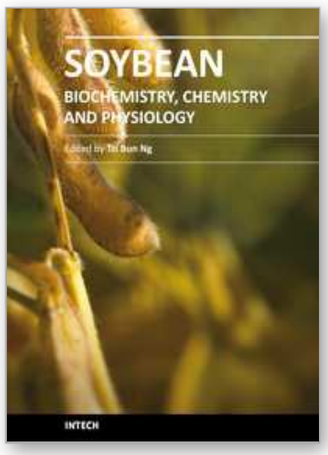

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Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

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