

New insights on proteomics of transgenic soybean seeds: evaluation of differential expressions of enzymes and proteins

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Abstract This work reports the evaluation of differentially expressed enzymes and proteins from transgenic and nontransgenic soybean seeds. Analysis of malondialdehyde, ascorbate peroxidase (EC 1.11.1.11), glutathione reductase (EC 1.6.4.2), and catalase (EC 1.11.1.6) revealed higher levels (29.8, 30.6, 71.4, and 35.3%, respectively) in transgenic seeds than in nontransgenic seeds. Separation of soybean seed proteins was done by two-dimensional polyacrylamide gel electrophoresis, and 192 proteins were identified by matrix-assisted laser desorption/ionization (MALDI) quadrupole time-of-flight (QTOF) mass spectrometry (MS) and electrospray ionization (ESI) QTOF MS. Additionally, the enzyme CP4 EPSPS, involved in the genetic modification, was identified by enzymatic digestions using either trypsin or chymotrypsin and ESI-QTOF MS/MS for identification. From the proteins identified, actin fragment, cytosolic glutamine synthetase, glycinin subunit G1, and glycine-rich RNA-binding protein were shown to be differentially expressed after analysis using the

two-dimensional difference gel electrophoresis technique, and applying a regulator factor of 1.5 or greater.

Keywords Two-dimensional difference gel electrophoresis · Mass spectrometry · Reactive oxygen species · Genetically modified organisms

Introduction

There is no doubt that soybean [*Glycine max* (L) Merrill] is of great economic importance for many countries. The total US soybean crop in 2009 was valued at 31 billion US dollars [1]. This economic importance has resulted in a worldwide increase in soybean production, which reached more than 200 million tons between 2005 and 2006 [1]. These increases in crop production can also be attributed to the use of transgenic plants, i.e., approximately 49 million hectares (60% of the worldwide cultivated area) is now planted with transgenic soybean [2].

Transgenic organisms are modified by the insertion of exogenous DNA fragments, and in terms of plants this process is done to improve the productivity, to enhance the tolerance to herbicides, and to synthesize new substances not present prior to genetic modification [3]. The literature reports that these genetic modifications change the proteome of the soybean seeds, as well as the metallome of the plant [4–7]. On the basis of these data, it was rationalized that if the metallome changes in these transgenic plants, then the ability of the plant to take in metals could also change, thus affecting the production of reactive oxygen species (ROS), and leaving the plant more susceptible to oxidative stress. The ROS molecules are scavenged by different antioxidative defense mechanisms [8] under steady-state conditions. The equilibrium between the

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production and the scavenging of ROS may be altered by biotic and abiotic stress factors such as UV radiation, temperature, air pollution, pathogen attacks, heavy metals, nutrient deficiency, and herbicides [9, 10]. Stress-induced ROS accumulation is counteracted by enzymatic oxidant systems that include a variety of scavengers, such superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), and catalase (CAT), and nonenzymatic low molecular weight metabolites, such as carotenoids and flavonoids [11–13]. It has also been shown that ROS influence the expression of a number of genes and signal transduction pathways, suggesting that cells have evolved strategies to use ROS as biological stimuli and signals that activate and control various genetic stress-response programs [13–15].

Therefore, the main hypothesis of the present work is that the genetic modification itself could be a stress factor, provoking changes in the activity of some enzymes involved in combating the ROS as well as alterations of the proteome. To corroborate this hypothesis, we evaluated transgenic and nontransgenic soybean seeds from an enzymatic and proteomic point of view, using enzymatic activity quantification, two-dimensional (2-D) difference gel electrophoresis (DIGE) [16], and mass spectrometry (MS) [17] techniques not only for protein evaluation in terms of their differential expressions when these organisms are compared, but also for the identification of the enzyme involved in the genetic modification (CP4 EPSPS). A proteomic map for transgenic soybean seeds was also obtained.

Materials and methods

Plant material

Transgenic (variety MSOY 7575 RR) and nontransgenic (variety MSOY 7501) soybean seeds were kindly donated by Siu Mui Tsai (CENA/USP, Piracicaba, SP, Brazil). The transgenic soybean confers resistance to the herbicide glyphosate—*N*-(phosphonomethyl)glycine). The seeds were originally obtained from Monsanto (Brazil) [18]. For handling the Roundup Ready-type transgenic soybean seeds, the Institute of Chemistry has a Biosafety Quality Certificate (issued on 24 July 2007, number 240/2007).

Enzyme extractions from soybean seeds and determination of their activities

The seeds were ground in a cryogenic mill for 1 min, and the material was homogenized in a mortar and pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol, and 4% (w/v) polyvinylpyrrolidone. The

homogenate was centrifuged (5,000g) at 4 °C for 30 min, and the supernatant was collected and stored at –80 °C for all enzyme activity determinations [19].

For CAT (EC 1.11.1.6) activity, the method described by Azevedo et al. [19] was used, where the enzyme was assayed spectrophotometrically at 25 °C using a 1 mL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) and 25 μ L of 30% (v/v) H₂O₂. The reaction was initiated by adding 25 μ L of seed extract and the CAT activity was determined by monitoring the H₂O₂ degradation after 1 min at 240 nm. The extinction coefficient used to calculate the CAT activity was 39.4 mM^{–1} cm^{–1}.

The ascorbate oxidation rate at 30 °C was used to determine APX (EC 1.11.1.11) activity, which was determined in a spectrophotometric assay at 290 nm [20]. For this reaction, 40 μ L of seed extract was added to a solution containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H₂O₂. The APX activity was expressed in millimoles of ascorbate per liter per minute per milligram of protein, and an extinction coefficient of 2.8 mM^{–1} cm^{–1} was used for ascorbate.

Glutathione reductase (GR; EC 1.6.4.2) activity was determined spectrophotometrically using the method described by Gomes-Junior et al. [21]. Potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 1 mM glutathione disulfide (GSSH), and 0.1 mM NADPH was used; to this 50 μ L of seed extract was added to start the reaction. The reduction rate of GSSH was monitored at 412 nm and 30 °C. The results were expressed in micromoles of GSSH per liter per minute per milligram of protein.

The SOD (EC 1.15.1.1) activity was determined by the capacity of the enzyme to inhibit the photoreduction of nitroblue tetrazolium (NBT) in the presence of light, according to the method of Giannopolis and Reis [22] with some modifications as described by Azevedo et al. [19]. The reaction was conducted in a volume of 3.0 mL (50 mM sodium phosphate buffer pH 7.8, 13 mM methionine, 75 μ M NBT, 0.1 mM EDTA, and 2 μ M riboflavin) containing 150 μ L of seed extract. The reaction was monitored by a fluorescence camera for 5 min. After this period, SOD activity was determined in a spectrophotometer at 560 nm. According to the method, six reactions for each sample were performed [one phormazan, one control (reaction in the dark, without seed extract), four replicates of the homogenate with seed extract in the presence of light]. The results were expressed in SOD units per milligram of protein.

Lipid peroxidation evaluation

For lipid peroxidation estimations, the malondialdehyde (MDA) content in the seed samples was determined. This

method was conducted as described by Gomes-Junior et al. [23] with some modifications: 100 mg of seeds was ground in a cryogenic mill and homogenized with a mortar and pestle with a solution containing 0.1% (v/v) trichloroacetic acid and 20% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 10,000g for 10 min, then 250 μ L of the supernatant was added to a solution composed of 1 mL of 0.5% (v/v) 2-thiobarbituric acid plus 20% (v/v) trichloroacetic acid and the mixture was incubated in a dry bath at 95 °C for 30 min. Then, the homogenate was maintained in an ice bath for 1 min and centrifuged at 10,000g for 10 min in order to separate some residues formed during the heating process. The concentration of MDA was determined at 532 nm using an extinction coefficient of 155 mM⁻¹ cm⁻¹, following a correction for unspecific turbidity determined by the absorbance at 600 nm.

Enzymatic activity in nondenaturing polyacrylamide gel electrophoresis

Electrophoresis experiments were performed according to the method of Laemmli [24] for nondenaturing gels, with some modifications. Mini gels (8.3 cm $\times$ 10.2 cm) were used in a 12% (w/v) final acrylamide concentration to improve gel resolution. Electrophoresis was performed at a constant current (15 mA) for 4 h for SOD activity. A protein mass of 60 μ g was applied to the SOD gel, and the gel staining was performed as described by Gomes-Junior et al. [23]. After electrophoresis, the gels were rinsed in deionized water and incubated for 30 min, with agitation, in a mixture of 70 mL of a buffer solution containing 100 mM potassium phosphate (pH 7.5), NBT, EDTA, *N,N,N',N'*-tetramethylethylenediamine, and 30 mL of a solution containing riboflavin (50 mg/100 mL deionized water). After the reaction, the gels were rinsed in deionized water and exposed to fluorescent light, and colorless bands of SOD activity appeared in the purple-stained gel. To stop the reaction, the gels were incubated in 70% (v/v) acetic acid for 15 min with agitation.

SOD isoenzymes were classified as described previously by Azevedo et al. [19]. The gel was cut into three slices: the first (control) was stained for SOD activity as described above. The second and the third slices were incubated for 20 min in a 100 mM potassium phosphate buffer solution (pH 7.5) containing 2 mM KCN and in a 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM H₂O₂, respectively. After this period, both slices were washed in deionized water and then stained for SOD activity. Gels pretreated with H₂O₂ and KCN before SOD activity evaluation were used to classify the enzymes into the various types: Cu/Zn-SOD, Fe-SOD, or Mn-SOD. Mn-SOD is resistant to both inhibitors, Fe-SOD isoenzyme is resistant to KCN and is inhibited by H₂O₂, and Cu/Zn-SOD is inhibited by both inhibitors.

Protein extraction from soybean seeds

The extraction was conducted on the basis of the method proposed by Sussulini et al. [25]. Seeds of transgenic or nontransgenic soybeans (about 500 mg) were frozen in liquid nitrogen and ground for 45 min. The powder obtained was then mixed with petroleum ether and gently agitated (about 15 min) to remove the oils. Protein extraction was performed using a buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl pH 8.8, 1.5 mM KCl, 10 mM dithiothreitol, 1.0 mM phenylmethanesulfonyl fluoride, and 0.1% (w/v) sodium dodecyl sulfate (SDS), in a 10:1 (v/w) ratio. After the mixture had been centrifuged at 5,000g at 4 °C for 5 min, the supernatant was collected and stored at –20 °C.

One-dimensional gel electrophoresis (SDS-PAGE)

The protein extract was submitted to SDS-PAGE separation in order to identify the CP4 EPSPS enzyme in transgenic soybean seeds. The separation was performed with a vertical slab gel apparatus (Hoefer SE 600 Ruby). The SDS-PAGE was conducted using a 12.5% (w/w) acrylamide gel at pH 8.8, prepared according to the method of Laemmli et al. [24]. Different amounts of total proteins (25, 50, 75, and 100 mg) were applied to the upper region of the gel. Electrophoresis was performed at 200 V and 25 mA for 9 h. As soon as the electrophoretic run had finished, the gel was stained with 1% Coomassie staining solution [20% (v/v) ethanol, 8% (v/v) ammonium sulfate, 1.6% (v/v) phosphoric acid, 0.4 gL⁻¹ Coomassie brilliant blue G-250]. The gel was scanned and its image was analyzed by GelPro Analyzer version 3.1 to estimate the molar mass of the proteins.

Two-dimensional gel electrophoresis separation of proteins

To precipitate the proteins, 1 mL of 0.1 M ammonium acetate in methanol was added to 200 μ L of the previously obtained protein extract. The precipitated proteins were then washed twice with ammonium acetate–methanol solution, twice with cold 80% (v/v) acetone, and finally once with cold 70% (v/v) ethanol. The proteins were quantified by the Bradford method [26].

For separation by 2-D PAGE, protein resolubilization was performed using 7 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 0.002% (w/v) bromophenol blue, and 0.5% (v/v) carrier ampholytes.

Protein separation by 2-D PAGE was performed according to the manufacturer's recommendations [27]. A total of 500 μ g of proteins, as previously optimized, was applied to immobilized pH gradient strips (pH range 4–7) and they were rehydrated at room temperature for at least 12 h.

Isoelectric focusing was performed with a Multiphore II system (GE Healthcare), for a total of 14,300 Vh. The second-dimension separation was conducted at 25 mA per gel and 100 W with an Ettan DALT system (GE Healthcare) on 1-mm 12.5% (w/v) SDS polyacrylamide gels. The buffer system consisted of a solution of 25 mM Tris-HCl, pH 8.8, 192 mM glycine, and 0.1% (w/v) SDS. After separation the gels were stained with colloidal Coomassie brilliant blue [28].

The gels were scanned using an ImageScanner II (GE Healthcare) and the images were processed by ImageMaster 2D Platinum 6.0 (GeneBio, Geneva, Switzerland), which estimated the number of spots, the pI values and molecular mass of the proteins.

Protein spot in-gel digestion and MS analysis

In-gel digestion of protein spots were performed using a Montage In-Gel Digest_{2P} kit (Millipore, Bedford, USA), containing a ZipPlate micro-solid-phase-extraction plate. The digestion and vacuum elution protocols applied were according to the manufacturer's recommendations. For vacuum elution, a Multiscreen[®] vacuum manifold (Millipore) was employed.

All samples obtained by tryptic digestion were prepared using the dried droplet method. For matrix-assisted laser desorption/ionization (MALDI) quadrupole time-of-flight (QTOF) MS and MALDI-QTOF MS/MS analysis, the samples were acidified by adding two sample volumes of 0.1% (v/v) trifluoroacetic acid and were left at room temperature for a few minutes to reduce the droplet volume via evaporation. The matrix [1% (w/v) α -cyano-4-hydroxycinnamic acid (CHCA) in a 1:1 (v/v) water–acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid] was added and the sample was allowed to dry at room temperature.

MS data were collected in the positive ion mode. Mass spectra were acquired with a Waters Q-ToF Premier[™] mass spectrometer (Waters-Micromass, Manchester, UK) operating in the positive mode (LDI+) with a fixed nitrogen ion source using the following main parameters: laser firing rate of 200 Hz, mass range of 800.0–3,000.0 Da, peak detection threshold for MS/MS of 1,500.0, mass threshold of 200.0 Da, scan time of 1 s, Np multiplier set to 0.70, resolution of 10,000 with “V” mode, trigger threshold of 700, signal threshold of 80, and microchannel plate set to 2,100 V. Each spectrum was collected using 2 s scans, and the spectra were accumulated over approximately 2 min. Argon gas was used in the collision cell and the typical collision energy used was in the range of 34–161 eV depending on the size of the ions. The instrument was controlled by MassLynx version 4.1.

For electrospray ionization (ESI) QTOF MS/MS analysis, the peptides obtained by enzymatic digestion were dried and resolubilized in formic acid 0.1% (v/v). An aliquot (4.5 μ L) of

the resulting peptide mixture was separated by C₁₈ (75 μ m $\times$ 100 mm) RP-nanoUPLC (nanoAcquity, Waters) coupled with a Q-ToF Ultima mass spectrometer (Waters) with a nano-electrospray source at a flow rate of 0.6 μ L/min. The gradient was 2–90% acetonitrile in 0.1% formic acid over 20 min. The instrument was operated in the “top three” mode, in which one MS spectrum is acquired followed by MS/MS of the top three most intense peaks detected. The spectra were acquired using MassLynx software version 4.1.

For identification of the CP4 EPSPS enzyme, the protein band corresponding to a mass of 47 kDa was cut, reduced, alkylated, and subjected to two enzymatic digestion protocols either using trypsin or chymotrypsin as cleavage enzymes. The peptides obtained by enzymatic digestion were dried and resolubilized in 0.1% (v/v) formic acid, and the analyses were performed by ESI-QTOF MS/MS. The acquisition of the MS/MS spectra and the identification of proteins were similar to what was described above with the exception of the run time to obtain the spectra, which was 40 min.

Two-dimensional DIGE analysis

The 2-D DIGE gels of transgenic and nontransgenic soybean seed proteins were prepared in triplicate. Protein samples were labeled according to the manufacturer's protocol for minimal labeling (GE Healthcare). Two-dimensional (2-D) DIGE was performed identically to the previous 2-D method; however, the precipitated proteins were resolubilized in a lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 20 mM Tris, pH 8.8, without a reducing agent] prior to dye labeling. For labeling with CyDye DIGE Fluors minimal dyes, 50 μ g (pH range 4–7) of each sample was labeled with 400 pM CyDye DIGE Fluors minimal dyes (GE Healthcare) and incubated on ice in the dark for 30 min. For each replicate reaction, one sample was labeled with Cy3, the other with Cy5, and the internal standard with Cy2. Two transgenic and two nontransgenic seed samples were labeled with Cy5 and Cy3, respectively. For the third replicate, the CyDyes were swapped to correct for the bias introduced by the different fluorescence characteristics of acrylamide at the different wavelengths of excitation for Cy3 and Cy5. The internal standard labeled with Cy2 consisted of a pooled sample comprising equal amounts of each transgenic and nontransgenic seed samples used for each replicate. The reactions were quenched by adding 1 μ L of 10 mM lysine and incubating the mixture for an additional 10 min. Each labeled protein sample was diluted with rehydration solution. Isoelectric focusing was performed with Immobiline DryStrips (pH 4–7, 13 cm, GE Healthcare) for 14.6 kV h.

The final gels were scanned with an Ettan DIGE imager scanner (GE Healthcare) and analyzed for proteome differences. DeCyder Differential Image Analysis (GE Healthcare) was used for image analysis, spot detection, matching between the gels, and normalization based on the pooled internal standard before quantification. Differential expression was considered to be statistically significant after the Student *t* test and if $p < 0.05$. After poststaining with colloidal Coomassie brilliant blue, spots of interest were excised manually and subjected to MS protein identification.

Protein identification

All mass spectra data files were converted to a peak list format (mgf) by Mascot Distiller version 2.2.1.0, 2008 (Matrix Science) and searched against nonredundant protein databases, Mass Spectrometry Data Bank (MSDB), National Center for Biotechnological Information (NCBI) database, and the nonredundant *Glycine max* database. The search engine MASCOT version 2.3 (Matrix Science) was used with the following parameters: carbamidomethylation as fixed modification, oxidation of methionine as variable modification, one trypsin missed cleavage, and a tolerance of 0.1 Da for both precursor and fragment ions. The search error tolerance was set at 0.1 Da with an $[M+H]^+$ charge state. Peptides from unmatched proteins were searched using identical search criteria, but within “all-plant” databases.

Results and discussion

Lipid peroxidation evaluation

The MDA concentration, which is an indicator of lipid peroxidation and oxidative stress, was 2.25 ± 0.04 and 1.56 ± 0.06 for transgenic and nontransgenic soybean seeds, respectively, and was 29.8% higher in transgenic seeds than in nontransgenic seeds. Such results suggest that the transgenic material naturally exhibited a level of stress higher than that of the nontransgenic material. The results agree with those reported by Sergiev et al. [29], who pointed out that plants treated with glyphosate increase lipid peroxidation, ion fluxes in leaves, and CAT, peroxidase, and GST activities, which are indirect indications of an oxidative stress condition.

Enzymatic activities

The spectrophotometric enzymatic assays revealed that the transgenic seeds exhibited higher activities than nontransgenic seeds (Table 1). Although APX, CAT, and GR exhibited 30.6%, 35.3%, and 71.4% activity increases, respectively, SOD activity was lower (47.7%) in transgenic seeds than in nontransgenic seeds (Table 1).

Table 1 Enzymatic activity determined by spectrophotometric assays for transgenic and nontransgenic soybean seed samples for ascorbate peroxidase (APX; mM ascorbate $\text{min}^{-1} \text{mg}^{-1}$ protein), catalase (CAT; nM $\text{H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein), glutathione reductase (GR; μM glutathione disulfide $\text{min}^{-1} \text{mg}^{-1}$ protein) and superoxide dismutase (SOD; SOD units mg^{-1} protein)

Soybean seed samples	Enzymatic activity			
	APX	CAT	GR	SOD
Transgenic	18.84 ± 0.003	3.37 ± 0.10	0.14 ± 0.01	42.6 ± 1.81
Nontransgenic	13.08 ± 0.002	2.25 ± 0.02	0.04 ± 0.01	81.5 ± 3.11
T/NT ^a	1.44	1.50	3.5	0.52

Average values $\pm$ standard deviation ($n=6$)

^a Ratio between transgenic and nontransgenic seed values

SOD activity staining showed the same overall pattern of reduction in SOD activity as that observed by the spectrophotometric assay (Fig. 1). Eight SOD isoenzymes were detected in all genotypes tested, one classified as Mn-SOD (SOD I), two as Fe-SOD (SOD II and SOD III), and five as Cu/Zn-SOD (SOD IV to SOD VIII) (Figs. 1, 2). However, the reduction in SOD activity detected for the transgenic seeds was a result of a reduction in activity of all isoenzymes, but with a more significant reduction in the activity of Fe-SOD isoenzymes II and III. Iron is not bound to SOD, and the results corroborate those previously obtained by Mataveli et al. [7], since iron was more bioaccessible in transgenic soybean seeds than in nontransgenic soybean seeds. SOD is the first line of the defense system against ROS, dismutating the superoxide radical. Fe-SOD is normally localized in the chloroplasts, and the more significant reduction in the two Fe-SOD isoenzymes may indicate a lower demand for SOD activity in the transgenic line under stress. These results also indicate

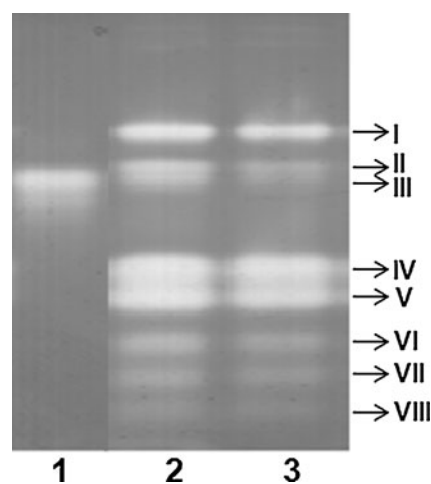


Fig. 1 Superoxide dismutase (SOD) activity staining following nondenaturing polyacrylamide gel electrophoresis (PAGE). Lane 1 bovine SOD standard, lane 2 nontransgenic soybean seed, lane 3 transgenic soybean seed. Arrows indicate sequentially numbered SOD bands (I–VIII)

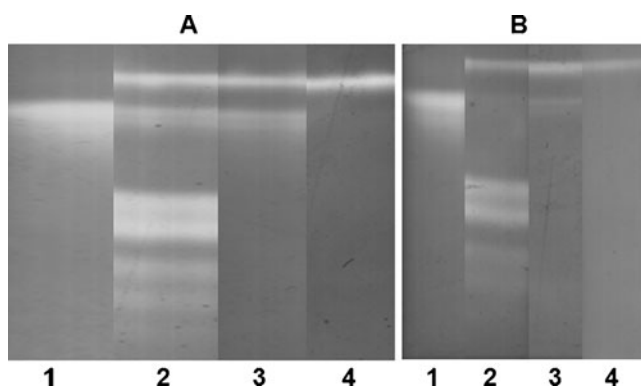


Fig. 2 SOD isoenzyme classification following nondenaturing PAGE. **A** Nontransgenic soybean seed and **B** transgenic soybean seed. Lane 1 bovine SOD standard, lane 2 control SOD activity, lane 3 plus 2 mM potassium cyanide, lane 4 5 mM hydrogen peroxide

that the stressful condition may be established differently among the organelles of the cells. Ferreira et al. [30] working with soybean seedlings under cadmium stress observed seven isoenzymes in leaves and eight in roots, corresponding to Mn-SOD and Cu/Zn-SOD isoenzymes.

On the other hand, considering the increased activity of the two peroxidases analyzed, CAT and APX, it appears that the oxidative stressful condition in transgenic seeds resulted in increased production of H_2O_2 which is possibly controlled by the action of these two enzymes and even GR. Yet, the participation of other antioxidant enzymes in the responses to stress in these genotypes cannot be ruled out.

It is well known that the oxidative response in plants can be exacerbated under stress conditions and these responses can differ among plant species, among organs, tissues, and even among varieties of the same species, and for different time length of exposure to the stress [13, 31]. For instance, the results reported by Ferreira et al. [30], who investigated the effects of cadmium on the activities of CAT and GR in roots and leaves of soybean seedlings, revealed that although an effect of cadmium on plant growth was observed, the CAT activity was not altered significantly in roots and leaves. On the other hand, an increase in GR activity in roots at 0.5 mM cadmium was observed. These authors considered two possibilities: GR was stimulated by cadmium in roots in order to produce reduced glutathione for the synthesis of phytochelatins or for detoxification of ROS which were being produced in excess.

It is important to consider that an increase in the antioxidant enzyme system can also be advantageous for plants exposed to herbicides. For example, Donahue et al. [32] evaluated APX and GR activities in leaves of *Pisum sativum* exposed to paraquat and found an interesting positive correlation between the resistance to paraquat and the increase in the activities of those enzymes. Geoffroy et al. [33] showed an increase in CAT, GST, GR, and APX activities in algal material exposed to the herbicide oxyfluorfen. Moldes et al. [18] observed that at certain points during their experiment,

some evaluated biochemical parameters responded differentially between transgenic and nontransgenic soybean cultivars. The results were more evident when CAT activity in roots and total soluble amino acid contents in leaves were considered. CAT is an important enzyme largely studied in plants with important roles in cell metabolism, particularly in metabolizing hydrogen peroxide produced in the peroxisome following the conversion of glycolate to glyoxylate during photorespiration [34] and in the metabolism of hydrogen peroxide liberated during the metabolism of fatty acids in germinating seeds [35]. Yet, the activity of peroxidases must also be considered in the context of the level of lipid peroxidation observed. In this study not only CAT, APX, and GR activities were increased in transgenic seeds, but the level of lipid peroxidation was also increased even when no herbicide was used, such as in most of the previous reported works. Such an aspect suggests that the genetic modification itself might have induced extra ROS generation since increases in CAT, APX, and GR activities were observed.

Protein identification by MS

Brandão et al. [5] evaluated the electrophoretic profiles of samples of transgenic and nontransgenic soybean seeds using 2-D PAGE and image analysis. The results of this study revealed that the number of protein spots present in the linear pH range from 4 to 7 was higher than the number of spots present in the linear range from 3 to 10 for transgenic soybean seeds. Therefore, for the range from 4 to 7, a higher number of spots was selected for comparison with the range from 3 to 10. In this pH range, in particular, the spots were selected, preferably located at pH 7–10, for the process of MS identification.

A total of 192 proteins from transgenic soybean seeds were identified, 179 of them identified within the pH range from 4 to 7 and 13 of them identified within the pH range from 3 to 10 (Table 2). The proteomic map in the two pH ranges tested can be seen in Fig. 3. Using the Mascot program to search protein identification databases generated multiple possible IDs for some of the spots. Thus, the criterion used to select the most likely related protein was based on the higher scores and proteins that most closely match the pI values and molecular mass observed.

An important point to consider when the goal is the development of a proteomic map is related to the efficiency (as a percentage) achieved in the identification process. In this study, 49% of the spots present in the gel were identified in the database. This result shows good efficiency when compared with similar studies involving soybean published in the literature. For example, Xu et al. [36] reported an efficiency of 46% in the identification of proteins from soybean leaves using 2-D PAGE and MS (MALDI time-of-flight MS and liquid chromatography–

Table 2 Proteins identified in transgenic soybean seeds using two-dimensional (2-D) polyacrylamide gel electrophoresis and mass spectrometry

Spot	Protein	Access code	Score	pI/MM (kDa)	Coverage (%)
1	Trypsin inhibitor KTi1 (Kunitz); soybean	JQ1091	85	4.20/17,622	13
2	Seed maturation protein PM41; <i>Glycine max</i> (soybean)	Q9SWB2_SOYBN	54	4.46/15,103	17
3	2S albumin precursor; soybean	T05710	119	4.58/16,351	20
4	Proteinase inhibitor (Bowman–Birk) D-II precursor; soybean	TISYD2	146	4.58/15,344	48
5	Proteinase inhibitor (Bowman–Birk) D-II precursor; soybean	TISYD2	48	4.80/14,400	24
6	Bowman–Birk-type proteinase inhibitor D-II precursor related cluster	SOYCIIPIB	48	4.86/15,449	34
7	Napin-type 2S albumin 1 precursor; <i>Glycine max</i> (soybean)	Q9ZNZ4_SOYBN	59	5.37/11,296	14
8	Embryonic abundant protein; soybean	T07087	147	5.35/16,166	24
9	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	63	5.41/14,342	5
10	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	102	5.86/14,342	8
11	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	63	6.37/15,034	5
12	Seed maturation protein PM31; <i>Glycine max</i> (soybean)	Q9XET1_SOYBN,	87	5.83/17,710	40
13	Ubiquitin precursor; soybean (fragment)	UQSY	63	7.07/11,974	25
14	Seed maturation protein PM31; <i>Glycine max</i> (soybean)	Q9XET1_SOYBN	105	6.26/18,949	24
15	Glycinin chain A2B1a precursor; soybean	FWSYG1	90	7.05/20,497	9
16	Glycinin G1 precursor; soybean	S10851	106	6.76/20,297	8
17	Glycinin chain A2B1a precursor; soybean	FWSYG1	71	6.56/20,040	7
18	Glycinin chain A1aBx precursor; soybean	FWSYG2	60	6.35/20,497	7
19	Glycinin G1 precursor; soybean	S10851	107	6.18/20,577	8
20	Glycinin chain A2B1a precursor; soybean	FWSYG1	53	5.94/20,417	7
21	Glycinin G1 precursor; soybean	S10851	52	5.77/20,417	3
22	Glycine G2 A2B1a subunit; <i>Glycine max</i>	AAA33963	89	6.88/23,624	37
23	Glycinin chain A2B1a precursor; soybean	FWSYG1	72	6.64/24,874	7
24	Seed maturation protein PM22; <i>Glycine max</i> (soybean)	Q9XER5_SOYBN	118	4.90/19,165	30
25	Trypsin inhibitor KTi1 (Kunitz); soybean	JQ1091	145	4.61/21,938	18
26	Trypsin inhibitor A (Kunitz) precursor; soybean	TISY	167	4.41/20,218	13
27	Trypsin inhibitor A (Kunitz) precursor; soybean	TISY	189	4.28/20,417	22
28	β -Conglycinin alpha chain precursor; soybean	FWSYBA	123	4.13/24,199	6
29	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	119	4.18/30,828	9
30	Gly m Bd 30 K allergen; <i>Glycine max</i> (soybean)	O64458_SOYBN	81	4.25/34,900	8
31	Gly m Bd 30 K allergen; <i>Glycine max</i> (soybean)	O64458_SOYBN	50	4.33/34,480	8
32	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	143	4.32/31,968	13
33	Seed maturation protein PM24; <i>Glycine max</i> (soybean)	Q9SEL0_SOYBN	66	4.46/31,488	14
34	Seed maturation protein PM25; <i>Glycine max</i> (soybean)	Q9SEK9_SOYBN	226	4.65/31,015	50
35	Glycinin A3B4 subunit; <i>Glycine max</i> (soybean)	Q7GC77_SOYBN	56	4.64/28,738	9
36	Glycinin chain A2B1a precursor; soybean	FWSYG1	47	4.84/29,650	2
37	Soybean agglutinin; soybean	1SBF	70	5.03/33,554	13
38	Seed maturation protein PM36; <i>Glycine max</i>	AAD51624	89	5.18/27,963	16
39	In2-1 protein; <i>Glycine max</i> (soybean)	Q9FQ95_SOYBN	130	5.27/30,991	54
40	Glycinin G1 precursor; soybean	S10851	54	5.31/34,255	10
41	Soybean agglutinin, fragment 2; soybean	1SBD2	58	5.20/32,357	22
42	Soybean agglutinin; soybean	1SBF	52	5.23/33,758	16
43	Glycinin G1 precursor; soybean	S10851	91	5.47/34,355	18
44	Soybean agglutinin; soybean	1SBF	50	5.49/31,679	24
45	Allergen Gly m Bd 28 K (fragment); <i>Glycine max</i> (soybean)	Q9AVK8_SOYBN	69	5.57/26,013	2
46	Dehydrin-like protein; <i>Glycine max</i> (soybean)	Q39805_SOYBN	79	5.73/24,389	42
47	Dehydrin; <i>Glycine max</i> (soybean)	Q70EM0_SOYBN	161	6.41/26,578	31
48	Lea protein; <i>Glycine max</i> (soybean)	Q7XAW0_SOYBN	129	6.47/29,305	22
49	Dehydrin; <i>Glycine max</i> (soybean)	Q70EM0_SOYBN	70	6.21/29,631	24
50	Dehydrin; <i>Glycine max</i> (soybean)	Q70EL8_SOYBN	57	6.23/30,991	36
51	Triosephosphate isomerase (EC 2.3.1.16); <i>Glycine max</i> (soybean)	Q6GW08_SOYBN	79	6.29/30,508	20
52	Lea protein; <i>Glycine max</i> (soybean)	Q7XAW0_SOYBN	47	6.35/29,941	19
53	Seed maturation protein PM34; <i>Glycine max</i> (soybean)	Q9LLQ6_SOYBN	78	6.77/36,651	28

Table 2 (continued)

Spot	Protein	Access code	Score	pI/MM (kDa)	Coverage (%)
54	Alcohol dehydrogenase 1 (fragment); <i>Glycine max</i> (soybean)	Q8LJR2_SOYBN	55	6.86/48,245	7
55	Putative glyceraldehyde 3-phosphate dehydrogenase	Q9FX54_ARATH	56	6.73/43,003	14
56	Alcohol dehydrogenase 1 (fragment); <i>Glycine max</i> (soybean)	Q8LJR2_SOYBN	96	6.66/45,813	10
57	Alcohol dehydrogenase (fragment); <i>Glycine max</i> (soybean)	Q9ZT38_SOYBN	61	6.16/47,002	8
58	35 kDa seed maturation protein; <i>Glycine max</i> (soybean).	Q9ZTY1_SOYBN	86	6.10/39,991	20
59	Cytosolic malate dehydrogenase (EC 2.3.1.16); <i>Glycine max</i> (soybean)	Q6RIB6_SOYBN	84	6.02/43,527	28
60	35 kDa seed maturation protein; <i>Glycine max</i> (soybean)	Q9ZTY1_SOYBN	56	5.97/41,667	15
61	Isoflavone reductase homolog 2; <i>Glycine max</i> (soybean)	Q9SDZ0_SOYBN	101	5.91/40,329	32
62	Isoflavone reductase homolog 2; <i>Glycine max</i> (soybean)	Q9SDZ0_SOYBN	73	5.70/39,391	14
63	Glycinin G1 precursor (contains glycinin A1a subunit, glycinin Bx subunit) related cluster	TA439_3847	69	5.55/39,391	8
64	Glycinin chain A1aBx precursor; soybean	FWSYG2	54	5.47/ 38799	9
65	Glycinin chain A1aBx precursor; soybean	FWSYG2	64	5.36/37,757	6
66	Glycinin G1 precursor; soybean	S10851	105	5.26/36,631	10
67	Glycinin chain A1aBx precursor; soybean	FWSYG2	62	5.15/35,864	6
68	Glycinin G1 precursor; soybean	S10851	55	5.06/36,631	10
69	Glycinin chain A2B1a precursor; soybean	FWSYG1	53	4.97/38,216	5
70	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	156	4.66/38,565	13
71	Glycinin A3B4 subunit; <i>Glycine max</i> (soybean)	Q7GC77_SOYBN	82	4.86/44,593	14
72	Glycinin G5 precursor; soybean	FWSYG3	83	5.00/43,527	10
73	Glycinin A3B4 subunit; <i>Glycine max</i> (soybean)	Q7GC77_SOYBN	74	5.08/44,459	19
74	Cytosolic glutamine synthetase alpha (fragment); <i>Glycine max</i> (soybean)	Q94K13_SOYBN	60	5.15/45,486	37
75	Glycinin G4 precursor; soybean	S11004	79	4.86/54,498	16
76	Glycinin A5A4B3; <i>Glycine max</i> (soybean)	Q9SB11_SOYBN	103	4.81/54,498	12
77	β -Conglycinin alpha prime subunit; <i>Glycine max</i> (soybean)	Q4LER6_SOYBN	131	4.15/84,770	14
78	β -Conglycinin alpha chain precursor; soybean	FWSYBA	79	4.70/10,4626	10
79	β -Conglycinin alpha chain precursor; soybean	FWSYBA	303	4.84/76,620	16
80	β -Conglycinin alpha chain precursor; soybean	FWSYBA	215	5.04/59,974	15
81	β -Conglycinin alpha chain precursor; soybean	FWSYBA	139	5.04/59,974	13
82	β -Conglycinin alpha chain precursor; soybean	FWSYBA	197	5.11/59,800	13
83	β -Conglycinin alpha chain precursor; soybean	FWSYBA	151	5.16/59,627	11
84	β -Amylase; <i>Glycine max</i> (soybean)	AMYB_SOYBN	86	5.36/59,110	10
85	Actin (fragment); <i>Glycine max</i> (soybean)	Q96445_SOYBN	65	5.25/51,006	9
86	Actin (fragment); <i>Glycine max</i> (soybean)	Q96447_SOYBN	103	5.34/50,281	16
87	β -Conglycinin beta subunit (fragment); <i>Glycine max</i> (soybean)	Q50JD8_SOYBN	246	5.42/55,616	22
88	β -Conglycinin beta subunit (fragment); <i>Glycine max</i> (soybean)	Q50JD8_SOYBN	210	5.51/55,134	22
89	Beta subunit of β -conglycinin (fragment); <i>Glycine max</i> (soybean)	Q22121_SOYBN	110	5.63/55,134	16
90	Beta subunit of β -conglycinin (fragment); <i>Glycine max</i> (soybean)	Q22121_SOYBN	175	5.73/54,974	16
91	Beta subunit of β -conglycinin (fragment); <i>Glycine max</i> (soybean)	Q22121_SOYBN	194	5.87/54,656	16
92	Beta subunit of β -conglycinin (fragment); <i>Glycine max</i> (soybean)	Q22121_SOYBN	207	5.99/55,134	16
93	Sucrose binding protein homolog S-64; <i>Glycine max</i> (soybean)	Q9SP11_SOYBN	53	6.34/57,922	4
94	Seed biotin-containing protein LEA; soybean	T07064	113	6.41/74,082	24
95	Lipoxygenase-3; soybean (fragments)	1BYT	48	6.37/93,336	4
96	Seed biotin-containing protein LEA; soybean	T07064	81	6.30/74,082	10
97	Seed biotin-containing protein LEA; soybean	T07064	109	6.21/74,799	30
98	62 K sucrose-binding protein precursor; soybean	JQ1730	94	6.16/69,924	8
99	Glycinin G1 precursor; soybean	S10851	81	6.10/63,006	17
100	Beta subunit of β -conglycinin (fragment); <i>Glycine max</i> (soybean)	Q22121_SOYBN	139	5.97/63,189	10
101	62 K sucrose-binding protein precursor; soybean	JQ1730	152	6.07/70,601	11
102	62 K sucrose-binding protein precursor; soybean	JQ1731	126	6.00/69,924	8
103	62 K sucrose-binding protein precursor; soybean	JQ1732	144	5.93/70,601	10
104	62 K sucrose-binding protein precursor; soybean	JQ1733	62	5.85/70,601	6
105	62 K sucrose-binding protein precursor; soybean	JQ1734	111	5.70/70,626	7

Table 2 (continued)

Spot	Protein	Access code	Score	pI/MM (kDa)	Coverage (%)
106	62 K sucrose-binding protein precursor; soybean	JQ1735	56	5.68/70,262	6
107	62 K sucrose-binding protein precursor; soybean	JQ1736	67	5.55/69,589	6
108	Enolase (EC 2.3.1.16); <i>Glycine max</i> (soybean)	Q6RIB7_SOYBN	103	5.47/60,850	18
109	62 K sucrose-binding protein precursor; soybean	JQ1736	93	5.45/69,924	10
110	β -Conglycinin alpha prime subunit; <i>Glycine max</i> (soybean)	Q4LER6_SOYBN	77	5.21/73,726	8
111	β -Conglycinin alpha prime subunit.- <i>Glycine max</i> (soybean)	Q4LER6_SOYBN	166	5.17/81,568	12
112	β -Conglycinin alpha prime subunit; <i>Glycine max</i> (soybean)	Q4LER6_SOYBN	97	5.16/73,020	5
113	Dehydrin; <i>Glycine max</i> (soybean)	Q70EL8_SOYBN	73	6.07/29,728	31
114	Nucleoside diphosphatase kinase (EC 2.7.4.6); <i>Glycine max</i> (soybean)	Q8GV24_SOYBN	151	6.37/17,295	18
115	Embryonic abundant protein; soybean	JQ2273	79	6.29/16,309	11
116	Allergen Gly m Bd 28 K (fragment); <i>Glycine max</i> (soybean)	Q9AVK8_SOYBN	146	6.93/24,289	7
117	Bowman-Birk-type proteinase inhibitor C-II precursor related cluster	TA5957_3847	35	4.42/16,270	7
118	Late embryogenic abundant protein related cluster	TA9647_3847	40	5.21/20,432	6
119	Formate dehydrogenase related cluster	TA1261_3847	75	6.56/51,810	12
120	Formate dehydrogenase related cluster	TA1261_3847	55	6.47/52,374	12
121	Hypothetical protein related cluster	AW307413	43	5.77/34,307	9
122	PreproMP73 related cluster	TA28641_3847	118	5.40/25,098	13
123	Glycinin G1 precursor	TA439_3847	37	4.86/31,500	5
124	Class III acidic endochitinase precursor related cluster	TA2420_3847	40	4.33/29,139	3
125	Putative dehydrin (fragment); <i>Glycine max</i> (soybean)	Q5K3Q9_SOYBN	49	6.43/27,691	21
126	β -Conglycinin alpha prime subunit; <i>Glycine max</i> (Soybean)	Q4LER6_SOYBN	153	4.53/82,304	7
127	Glycinin chain A1aBx precursor; soybean	FWSYG2	81	5.65/15,022	4
128	Dehydrin-related cluster	TA266_3847	49	5.53/19,496	11
129	LEA protein (fragment)	Q4U4M1_ARAHY	56	5.22/22,666	11
130	Glycinin chain A1aBx precursor; soybean	FWSYG2	49	5.57/38,756	6
131	β -Amylase; <i>Glycine max</i> (soybean)	AMYB_SOYBN	103	5.32/61,399	5
132	Alcohol dehydrogenase 1 related cluster	TA1574_3847	56	5.69/32,277	3
133	Sucrose-binding protein 2; <i>Glycine max</i> (soybean)	Q84V19_SOYBN	109	5.19/62,068	5
134	β -Conglycinin alpha chain precursor; soybean	S20007	175	4.73/52,185	7
135	β -Conglycinin alpha chain precursor; soybean	FWSYBA	260	5.07/75,813	9
136	β -Conglycinin alpha subunit; <i>Glycine max</i> (soybean)	Q948X9_SOYBN	131	5.27/30,183	7
137	18.5 kDa class I heat shock protein related cluster	TA4152_3847	54	6.37/19,268	6
138	35 kDa seed maturation protein; <i>Glycine max</i> (soybean)	Q9ZTY1_SOYBN	90	5.71/31,308	9
139	Hypothetical protein P0456A01.21 related cluster	TA3824_3847	75	6.68/28,927	14
140	Cytosolic glutamine synthetase (<i>Cucumis melo</i>)	gi 113171384	198	5.31/49,752	13
141	Glycinin A1aBx	gi 225651	91	6.00/17,928	4
142	Glycine-rich RNA-binding protein (<i>Glycine max</i>)	gi 18643	242	5.08/17,569	6
143	Napin-type 2S albumin 1 precursor (<i>Glycine max</i>)	gi 4097894	367	5.43/14,128	40
144	Unnamed protein product (<i>Glycine max</i>)	gi 296510449	314	6.90/18,893	26
145	Unnamed protein product (<i>Glycine max</i>)	gi 218319206	475	6.69/34,675	
146	RecName: Full = β -conglycinin, alpha' chain;Flags: Precursor	gi 121286	40	4.88/27,934	1
147	Unknown (<i>Glycine max</i>)	gi 255630413	40	5.60/25,654	8
148	Unknown (<i>Glycine max</i>)	gi 255633070	602	4.68/18,078	60
149	Unnamed protein product (<i>Glycine max</i>)	gi 227247706	166	5.34/19,279	19
150	Glycine-rich RNA-binding protein (<i>Glycine max</i>)	gi 5726567	171	5.31/17,506	20
151	RecName: Full = P34 probable thiol protease; Flags: Precursor	gi 129353	173	4.84/36,411	4
152	Unknown (<i>Glycine max</i>)	gi 255628279	365	7.05/30,493	34
153	Unnamed protein product (<i>Glycine max</i>)	gi 253775905	380	6.79/43,557	30
154	Maturation protein pPM32 (<i>Glycine max</i>)	gi 5733686	312	5.23/24,108	37
155	Unnamed protein product (<i>Glycine max</i>)	gi 227247706	205	5.60/18,893	19
156	Unknown (<i>Glycine max</i>)	gi 255627799	29	5.18/21,537	19
157	Maturation protein pPM32 (<i>Glycine max</i>)	gi 5733686	199	5.12/22,812	31
158	Trypsin inhibitor (<i>Glycine max</i>)	gi 9367042	57	5.04/21,786	6

Table 2 (continued)

Spot	Protein	Access code	Score	pI/MM (kDa)	Coverage (%)
159	2S albumin pre-propeptide (<i>Glycine max</i>)	gi 2305020	121	4.35/15,689	19
160	Glycinin (<i>Glycine max</i>)	gi 18641	341	4.67/28,617	14
161	Dehydrin (<i>Glycine max</i>)	gi 2270990	135	5.46/24,108	21
162	Glycinin subunit G2 (<i>Glycine max</i>)	gi 18637	261	6.00/34,487	14
163	51 kDa seed maturation protein (<i>Glycine max</i>)	gi 414977	1056	7.00/63,520	45
164	Bowman–Birk proteinase inhibitor	gi 157833497	80	5.70/13,359	44
165	Manganese superoxide dismutase (<i>Glycine max</i>)	gi 147945633	217	6.84/26,677	17
166	Unnamed protein product (<i>Glycine max</i>)	gi 296524578	180	4.40/67,840	25
167	Late embryogenesis abundant protein (<i>Glycine max</i>)	gi 170010	920	6.68/71,808	41
168	LEA protein (<i>Glycine max</i>)	gi 1389897	529	6.61/80,306	36
169	Glutathione <i>S</i> -transferase GST 14 (<i>Glycine max</i>)	gi 11385443	317	6.31/28,063	28
170	Glycinin subunit G2 (<i>Glycine max</i>)	gi 18637	191	4.79/30,714	7
171	Unknown (<i>Glycine max</i>)	gi 255637485	206	6.24/18,580	18
172	2S albumin pre-propeptide (<i>Glycine max</i>)	gi 2305020	221	4.77/16,010	19
173	Chain A, soybean agglutinin complexed with 2,6-pentasaccharide	gi 6729836	240	5.53/35,372	32
174	Glycinin (<i>Glycine max</i>)	gi 18641	191	4.63 / 35758	12
175	Glycinin (<i>Glycine max</i>)	gi 18642	222	4.78/24,584	12
176	RecName: Full = β -conglycinin, beta chain; Flags: Precursor	gi 121282	42	6.96/56,823	1
177	RecName: Full = β -conglycinin, beta chain; Flags: Precursor	gi 121282	50	6.85/55,455	1
178	Unnamed protein product (<i>Glycine max</i>)	gi 218336143	337	6.60/51,098	31
179	Malate dehydrogenase (<i>Glycine max</i>)	gi 5929964	454	6.39/45,950	54
180	Glycinin chain A2B1a precursor; soybean	FWSYG1	165	7.45/19,314	7
181	Basic 7S globulin; <i>Glycine max</i>	BAA03681	125	7.88/15343	7
182	Glycinin A1bB2-784; <i>Glycine max</i> (soybean)	Q852U4_SOYBN	151	8.49/19,039	7
183	Glycinin chain A5A4B3 precursor; soybean	FWSYG5	272	9.17/19,693	11
184	Glycinin chain A5A4B3 precursor; soybean	FWSYG5	285	9.38/20,100	11
185	Glycinin chain A5A4B3 precursor; soybean	FWSYG6	121	9.73/21,122	8
186	Glycinin A3B4 subunit; <i>Glycine max</i> (soybean)	Q7GC77_SOYBN	318	9.88/19,314	14
187	Basic 7S globulin precursor; soybean	S06750	74	9.84/29,411	9
188	Glyceraldehyde 3-phosphate dehydrogenase; <i>Glycine max</i> (soybean)	Q2I0H4_SOYBN	92	7.24/36,815	8
189	Glycinin chain A2B1a precursor; soybean	FWSYG1	113	7.44/78,920	7
190	Sucrose-binding protein precursor related cluster	TA1389_3847	41	5.35/57,373	2
191	Glycinin precursor (contains glycinin A3 subunit, glycinin B4 subunit)	TA389_3847	32	4.55/37,255	2
192	Glycinin G1 precursor; soybean	S10851	126	7.56/38,864	3

MM molecular mass

MS/MS). A higher efficiency was obtained in the work of Brechenmacher et al. [37], who established a proteomic reference map for cells present in hair roots of soybean plants, yielding an efficiency of 57% using 2-D PAGE and MS. However, in that work, 1,165 spots were detected, which can lead to better identification.

Functional evaluation of the proteins identified

The proteins identified were classified into categories according to their biological activities [38] (Fig. 4). In our study, 50% (96 spots) of the proteins identified were related to storage function. Proteins such as glycinin and β -conglycinin and its subunits are included in this class [39]. Owing to the remarkable presence of these proteins, losses in the detection

of low-abundance proteins present in seeds of transgenic soybean are reported [39]. Kunitz trypsin inhibitors (spots 1, 25, 26, 27, and 158) act as an inhibitor of the enzyme trypsin and Bowman–Birk protease inhibitors (spots 4, 5, 6, 117, and 164), which belong to a class of protease inhibitors specific for trypsin, chymiotrypsin, and elastase [40], are other proteins within this class.

The second functional class of proteins (18%, 34 spots of the total proteins identified) is the most abundant class of proteins involved in the growth/cell division processes. This result is particularly important since a stressful condition is likely to cause growth pattern changes such as in the seed aging process [41]. Two proteins and their subclasses were identified: late embryonic abundant protein (spots 8, 48, 52, 94, 96, 97, 115, 118, 129, 167, and 168)

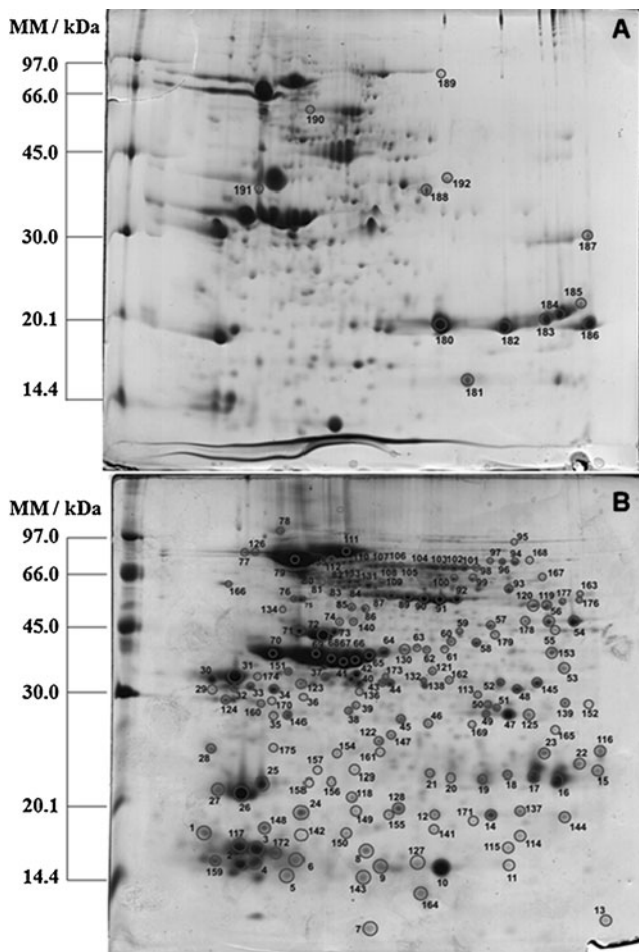
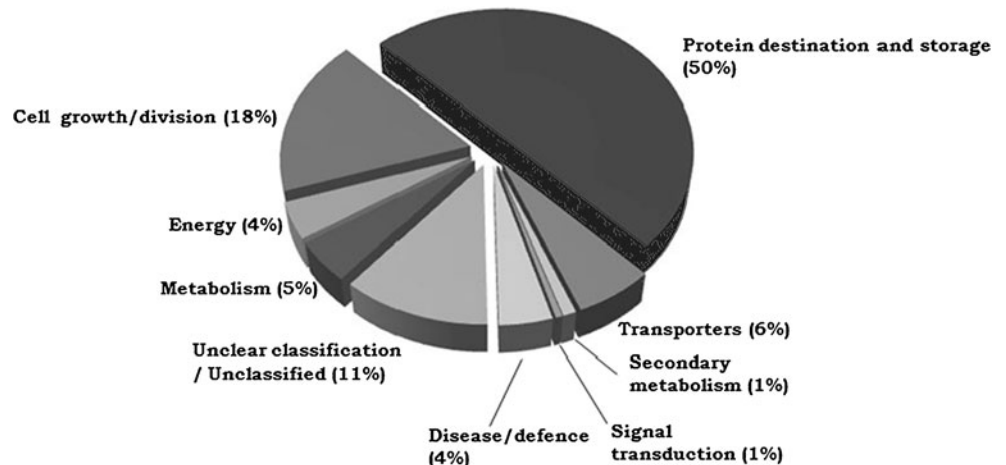


Fig. 3 Proteins identified in transgenic soybean seeds: **A** pH 3–10, 13 proteins identified, and **B** pH 4–7, 179 proteins identified. *MM* molecular mass

and seed maturation protein (14 spots). These proteins are accumulated in the last stages of embryogenesis from seed, and are related to processes of response to osmotic and water stress [42, 43].

Fig. 4 Functional distribution of proteins identified in transgenic soybean seeds using the classification described by Bevan et al. [38]. A total of 192 spots were classified



In relation to seed maturation protein, nine varieties were identified, namely, PM22 (spot 24), PM24 (spot 33), PM25 (spot 34), PM31 (spots 12 and 14), PM32 (spots 154 and 157), PM34 (spot 53), PM36 (spot 38), 35 kDa seed maturation protein (spots 58, 60, and 138), and 51 kDa seed maturation protein (spot 163). Dehydrin LEA type (spots 46, 47, 49, 50, 113, 125, 128, and 161) and the class III acidic endochitinase precursor (spot 124) were also identified. Dehydrin LEA is involved in the mechanisms of response to abiotic stress such as dehydration and low temperatures [44, 45], whereas the class III acidic endochitinase is specifically expressed during the developmental stage of the soybean seed [46].

Seventeen proteins identified (9%) are involved in metabolic/energy processes (carbohydrate metabolism and degradation of polysaccharides [43]), including agglutinin (spots 37, 41, 42, 44, and 173), triosephosphate isomerase (spot 51), glyceraldehyde 3-phosphate dehydrogenase (spots 55 and 118), cytosolic malate dehydrogenase (spot 59), cytosolic glutamine synthetase (spot 74), β -amylase (spots 84 and 131), enolase (spot 108), formate dehydrogenase (spots 119 and 120), and lipoxygenase (spot 95). The latter is involved in growth and development, resistance to pests, and plant response to injury [44], once again important processes that may also be related to stress response. It is also important to mention that seed aging could also account for some of the observations reported in this study as previously reported for soybean seeds [41].

Approximately 6% (13 spots) are related to protein transport. The sucrose-binding protein (spots 93, 98, 101, 102, 103, 104, 105, 106, 107, 108, 109, 133, and 190), the unique protein found, is responsible for the transport of sucrose during the seed developmental stage and can act to store nutrients [44].

About 4% (8 spots) correspond to proteins involved in the disease/defense category, and most spots detected belong to the alcohol dehydrogenase enzyme (spots 54, 56,

57, and 132), a zinc-ligand enzyme involved in catalytic activity of redox reactions [44]. Mn-SOD (spot 165), an antioxidant metalloenzyme acting on plant defense mechanisms against ROS [12], also belongs to this class, as do In2-1 protein (spot 39), 18.5 kDa class I heat shock protein (spot 137), and GST (spot 169), another enzyme involved in stress response. GSTs are dimeric proteins and are part of an enzyme superfamily found in all organisms and are involved primarily in detoxification mechanisms [47]. Recently, a sugarcane GST was modeled and molecularly docked to investigate the contributions of the active site residues to the binding of reduced glutathione and 1-chloro-2,4-dinitrobenzene as a way to better understand the mechanisms of interaction between the GST enzyme and its substrates. The information might help in the engineering of new GSTs with improved detoxification efficiency [48].

Nucleoside diphosphatase kinase (spot 114) was the unique enzyme related to the functional category of signal transduction. It binds ATP and magnesium and is involved in biochemical reactions of nucleotides (transfer of phosphate from ATP molecules normally for secondary substrates [44]). Isoflavone reductase enzyme homolog 2 (spots 61 and 62) has been classified in the secondary metabolism class and it is involved in the biosynthesis of isoflavones, which are a subclass of flavonoids widely known for their high concentration in soybean. These compounds also play an important role in plants during the stages of growth, development, nodulation, and defense against microorganisms and abiotic stress [49].

A total of 21 proteins were identified in the category of nonclassified proteins: Gly m Bd 30 K allergen (spots 30 and 31), allergen Gly m Bd 28 K (spots 45 and 116), hypothetical protein related (spots 121 and 139), related PreproMP73 (spot 122), glycine-rich RNA-binding protein (spots 142 and 150), and unknown (spots 140, 144, 145, 147, 148, 149, 150, 152, 153, 155, 156, 166, 171, and 178).

Identification of the enzyme CP4 EPSPS by MS

The identification of the enzyme CP4 EPSPS by 2-D PAGE was not possible owing to its position in the gel (Fig. 3) since it matches the region where very intense spots of glycinin subunits (spots 71, 72, and 73) [50] are located. Posttranslational modifications may have contributed to the

difference of the spot position in both gels, since these changes could alter the pI values and/or molar mass compared with the theoretical values [51]. Nevertheless, the enzyme CP4 EPSPS was identified using the SDS-PAGE technique and either trypsin or chymotrypsin as a cleavage enzyme. However, trypsin showed the best results in terms of score and coverage (as a percentage). This method proved to be simple and very efficient since, in a similar study reported in the literature [52], there was a need for sample prefractionation using two chromatographic columns (size exclusion and ion exchange).

To confirm the origin of the gene that was used in the genetic modification process for synthesis of the enzyme CP4 EPSPS in transgenic soybean seeds, a search of the database (NCBI) containing sequences from *Agrobacterium* sp. was performed. The results led to the enzyme being identified in this organism, confirming the fact that the gene originated from the soil bacterium *Agrobacterium* sp. The identification results from both databases can be seen in Table 3.

Identification of protein abundance changes by the DIGE technique

The 2-D DIGE technique was used to visualize the expression of protein profiles in samples from transgenic and non-transgenic soybean seeds. Three biological replicates were analyzed. A regulation factor of 1.5 or greater (50% variation) was chosen as determined by the image analysis program DeCyder 2-D, and considering statistically significant differences in expression ($p \leq 0.05$, according to the Student *t* test). The results in terms of the volume ratio using the regulation factor of 1.5 and the statistical results are presented in Table 4. The analysis of variance values were used to test for differences in standardized abundance among the samples, with the most significant results being obtained for spots 1–4, which were differentially expressed between transgenic and nontransgenic soybean seeds (see Fig. 5).

In this work, the program DeCyder 2-D version 7.0 (GE Healthcare) was used for comparative analysis of gels images. This program uses an algorithm for codetection that detects both protein spots labeled in different images but in the same gel. This codetection increases the accuracy in a comparative proteomic study; in addition, equivalent areas of each image can be compared more accurately [53,

Table 3 Results for identification of the enzyme CP4 EPSPS in transgenic soybean seeds from a general database (National Center for Biotechnological Information, NCBI) for *Glycine max* and *Agrobacterium* sp

Protein	Database	Score	NCBI access code	MM (kDa)/pI	Coverage (%)
CP4 EPSPS	NCBI (<i>Glycine max</i>)	868 (27 ^a)	gi 18266432	47,699/5.13	40
5-Enolpyruvylshikimate 3-phosphate synthase	NCBI (<i>Agrobacterium</i> sp.)	573 (53 ^a)	gi 8469107	47,673/5.13	34

^a Minimal score for identification in database

Table 4 Statistical data for the differential spots obtained with the 2-D difference gel electrophoresis technique (DIGE)

Spot	Volume ratio ^a	1-ANOVA ^b
1	1.89±0.14	0.0087
2	1.63±0.39	0.069
3	3.34±1.79	0.0096
4	1.51±0.06	0.069

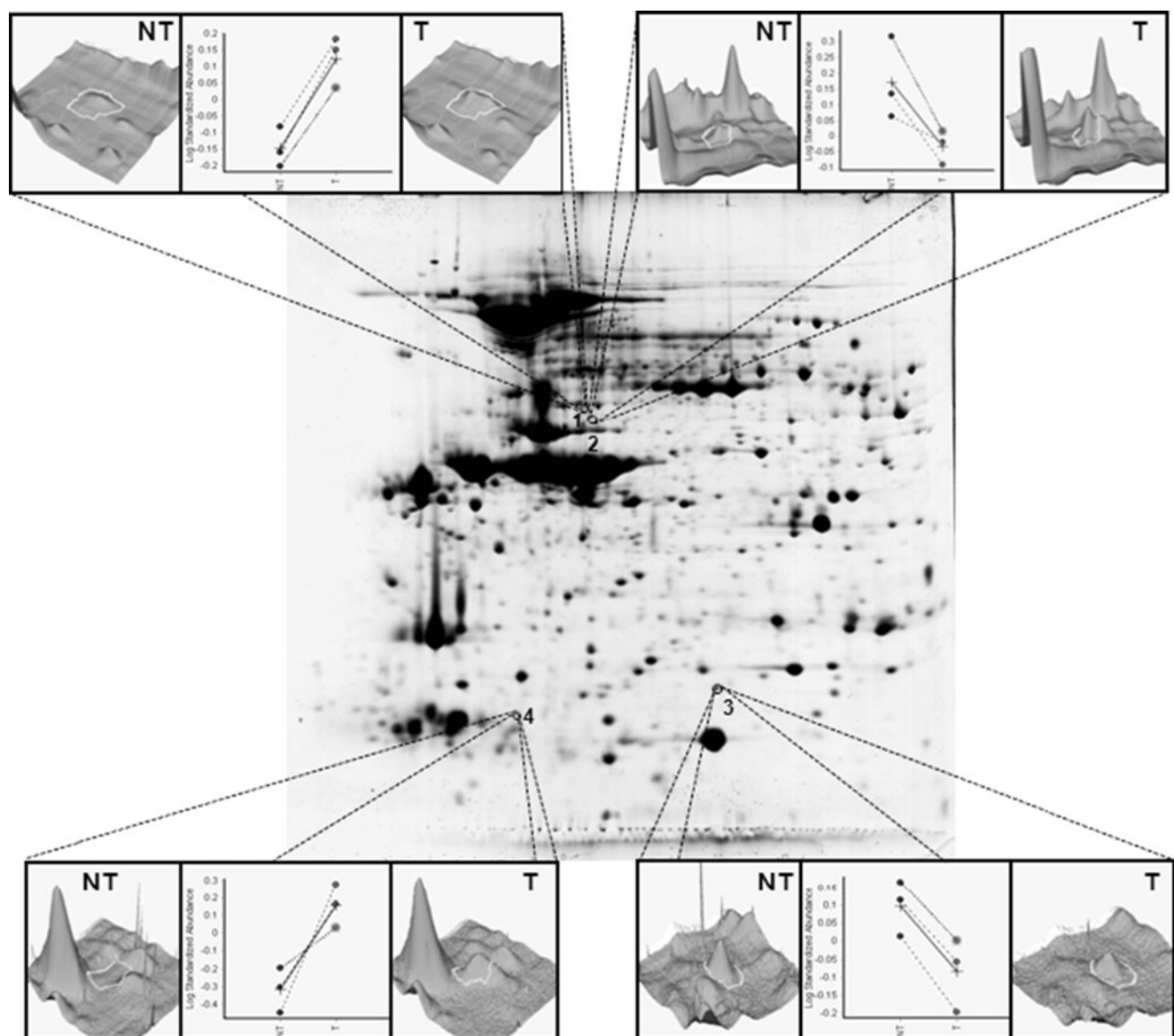
Regulation factor of 1.5

1-ANOVA: one-way analysis of variance

^a Mean ± standard deviation ($n=3$)^b Significant difference between all spots and samples

54]. Thus, the program allows the automatic detection of the spots, and does not require any manual editing, either in adding or in altering the area defining the spots, in contrast to other programs for image 2-D PAGE [53].

The advantage of this detection method is that as two samples are run on the same gel, the delimitation of areas of the spots is correlated between samples [55]. However, a possible limitation of this method of selection is that some spots may be identified incorrectly in the gel image. This can be evidenced for spot 1, where the area for the spot in nontransgenic soybean was less than the area that would be defined manually. Moreover, for spot 3, the area for the transgenic soybean could be greater, since there is a clear distinction between two spots, as shown in Fig. 5.

**Fig. 5** Spots with expression variation among samples of transgenic (*T*) and nontransgenic (*NT*) soybean seeds. Underexpressed proteins (2 and 4) and overexpressed proteins (1 and 3) are shown. The numbers are related to the identification of proteins (see also Table 5)

With respect to differential spots, two were overexpressed, being more highly expressed in transgenic soybean, and two were underexpressed, being less expressed in transgenic soybean. Thus, these four spots were selected, removed from the gel, digested with trypsin, and analyzed by ESI-QTOF MS/MS. The results of protein identification are shown in Table 5. All of the proteins identified had values of the score which were statistically significant ($p < 0.05$).

Protein spot 1 was identified as actin protein (fragment). This protein had previously been identified in the proteomic map and was classified in the storage category. This protein is involved in various types of cell motility and is widely expressed in all eukaryotic cells. Furthermore, this protein also binds to ATP and other proteins [43]. It is also noteworthy that this protein was also detected by Brandão et al. [5] working with 2-D PAGE, image analysis, and the same sample.

Protein spot 2 was identified as cytosolic glutamine synthetase and was classified in the metabolism category. It is considered as a ligand enzyme, being highly expressed in many types of roots, mainly in the capillary part. This enzyme binds ATP molecules, is responsible for the primary assimilation of ammonia in all living organisms, participates in the nitrogen fixation and in the biosynthesis of glutamic acid [43]. This is particularly important since this is a key enzyme in nitrogen metabolism, which has been shown to be affected by a range of biotic stresses and is a target of some herbicides [56–58].

Protein spot 3 is the glycinin G1 subunit. This protein, classified in the storage protein category, is a subunit of one of the most abundant storage proteins of soybean, glycinin. This protein is responsible for the nutritional, physico-chemical, and physiological characteristics of soybean seeds [59].

Protein spot 4 was identified as glycine-rich RNA-binding protein. This protein belongs to a class of RNA-binding proteins in plants and is involved in cellular response to environmental and developmental conditions. However, its relation to RNA metabolism remains unknown [43, 60]. Thus, this protein has an unknown classification.

Correlation between enzymatic activities and differential proteins found

Exposure of an organism to specific conditions that are different from its normal environment can increase the production of ROS. To protect themselves against these toxic oxygen intermediates (e.g., H_2O_2 , $OH^\bullet$, $O_2^{\bullet-}$, among others), cells and their organelles employ antioxidant defense systems (enzymes such as CAT, SOD, GR, GPX, among others) and nonenzymatic antioxidants. Additionally, lipid peroxidation is considered as the most damaging process known to occur in every living organism [9]. Our results showed higher levels of MDA production in the transgenic soybean seeds than in the nontransgenic ones, indicating a differential condition of oxidative stress for both genotypes. Even though there is no information in the literature regarding ROS production and genetic modification, particularly when seeds are concerned, it is well known that plants are highly adapted to respond quickly to biotic or abiotic stresses by altering their gene expression and metabolism as a result of cell signaling, which may be mediated by ROS [61]. In the case of this work, there was a previous genetic modification, achieved by inserting an exogenous gene, which conferred the plant with resistance to glyphosate. In plants, posttranscriptional control mechanisms are growing in importance, and proteins involved in the RNA processing and alternative splicing, RNA trans-

Table 5 Results obtained for the identification of proteins with differences in expression between the transgenic and nontransgenic soybean seeds using 2-D DIGE

Spot	Protein	Access code	MM(kDa)/pI	Score	Coverage (%)	Peptides sequenced
1	Actin (fragment) (<i>Glycine max</i>)	Q96445_SOYBN (MSDB)	37,236/5.57	65 (35 ^a)	9	K.SYELPDGQVITIGDER.F K.LSYIALDYEQELETAR.T
2	Cytosolic glutamine synthetase (<i>Cucumis melo</i>)	gi 113171384 (NCBI)	39,338/5.23	198 (54 ^a)	13	K.GYFEDR.R R.DIVDAHVK.A R.EEGGYEVIK.K K.EHIAAYGEGNER.R K.SMREEGGYEVIK.K
3	Glycinin subunit G1 (<i>Glycine max</i>)	gi 225651 (NCBI)	17,928/6.00	91 (54 ^a)	4	R.VFDGELQEGR.V R.SQSDNFEYVSFK.T
4	Glycine-rich RNA-binding protein (<i>Glycine max</i>)	gi 5726567 (NCBI)	15,894/6.58	30 (27 ^a)	6	R.NITVNEAQRS.G

MSBD Mass Spectrometry Data Bank

^a Minimal score for identification

port, messenger RNA (mRNA) translation, mRNA stability, and mRNA silencing mechanisms have been shown to be required for normal plant development and the responses of plants to altered environments [62–64]. In our case, just the glycine-rich RNA-binding protein was differentially found after DIGE analysis, and this protein correlated to ROS production according to different articles [61, 65].

As already mentioned in the previous section, the cytosolic glutamine synthetase (another differentially expressed protein found through 2-D DIGE analysis) is involved in nitrogen fixation and is a target of herbicides. In a recent article [66] it was demonstrated that oxidative stress can also control the expression of nitrogen-metabolism genes. Then, it is easy to show that cytosolic glutamine synthetase can be altered because of the oxidative stress observed in the transgenic soybean line, mainly when higher levels of H_2O_2 are present [66], which is correlated to higher activities of CAT.

It is also important to mention that even though the expression of genes and posttranslational alterations may occur as a result of the response of the cell to any stressful situation to which it is subjected, some of the antioxidant enzymes, including those tested in our study, may have simply undergone a change in their activity, and may not necessarily be due to changes in *de novo* protein synthesis, which would not result in any specific changes in the proteomic analyses performed. A more detailed kinetic study with these enzymes in the soybean genotypes may help to further elucidate these aspects and the responses observed.

Finally, as far as we are aware, there is no report in the literature concerning actin or glycinin G1 subunit and ROS production, which might open up a new possibility for future studies.

Conclusions

The results obtained in this work indicate that the initial hypothesis formulated, that the genetic modification itself could be a stress factor, provoking changes in the activity of some enzymes as well as alterations in the soybean seed proteome, makes sense. MDA production, which is an indicator of lipid peroxidation and oxidative stress, was higher in transgenic soybean seeds than in nontransgenic seeds. Furthermore, higher enzyme activities for APX, GR, and CAT were found in transgenic seeds. These results together suggest a higher level of stress in the transgenic soybean seeds, even when no herbicides were used, since the gene conferring resistance to herbicide is inserted into the DNA of the soybean seeds. This fact now explains some results of our research group previously reported in the literature since the transgenic seeds have greater ability to take up metals from the soil and they have higher metal

bioaccessibility than nontransgenic soybean seeds [5–7]. Moreover, Fe-SOD isoenzymes appear to be more significantly reduced in transgenic seeds.

In terms of protein analysis, after separation of the soybean seed protein by 2-D PAGE, a successful identification by MALDI Q-TOF-MS of approximately 200 proteins was possible, with about 50% of them being related to storage functions. Additionally, the enzyme involved in the genetic modification (CP4 EPSPS) was also easily and successfully identified by ESI-QTOF MS/MS and using trypsin as a cleavage enzyme. Finally, among 192 proteins identified, four of them (actin fragment, cytosolic glutamine synthetase, glycinin subunit G1, and glycine-rich RNA-binding protein) were shown to be differentially expressed through 2-D DIGE analysis and by applying a regulation factor of 1.5 or greater.

If one takes into account the correlation between the differentially expressed proteins and the enzyme activities found, it is not difficult to rationalize that the genetic modification induced an oxidative stress condition in those seeds.

Finally, from our results the organism studied is apparently searching for a new equilibrium in a way that the genetic modification could be producing alterations, in a cascade manner, to maintain its metabolism. However, plants with the ability to control the cellular ROS level may be particularly useful for future use under harsh environmental conditions. This fact is moving our research group to work with genetically modified organisms, and some other studies are in progress in our laboratory to better understand the specific aspects of the metabolism and to identify key proteins that may eventually be the subject of genetic modification so that soybean, and eventually other plant species, can exhibit higher tolerances to xenobiotics.

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References

1. SoyStats, The American Soybean Association. <http://www.soystats.com>. Accessed 16 Jun 2011
2. Monsanto do Brasil. <http://www.monsanto.com.br>. Accessed 16 Jun 2011
3. Uzogara SG (2000) *Biotechnol Adv* 18:179–206
4. Kim Y, Choi SJ, Lee H, Moon TW (2006) *J Microbiol Biotechnol* 16:25–31

5. Brandão AR, Barbosa HS, Arruda MAZ (2010) *J Proteomics* 73:1433–1440
6. Sussulini A, Souza GHMF, Eberlin MN, Arruda MAZ (2007) *J Anal At Spectrom* 22:1501–1506
7. Mataveli LRV, Pohl P, Mounicou S, Arruda MAZ, Szpunnar J (2010) *Metallomics* 2:800–805
8. Foyer CH, Noctor G (2005) *Plant Cell* 17:1866–1875
9. Gill SS, Tuteja N (2010) *Plant Physiol Biochem* 48:909–930
10. Arruda MAZ, Azevedo RA (2009) *Ann Appl Biol* 155:301–307
11. Gill SS, Khan NA, Anjum NA, Tuteja N (2011) *Plant Stress* 5: 1–23
12. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) *Trends Plant Sci* 9:490–498
13. Gratão PL, Polle A, Lea PJ, Azevedo RA (2005) *Funct Plant Biol* 32:481–494
14. Gratão PL, Monteiro CC, Antunes AM, Peres LEP, Azevedo RA (2008) *Ann Appl Biol* 153:321–333
15. Dalton TP, Shertzer HG, Puga A (1999) *Annu Rev Pharmacol Toxicol* 39:67–101
16. Marouga R, David S, Hawkins E (2005) *Anal Bioanal Chem* 382:669–678
17. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B (2007) *Anal Bioanal Chem* 389:1017–1031
18. Moldes CA, Médiçi LO, Abrahão OS, Tsai SM, Azevedo RA (2008) *Acta Physiol Plant* 30:469–479
19. Azevedo RA, Alas RM, Smith RJ, Lea PJ (1998) *Physiol Plant* 104:280–292
20. Nakano Y, Asada K (1981) *Plant Cell Physiol* 22:867–880
21. Gomes-Junior RA, Moldes CA, Delite FS, Gratão PL, Mazzafera P, Lea PJ, Azevedo RA (2006) *Plant Physiol Biochem* 44:420–429
22. Giannopolis CN, Reis SK (1977) *Plant Physiol* 59:309–314
23. Gomes-Junior RA, Gratão PL, Gaziola SA, Mazzafera P, Lea PJ, Azevedo RA (2007) *Funct Plant Biol* 34:449–456
24. Laemmli UK (1970) *Nature* 227:680–685
25. Sussulini A, Garcia JS, Mescio MF, Moraes DP, Flores EMM, Pérez CA, Arruda MAZ (2007) *Microchim Acta* 158:173–180
26. Bradford MM (1976) *Anal Biochem* 72:248–254
27. Berkelman T, Stenstedt T (1998) *2-D electrophoresis - principles and methods*. Amersham Biosciences, Uppsala
28. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004) *Electrophoresis* 25:1327–1333
29. Sergiev IG, Alexieva VS, Ivanov SV, Moskova II, Karanov EN (2006) *Pestic Biochem Physiol* 85:139–146
30. Ferreira RR, Fornazier RF, Vitória AP, Lea PJ, Azevedo RA (2002) *J Plant Nutr* 25:327–342
31. Yu W, Zhang R, Li R, Guo S (2007) *Plant Sci* 172:497–504
32. Donahue JL, Okpodu CM, Cramer CL, Grabau EA, Alscher AG (1997) *Plant Physiol* 113:249–257
33. Geoffroy L, Teisseire H, Coudrchet M, Vernet G (2002) *Pestic Biochem Physiol* 72:178–185
34. Kendall AC, Keys AJ, Turner JC, Lea PJ, Mifflin BJ (1983) *Planta* 159:505–511
35. Holtman WL, Heistek JC, Mattern KA, Bakhuizen R, Douma AC (1994) *Plant Sci* 99:43–53
36. Xu C, Garrett WM, Sullivan J, Caperna T, Natarajan SS (2006) *Phytochemistry* 67:2431–2440
37. Brechenmacher L, Lee J, Sachdev S, Song Z, Nguyen THN, Joshi T, Oehrle N, Libault M, Mooney B, Xu D, Cooper B, Stacey G (2009) *Plant Physiol* 149:670–682
38. Bevan M, Bancroft I, Bent E, Love K, Goodman H, Dean C, Bergkamp R, Dirkse W, Van Staveren M, Stiekema W, Drost L, Ridley P, Hudson HA, Patel K, Murphy G, Piffanelli P, Wedler H, Wedler E, Wambutt R, Weizenegger T, Pohl TM, Terry N, Gielen J, Villarroel R, De Clerck R, Montagu MV, Lecharny A, Auborg S, Gy I, Kreis M, Lao N, Kavanagh T, Hempel S, Kotter P, Entian KD, Rieger M, Schaeffer M, Funk B, Mueller-Auer S, Silvey M, James R, Montfort A, Pons A, Puigdomenech P, Douka A, Voukelatou E, Milioni D, Hatzopoulos P, Piravandi E, Obermaier B, Hilbert H, Düsterhöft A, Moores T, Jones JDG, Eneva T, Palme K, Benes V, Rechman S, Ansorge W, Cooke R, Berger C, Delseny M, Voet M, Volckaert G, Mewes HW, Klosterman S, Schueller C, Chalwatzis N (1998) *Nature* 391:485–488
39. Natarajan SS, Xu C, Bae H, Caperna TJ, Garret W (2006) *J Agric Food Chem* 54:3114–3120
40. Krishnan HB, Oehrle NW, Natarajan SS (2009) *Proteomics* 9:3174–3188
41. Stewart RRC, Bewley JD (1980) *Plant Physiol* 65:245–248
42. Krishnan HB (2001) *Plant Sci* 160:979–986
43. Wang W, Vinocur B, Altman A (2003) *Planta* 218:1–14
44. ExPASy proteomics server. <http://www.expasy.org>. Accessed 10 Jun 2011
45. Campbell SA, Close T (1997) *New Phytol* 137:61–74
46. Yeboah NA, Arahira M, Nong VH, Zhang D, Kadokura K, Watanabe A, Fukazawa C (1998) *Plant Mol Biol* 36:407–415
47. Dixon DP, Skipsey M, Edwards R (2010) *Phytochemistry* 71: 338–350
48. Ghelfi A, Gaziola SA, Cia MC, Chabregas SM, Falco MC, Kuser-Falcão PR, Azevedo RA (2011) *Ann Appl Biol* 159:267–280
49. Alam I, Lee DG, Kim KH, Park CH, Sharmin SA, Lee H, Oh KW, Yun BW, Lee BH (2010) *J Biosci* 35:49–62
50. Kim VH, Choi SJ, Lee HA, Moon TW (2006) *J Microbiol Biotechnol* 16:25–31
51. Sickmann A, Marcus K, Schäfer H, Dörje EB, Lehr S, Herkner A, Suer S, Bahr I, Meyer HE (2001) *Electrophoresis* 22:1669–1676
52. Ocana MF, Fraser PD, Patel RKP, Halket JM, Bramley PM (2007) *Rapid Commun Mass Spectrom* 21:319–328
53. Heinemeyer J, Scheibe B, Schmitz UK, Braun HP (2009) *J Proteomics* 72:539–544
54. Eravci M, Fuxius S, Broedel O, Weist SE, Mansmann U, Schluter H, Tiemann J, Baumgartner A (2007) *Proteomics* 7:513–523
55. Arruda SCC, Barbosa HS, Azevedo RA, Arruda MAZ (2011) *Analyst*. doi:10.1039/c1an15513j
56. Shin JS, Kim KM, Lee DJ, Burgos NR, Kuk YI (2011) *Field Crop Res* 121:324–332
57. Forde BC, Lea PJ (2007) *J Exp Bot* 58:2339–2358
58. Lea PJ, Mifflin BJ (2003) *Plant Physiol Biochem* 41:555–564
59. Thanh VH, Shibasaki K (1978) *J Agric Food Chem* 26:692–695
60. Kim JY, Kim WY, Kwak KJ, Oh SH, Han YS, Kang H (2010) *J Exp Bot* 61:2317–2325
61. Schmidt F, Marnef A, Cheung MK, Wilson I, Hancock J, Staiger D, Ladomery M (2010) *Mol Biol Rep* 37:839–845
62. Kazan K (2003) *Trends Plant Sci* 8:468–471
63. Cheng Y, Chen X (2004) *Curr Opin Plant Biol* 7:20–25
64. Wang QL, Li ZH (2007) *Plant Biosci* 12:3975–3982
65. Kim YO, Pan S, Jung CH, Kang H (2007) *Plant Cell Physiol* 48:1170–1181
66. Pageau K, Reisdorf-Cren M, Morot-Gaudry JF, Masclaux-Daubresse C (2006) *J Exp Biol* 57:547–557