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PROTEOMIC ANALYSIS OF AMARANTH (*AMARANTHUS HYPOCHONDRIACUS* L.) LEAVES UNDER DROUGHT STRESS

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Amaranth (*Amaranthus hypochondriacus* L.) is a plant that produces seed with high protein content, is rich on nutraceutical compounds, and can grow under environmental conditions where most of the basic crops are not able to develop. But little is know about the amaranth stress-responsive genes/proteins. The aim of this work was to apply the comparative proteomics approach to study the differential expression of amaranth leaf proteins under drought stress. However, the protein extraction from amaranth tissues is difficult as a result of high endogenous concentrations of interfering compounds; we have made some modifications of the classical trichloroacetic acid–acetone precipitation method to improve the quantity and quality of extracted proteins. Satisfactory and reproducible two-dimensional electrophoresis protein profiles were obtained; the method was also tested for*Agave tequilana* and *Opuntia* spp., two more examples of plants that are tolerant to drought stress. Drought-responsive proteins in amaranth leaves were identified by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). The upregulated proteins identified included chloroplast chaperonins involved in refolding and protein complexes protection. Downregulated proteins include Rubisco large subunit, cytochrome *b6f*, oxygen evolving complexes, and the ascorbate peroxidase mitochondrial. The results have shown that chloroplasts and mitochondria may play a central role in amaranth adaptation to abiotic stress, and further studies should be done at the subcellular level.

Keywords: abiotic stress, agave, LC/ESI-MS/MS, nonmodel plants, *Opuntia* spp., recalcitrant plants.

Introduction

Drought and salinity are the most common problems in agriculture worldwide. Plant survival and hence crop productivity depend greatly on the ability to adapt to, respond to, and tolerate variable environmental conditions (Rossignol et al. 2006). When plants are subjected to stress, genes are turned on or off, with the resulting changes of the transcripts levels and their translated proteins responsible for the plant adjustments in the adaptation to the environmental conditions. Amaranth (*Amaranthus hypochondriacus* L.) produces seeds with high-quality protein, and according to recent reports, the seeds contain nutraceutical compounds and peptides with anticarcinogenic and antihypertensive activities (Silva-Sánchez et al. 2008; Barba de la Rosa et al. 2009). In areas of subtropical and tropical Asia, Africa, and Central America, *Amaranthus* ssp. are grown as green vegetables. The leaves are especially high in calcium and, on a fresh-weight basis, contain more fiber, niacin, and vitamin C than spinach (Abbot and Campbell 1982). The plant can grow at high temperatures with small quantities of water, and its cultivation has been extended worldwide (Sellers et al. 2003; Gimplinger et al. 2008).

Proteomics approach has a great potential for research using nonmodel plant species, where no genomic sequencing data are available and the identification of candidate genes can be difficult because of the divergence between orthologous genes. However, protein sequences are better conserved and make possible the identification of proteins in nonsequenced organisms (Carpentier et al. 2008). Not only is the recent progress in crop proteomics giving clues about nutritional value, but also major emphasis is taking on crop responses to abiotic stresses (Salekdeh and Komatsu 2007). Protein sample preparation is the most critical step for any proteomic study based on two-dimensional electrophoresis (2-DE) analyses (Görg et al. 2004), and researchers have turned special attention to the development of optimal protocols for 2-DE and proteomic analysis of different recalcitrant plant tissues (Giavalisco et al. 2003; Saravanan and Rose 2004; Carpentier et al. 2005; Song et al. 2006; Xie et al. 2007). Some of these protocols are known as standard or universal protocols (Wang et al. 2006), but they are not as useful for plants that have not been tested previously, where the best choice is to develop a specific optimized protocol rather than applying a given universal protocol (Wang et al. 2007; Gómez-Vidal et al. 2008; Sheoran et al. 2009).

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The aim of this work was to identify proteins in response to drought stress in amaranth leaves. We have optimized a protocol for amaranth leaf protein extraction; the protocol was also useful for *Agave tequilana* Weber var. azul and *Opuntia* spp., two examples of plants tolerant to abiotic stress with high biotechnological interest but which also contain different compounds that interfere with protein extraction. Amaranth, agave, and nopal could play an important role in the search of novel proteins/genes related to abiotic stress.

Material and Methods

Plants

Amaranth (*Amaranthus hypochondriacus* L.) cv. Nutrisol leaves were harvested from 45-d-old plants. Leaves were frozen and powdered under liquid nitrogen using a mortar and pestle. Six-month-old agave (*Agave tequilana* Weber var. azul) plants were kindly donated by the Herradura Company, and the leaves were frozen and milled. Two wild types of nopal (*Opuntia* spp.), locally named tapon morado and tempranillo, were collected from the Mexican High Plateau. The young cladodes were cleaned of spines, cut into small pieces, and freeze-dried. The dried plant was pulverized and sieved through a 100-mesh screen.

Amaranth Plants Drought Stress Treatment

Amaranth (*Amaranthus hypochondriacus* L.) cv. Nutrisol seeds were germinated on soil for horticulture (Sunshine Premix 3, Sun Gro Horticulture, Bellevue, WA). After germination, seedlings were transferred into 2000-mL pots with the same soil and grown in a greenhouse at 30°/27°C day/night temperatures with a 12L : 12D photoperiod for 29 d. Soil water content was monitored in the afternoon (4:00 p.m.) daily and maintained in humidity levels ~30% (Hydrosense, Campbell Scientific). Drought stress was initiated on day 22 by cessation of watering. On day 29, leaves were harvested from control and drought-stressed plants in parallel; leaves were flash frozen in liquid nitrogen and stored at −80°C until protein extraction. Leaf relative water content (RWC) was measured according to Barrs and Weatherley (1962) and calculated as follows:

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100.$$

Protein Extraction Procedures

The classic trichloroacetic acid–acetone (TCA-C) extraction protocol described by Damerval et al. (1986) was tested. Three grams of the powdered plant tissue were mixed with 10 mL of cold acetone containing 10% TCA and 0.07% 2-mercaptoethanol (2-ME). The suspension was sonicated (GE-505, Ultrasonic Processor) for 15 min at 4°C and centrifuged for 2 min at 3,000 *g* (Super T21, Sorvall), and the supernatant was incubated at −20°C for 2 h and then centrifuged at 10,000 *g* for 20 min. The protein pellet was resuspended and rinsed with ~1 mL of cold acetone containing 0.07% 2-ME to remove pigments and lipids. Washes were repeated until the pellet was colorless.

The TCA-C method was improved with the addition of acetone-ethanol washes (TCA-E protocol). Three grams of plant tissue were mixed with cold acetone containing 10% TCA and 0.07% 2-ME (1 mL for 0.3 g of tissue). The mixture was sonicated, centrifuged, and incubated at −20°C as for the TCA-C protocol. The pellet was washed with 1 mL of cold acetone containing 0.07% 2-ME, and one final wash was done with cold absolute ethanol.

In the third protocol, named TCA-A, 3 g of plant tissue were mixed with 4 volumes of ice-cold acetone containing 10% TCA, 1% PVPP, and 2% 2-ME (Saravanan and Rose 2004; Yao et al. 2006). The mixture was sonicated for 15 min and centrifuged as described above. The supernatant was incubated at −20°C for 2 h, and protein was pelleted by centrifugation at 13,000 *g* for 20 min at 4°C. The pellet was washed two times with 1 mL of cold acetone containing 0.07% 2-ME, once with cold absolute methanol, and once with cold acetone. Protein concentration was determined using Protein Assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard, according to the manufacturer's instructions.

Two-Dimensional Polyacrylamide Gel Electrophoresis

The protein samples obtained from the different protocols described above were resuspended in rehydration buffer: 8 M urea, 2% CHAPS (3-[3-(chloroamidopropyl)dimethylammonio]-1-propanesulfonate), 0.002% bromophenol blue, 0.28% DTT (dithiothreitol), and 0.5% w/v IPG buffer. Protein samples (350 μg for amaranth and agave leaves) were loaded onto an IPG strip holder with 13-cm linear gradient IPG strips, pH 3–10 or pH 4–7 (GE Healthcare). *Opuntia* spp. cladodes protein samples (25 μg) were loaded onto 7-cm pH 3–10 linear gradient IPG strips. The strips were rehydrated for 14–16 h at room temperature. Focusing was carried out in an Ettan IPGphor system (GE Healthcare) at a constant 50 mA per strip at 20°C, with the following program for 7-cm strips: (1) 500 V gradient until 0.01 kVh, (2) 4000 V gradient until 5.6 kVh, and (3) hold at 5000 V until 2.5 kVh. For 13-cm strips, the program was (1) 500 V gradient until 0.01 kVh, (2) 4000 V gradient until 5.6 kVh, and (3) hold at 8000 V until 15.2 kVh. After isoelectrofocusing (IEF), the IPG strips were immediately equilibrated for 15 min with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 0.002% bromophenol blue) containing 1% w/v DTT. The strips were then transferred to 13% SDS-PAGE gels for two-dimensional electrophoresis with a Hoefer SE 600 Ruby Cell (GE Healthcare) for 13-cm strips or to a Bio-Rad mini-protean III apparatus (Bio-Rad) for 7-cm strips, using SDS electrophoresis buffer (25 mM Tris pH 8.3, 192 mM glycine, and 0.1% SDS) and resolved at 20 mA/gel until the dye (bromophenol blue) reached the bottom of the gels. Two-dimensional electrophoresis gels were stained with Sypro Ruby (Molecular Probes) or Coomassie brilliant blue R-250.

The stained gels were scanned with the Gel Doc (Bio-Rad), 16-bit TIF images were analyzed, and spot detection was carried out with PDQuest (ver. 7.2.0; Bio-Rad). All gels were

produced in triplicate. Gel image analysis of control and drought-stressed amaranth plants included spot detection, spot measurement, background subtraction, and spot matching. To correct the variability and to reflect quantitative variation, the spot volumes were normalized as the percentage of the total volume in all of the spots in the gel. The molecular masses of proteins on gels were determined by coelectrophoresis of molecular mass standards, and the isoelectric point of proteins was determined by migration of protein spots on 13-cm IPG linear gradient strips (pH 4–7). Data analysis of differential expressed proteins was performed by one-way ANOVA (Statgraphics Plus, ver. 5.0); the effect of the treatment was significant when $P < 0.05$.

Liquid Chromatography Tandem Mass Spectrometry

The protein spots were carefully excised from the Coomassie stained gels, distained, washed, digested with modified porcine trypsin (Promega, Madison, WI), and extracted as described previously (Xolalpa et al. 2007). The volume of the extracts was reduced by evaporation in a vacuum centrifuge at ambient temperature and then adjusted to 20 mL with 1% formic acid.

Mass spectrometric analysis was carried out on a 3200 Q TRAP hybrid tandem mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario), equipped with a nanoelectrospray ion source (NanoSpray II) and a MicroIonSpray II head. The instrument was coupled online to a nanoAcquity Ultra Performance LC system supplied by Waters México (Waters, Milford, MA). Mass calibration of the hybrid triple quadrupole linear ion trap spectrometer was done with polypropylene glycol standard solutions. The instrument was then tuned and tested using [Glu1]-fibrinopeptide B (Sigma). Samples were desalted by injection onto a Symmetry C_{18} UPLC trapping column (5 μm, 180 μm $\times$ 20 mm; Waters) and washed with 0.1% formic acid in Milli Q water at a flow rate of 15 μL/min. After 3 min, the trap column was switched in line with the analytical column. Peptides were separated on a BEH C_{18} UPLC column (1.7 μm, 75 μm $\times$ 100 mm; Waters) equilibrated with 2% acetonitrile/0.1% formic acid using a linear gradient to 0.1% formic acid/70% acetonitrile over a 60-min period at a flow rate of 0.25 μL/min. Spectra were acquired in automated mode using information-dependent acquisition. Precursor ions were selected in Q1 using the enhanced MS mode (EMS) as a survey scan. The scan range for EMS was set at m/z 300–1500 and 4000 amu/s, with an ion spray voltage of +2.2 kV applied to a Picotip emitter FS150-20-10-N (New Objective, Woburn, MA). The interface heater for desolvation was held at 150°C. The survey scan was followed by an enhanced resolution scan (ER) of the three most intense ions at the low speed of 250 amu/s over a narrow (30 amu) mass range to determine the ion charge states. Two enhanced product ion scans (EPI) of the three most intensive peptide signals were performed at 4000 amu/s. The precursor ions were fragmented by collisionally activated dissociation (CAD) in the Q2 collision cell. Collision voltages were automatically adjusted on the basis of the ion charge state and mass using rolling collision energy. The fragment ions generated were captured and mass analyzed in the Q3 linear ion trap.

Data interpretation and protein identification were performed from the MS/MS spectra data sets using the MASCOT search algorithm (ver. 1.6b9; Matrix Science, London; available at http://www.matrixscience.com). Searches were conducted using the *Viridiplantae* subset of the National Center for Biotechnology Information nonredundant database (http://www.ncbi.nih.gov). Trypsin was used as the specific protease, and one missed cleavage was allowed with tolerances of 0.5 Da for the precursor and 0.3 Da for the fragment ion masses.

Results and Discussion

Total Protein Extraction and 2-DE Patterns

Yield of total protein extracted from amaranth leaves varied according to the method used. The TCA-A method gave the highest protein yields (0.99 $\pm$ 0.03 mg protein/g fresh tissue). TCA-C and TCA-E gave similar values (0.11 $\pm$ 0.03 mg protein/g fresh tissue. Equal amounts of the protein extracted from each of the three methods were separated by 2-DE (fig. 1). The 2-DE profiles of amaranth leaf proteins using the classic method (TCA-C) showed poor quality of electrophoretic patterns (fig. 1*a*). Vertical streaking was observed, and the average number of protein spots observed was 319 spots, with few spots with a molecular mass greater than 50 kDa. With the TCA-E method, the protein profile was somewhat improved, but there was still some horizontal streaking. The protein spots increased to 384 and were distributed all along the pH gradient, but only a few spots with a molecular mass greater than 50 kDa were observed (fig. 1*b*). The TCA-A protocol, which included polyvinylpolypyrrolidone (PVPP), greatly improved the quality of gels, with an increasing protein spot count to 453 and with improved resolution of spots above 50 kDa (fig. 1*c*). Basically, with the three methods tested, the 2-DE amaranth leaf profiles are the same, with the only changes being the resolution and number of spots as a result of the increased gel resolution. Sheoran et al. (2009) described differences on protein spots depending of the tested method, but Natarajan et al. (2005) reported only few differences from soybean extracts using different methods, and Saravan and Rose (2004) reported large differences in 2-DE spot patterns in tomato samples. The contrary results could be mainly due to the nature of the sample: it is well known that glycosylations and other posttraductional modifications affect the protein solubility, size, and charge under different extraction conditions (Saravan and Rose 2004). In amaranth samples, PVPP seems to help to extract proteins of high molecular mass.

Proteomic analysis is becoming an important tool for protein characterization of nonmodel plants (Carpentier et al. 2008). We tested the TCA-A protocol for total protein extraction from *Agave tequilana* Weber var. azul leaves and *Opuntia* spp. cladodes. Agave and nopal are two examples of plants that contain several interfering compounds and plants of high biotechnological potential as amaranth. The protein extraction from agave leaves using the TCA-A protocol resulted in 2-DE gels of good quality, and no background was observed. Using 13-cm strips at pH 3–10, there was a count of 269 spots

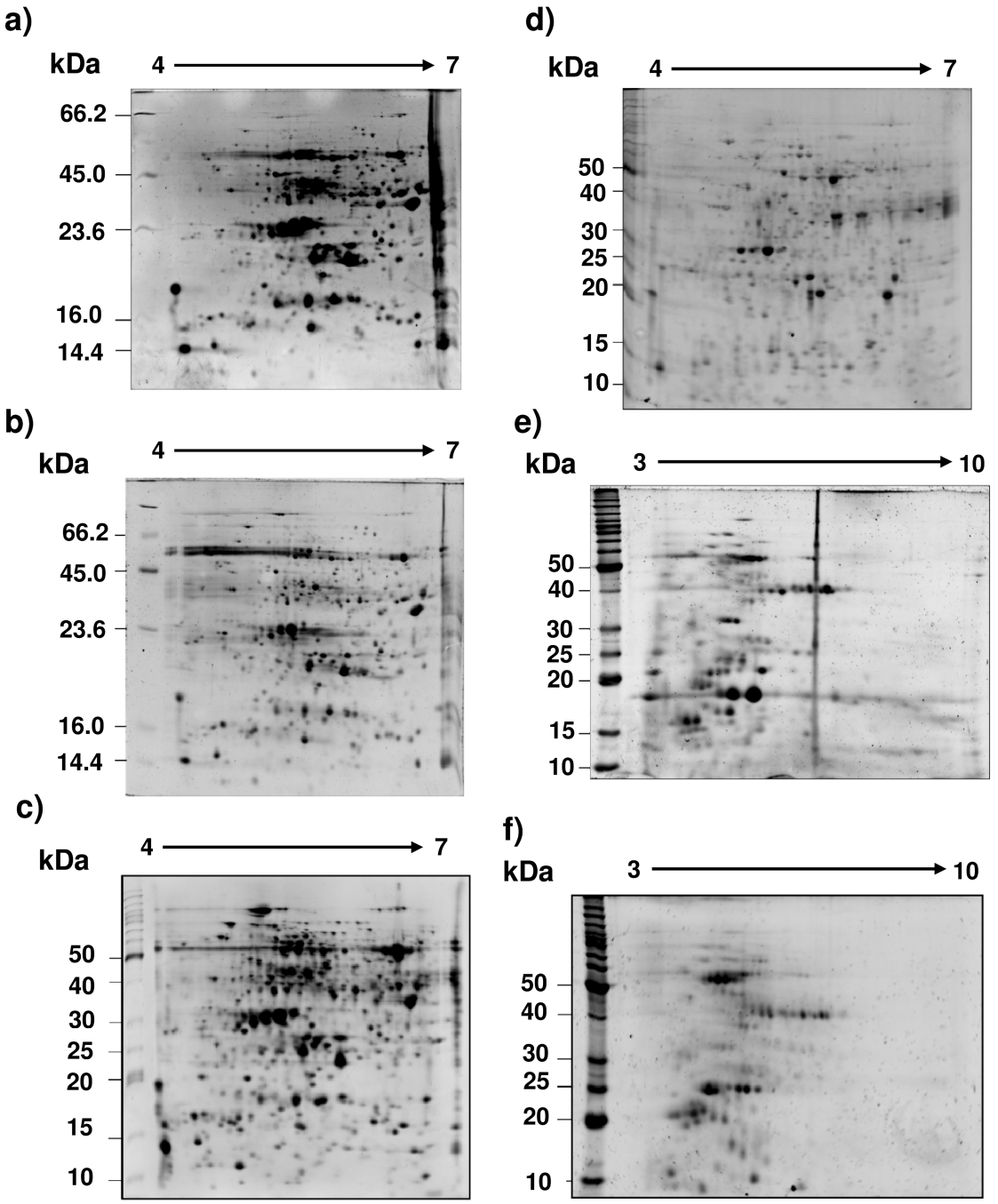

Fig. 1 Two-dimensional electrophoresis (2-DE) of total proteins from amaranth leaves extracted with different protocols: TCA-C, traditional TCA method (*a*); TCA-E, based on TCA-C with ethanol washes (*b*); TCA-A, based on PVPP extraction with methanol washes and cold acetone (*c*). The TCA-A method was tested in *Agave tequilana* leaves (*d*), *Opuntia* spp. tapon morado cladodes (*e*), and *Opuntia* spp. tempranillo cladodes (*f*).

(figure not shown), where the majority of spots were located in the pH range of 4–7. The 2-DE patterns of agave in the strips in the range of pH 4–7 gave a count of 369 protein spots (fig. 1*d*). This is mainly due to the fact that in a narrow pH range, more proteins of close p*I* could be separated. The 2-DE gel pattern for the *Opuntia* tapon morado sample was also of good quality, with 118 protein spots counted (fig. 1*e*), while in the *Opuntia* tempranillo sample 85 protein spots were counted (fig. 1*f*). The smaller number of spots resolved in *Opuntia* spp. samples is mainly due to the smaller amount of protein present in cladodes. Comparing the 2-DE profiles from the two *Opuntia* spp. samples, clear differences were found, and these patterns could be used as biomarkers for sample identification.

Comparative Proteomics of Amaranth Leaf Proteins under Drought Stress

The drought stress in amaranth was done by stopping the watering; soil water content decreased from levels of 16% ± 2% to 6% ± 1%. In this stage, the leaves of stressed plants started to roll. On day 29, the soil water content decreased to 4% ± 1%. The RWC declined from 95% in control plants to 45% in drought-stressed plants. When the plants were rewatered, the RWC in leaves returned to 90% in 5 h. The rapid recovery observed on amaranth after severe drought conditions is a distinctive characteristic of drought-tolerant plants. This is important to analyze the protein changes under abiotic stress, and comparative proteomics is the tool used for the analysis of the changes of plants under different environmental conditions (Salekdeh et al. 2002; Hajheidari et al. 2005; Salekdeh and Komatsu 2007; Xu and Huang 2008). We applied the comparative proteomics approach for the study of protein changes in amaranth leaves when the plant is grown under severe drought stress.

Leaf samples from control plants and stressed plants were taken, and total protein was extracted using the TCA-A method; protein profiles were observed by 2-DE gels. Digital image analysis of 2-DE gels profiles (p*I* range 4–7) showed that 453 spots were reproducibly detected. Figure 2 shows the amaranth leaves 2-DE pattern, and zoomed images compare the protein spots between the control (C) and the drought (D) treatment. The histograms in figure 3 show the changes in the amount of protein from the 22 differentially expressed proteins. Those spots were excised and tryptic digested, and peptides were characterized by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). From these, 17 proteins spots (77%) were successfully identified (table 1), with a significant score greater than 44 indicating the identity (homology at $P < 0.05$), while in five cases (23%) the identification was not possible (spots 5, 8, 9, 17, and 22 in fig. 2).

Identification of Drought-Responsive Proteins in Amaranth Leaves

Three spots that increased in leaves in response to drought were identified as chloroplast chaperone subunits. The chloroplast chaperonins consist of two distinct polypeptides, Cpn 60α and Cpn 60β, which share only ~50% amino acid identity. Spot 6 was identified as the α subunit of the chaperonin of 60 kDa (Cpn 60α), while spots 3 and 7 corresponded to the β subunit of this protein (Cpn 60β). Functional characterization of plant chaperonins is limited, but it is generally agreed that they are important in assisting plastid proteins as Rubisco (Boston et al. 1996). Spot 13 increased in response to drought stress and was identified as a heat shock protein (Hsp) of 70 kDa. Hsp 70 chaperones, together with their cochaperones, have essential functions in preventing aggregation and assisting refolding of nonnative proteins under normal and stress conditions (Hartl 1996). Some members of the Hsp 70 family are constitutively expressed and are involved in assisting the folding of the de novo synthesized polypeptides and the import/translocation of precursor proteins. Therefore, they are more involved in the refolding and proteolytic degradation of nonnative proteins under stress conditions (Wang et al. 2004). Spot 14, which appeared only after the drought treatment, was identified as an Hsp 70. Research into the cross-talk between Hsp/chaperones and other stress-responsive mechanisms in plants will provide a better understanding of acquired stress tolerance (Wang et al. 2004).

The majority of cytosolic protein degradation in eukaryotes occurs through the ubiquitin proteasome system. In this process, cellular proteins targeted for degradation are tagged by multimers of ubiquitin and are degraded by the 26S proteasome, a giant cytosolic protease. Proteins may display a hydrophobic patch as a result of denaturation that is recognized and routed for degradation. Spot 4 was identified as a member of the ubiquitin-conjugating enzymes family. These kinds of enzymes catalyze the second step in the ubiquitination reactions that label proteins for proteasome degradation (Nandi et al. 2006).

Spots 1, 2, and 21 decreased their concentration in response to drought stress and were identified as Rubisco large subunits. Rubisco quantity in leaves is controlled by its synthesis and degradation rates. Even in early drought stages, this protein still is relatively stable as a holoenzyme and has a half-life of some days (Webber et al. 1994). However, under drought stress, a rapid decrease in its transcription rate has been found in tomato, *Arabidopsis*, and rice (Bartholomew et al. 1991; Williams et al. 1994; Vu et al. 1999). Using the proteomic approach, the increased abundance of Rubisco fragmentation under drought stress has been reported in rice (Salekdeh et al. 2002), sugar beet (Hajheidari et al. 2005), and *Elymus elongatum* (Gazanchian et al. 2007). It is possible that fragmentation occurs during protein solubilization in vitro. In this work, no Rubisco fragmentation spots were identified between the analyzed proteins.

In oxygenic photosynthesis carried out by cyanobacteria, algae, and higher plants, the multisubunit cytochrome *b6f* integral membrane protein complex mediates electron transfer between the photosystem II, in which H_2O is the electron donor, and the photosystem I reaction centers. The electron transfer through the *b6f* complex is coupled to proton translocation across the membrane, which establishes an electrochemical potential gradient (Cramer et al. 2004). This complex is the most likely point of regulation in the electron transport chain (Golding and Johnson 2003). Cytochrome *b6f* complex iron sulfur subunit 2 (spot 11) was decreased in response to drought. Downregulation of this protein during drought may help to protect photosystem II by downregulating the electron transport to match the demands of carbon fixation by the Calvin cycle. Electron transport to oxygen and thus the production of reactive oxygen species will be minimized (Golding and Johnson 2003).

Spots 10 and 20, which decreased under drought, were identified as the oxygen evolving complex (OEC) 23-kDa subunit. These two forms are probably due to posttranslational modifications. The photosynthetic OEC is the enzyme in green plants that participates in the use of oxidants produced in the light to oxidize water, obtaining molecular oxygen as a by-product. This multimeric protein consists of at least three subunits named for their molecular masses (OE 33, OE 23, and OE 17) that are attached at the inner surface of the thylakoid membrane to the photosystem II reaction center (Hashimoto et al.

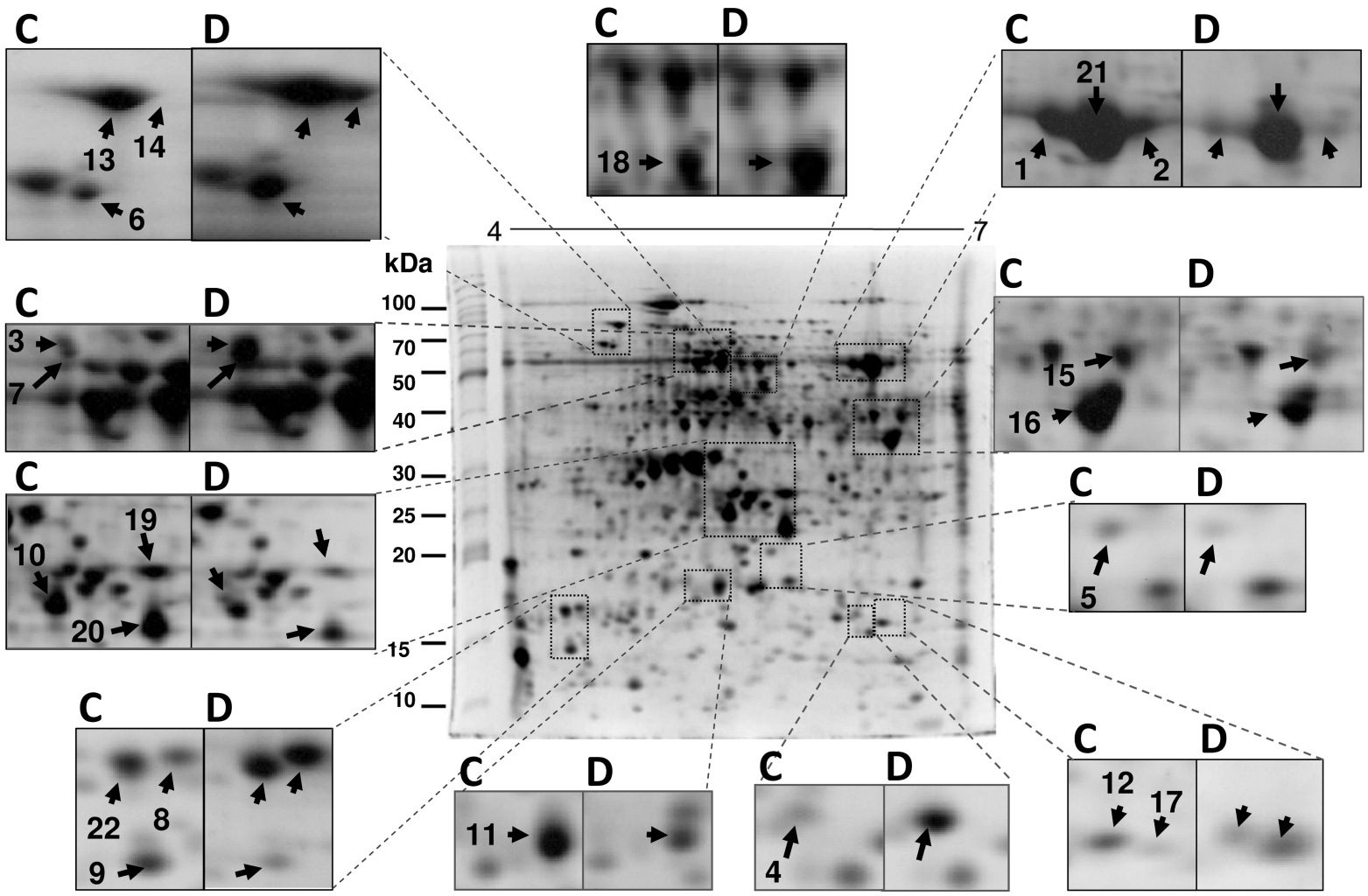

Fig. 2 Changes in protein abundance in amaranth leaves between control (*C*) and drought (*D*) conditions. Zoomed images show the differential protein spots characterized by LC/ESI-MS/MS.

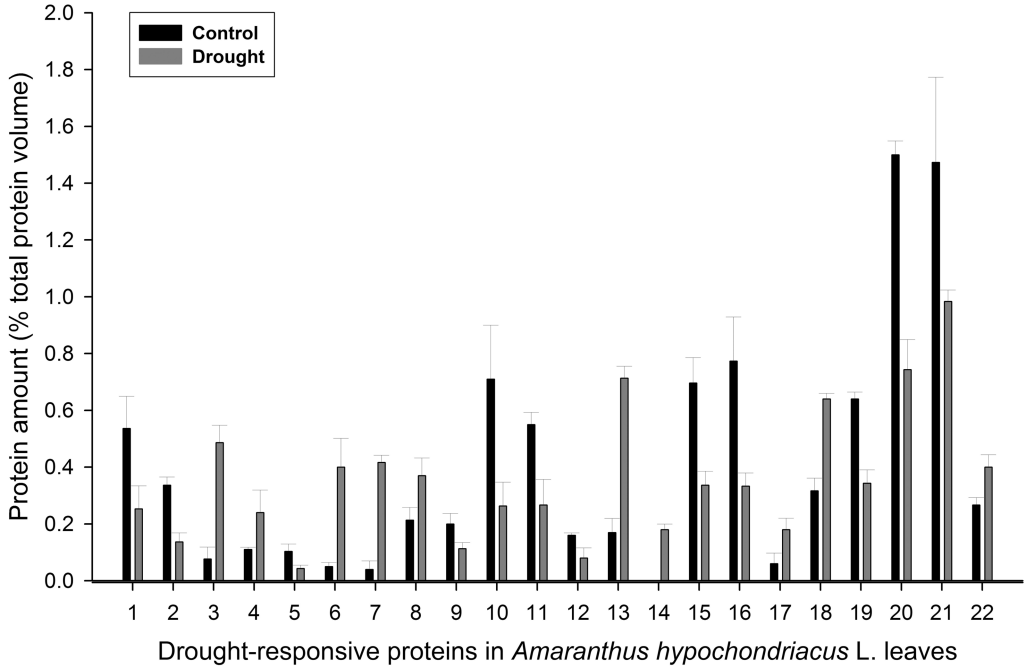

Fig. 3 Histogram showing changes in the amounts of differentially displayed proteins analyzed by LC/ESI-MS/MS.

1997). Under desiccation conditions, inhibition of the OEC occurs (Skotnica et al. 2000).

S-adenosylmethionine sintetase (SAMS, spot 18) increased in abundance following drought stress in amaranth leaves, and it catalyzes the production of S-adenosyl-L-methionine (SAM) from L-methionine and ATP. SAM serves as a methyl group donor in numerous transmethylation reactions. SAMS also play a role in the stress resistance and are encoded by small gene families that contain members that are differentially transcribed in response to various stress conditions (Sánchez-Aguado et al. 2004).

Spot 12, which decreased under drought stress, was identified as a hypothetical nucleoside diphosphate kinase (NDPK). This type of protein plays a significant role in hormone responses and MAPK-mediated H_2O_2 signaling, development, and growth. Yang et al. (2006) suggests that there might be a general stress response mechanism in relation to NDPK. The primary function of ascorbate peroxidase (APX) involves the reduction of hydrogen peroxide (H_2O_2), with the consequent oxidation of ascorbate to dehydroascorbate. H_2O_2 can act as a signaling molecule for the programmed cell death pathway and expression of defense-related genes that help to protect the cell from reactive oxygen species. Hossain et al. (2006) found a significant decrease in APX activity at the beginning of senescence, with a good correlation to an increase in the H_2O_2 content. This downregulation of APX seems to be a prerequisite for inducing senescence. In this work, spot 19, identified as APX, was decreased after severe drought stress in amaranth leaves. The increased expression of defense-related proteins cannot overcome the negative effects on the plant, and we saw that the leaves rolled and the plant started to wilt, but after rewatering, the plant recover its normal appearance.

Spot 16, found in decreased concentration, was identified as the mitochondrial malate dehydrogenase (mMDH). This enzyme catalyzes the reversible reduction of oxaloacetate to malate and is important in multiple metabolic pathways. Reduction of the activity of this enzyme showed an enhanced photosynthetic activity and aerial growth in transgenic tomatoes (Nunes-Nesi et al. 2005). Mitochondria are a key site of oxidative stress and play important roles in cell survival. It has been described that although plant cell bioenergetics is strongly affected by abiotic stresses, mitochondrial metabolism under stress is still largely unknown but may play a central role in cell adaptation to abiotic stresses that are known to induce oxidative stress at a cellular level (Pastore et al. 2001).

Spot 15 (fig. 2) was identified as the fructose-bisphosphate aldolase. The fructose-1,6 bisphosphate aldolase is an enzyme catalyzing a key reaction of glycolysis and energy production, converting D-fructose-1,6-bisphosphate into dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. It is documented that both genes of fructose-bisphosphatase are downregulated by salt stress. The downregulation of proteins involved in respiration as fructose-bisphosphate aldolase may contribute to tissue adaptation to stress by lowering respiratory energy consumption (Chaves et al. 2009).

Conclusion

The method used in this work (TCA-A) was efficient for protein extraction from amaranth, agave, and nopal samples. The TCA-A method was efficient for comparative proteomic analysis of amaranth leaves when the plant was grown in normal conditions compared with leaves from plants grown

Table 1

Identification of Drought-Responsive Proteins in *Amaranthus hypochondriacus* L. Leaves by LC/ESI-MS/MS Analysis

Spot no.[a]	Proteins	Peptides matched/ sequence coverage	Mascot score[b]	Protein name	Accession no.[c]	Experimental mass (kDa)/p*I*	Theoretical mass (kDa)/p*I*	Species
1*	D	8/15%	339	Rubisco large subunit	gil1346961	52/6.27	50.4/6.51	*Amaranthus tricolor*
2*	D	7/15%	277	Rubisco large subunit	gil1346961	52/6.52	50.4/6.51	*Amaranthus tricolor*
3*	U	1/2%	54	Chaperonin-60 β subunit	gil1762130	62/5.20	63.3/5.72	*Solanum tuberosum*
4*	U	2/20%	74	Ubiquitin-conjugating enzyme-like protein	gil76160962	16.3/6.28	16.5/6.20	*Solanum tuberosum*
6*	U	3/5%	174	Chaperonin-60 α subunit	gil1710807	62/4.73	61.9/5.15	*Pisum sativum*
7*	U	1/2%	53	Chaperonin-60 β subunit	gil1762130	60/5.20	63/5.72	*Solanum tuberosum*
10*	D	5/26%	130	23-kDa oxygen evolving complex protein	gil148535011	24.4/5.47	21.5/5.94	*Salicornia veneta*
11*	D	4/41%	145	Cytochrome *b6f* complex iron sulfur subunit 2	gil146454654	18/5.40	16.6/6.04	*Sonneratia alba*
12*	D	5/21%	223	Hypothetical NDPK	gil125595441	15.8/6.45	21.5/8.38	*Oryza sativa*
13*	U	8/10%	335	Hsp 70 kDa	gil1143427	75/4.76	75.4/5.15	*Cucumis sativus*
14[d]		2/3%	58	Hypothetical protein (Hsp 70 kDa)	gil147805297	75/4.83	69.1/5.11	*Vitis vinifera*
15*	D	2/7%	107	Fructose-bisphosphate aldolase	gil113624	38.7/6.58	38.5/5.96	*Spinacia oleracea*
16*	D	4/14%	260	Malate dehydrogenase, mitochondrial precursor	gil126896	34.6/6.51	36.2/8.88	*Citrullus lanatus*
18*	U	7/23%	391	S-adenosylmethionine synthetase	gil48928010	46/5.69	43.2/5.52	*Solanum brevidens*
19*	D	6/34%	223	Ascorbate peroxidase	gil559005	27/5.83	27.4/5.43	*Nicotiana tabacum*
20*	D	6/26%	155	23-kDa oxygen evolving complex protein	gil148535011	23/5.83	21.5/5.94	*Salicornia veneta*
21	D	8/17%	351	Rubisco large subunit	gil7240502	52/6.38	51.7/6.12	*Achlys triphylla*

Note. U, upregulated proteins; D, downregulated proteins; Hsp, heat shock protein.

[a] Numbers correspond to spots in figure 2.

[b] Scores >44 indicate identity or extensive homology ($P < 0.05$).

[c] Accession numbers are from the National Center for Biotechnology Information Entrez database.

[d] Only under drought stress.

* Treatment effect is significant, $P < 0.05$.

under drought conditions. The results show that stress-responsive proteins in amaranth leaves are mainly chloroplast chaperonins. The decreased abundance of Rubisco large subunit, OEC, and cytochrome *b6f* complex, as well as the enzyme fructose-bisphosphate aldolase, show that the effect of drought on electron transport chain could be related to the reduction of carbon metabolism. The decreased abundance of the malate dehydrogenase mitochondrial could help to enhance the photosynthetic activity.

Although the genomes of the three plants used in this work are not sequenced, we have shown that proteomic analysis was successful for protein identification of differential protein spots from amaranth leaves. This work is expected to accelerate proteomic studies of *Amaranthus hypochondriacus* L., *Agave tequilana* Weber var. azul, and *Opuntia* spp., three nonmodel plants that are essential as food and feed resources in several places worldwide that grow under stress environments.

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