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Sorghum 2-Dimensional Proteome Profiles and Analysis of HSP70 Expression Under Salinity Stress

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ABSTRACT

Sorghum (*Sorghum bicolor*), a drought-tolerant cereal, is the second most important grain crop in Africa after maize (*Zea mays*). In this study, sorghum proteomes from whole plant organs (leaves, sheaths and roots), as well as cell suspension culture systems were resolved successfully using gel-based proteomics tools. Total soluble proteins (TSP) were extracted from leaves, sheaths and roots, as well as cultured cells. These proteomes were resolved via two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Proteins were visualized by Coomassie Brilliant Blue (CBB) and identified by MALDI-TOF mass spectrometry (MS). Five different proteomes were studied: total soluble proteome and secretome from cell suspension culture, as well as leaf, sheath and root proteomes from whole plants. Unique protein expression profiles were observed for each of the five proteomes, suggesting specialization of the proteins. Western blot analysis indicated that, as expected, HSP70 protein expression was induced in the roots of the experimental material following salt stress treatments. Cell suspension cultures provide a special research material, particularly for studies related to secreted proteins. The expected secretion of an alpha-galactosidase was also observed. On completion of spot mapping and identification, this proteome data could be used as a research/reference resource by many sorghum and grain scientists worldwide.

Keywords: proteomics, sorghum, cell suspension cultures, proteome, secretome, extracellular matrix, 2D-PAGE, galactosidase, HSP70, MALDI-TOF MS

INTRODUCTION

Sorghum (*Sorghum bicolor*) is a drought-tolerant cereal crop (Rosenoww *et al*., 1983), which survives well under hot and dry conditions. In Africa, based on production yield, after maize (*Zea mays*), sorghum is ranked the second most important cereal (FAO, 2006). It is mainly used as a food source for humans in Africa and Asia, as well as for stock feed and as a source of bioethanol in the USA. Sorghum is not only an important food source in the semi-arid regions of the world, but is also a potential model crop for studying and gaining a better understanding of the molecular mechanisms of drought tolerance in cereals. Proteomics, the large-scale analysis of protein from a particular organism, tissue or cell (Blackstock and Weir, 1999; Pandey and Mann, 2000; van Wijk, 2001), has been used to study global changes in the protein expression of plant tissues, cells and sub-cellular compartments. Knowledge of plant proteomics has been gained using plants like tobacco (*Nicotiana tabacum*) (Okushima *et al*., 2000), rice (*Oryza sativa*)

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(Rakwal and Agrawal, 2003), maize (Riccardi *et al*., 1998) or Arabidopsis (*Arabidopsis thaliana*) (Ndimba *et al*., 2005), among others. Other studies have targeted specific compartments, like cell walls (Chivasa *et al*., 2002; Boudart *et al*., 2005) or the extracellular matrix (Borderies *et al*., 2003; Ndimba *et al*., 2003; Oh *et al*., 2005). Cell suspension cultures are a homogenous group of undifferentiated cells (Evans *et al*., 2003) and have been used extensively in proteomics (Okushima *et al*., 2000; Ndimba *et al*., 2005; Oh *et al*., 2005). Whole plant systems have also been used to understand tissue specific protein expression profiles at specific physiological states (Riccardi *et al*., 1998, Watson *et al*., 2003; Parker *et al*., 2006; Jiang *et al*., 2007).

Plants have developed tolerance mechanisms against the ionic effects of salinity, but they differ greatly among plant species, tissues and cell types. These mechanisms are diverse and complex, usually occurring simultaneously at different levels within the plant. Therefore, the study of tissue-specific protein expression in response to salinity stress through proteomics would give an insight towards the understanding of these tolerance mechanisms.

Despite the economic potential of sorghum in the semi-arid regions of Africa, as well as the promise offered by proteomics towards the understanding of plant biological systems, no proteomic studies on sorghum have been reported. Therefore, this study aimed to report on protein expression profiles from both whole plant tissue and cell suspension cultures resolved via 2D-PAGE and demonstrate the feasibility of this system in stress response studies. The study showed, as expected, that stress responsive proteins, such as HSP70, are induced following salinity stress treatments. This work was encouraged by the completion of the sorghum genome sequencing (Paterson *et al*., 2009) which made protein identification more feasible.

MATERIALS AND METHODS

Plant material

Plant culture and Salinity stress application: Surface-sterilised seeds were germinated on MS media only (control) or MS supplemented with 100 mM NaCl (salinity stress). Seeds were germinated and grown at 25°C under a regime involving 16 h light and 8 h darkness. At day 14 post-plating, the seedlings were harvested and the leaf, sheath and root tissues were separately flash-frozen in liquid nitrogen and stored at -20° C until further use.

Cell suspension culture: Sorghum calluses and cell suspension cultures were initiated and maintained in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) as described by Ngara *et al*. (2008).

Protein extraction from whole plant and cell suspension culture and protein quantification

Cell suspension culture: Cell suspension cultures (10 d old) were separated from the CF by filtering through four layers of Miracloth (Merck, Darmstadt, Germany). Proteins from both the CF and cells were extracted separately as described by Ngara *et al*. (2008).

Whole plant: Leaf and sheath protein extracts were prepared from approximately 10 plantlets, while root proteins were extracted from a bulk of a minimum of 20 seedlings. Leaves, sheaths and roots were separately ground in liquid nitrogen with a pestle and mortar and precipitated with 10% (w/v) trichloroacetic acid. Proteins were collected by centrifugation at 13,400 × *g* for 10 min at room temperature. The protein pellet was washed three times with 10 ml of ice-cold 80% (v/v) acetone by centrifugation at 13,400 × *g* for 10 min for each wash, air dried at room temperature and resuspended in 2 ml of urea buffer [9 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)] for at least 1 hr with

vigorous vortexing at room temperature. Soluble proteins were cleared out by centrifugation at 15,700 × *g* for 10 min. The protein content of the extracts was estimated by a modified Bradford assay using BSA as standard, as described by Ndimba *et al*. (2003). The quality of protein extracts was evaluated by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE).

Protein separation by 2D-PAGE

Leaf, sheath, root, cell suspension culture TSP and CF protein extracts (100-150 μg) were each separately subjected to 2D-PAGE as described by Ngara *et al*. (2008). Gels were stained with Coomassie Brilliant Blue (CBB) for protein visualization.

Detection of heat shock proteins by western blotting

Control and NaCl-treated root extracts (20 μg) were separated by 1D-PAGE and transferred onto PVDF transfer membrane (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) as described by Towbin *et al*. (1979) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). The transfer was performed at 36 V, overnight at 4°C with constant stirring. All incubation steps were performed with gentle agitation at room temperature. After protein transfer, the membrane was washed once in Tris-buffered saline (TBS) (50 mM Tris and 150 mM NaCl, pH 7.5) for 10 min and incubated in blocking solution [1% (w/v) fat free milk powder in TBS] for 1 h. The membrane was then incubated for 1 h with the primary antibody [human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody raised in mouse (Stressgen Bioreagents Corp., Victoria, Canada)] diluted to 1:2,500 with 0.5% (w/v) blocking solution. The membrane was then washed three times with TBST ([TBS containing 0.1% (v/v) Tween 20)] for 10 min per wash and once with 0.5 % (v/v) blocking solution for 10 min. Following this, the membrane was incubated for 1 h with the secondary antibody [Goat anti-mouse IgG (H & L) horseradish peroxidase conjugate (Invitrogen Corp., Carlsbad, CA, USA)] diluted to 1:1,000 with 0.5 % (w/v) blocking solution. The membrane was washed three times in TBST for 15 min each time. Heat shock proteins were detected using a SuperSigna® West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's instructions. The X-ray film was exposed on the membrane and developed using a Curix 60 (Agfa- Gevaert, N.V., Mortsel, Belgium).

Protein digestion and identification by MALDI-TOF MS

Following CBB staining, gels were imaged using Molecular Imager PharosFX Plus System (Bio-Rad). Where necessary, protein spots were robotically excised from gels with an ExQuest (Bio-Rad) spot cutter and transferred into sterile tubes. Gel pieces were washed twice with 50 mM ammonium bicarbonate for 5 min each time and a third time for 30 min, with occasional vortexing and then destained with 50% (v/v) 50 mM ammonium bicarbonate and 50% (v/v) acetonitrile for 30 min twice, vortexing occasionally. Gel pieces were dehydrated with 100 μL (v/v) acetonitrile for 5 min, and then completely dessicated using a Speed Vac SC100 (ThermoSavant, Waltham, MA, USA). Proteins were in-gel digested with approximately 120 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) dissolved in 25 mM ammonium bicarbonate for 6 h at 37°C.

Prior to spotting, the samples were cleaned-up by reverse phase chromatography using ZipTip $_{C18}$® (Millipore, Billerica, MA, USA) pre-equilibrated first in 100% (v/v) acetonitrile and then in 0.1% (v/v) TFA and eluted out with 50% (v/v) acetonitrile. One microlitre from each sample was mixed with the same volume of α-cyna-hydroxy-cinnamic acid (CHCA) matrix and

spotted onto a MALDI target plate for analysis using a MALDI-TOF MS, the Voyager DE Pro Biospectrometry workstation (Applied Biosystems, Forster City, CA, USA) to generate a peptide mass fingerprint. All spectra were calibrated using the sequazyme calibration mixture II, containing angiotensin I, ACTH/1-17 clip, ACTH/18-39 clip and ACTH/7-38 clip (Applied Biosystems). The NCBI and MSDB peptide mass databases were searched using MASCOT ([cited 2010 Feb 23]. Available from: http://www.matrixscience.com/search_form_select.html).

RESULTS AND DISCUSSION

Sorghum leaf, sheath and root proteomes

As an initial attempt towards mapping of the entire sorghum proteome, TSP (100-150 mg) from leaves, sheaths and roots was extracted, separated by 2D-PAGE and visualized by staining with CBB (Figure 1). Over 300 protein spots were visualized. In all three organs, the majority of proteins were resolved between pH 4 and 7 and between 90 and 10 kDa. Within the parameters, more soluble proteins were detected in sheaths and roots than in leaves, particularly low abundance proteins in the pH 7 region of the gel.

The difference in protein spot profiles demonstrates the tissue specificity and specialisation of some proteins. Once protein spots are identified, they may provide supporting information on the functions of these proteins. For example, photosynthesis-related proteins are expected in leaves while mineral-uptake related and soil-microbe symbiosis related proteins are predicted to occur in roots. Since the ultimate goal is to map the entire sorghum proteome, it should be possible to confirm these suggestions and possibly identify novel proteins that could lead to new biological paradigms.

However, 2D-PAGE proteomics approaches have their limitations, with one being the under-representation of insoluble, hydrophobic and low abundance proteins, like membrane proteins and some transcription factors. Proteins with extreme pI's and/or molecular weights will not be represented here (Lin *et al*., 2003).

Salinity stress induces HSP 70 expression in roots

To test the effects of salt stress on sorghum seedlings, the expression of Hsp70, a classical stress responsive protein, was observed by western blotting (Ndimba *et al*., 2005). When

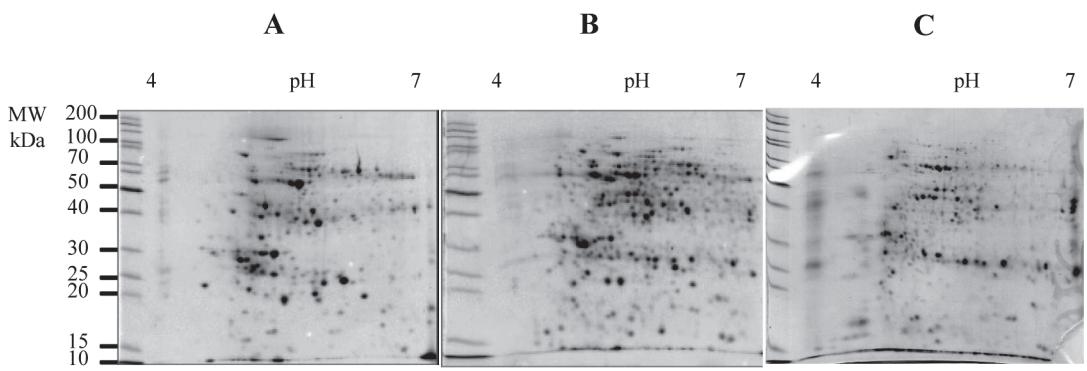

Figure 1 Detection of total soluble proteins from sorghum leaf (A), sheath (B) and root (C), as indicated, after separation by 2D-PAGE.
Total soluble protein from leaf (100 mg), sheath (150 mg) and root (100 mg) were separated using 7 cm linear IPG strips, pH range 4-7 in the first dimension and by 12% SDS-PAGE in the second dimension and stained with Coomassie Brilliant Blue.

grown in MS containing 100 mM NaCl, HSP70 protein levels in roots increased when compared to plants grown in MS only (Figure 2). This information shows that the sorghum plant material resembles a typical plant stress response and is therefore crucial in future experiments that endeavour to simulate natural physiological effects.

Sorghum cell suspension culture proteomes

The establishment of sorghum cell suspension culture system for proteomics studies and developed proteome maps from both cellular TSP and CF proteins were reported by Ngara *et al*. 2008. Figure 3 highlights the expected differences in proteome complexity between TSP and CF.

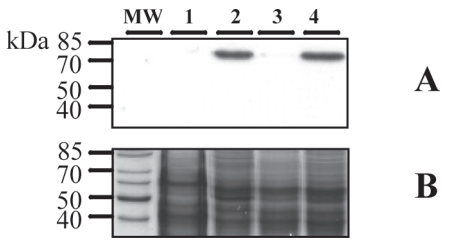

Figure 2 Detection of HSP70 accumulation in root extracts by Western blot.
Total soluble proteins (20 mg) from roots of plants grown in MS medium only (lanes 1 and 3) or MS medium supplemented with 100 mM NaCl (lanes 2 and 4) were resolved by 12% SDS-PAGE gel and transferred onto PVDF membrane. HSP70 accumulation was detected by western blot (A) and total soluble proteins were stained by Coomassie Brilliant Blue.

To further characterize the secretome, two CF protein spots were randomly selected (spot 1 and 2) for MS identification (Figure 3D). The peptide mass fingerprint for both spots matched that of a rice alpha-galactosidase with the Swiss-Prot database accession Q9FXT4 and a molecular weight search (MOWSE) score of 80 and 86, respectively (Table 1). The polypeptide sequence of the matched alpha-galactosidase is shown in Figure 4. According to the phytozome sorghum database ([cited date in format YYYY MMM DAY]. Available from: http://www.phytozome.net), this polypeptide has a 91% identity with a sequence of a putative sorghum alpha-galactosidase 7.396 kbp gene (ASb01g018400.1) located in chromosome 1, positions 19,132,571 to 19,139,966. The theoretical pI (pH 5.9) of this protein was calculated using the EMBOSS pK Value Model ([cited date in format YYYY MMM DAY]. Available from: http://isoelectric.ovh.org/). These two protein spots, which displayed the same molecular weight of approximately 40 kDa, but had different pI's, may be isoforms of the same alpha-galactosidase. Previous proteomic-based studies demonstrated that proteins may exist in multiple spots on 2D-PAGE (Chivasa *et al*., 2002; Ndimba *et al*., 2005; Oh *et al*., 2005), which can be the result of post-translational modifications, like phosphorylation or glycosylation.

Alpha galactosidases are bona fide residents of plant apoplasts and are known to play an important role in the cell wall metabolism, and therefore in plant growth and development. Using the SignalP 3.0 prediction server (([cited date in

Table 1 Identified proteins from sorghum cell suspension culture filtrate.
The protein spot number corresponds to the numbers given in Figure 3.

Spot	Protein name	Accession No.	MOWSE score	Theoretical Mass/ pI	Experimental Mass/ pI
1	Rice alpha-galactosidase	Q9FXT4	80	39 926/ 5.9	40 000/ 6.1
2	Rice alpha-galactosidase	Q9FXT4	86	39 926/ 5.9	40 000/ 6.3

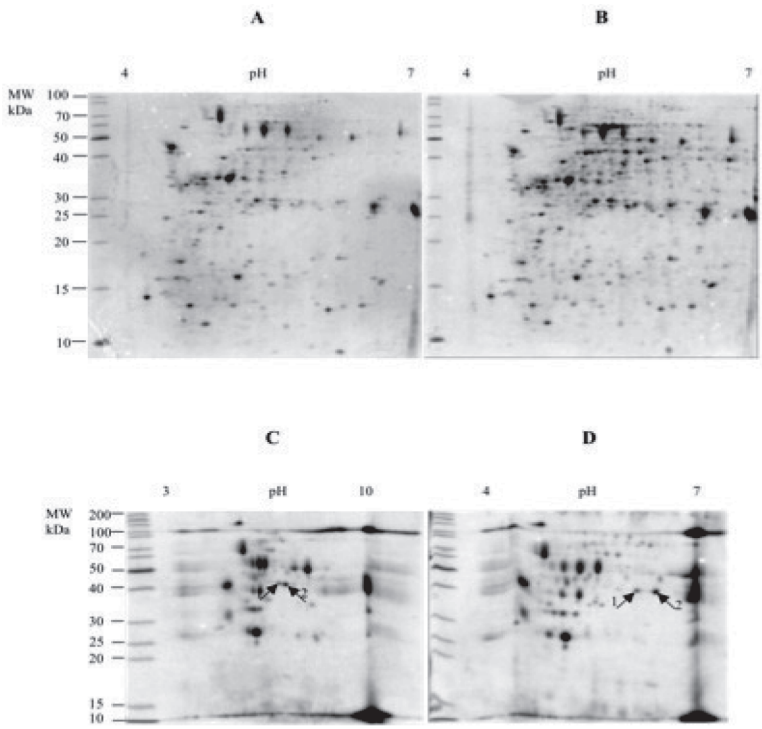

Figure 3 Detection of total soluble proteins from sorghum cell suspension cultures and culture filtrate after separation by 2D-PAGE.

Total soluble proteins were separated using linear IPG strips in the first dimension and by 12% SDS-PAGE in the second dimension and stained with Coomassie Brilliant Blue. Cell suspension culture proteins were resolved using 18 cm strips, pH range 4-7. A total of 400 mg (A) and 800 mg (B) proteins were loaded onto the strips. Culture filtrate proteins (100 mg) were resolved using 7 cm strips, pH range 3-10 (C) and pH range 4-7(D).

MARASSSSSP PSPRLLLLLL VAVAATLLPE AAALGNFTAE SRGARWRSRR
ARRRAFENGL GRTPQMGWNS WNHFYCGINE QIIRETADAL VNTGLAKLGY
QYVNIDDCWA EYSRDSQGNF VPNRQTFPSG IKALADYVHA KGLKLGIYSD
AGSQTCSNKM PGSLDHEEQD VKTFASWGVD YLKYDNCNDA GRSVMERYTR
MSNAMKTYGK NIFFSLCEWG KENPATWAGR MGNSWRTTGD IADNWGSMTS
RADENDQWAA YAGPGGWNDP DMLEVGNGGM SEAEYRSHFS IWALAKAPLL
IGCDVRSMSQ QTKNILSNSE VIAVNQDSLG VQGKKVQSDN GLEVWAGPLS
NNRKAVVLWN RQSYQATITA HWSNIGLAGS VAVTARDLWA HSSFAAQGQI
SASVAPHDCK MYVLTPN

Figure 4 A polypeptide sequence of an alpha-galactosidase identified from sorghum culture filtrate secreted proteins.

Spots 1 and 2 were selected for mass spectrometric identification from the 2-dimensionally separated and Commassie stained gel spots illustrated in Figure 3D. Both spots were positively identified by MALDI-TOF mass spectrometry as an alpha-galactosidase (Q9FXT4). The N-terminal cleavable secretory peptide is underlined.

format YYYY MMM DAY]. Available from: www.cbs.dtu.dk/services/SignalP/), a cleavable secretory N-terminal (33 amino acids) signal peptide sequence domain (MARASSSSSPPSPR LLLLLLVAVAATLLPEAAA), underlined in Figure 4, was detected on the sequence of the alpha-galactosidase polypeptide. This bioinformatics analysis further demonstrates evidence for the secretion of this protein to the extracellular matrix. These data add to the validation of the experimental system.

CONCLUSION

Sorghum soluble proteins were successfully extracted from both whole plants and cell suspension cultures. High quality 2D gels were developed, which allowed the visualization of hundreds of proteins, some of which were tissue specific. Using western blotting, the expression of HSP70 was induced in roots from plants grown in medium supplemented with NaCl. The results showed that this experimental workflow could be used for further salinity stress response studies.

Additionally, protein extracts from cell suspension culture and CF were resolved on 2D-PAGE. Two of the protein spots were positively identified as galactosidase, a bona fide apoplastic protein, suggesting that the extraction method was suitable for the isolation of the secretome. However, further verification that no cytosolic proteins contaminate the extracts is desirable. The identification of galactosidase isoforms also suggests the occurrence of post-translational modification. The results thus show that this material could be used for further studies, such as the mapping and characterization of the secretome that are more challenging using whole plant tissue systems.

Future work will include the large-scale identification of sorghum proteins towards the creation of reference maps, which will be a valuable resource for sorghum and other cereal researchers worldwide. The identification and characterisation of the hyperosmotic stress responsive proteins is also of interest, to gain knowledge on the effects of salinity and drought on the protein expression.

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