View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by University of the Western Cape Research Repository

Ngara, et al. (2012). Identification and profiling of salinity stress-responsive proteins in Sorghum bicolor seedlings. JOURNAL OF PROTEOMICS, 75: 4139-4150

Identification and Profiling of Salinity Stress-Responsive Proteins in *Sorghum bicolor* Seedlings

Rudo Ngara [a], Roya Ndimba [b], Jonas Borch-Jensen [c], Ole Nørregaard Jensen [c], Bongani [a,d,*]Ndimba

[a] Proteomics Research Group, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville, 7535, South Africa

[b] iThembaLABS, Materials Research Department, Old Faure Road Somerset West, South Africa

[c] Department of Biochemistry and Molecular Biology, University of Southern Denmark, Denmark

[d] Proteomics Unit, Biotechnology Platform, Agricultural Research Council, Infruitec Campus, Stellenbosch, South Africa

*Corresponding author Tel: +27 21 959 2215; Fax+27 21 959 1551; Emails: NdimbaB@arc.agric.za cc. bndimba@uwc.ac.za

Key words: Sweet Sorghum; MN1618; salinity stress; MALDI-TOF/TOF; mass spectrometry; Biofuels

Abstract

Sorghum bicolor, a drought tolerant cereal crop, is not only an important food source in the semi arid/arid regions but also a potential model for studying and gaining a better understanding of the molecular mechanisms of drought and salt stress tolerance in cereals. In this study, seeds of a sweet sorghum variety, MN1618, were planted and grown on solid MS growth medium with or without 100 mM NaCl. Heat shock protein expression immunoblotting assays demonstrated that this salt treatment induced stress within natural physiological parameters for our experimental material. 2D PAGE in combination with MS/MS proteomics techniques were used to separate, visualise and identify salinity stress responsive proteins in young sorghum leaves. Out of 281 Coomassie stainable spots, 118 showed statistically significant responses ($p<0.05$) to salt stress treatments. Of the 118 spots, 79 were selected for tandem mass spectrometric identification, owing to their good resolution and abundance levels, and of these, 55 were positively identified. Identified proteins were divided into six functional categories including both known and novel/putative stress responsive proteins. Molecular and physiological functions of some of our proteins of interest are currently under investigation via bioinformatic and molecular biology approaches.

Introduction

Abiotic stresses such as salinity and drought affect plant growth, development and productivity by imposing hyperosmotic and oxidative stresses, ion toxicity and nutrient deficiency [1-3]. These environmental stresses are more prevalent in arid and semi-arid areas. Although irrigation is often used as a remedy to supplement inadequate rainfall in drought prone areas, over-irrigation of arable lands may increase soil salinity in the long

term, thus worsening the situation [4]. Agriculturally important cereals such as rice and maize, like many other food crops, are sensitive to hyperosmotic stresses [5-7]; and thus show reduced productivities when cultivated in saline soils. With the increasing soil salinisation that is projected to affect more than 50% of all arable lands by the year 2050 [8], as well as the growing world population, there is increasing need to develop crops that are well adapted to salt stress. The cultivation of salt tolerant crops on saline soils would aid towards the full utilisation and reclamation of salt affected soils, which would otherwise be non-productive.

The physiological impact of salt stress at both tissue and cellular levels in plants has been shown to resemble that of other hyperosmotic stresses such as drought, cold and freezing [9, 10]. In addition to causing tissue and cellular dehydration, salt stress imposes ionic stress in plants [1, 11]. Some of the biological processes and metabolic pathways that are involved in salt stress response and tolerance, or those that are affected by salt stress, have been identified in gene expression studies. This is in line with the knowledge that salt stress, like all abiotic stresses, causes changes in gene expression [12, 13], which ultimately affects the expression of gene products, the proteins [9, 12-16].

Proteomics, the large-scale analysis of proteins from a particular organism, tissue or cell at a given time [17-19] has been used to study salt stress responsive protein expression in crops such as rice (*Oryza sativa*; [20]), potato (*Solanum tuberosum*; [21]) and foxtail millet (*Setaria italica* L. cv Prasad; [22]) amongst others. Sobhanian and co-workers reviewed some of these studies, giving a description of unique proteome changes of several other economically important food crops under salt stress [7]. Despite these reported proteomics advances in food crops under salt stress, comparable studies on sorghum, one of the most stress tolerant commercial grain crops are still limited. Kumar Swami and co-workers reported on the first attempt in cataloging differential proteome expression changes in sorghum leaf tissue in response to salt stress [23].

Sorghum is considered to be moderately tolerant to salt, being particularly more tolerant than maize [24], the most widely produced grain crop worldwide. As such, sorghum offers great potential as a food source in both dry and relatively more saline regions. Several studies have been reported on the large scale screening of sorghum varieties for salt tolerance [24], transcriptome changes in response to dehydration and high salinity [25], the evaluation of growth parameters and ion accumulation [26], and soluble carbohydrate contents [27, 28] among various cultivars of sorghum. Although these studies provide valuable information on the effect of salt stress on gene expression, plant growth and the accumulation of soluble sugars which may function as osmoprotectants, there is need to identify specific proteins that contribute to sorghum's salt tolerance mechanisms. The measurement of protein expression using proteomics tools would therefore provide a better indication of cellular activities under salt stress. Sorghum proteomics is further encouraged by the published sorghum genome sequence [29], which will help in downstream protein identification steps by mass spectrometry analysis. The current study aims at identifying salt stress responsive proteins in sorghum leaf extracts using 2-DE and MS. The data obtained will be useful in understanding how sorghum, a potential fail-safe crop grown in arid regions, copes with salinity stress when cultivated on saline soils.

2 Materials and methods

2.1 Plant growth and salt treatment

The MN1618 sorghum seed variety used in this study was obtained from Dr Pangirai Tongoona, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The seeds were first surface decontaminated with 70% (v/v) ethanol for a minute followed by absolute commercial bleach (12% sodium hypochlorite solution) for 20 min before plating in 175 ml capacity plant tissue culture vessels (Sigma-Aldrich, Saint Louis, MO., USA) containing 50 ml of sorghum seed growth media [2.2g/l Murashige and Skoog basal medium (MS; 1% (w/v) sucrose, 5 mM MES and 0.8% (w/v) agar, pH 5.8]. The media was supplemented with either 100 mM NaCl (salt stressed treatment group) or without NaCl (unstressed treatment group). Both treatment experiments were performed in triplicate. Culture vessels were incubated at 25°C under a 16 h light/8 h dark regime for 14 days after which leaf material was excised from the seedlings and immediately flash frozen in liquid nitrogen.

2.2 Protein extraction from sorghum leaf tissue and quantification

Sorghum leaf protein extracts were prepared from an average of ten, 14 day old sorghum seedlings. The leaf material was ground in liquid nitrogen using a mortar and pestle, and precipitated with 10% (w/v) TCA. The homogenate was centrifuged at 13,400 x *g* for 10 min at room temperature and the resultant pellet was washed three times with ice-cold 80% (v/v) acetone by centrifuging at 13,400 x *g* for 10 min per wash. The pellet was air dried for 5 min at room temperature and resuspended in urea buffer (9 M urea, 2 M thiourea and 4% CHAPS) for at least 1 h with vigorous vortexing at room temperature. After vortexing, the homogenate was centrifuged at 15,700 x *g* for 10 min and the supernatant containing soluble leaf proteins was collected and stored at -20□C. Extracted leaf proteins were quantified using a modified Bradford assay [30] as previously described [31] using BSA as a standard.

2.3 1-DE and 2-DE

One-dimensional gel electrophoresis of approximately 10 μg of leaf protein extracts was carried out to evaluate both the quality and loading quantities of the extracts prior to 2-DE. For 2-DE, mini format SDS-PAGE gels [10.1 cm (width) x 8.3 cm (height)] were run for use in comparative gel analysis, while the large format gels [27.6 cm (width) x 21.6 cm (height)] were run for spot picking. For mini gels, 100 μg of the leaf protein extracts were isoelectrophoresed on 7 cm IPG strips of pH range 4-7 (Bio-Rad, Hercules, CA, USA), equilibrated in SDS-containing buffers and run on 12 % (w/v) SDS polyacrylamide gels as previously described [32]. For large format gels, 750 μg leaf protein extracts were isoelectrophoresed on 18 cm IPG strips of pH range 4-7 (Bio-Rad), equilibrated in SDS-containing buffers and run on 12 % (w/v) SDS polyacrylamide gels as previously described [33]. Isoelectric focusing of both the 7 and 18 cm strips were separately performed on an Ettan™ IPGphor II™ (GE Healthcare, Amersham, UK) using stepwise programmes as described below. For 7 cm strips; 250 V for 15 min, followed by 4 000 V for 1 h and finally, 4 000 V until it reached 12 000 Vh. For 18 cm strips; 250 V for 15 min, followed by 8 000 V

for 2 h and finally, 8 000 V until it reached 60 000 Vh. The 2-DE gels were stained with CBB R-250 and imaged using the Molecular Imager® PharosFX™ Plus System (Bio-Rad).

2.4 Immunoblotting for Heat shock protein 70 (Hsp70)

Immunoblotting analysis for Hsp70 on sorghum leaf protein extracts separated on 1DE gels was performed as previously described [34].

2.5 Comparative analysis of 2-DE gels

Comparative analysis of protein expression on 2-DE gels between stressed and unstressed treatment groups was carried out using the PDQuest™ Advanced 2D Analysis Software version 8.0.1 build 055 (Bio-Rad) according to the user manual. Each treatment group had three biological replicates. Gels were normalised using the local regression model and spots were manually edited using the group consensus tool. Differentially expressed protein spots were statistically significant using the Student's *t*-test at a 95% significance level. Protein spots of interest were picked from large format CBB stained gels using the ExQuest™ spot cutter (Bio-Rad) for identification by MS/MS.

2.6 Protein identification by MALDI-TOF-TOF MS

The protein spots were destained with 30% ACN in aqueous ammonium bicarbonate (70 mM final concentration), reduced with DTT, alkylated with iodoacetamide and digested with sequencing grade trypsin (Promega, Madison, USA) according to Shevshenko et al. [35]. The tryptic peptides were either spotted directly on the MALDI target as a dried droplet preparation with 10 mg/ml CHCA or, in cases where MS signals of dried droplet preparations were low, concentrated and desalted on hand held C18 microcolumns and eluted with 10 mg/ml CHCA in 50% ACN, 0.1% TFA. The MS and MS/MS mass spectra were recorded on an ABI 4800 *plus* (Applied Biosystems, Foster City, CA) MALDI-TOF/TOF mass spectrometer in the positive ion mode with internal calibration of the MS spectra employing masses of commonly observed trypsin peaks. The 10 most abundant peptide ions from each spot were selected for CID (gas pressure of

$\sim 1 \times 10^{-6}$ Torr) TOF/TOF MS/MS. In cases where commonly observed keratin, trypsin or project specific contaminants dominated the MS spectra, these were excluded from analysis by an exclusion list, which was built by PeakErazor [36] after the first round of analysis.

The spectra were annotated and analyzed using Data Explorer v. 4.5 (Applied Biosystems) without smoothing or noise reduction. The MS and MS/MS data from each gel band was combined into a single mass list (.mgf file) using an in-house developed script (Jakob Bunkenborg, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Denmark).

The .mgf files of each spot were searched against protein sequences from *Viridiplantae* (green plants) (846849 sequences) in the NCBI non-redundant database, release NCBInr 20101126. The peptide MS and MS/MS tolerances were set to 15 ppm and 0.6 Da, respectively. The MASCOT search engine located on an in-house server (version 2.2.06,

Matrix Science, London, UK) was used for searching and scoring the identified proteins. The search parameters were as follows: Specificity of protease digestion was set to trypsin with one missed trypsin site allowed. Oxidation of Met and deamidation of Asn and Gln were set as variable modification for all Mascot searches, and carbamidomethyl on Cys was set as a fixed modification. Individual peptides with a MASCOT score >35 (probability value of $p < 0.05$) were accepted as identified. Using these parameters, a false discovery rate of 0.00% was obtained as judged by MASCOT's decoy database searching.

2.7 Bioinformatic analysis

Theoretical M*r* and p*I* of MS identified proteins were estimated using the Compute pI/MW tool available on ExPASy (http://expasy.org). Proteins were grouped into functional categories [37] using data available on the UniProt database (www.uniprot.org) as well as literature sources. Hypothetical proteins were annotated with function by extracting information from the Conserved Domain Database (CDD) entries of the NCBI protein database.

3 Results and discussion

3.1 Choice of salt concentration for stress treatment and its effect on Hsp70 protein expression.

A preliminary experiment was conducted to establish the appropriate salt concentration for use in this study. In the experiment, surface decontaminated white sorghum seeds (purchased from Agricol, Brackenfell, South Africa) were plated and grown on sorghum seed growth media supplemented with 0 mM (control), 50, 100, 150 and 200 mM NaCl for 14 days. As illustrated in Figure 1, the seeds were able to germinate and grow albeit at different degrees across the treatment regime. However, from 150 mM NaCl, the efficiency of seed germination and seedling growth was greatly reduced. On the basis of this preliminary experiment, 100 mM NaCl was selected as the concentration for use in subsequent salt treatment experiments.

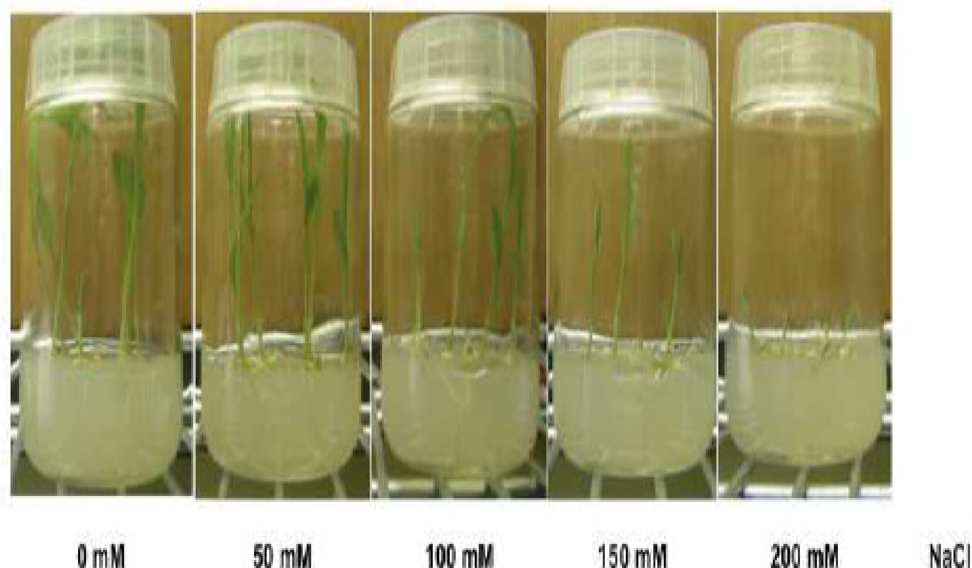

Fig. 1 – Effects of different levels of salt stress on the germination and growth of sorghum. Surface decontaminated white sorghum seeds were germinated on MS medium supplemented with 0–200mM NaCl for a total of 14 days. The picture was taken on day 10 post planting.

In order to adapt to and/or tolerate salt stress, plant cells may alter their gene expression resulting in an increase, decrease, induction or total suppression of some stress responsive proteins [13-16]. To establish whether or not our salt stress treatment regime of 100 mM NaCl for 14 days was within the physiological range of our experimental system, we investigated its effect on the expression of Hsp70 protein, a known stress responsive protein, using immunoblotting analysis. Immunoblotting was conducted on protein extracts from both salt stressed (100 mM NaCl) and unstressed (0 mM NaCl; control) MN1618 leaves using a human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody. Figure 2 illustrates that the levels of Hsp70 were enhanced in the salt stressed samples in comparison with the control. Heat shock protein 70 is a known stress responsive protein [38], and is expressed in response to abiotic stresses such as heat, cold, drought, salinity and oxidative stress [39]. The protein prevents aggregation of stress-denatured proteins and facilitates the refolding of proteins in order to restore their native biological functions [39, 40]. As such, Hsp70s are broadly known to function as molecular chaperones [41]. Overall, the Hsp70 immunoblotting experiment on salt stressed sorghum material demonstrates that our stress treatment conditions were within the physiological parameters of our experimental system, inducing known stress responses. This observation was indicative of the fact that our system was sufficient for application in further experimentation procedures.

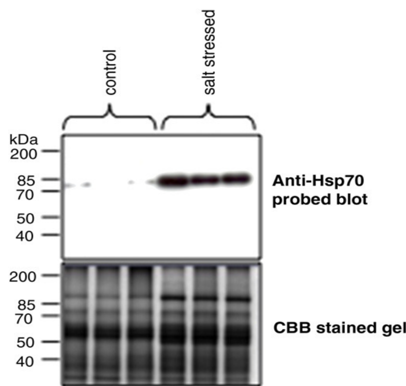

Fig. 2 – Immunoblotting analysis of Hsp70 expression patterns on salt stressed MN1618 sorghum leaf protein extracts. Three biological replicates of control (0 mM NaCl) and salt stressed (100mM NaCl) treatment are shown.

3.2 2-DE image analysis and annotation of stress-responsive protein spots.

One dimensional gel electrophoresis of the protein extracts showed that protein expression, abundance and loading across biological replicates was relatively uniform in both the control and salt stressed treatment groups (results not shown). To determine salt stress responsive sorghum leaf proteins, 2-DE expression profiles of leaf protein extracts from stressed (100 mM NaCl) and unstressed (control) 14-day old plants were compared. One hundred micrograms of protein extracts from three independent biological replicates of stressed and unstressed sorghum plant leaves were resolved on 7 cm IPG strips of pH range 4-7 and 12% (v/v) SDS PAGE gels. Gel electrophoresed proteins were visualised after CBB staining and imaged. The inclusion of biological replicates in comparative proteomic studies is important for accounting for the normal biological variation expected within each treatment group as well as for reducing chances of detecting non-reproducible protein expression differences between experiments. For this reason, all spots included in the analysis were reproducibly expressed amongst the three biological replicates of each treatment group. Differential protein expression between the two treatment groups was assessed using PDQuest^{TM} software. This resulted in the visualisation of 233 and 281 CBB stainable leaf protein spots in the control and salt stressed treatment groups, respectively. From the analysis of differential protein expression between the control and salt treatment groups, a total of 118 protein spots were statistically significant using the Student's *t*-test at a 95% significance level. Some examples of the differentially expressed proteins as predicted by the PDQuest^{TM} software are shown in Figure 3. The expression patterns of these spots amongst the three biological replicates per treatment also showed a consistent pattern (Figure 3). Representative control and salt treated sorghum leaf proteomes are shown in Figure 4A and 4B respectively.

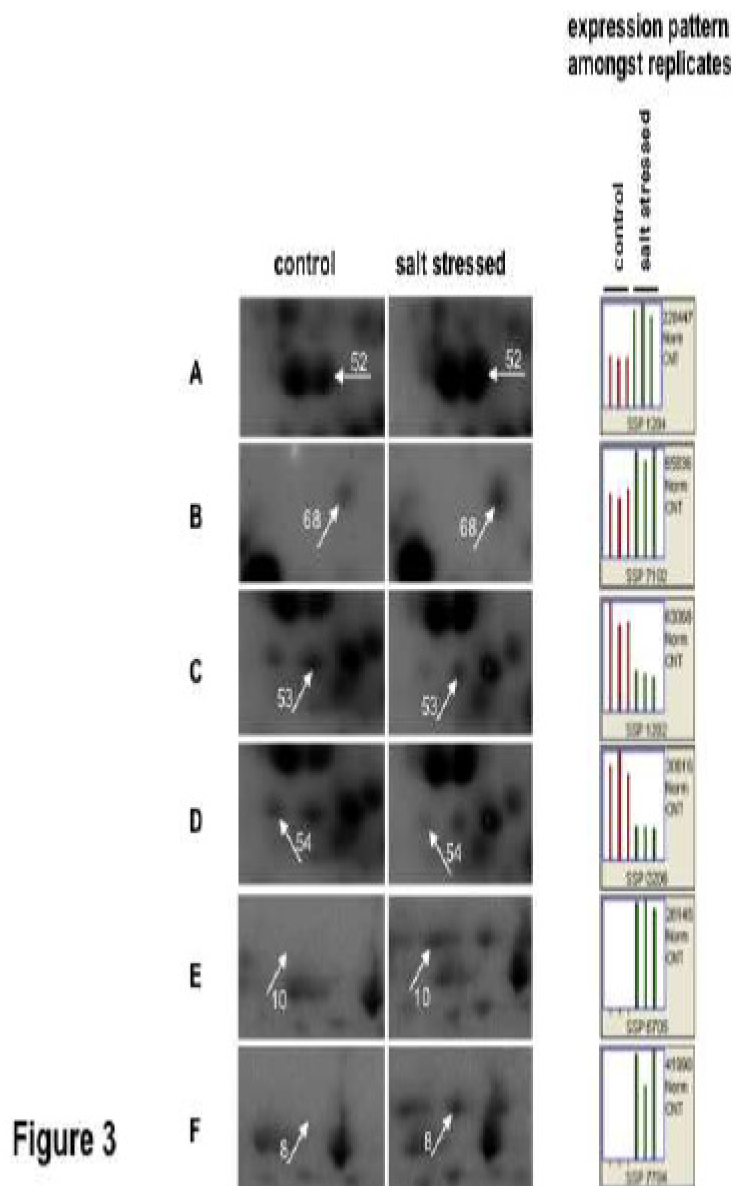

Fig. 3 – Zoomed in gel sections of representative spots showing differential expression patterns following salt treatment. Responsive spots were either up-regulated (A and B), down-regulated (C and D) or induced (E and F) following salt stress. The overall expression patterns of these representative spots amongst the three biological replicates used in the analysis are also shown in PDQuest™ software generated bar graphs.

Overall, most protein spots were confined between the M*r* range of 10-110 kDa with an experimental IEF pH restriction of 4-7. Protein separation by 2-DE is known to have physical limitation in separating proteins, where proteins of extreme p*I*s and M*r* are excluded from the 2-DE profiles [42, 43]. Limited M*r* range of 2-DE electrophoresed spots has also been reported in other studies of the proteome of maize (*Zea mays*) leaves [44] and cell suspension cultures [45] as well as different tissues

[46] of barrel medic (*Medicago truncatula*), amongst others. The IEF pH restriction of 4-7 in sorghum leaf proteome has also been previously reported [23].

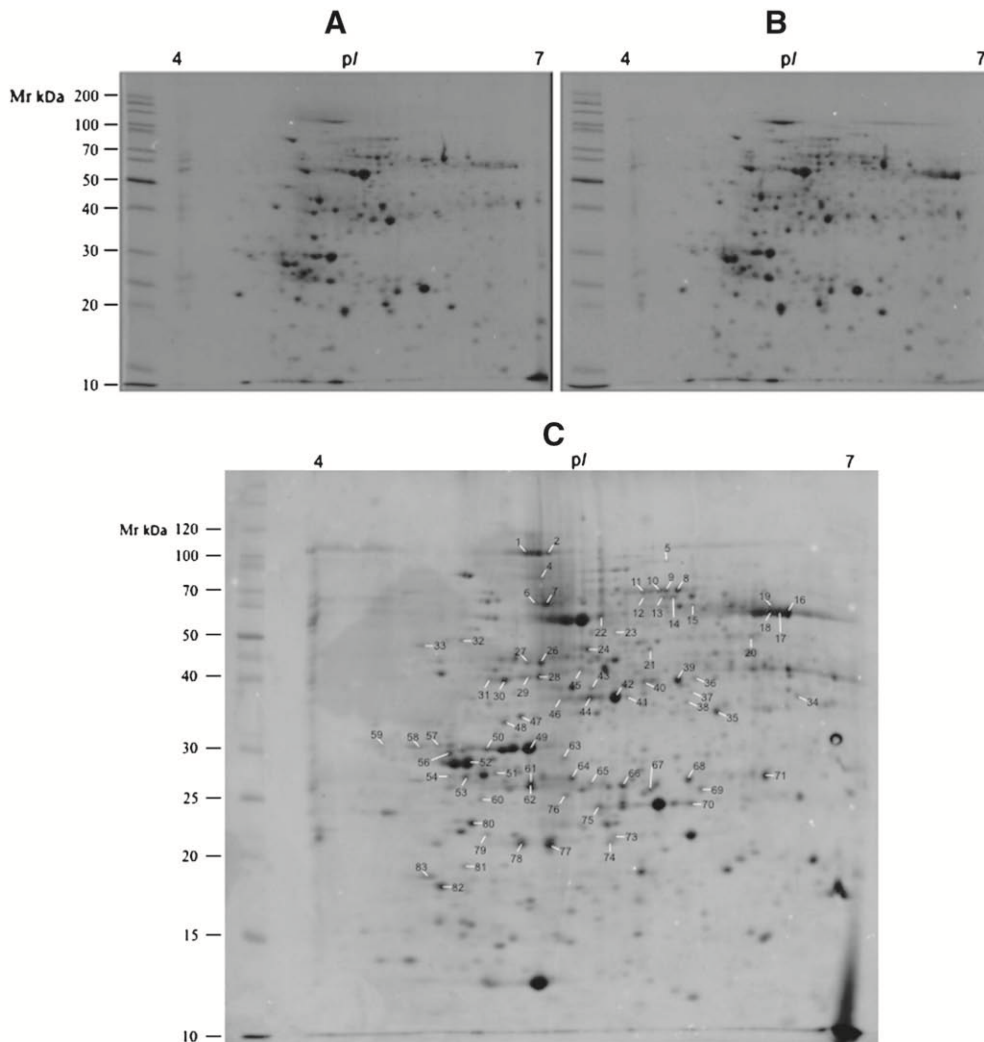

Fig. 4 – Coomassie brilliant blue R-250 stained 2-DEgels of MN1618 sorghum leaf proteins. Representative mini format gels of control (A) and salt stressed (B) sorghum leaf proteome showing the general protein expression within the two treatment groups. The large format gel (C) shows a total of 79 annotated sorghum salt stress responsive proteins that were selected for MS/MS analysis.

3.3 Mass spectrometric identification of salt stress-regulated sorghum proteins

From the 118 statistically significant differentially expressed protein spots as determined by PDQuest™ software ($p<0.05$), a total of 79 abundant, well-resolved and reproducible spots (numbered spots; Figure 4C) were selected from large format CBB stained gels for trypsinisation and identification by MALDI TOF/TOF MS. Of these 79 statistically significant spots, 17 (spots 1; 4-6; 8-10; 17; 20; 29; 33; 38; 65; 69; 71; 78; 83; Figure 4C) were only present in the salt stressed treatment group, suggesting that they were only synthesised in response to salt stress. In comparison with a similar study conducted in abiotic-stress sensitive rice plants [47], only two such proteins were observed. Salinity induced proteins might possibly contribute towards a plant's tolerance mechanism to salt

and thus could be useful candidates for further investigation of their molecular mechanisms of action.

The 79 protein spots (Figure 4C) whose abundance changed with a ≥ 95% statistical confidence (Student's *t*-test) were picked from CBB stained gels, trypsinised and analysed using MALDI TOF-TOF MS. MS and MS/MS spectra were searched by MASCOT against NCBInr protein database [taxonomy: *Viridiplantae* (green plants)] to identify the proteins according to the criteria stated in the "Materials and Methods" section.

Out of the 79 salt stress responsive protein spots that were selected for identification by MS (Figure 4C), 55 gave positive hits in the searched sequence database, matching those from sorghum (*S. bicolor)* and orthologs from other monocots such as sugarcane (*Saccharum officinarum)*, maize (*Z. mays)*, rice (*Oryza sativa),* wheat *(Triticum aestivum),* barley *(Hordeum vulgare)* amongst others. Table 1 illustrates spot numbers, their protein identities, number of matching peptides, theoretical/experimental M*r*/p*I* as well as their abundance changes. Refer to supplementary material, Table SM1 for quantity values of all differentially expressed spots annotated in Figure 4C, and Table SM2 for detailed information on the peptide identification summary of all the 55 positively identified protein spots.

[Table 1: at end of document]

Out of the 55 positively identified proteins, 22 matched *S. bicolor* hypothetical proteins (Table 1). Following the completion of the sorghum genome-sequencing project [29], most predicted sorghum gene products remain experimentally uncharacterised and are submitted as hypothetical proteins. By definition, hypothetical proteins are proteins predicted from genome sequences but whose existence has not been experimentally proven at the protein level [48]. As such, sorghum proteomic studies such as the current one and others [23] have been confronted with incomplete sorghum sequencing data that awaits full characterisation. If proteomic technologies are to be fully utilised in unravelling the array of proteins, and thus genes and biological processes at work in sorghum under a range of abiotic stresses, the scientific community worldwide is encouraged to begin viewing sorghum as a potential model plant of cereals, and thus investing resources in further characterising sorghum proteins.

The remaining 33 (55-22=33) positively identified proteins represented 19 unique protein identities due to the occurrence of similar proteins in multiple spots on the gel, differing in M*r*, p*I* or both. Such observed spotting patterns have been reported in previous studies [32, 38, 49, 50]. Multiple spotting of the same protein in different locations on a 2-DE gel may be a reflection of protein isoforms due to PTMs or multigene families, products of proteolytic activities, the presence of multiple subunits of a single protein and/or the chemical modification of proteins during sample preparation [51]. Examples of possible protein isoforms in our sorghum leaf proteome, which appeared in multiple spots with similar M*r* but different p*I*s (Figure 4C, Table 1), include cyanogenic beta-glucosidase dhurrinase-2 (spots 8 and 9), ATP synthase CF1 alpha subunit (spots 13 and 14) and hydroxynitrile lyase (spots 77 and 78). Components of the ATP synthase protein complex, namely the alpha (spots 7, 13 and 14), beta (spot 22) and gamma (spot 21 and 40) subunits

were also identified in the current study. Such component subunits of protein complexes have also been previously reported in *Brassica napus* stem proteome [51].

3.4 Functional classification of identified sorghum salt stress responsive proteins

The 55 positively identified proteins were grouped into functional categories as previously described [37] using a combination of similarity searches on UniProt, literature sources and information available on the CDD entries of the NCBI protein database for hypothetical proteins. This functional classification remains putative until the actual biological roles of the identified proteins are experimentally proven under similar experimental conditions. The proteins in each functional category are listed in Table 1, while a graphical representation of this distribution is illustrated in Figure 5. Below, a brief account of the possible biological roles of some of these proteins in response to salt stress in sorghum leaves is given.

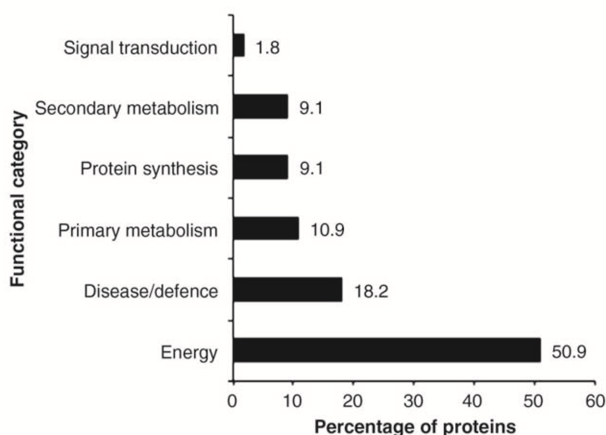

Fig. 5 – Functional characterisation of the positively identified MN1618 sorghum salt stress responsive leaf proteins.

3.4.1 Energy

The majority of the identified salt stress responsive sorghum leaf proteins (28 spots) were energy related proteins (50.9%; Figure 5). These included photosynthesis associated proteins such as Rubisco (spots 16-19), sedoheptulose bisphosphatase (spots 30 and 31), phosphoribulokinase (spot 28), ribulose 5-phosphate isomerase (spot 53) and the oxygen-evolving enhancer protein 1 (spot 53). Malate dehydrogenase (spots 26, 27 and 39), which is involved in the malate/oxaloacetate shuttling system, was also identified. Rubisco, sedoheptulose bisphosphatase and phosphoribulokinase proteins are all involved in the carboxylation, reduction and regeneration phases of the Calvin's cycle, a metabolic pathway that produces pentose sugars [52]. Rubisco which catalyses the carbon fixation reaction in photosynthetic plants [52] has an increased abundance in all four spots (spots 16-19; Table 1) in response to salt stress. In contrast, increased degradation of Rubisco was reported in the proteomic analysis of potato under salt stress [21], sugar beet under drought stress [53],

rice under chilling stress [54], and maritime pine seedlings under water stress [55]. However, in another proteomic study involving sorghum leaf under salt stress, the expression of Rubisco was not shown to be altered in response to salt stress [23].

This observed contrast in salt stress responsiveness of a single protein in two different sorghum varieties might confirm the wide genetic variation in salt tolerance amongst different sorghum varieties as previously reported [24]. The increased levels of Rubisco in our plant system might be indicative of sorghum MN1618 varietal needs for increased carbon fixation as well as increased energy needs under periods of salt stress. However, this speculation is complicated by the observation that proteins that are involved in the regeneration phase of the Calvin's cycle (which include sedoheptulose bisphosphatase and phosphoribulokinase) have reduced abundances following salt stress (Table 1). The observed different protein expression pattern of proteins involved in different phases of the same metabolic pathway may point out to differential effects of salt stress at different phases of a pathway and its regulatory systems. However, these speculations are yet to be proven experimentally.

A total of six proteins, all being subunit components of the ATP synthase chloroplastic protein complex that is involved in ATP synthesis, were identified in this study. These included the alpha (spots 7, 13, 14), gamma (spots 21 and 40) and beta (spot 22) subunits (Table 1). Of these, spots 7, 21, 22 and 40 had increased abundance levels, while spots 13 and 14 had reduced abundance levels following salt stress. The upregulation of protein expression observed here has also being documented in other studies on the proteomic analysis of potato shoots [21] and rice leaf lamina [20] under salt stress. The ATP synthase complex produces ATP from ADP in the presence of a proton gradient across the thylakoid membrane [56, 57], which is in turn used for various energy demanding activities during periods of stress. It is unclear, however, why two of the six subunits identified had contradictory expression levels from the rest.

3.4.2 Disease/defence

It is known that abiotic stresses such as drought, salinity, cold and heat are interconnected and all produce secondary stresses such as osmotic and oxidative stresses [8]. In turn, the plant responds to these primary and secondary stresses in a complex manner, resulting in similar proteins being responsive to different stresses [58]. Some abiotic stress inducible genes include those that directly protect the plant cell against stresses such as chaperones as well as enzymes involved in detoxification systems and the synthesis of osmoprotectants [13, 16]. In the current study, 10 (18.2%; Figure 5) disease/defence related proteins were identified as being salt stress responsive in the sorghum leaf proteome.

Salt stress induces oxidative stress, which is characterised by the accumulation of ROS that are toxic to cells, damaging membranes and macromolecules. To scavenge for and eliminate these ROS, plants under stress express antioxidant enzymes such as GST, superoxide dismutase, catalase and ascorbate peroxidase [8, 53, 59]. In the current sorghum study, GST (spots 68; 69;) and ascorbate peroxidase (spots 64; 66) showed an increased abundance following salt stress (Table 1; Figure 4C). Similar expression patterns have also been reported in Arabidopsis [38, 60] and sorghum [23, 61] for GST, and in Arabidopsis for

ascorbate peroxidase [60]. The increased accumulation of these enzymes in plants is thought to be a part of the cell's detoxification system for scavenging excess ROS during oxidative stress, a secondary stress to most abiotic stresses.

3.4.3 Secondary metabolism

Cyanogenic beta-glucosidase dhurrinase-2 (spots 8 and 9), p-(S)hydroxymandelonitrile lyase (spot 52) and hydroxynitrile lyases (spots 77 and 78) all showed increased abundance levels following salt stress in the current study (Table 1). Cyanogenic beta-glucosidases and hydroxynitrile lyases are both involved in the catabolism of cyanogenic glycosides [62], secondary metabolites, which play a role in the chemical defense system in plants and may also serve as nitrogen storage compounds [63, 64]. During their catabolism, cyanogenic glycosides are hydrolysed by beta-glucosidases into alpha-hydroxynitrile, which are further broken down by alpha-hydroxynitrile lyases at low pH into keto compounds and hydrogen cyanide [62, 65, 66]. Hydrogen cyanide is toxic and its production (cyanogenesis) acts as a defence mechanism against herbivores and microbial attack. It is however unclear at this point what role cyanogenic glycoside catabolism plays in sorghum leaves under salt stress and none of these proteins have been reported in a similar study [23]. A question that warrants further investigation would be whether or not the levels of both the cyanogenic glucosides and hydrogen cyanide increase in the sorghum tissues under salt stress.

3.4.4 Protein synthesis

Chloroplast translational elongation factor Tu (spot 24), cysteine synthase (spot 38), elongation factor 1-delta (spots 56 and 58) and a ribosomal protein (spot 82) were all identified as upregulated proteins in the current study (Table 1). These proteins are all involved in protein synthesis. Abiotic stresses including salt stress cause protein damage and/or degradation either due to oxidative damage or proteolytic activities. Increased levels of protein synthesis are therefore important in order to restore the damaged proteins for full restoration of the plant cell's metabolic activities and general growth. Increased levels of some of these protein synthesis related proteins have also been reported in Arabidopsis cell cultures under salt stress [38].

3.4.5 Other functional categories

Other identified salt stress responsive proteins in this study were classified as having functions in primary metabolism (spots 20, 67, 41, 61-63) and signal transduction (spot 81). Protein spot 81, containing a forkhead associated domain showed reduced abundance levels following salt stress. In prokaryotes and eukaryotes, a range of proteins involved in processes such as signal transduction, protein transport and degradation, transcription and DNA repair are known to contain this domain [67]. In this study, this protein was loosely grouped as a signal transduction related protein although further research work would need to be conducted to find out its molecular function in plants under salt stress.

4. Conclusion

The negative impact of adverse environmental stresses, such as salinity and drought on the planet is now well known and poses serious threats to societies, particularly to the resource poor rural farmers in the developing world. Sorghum, despite its well-known natural stress tolerance qualities, remains understudied at molecular level. This work is a part of our long-term vision that seeks to elevate this African crop as an important model plant to study stress avoidance and tolerance mechanisms in grain crops.

Our Hsp70 expression assays showed that our experimental setup and system mimicked expected physiological response and that plant growth, stress treatments and protein extraction and staining methods gave reproducible and high quality 1-and 2-dimensional SDS PAGE results. Our proteins of interest, identified here were found to belong to a number of important functional categories that include stress (abiotic/biotic) defence, energy metabolism, protein synthesis, primary and secondary metabolism as well as signal transduction. As expected, most of these proteins have also been identified in other “omics” studies, thus validating our experimental approach, while others such as cyanogenic beta-glucosidase dhurrinase and hydroxynitrile lyases have not been previously reported as being salt stress responsive. Current and future work is aimed at further understanding the molecular functional interactions of our protein candidates, particularly those that show sorghum unique features, and towards evaluating the mechanisms that make this crop more stress tolerant in comparison to other grain crops.

Acknowledgements

The authors are grateful to Professor Antoni Slabas and Dr William Simon of Durham University for preliminary tandem mass spectrometric identification of the spots, which were further confirmed here. The National Research Foundation and the Department of Science and Technology of South Africa funded this work. The authors declare no conflict of interest.

5. References

[1] Zhu JK. Plant salt tolerance. Trends Plant Sci 2001;6:66-71.

[2] Wyn Jones RG. Salt tolerance. In: Johnson CB, editor. Physiological processes limiting plant productivity. London: Butterworths; 1981, p. 271-92.

[3] Hurkman WJ. Use of two dimensional gel electrophoresis to characterise changes in gene expression associated with salt stress on barley. In: Katterman F, editor. Environmental injury to plants. London: Academic Press; 1990, p. 205-29.

[4] Flowers TJ. Improving crop salt tolerance. J Exp Bot 2004;55:307-19.

[5] Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 2005;444:139-58.

[6] Munns R, Tester M. Mechanisms of salinity tolerance. Annu Rev Plant Biol 2008;59:651-81.

[7] Sobhanian H, Aghaei K, Komatsu S. Changes in the plant proteome resulting from salt stress: Toward the creation of salt-tolerant crops? J Proteomics 2011;74:1323-37.

[8] Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 2003;218:1-14.

[9] Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 1999;17:287-91.

[10] Xiong L, Schumaker KS, Zhu JK. Cell signaling during cold, drought, and salt stress. Plant Cell 2002;14:S165-83.

[11] Hale MG, Orcutt DM. The physiology of plants under stress. New York: John Wiley and Sons; 1987.

[12] Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ. Plant Cellular and Molecular Responses to High Salinity. Annu Rev Plant Physiol Plant Mol Biol 2000;51:463-499.

[13] Seki M, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K. Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr Opin Biotechnol 2003;14:194-99.

[14] Sachs MM, Ho T-HD. Alteration of gene expression during environmental stress in plants. Annu Rev Plant Physiol 1986;37:363-76.

[15] Ho T-HD, Sachs MM. Environmental control of gene expression and stress proteins in plants. In: Jones HG, Flowers TJ, Jones MB, editors. Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge: Cambridge University Press 1989, p.157-80.

[16] Shinozaki K, Yamaguchi-Shinozaki K, Seki M. Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 2003;6: 410-7.

[17] Blackstock WP, Weir MP. Proteomics: quantitative and physical mapping of cellular proteins. Trends Biotech 1999; 17:121-7.

[18] Pandey A, Mann M. Proteomics to study genes and genomes. Nature 2000; 405:837-46.

[19] van Wijk KJ. Challenges and prospects of plant proteomics. Plant Physiol 2001; 126:501-8.

[20] Parker R, Flowers TJ, Moore AL, Harpham NV. An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. J Exp Bot 2006;57:1109-18.

[21] Aghaei K, Ehsanpour AA, Komatsu S. Proteome analysis of potato under salt stress. J Proteome Res 2008;7:4858-68.

[22] Veeranagamallaiah G, Jyothsnakumari G, Thippeswamy M, Reddy PCO, Surabhi GK, Sriranganayakulu G, et al. Proteomic analysis of salt stress responses in foxtail millet (*Setaria italica* L. cv. Prasad) seedlings. Plant Sci 2008;175:631-41.

[23] Kumar Swami A, Alam SI, Sengupta N, Sarin R. Differential proteomic analysis of salt stress response in *Sorghum bicolor* leaves. Environ Exp Bot 2011;71:321-8.

[24] Krishnamurthy L, Serraj R, Hash CT, Dakheel AJ, Reddy BVS. Screening sorghum genotypes for salinity tolerant biomass production. Euphytica 2007;156:15

24.

[25] Buchanan CD, Lim S, Salzman RA, Kagiampakis I, Morishige DT, Weers B, et al. *Sorghum bicolor*'s transcriptome response to dehydration, high salinity and ABA. Plant Mol Biol 2005;58:699-720.

[26] Netondo GW, Onyango JC, Beck E. Sorghum and salinity: I. Response of growth, water relations, and ion accumulation to NaCl salinity. Crop Sci 2004;44: 797-805.

[27] Almodares A, Hadi MR, Ahmadpour H. Sorghum stem yield and soluble carbohydrates under different salinity levels. Afr J Biotechnol 2008;7:4051-5.

[28] Almodares A, Hadi MR, Dosti B. The effects of salt stress on growth parameters and carbohydrate contents in sweet sorghum. Res J Environ Sci 2008;2:298-304.

[29] Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, et al. The Sorghum bicolor genome and the diversification of grasses. Nature 2009; 457:551-6.

[30] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

[31] Ndimba BK, Chivasa S, Hamilton JM, Simon WJ, Slabas AR, Proteomic analysis of changes in the extracellular matrix of Arabidopsis cell suspension cultures induced by fungal elicitors. Proteomics 2003;3:1047-59.

[32] Ngara R, Ndimba BK. Mapping and characterisation of the sorghum cell suspension culture secretome. Afr J Biotechnol 2011;10,:253-66.

[33] Ngara R, Jasper D, Rees G, Ndimba BK. Establishment of sorghum cell suspension culture system for proteomics studies. Afr J Biotechnol 2008;7:744-9.

[34] Ndimba BK, Thomas LA, Ngara R. Sorghum 2-dimensional proteome profiles and analysis of Hsp70 expression under salinity stress. Kasetsart J (Nat Sci) 2010;44: 768-75.

[35] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850-8.

[36] Hjerno K, Hojrup P. PeakErazor: A Windows-based program for improving peptide mass searches. In Kamps RM, Calvete JJ, Choli-Papadopoulou T, editors. Methods in Proteome and Protein analysis. New York: Springer Verlag; 2004, p. 359

70.

[37] Bevan M, Bancroft I, Bent E, Love K, Goodman H, Dean C, et al. Analysis of

1.9 Mb of contiguous sequence from chromosome 4 of Arabidopsis thaliana. Nature 1998;391:485-8.

[38] Ndimba BK, Chivasa S, Simon WJ, Slabas AR. Identification of Arabidopsis salt and osmotic stress responsive proteins using two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 2005; 5:4185-96.

[39] Wang WX, Vinocur B, Shoseyov O, Altman A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 2004;9:244

52.

[40] Sung DY, Kaplan F, Guy CL. Plant Hsp70 molecular chaperones: Protein structure, gene family, expression and function. Physiol Plant 2001;113:443-51

[41] Miernyk JA. The 70 kDa stress-related proteins as molecular chaperones. Trends Plant Sci 1997;2:180-7.

[42] Lin D, Tabb DL, Yates JR 3rd. Large-scale protein identification using mass spectrometry. Biochim Biophys Acta 2003;1646:1-10.

[43] Jain R, Katavic V, Agrawal GK, Guzov VM, Thelen JJ. Purification and proteomic characterization of plastids from *Brassica napus* developing embryos. Proteomics 2008;8:3397-405.

[44] Porubleva L, Vander Velden K, Kothari S, Oliver DJ, Chitnis PR. The proteome of maize leaves: use of gene sequences and expressed sequence tag data for identification of proteins with peptide mass fingerprints. Electrophoresis 2001;22: 1724-38.

[45] Lei Z, Elmer AM, Watson BS, Dixon RA, Mendes, PJ, Summer LW. A two-dimensional electrophoresis proteomic reference map and systematic identification of 1367 proteins from a cell suspension culture of the model legume *Medicago truncatula*. Mol Cell Proteomics 2005;4:1812-25.

[46] Watson BS, Asirvatham VS, Wang L, Sumner LW. Mapping the proteome of barrel medic (*Medicago truncatula*). Plant Physiol 2003;31:1104-23.

[47] Yan S, Tang Z, Su W, Sun W. Proteomic analysis of salt stress-responsive proteins in rice root. Proteomics 2005;5:235-44.

[48] Lubec G, Afjehi-Sadat L, Yang JW, John JP. Searching for hypothetical proteins: theory and practice based upon original data and literature. Prog Neurobiol 2005;77:90-127.

[49] Chivasa S, Ndimba BK, Simon WJ, Robertson D, Yu XL, Knox JP. Proteomic analysis of the *Arabidopsis thaliana* cell wall. Electrophoresis 2002;23:1754-65.

[50] Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, et al. Secretome analysis reveals an Arabidopsis lipase involved in defense against *Alternaria brassicicola*. Plant Cell 2005;17:2832-47.

[51] Albertin W, Langella O, Joets J, Negroni L, Zivy M, Damerval C, et al. Comparative proteomics of leaf, stem, and root tissues of synthetic *Brassica napus*. Proteomics 2009;9:793-9.

[52] Heldt HW. Plant biochemistry and molecular biology. Oxford: Oxford University Press; 1997.

[53] Hajheidari M, Abdollahian-Noghabi M, Askari H, Heidari M, Sadeghian SY, Ober ES. Proteome analysis of sugar beet leaves under drought stress. Proteomics 2005;5:950-60.

[54] Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN. Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol Cell Proteomics 2006;5:484-96.

[55] Costa R, Bahrman N, Frigerio J-M, Kremer A, Plomion C. Water-deficitresponse proteins in maritime pines. Plant Mol Biol 1998;38:587-96.

[56] McCarty RE. A plant biochemist's view of H+-ATPases and ATP syntheses. J Exp Bot 1992;172:431-41.

[57] von Ballmoos C, Dimroth P. Two distinct proton binding sites in the ATP synthase family. Biochem 2007;46:11800-9.

[58] Levitt J. Responses of plants to environmental stresses., London: Academic Press; 1980.

[59] Xiong L, Zhu JK. Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ 2002;25:131-9.

[60] Jiang Y, Yang B, Harris NS, Deyholos MK. Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. J Exp Bot 2007; 58:3591-607.

[61] Jogeswar G, Pallela R, Jakka NM, Reddy PS, Rao JV, Sreenivasulu N. Antioxidative response in different sorghum species under short-term salinity stress. Acta Physiol Plant 2006;28:465-75.

[62] Ganjewala D, Kumar S, Devi A, Ambika K. Advances in cyanogenic glycosides biosynthesis and analysis in plants: A review. Acta Biol Szeged 2010;54:1-10.

[63] Hickel A, Hasslacher M, Griengl H. Hydroxynitrile lyases: Functions and properties. Physiol Plant 1996;98:891-8.

[64] Fechter MH, Griengl H. Hydroxynitrile lyases: Biological sources and application as biocatalysts. Food Technol Biotechnol 2004;42:287-94.

[65] Lauble H, Miehlich B, Forster S, Wajant H, Effenberger F. Crystal structure of hydroxynitrile lyase from *Sorghum bicolor* in complex with the inhibitor benzoic acid: a novel cyanogenic enzyme. Biochem 2002;41:12043-50.

[66] Purkarthofer T, Skranc W, Schuster C, Griengl H. Potential and capabilities of hydroxynitrile lyases as biocatalysts in the chemical industry. Appl Microbiol Biotechnol 2007;76:309-320.

[67] Durocher D, Jackson SP. The FHA domain. FEBS Lett 2002;513:58-66.

Table

Table 1: Sorghum leaf salt stress responsive proteins identified by MALDI-TOF-TOF mass spectrometry.

Spot No.[a)]	Protein Name and Species	gi[b)]	Accession [c)]	Score[d)]	Theo. Mr (kDa)/pI[e)]	Exp. Mr (kDa)/pI[f)]	Expression change [g)]	Matching peptides
	Primary Metabolism							
20	GDP-mannose 3,5-epimerase 1 [*Zea mays*]	195620882	ACG32271	105	42.96/5.94	52.0/6.42	up*	1
67	Unknown [*Zea mays*] Ribulose- 5-phosphate 3-epimerase	223945381	ACN26774	82	26.26/6.59	26.5/5.85	down (64%)	1
41	Adenosine kinase [*Zea mays*]	4582787	CAB40376	273	36.03/5.23	38.5/5.74	up (101%)	4
61	Hypothetical protein SORBIDRAFT_07g000980 [*Sorghum bicolor*] Adenylate kinase	242080247	XP_002444892	302	31.06/7.72	27.5/5.19	up (67%)	3
62	Hypothetical protein SORBIDRAFT_07g000980 [*Sorghum bicolor*] Adenylate kinase	242080247	XP_002444892	349	31.06/7.72	27.0/5.19	up (67%)	4
63	Hypothetical protein SORBIDRAFT_09g001130 [*Sorghum bicolor*] Atypical (a) short-chain dehydrogenase/reductase (SDRs) NAD(P) binding	242086601	XP_002439133	127	31.77/6.98	29.5/5.38	down (34%)	1
	Energy							
1	Pyruvate phosphate dikinase [*Sorghum bicolor*]	30385668	AAP23874	234	102.29/5.68	101.0/5.15	up*	4
16	Unnamed protein product [*Hordeum vulgare*] Ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit	11587	CAA25265	165	53.08/6.22	58.0/6.64	up (124%)	3
17	Ribulose-1.5-bisphosphate carboxylase large subunit [Saccharum hybrid cultivar SP-08-3280]	48478779	YP_024387	215	52.73/6.33	58.0/6.58	up*	3
18	Ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit [*Pharus parvifolius*]	131984	P28428	160	51.72/5.83	58.0/6.53	up (110%)	1
19	Unnamed protein product [*Hordeum vulgare*] Ribulose-1.5-bisphosphate carboxylase large chain	11587	CAA25265	126	53.08/6.22	60.0/6.53	up (278%)	1
28	Phosphoribulokinase [*Zea mays]*	195645472	ACG42204	91	45.71/5.75	41.5/5.25	down (18%)	1
26	Malate dehydrogenase (NADP(+))[*Sorghum bicolor*]	755781	CAA37531	207	46.46/5.77	44.0/5.26	up (134%)	2

27	Hypothetical protein SORBIDRAFT 01g019280 [*Sorghum bicolor*] Malate dehydrogenase	242039369	XP_002467079	328	35.46/5.76	44.0/5.18	up (107%)	4
39	Malate dehydrogenase (NADP(+))[*Sorghum bicolor*]	755781	CAA37531	360	46.46/5.77	41.0/6.00	up (39%)	4
30	Sedoheptulose-1,7-bisphosphatase [*Triticum aestivum*]	1173347	P46285	49	42.06/6.04	40.5/5.07	down (30%)	1
31	Sedoheptulose-1,7-bisphosphatase, chloroplastic [*Triticum aestivum*]	1173347	P46285	172	42.06/6.04	40.5/4.98	down (52%)	1
34	Hypothetical protein SORBIDRAFT_10g000720 [*Sorghum bicolor*] Ferredoxin-NADP+ reductase	242094386	XP_002437683	217	39.96/8.31	38.5/6.68	up (229%)	3
35	Os06g0107700 [*Oryza sativa* Japonica group] Ferredoxin-NADP+ reductase	115465942	NP_001056570	81	40.01/8.72	36.0/6.23	up (318%)	1
42	Hypothetical protein SORBIDRAFT_ 08g004500 [*Sorghum bicolor*] Fructose-1,6-biphosphate aldolase	242084936	XP_002442893	918	41.87/6.39	38.5/5.66	down (18%)	10
44	Hypothetical protein SORBIDRAFT_08g004500 [*Sorghum bicolor*] Fructose-1,6-biphosphate aldolase	242084936	XP_002442893	472	41.87/6.39	38.5/5.54	down (45%)	7
43	Hypothetical protein SORBIDRAFT_10g031120 [*Sorghum bicolor*] Photosystem II stability/assembly factor	242097170	XP_002439075	336	43.15/8.67	39.0/5.54	down (45%)	4
45	Hypothetical protein SORBIDRAFT_05g027870 [*Sorghum bicolor*] Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic precursor	242072103	XP_002451328	243	48.47/6.98	43.0/5.49	down (56%)	3
49	Oxygen-evolving enhancer protein 1 [*Zea mays*]	195619530	ACG31595	322	34.52/5.59	31.0/5.19	down (13%)	4
53	LOC100284771 ribose-5-phosphate isomerase [*Zea mays*]	226506270	NP_001151138	146	28.78/5.53	28.0/4.83	down (61%)	2
75	Hypothetical protein SORBIDRAFT_02g002690 [*Sorghum bicolor*] Required for photosystem II activity (PspB)	242047384	XP_002461438	248	27.56/8.64	24.5/5.57	down (26%)	4
76	Hypothetical protein SORBIDRAFT_03g037420 [*Sorghum bicolor*] Required for photosystem II activity (PspB)	242054707	XP_002456499	259	28.00/8.94	25.0/5.40	down (40%)	3
79	Os08g0504500 [*Oryza sativa japonica group*] Required for photosystem II activity (PspB)	115477166	NP_001062179	87	25.51/6.85	22.5/4.96	down (74%)	1
7	ATP synthase CF1 alpha subunit [Saccharum hybrid cultivar SP-08-3280]	48478769	YP_024377	189	55.75/5.87	64.0/5.28	up (95%)	2
13	ATP synthase CF1 alpha subunit [Saccharum hybrid cultivar SP-08-3280]	48478769	YP_024377	154	55.75/5.87	65.0/5.93	down (26%)	2
14	ATP synthase CF1 alpha subunit [Saccharum hybrid cultivar SP-08-3280]	48478769	YP_024377	170	55.75/5.87	65.0/6.00	down (39%)	2

21	ATP synthase subunit, gamma, chloroplastic precursor [*Zea mays*]	226533016	NP_001150872	120	39.79/8.44	46.0/5.87	up (47%)	1
22	ATPase, beta subunit [*Hordeum vulgare*]	11583	CAA25114	70	53.87/5.17	58.0/5.60	up (26%)	1
40	ATP synthase subunit gamma, chloroplastic precursor [*Zea mays*]	226533016	NP_001150872	260	39.79/8.44	40.0/5.83	up (156%)	4
	Protein Synthesis							
24	Hypothetical protein SORBIDRAFT_04g024850 [*Sorghum bicolor*]	242062202	XP_002452390	177	50.72/6.06	48.0/5.51	up (329%)	2
	Chloroplast translational elongation factor Tu							
38	Cysteine synthase precursor [*Zea mays*]	162458737	NP_001105469	302	34.21/5.91	37.0/6.07	up*	3
56	Elongation factor 1-delta 1 [*Zea mays*]	226505926	NP_001149753	153	24.78/4.39	30.0/4.74	up (118%)	3
58	Elongation factor 1- delta [*Zea mays*]	195605696	ACG24678	137	24.88/4.39	31.0/4.58	up (26%)	2
82	Hypothetical protein SORBIDRAFT_03g030270 [*Sorghum bicolor*]	242053887	XP_002456089	413	18.75/5.55	18.5/4.70	up (77%)	4
	Ribosomal protein L7/L12							
	Secondary Metabolism							
8	Cyanogenic beta-glucosidase dhurrinase-2 [*Sorghum bicolor*]	13924741	AAK49119	97	64.66/6.22	68.0/6.01	up*	1
9	Cyanogenic beta-glucosidase dhurrinase-2 [*Sorghum bicolor*]	13924741	AAK49119	165	64.66/6.22	68.0/5.94	up*	2
52	p-(S)-hydroxymandelonitrile lyase [*Sorghum bicolor*]	666089	CAA58876	234	56.32/4.97	29.0/4.89	up (97%)	4
77	Hydroxynitrile lyase [*Sorghum bicolor]*	24987267	1GXS_B	265	17.70/5.12	22.0/5.29	up (26%)	3
78	Hydroxynitrile lyase [*Sorghum bicolor]*	24987267	1GXS_B	312	17.70/5.12	22.0/5.15	up*	5
	Disease/defence							
36	Aldo-keto reductase/oxidoreductase [*Zea mays*]	226528361	NP_001149144	129	40.88/7.55	41.5/6.11	up (62%)	3
46	Hypothetical protein SORBIDRAFT_02g004000 [*Sorghum bicolor*]	242043050	XP_002459396	191	38.13/6.03	38.5/5.37	down (65%)	3
	Contains tetratricopeptide repeat domain (TTR)							
50	Hypothetical protein SORBIDRAFT_05g023220 [*Sorghum bicolor*]	242069013	XP_002449783	155	30.50/8.84	31.5/4.94	down (29%)	2
	Plastid lipid-associated protein/fibrillin							
64	Hypothetical protein SORBIDRAFT_02g044060 [*Sorghum bicolor*]	242051414	XP_002463451	228	27.16/5.18	28.5/5.43	up (61%)	4
	Ascorbate peroxidase							
66	Hypothetical protein SORBIDRAFT_01g038760 [*Sorghum bicolor*]	242041317	XP_002468053	225	27.22/5.55	27.0/5.70	up (37%)	3
	Ascorbate peroxidase							

68	Hypothetical protein SORBIDRAFT_03g035420 [*Sorghum bicolor*] Glutathione S-transferase	242058791	XP_002458541	543	23.88/5.62	27.5/6.07	up (61%)	7
69	Hypothetical protein SORBIDRAFT_01g005990 [*Sorghum bicolor*] Glutathione S-transferase	242032767	XP_002463778	354	25.47/6.02	26.0/6.13	up*	5
74	Hypothetical protein SORBIDRAFT_09g004970 [*Sorghum bicolor*] Cupin	242087035	XP_002439350	163	22.75/6.02	22.0/5.62	down (40%)	2
80	Os02g0537700 [*Oryza sativa japonica group*] Peroxiredoxin	115446541	NP_0010470	103	28.10/5.67	23.0/4.87	down (23%)	2
83	Hypothetical protein SORBIDRAFT_04g021590 [*Sorghum bicolor*] contains heavy metal-associated domain (HMA)	242065280	XP_002453929	209	11.48/4.71	19.0/4.62	up*	3
	Signal transduction							
81	Hypothetical protein SORBIDRAFT_08g001890 [*Sorghum bicolor*] contains Forkhead associated (FHA) domain	242084608	XP_002442729	226	24.48/8.31	19.5/4.83	down (24%)	3

Proteins were grouped into functional categories according to Bevan et al. [37].

a) Spot numbers as illustrated in Figure 4C.

b) Gene identification number.

c) NCBI protein accession numbers.

d) Mascot score.

e) Theoretical M*r* and p*I* as calculated using Compute pI/Mw tool available on ExPASy (http://web.expasy.org/compute_pi).

f) Experimental M*r* and p*I* of the proteins we estimated from the 2D gel images (Figure 4C).

g) Expression changes of protein spots as measured by PDQuest software. Values in brackets indicate the percentage change of protein expression after salt treatment relative to the control group. * Indicates that spot under review was only present in the salt treated group. All reported changes were statistically significant using Student's *t*-test ($p<0.05$).