A PROTEOMIC ANALYSIS OF DROUGHT AND SALT STRESS RESPONSIVE PROTEINS OF DIFFERENT SORGHUM VARIETIES

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae, in the Department of Biotechnology, University of the Western Cape.

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May 2009

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KEYWORDS

Sorghum proteomics

Cereals

Drought and salinity stresses

Sorghum cell suspension cultures

Secretome

2D SDS-PAGE

MALDI-TOF MS

MALDI-TOF-TOF MS

Protein identification

Proteome reference maps

ABSTRACT

A proteomic analysis of drought and salt stress responsive proteins of different sorghum varieties

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Sorghum (*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. This study reports on a proteomic analysis of sorghum proteomes in response to salt and hyperosmotic stresses. Two-dimensional gel electrophoresis (2DE) in combination with mass spectrometry (MS) was used to separate, visualise and identify sorghum proteins using both sorghum cell suspension cultures and whole plants. The sorghum cell suspension culture system was used as a source of culture filtrate (CF) proteins. Of the 25 visualised CBB stained CF spots, 15 abundant and well-resolved spots were selected for identification using a combination of MALDI-TOF and MALDI-TOF-TOF MS, and database searching. Of these spots, 14 were positively identified as peroxidases, germin proteins, oxalate oxidases and alpha-galactosidases with known functions in signalling processes, defense mechanisms and cell wall metabolism. Following 200 mM NaCl and 400 mM sorbitol stress treatments, the expression/abundance of a protein spot similar to a rice wall-associated protein kinase

was upregulated in the sorghum secretome in response to both stresses. Amino acid sequence alignment of the matching peptides between these two proteins showed that the sorghum CF spot possesses a protein kinase domain. Therefore, this protein could possibly participate in cell signalling functions, which link the external environment with the cell's cytoplasm. Using whole plant systems, a comparative study of leaf protein expression between two sorghum varieties, AS6 (salt sensitive) and MN1618 (salt tolerant) was conducted. Forty well resolved spots of varying abundances were picked for MS analysis. Of these, 28 were positively identified, representing proteins with functions in carbohydrate metabolism (60.7%), proton transport (17.9%), protein synthesis (7.1%), hydrolytic functions (7.1%), nucleotide metabolism (3.6%) and detoxification (3.6%). Using PDQuest^{TM} Advanced 2D Analysis Software version 8.0.1 (BIO-RAD), a comparative analysis of leaf proteome expression patterns between the two sorghum varieties was conducted. The results indicated proteins with similar expression patterns as well as qualitative and quantitative differences between the two leaf proteomes. The effect of 100 mM NaCl on leaf proteome expression between the two sorghum varieties was also studied. Western blotting analysis of leaf, sheath and root tissues using Hsp70 antibodies showed that this treatment induced Hsp70 expression, a known stress protein, in both varieties. Thereafter, the partially annotated leaf proteome map was used to landmark other salt responsive proteins. Examples of differential expression patterns included glutathione S transferase and hydroxynitrile lyase proteins whose abundances were upregulated in both varieties, while the large subunit of RuBisCo was downregulated in AS6 but upregulated in MN1618. Qualitative spot expression differences in response to salt stress were also observed between the two sorghum

varieties but these remained unidentified after both MALDI-TOF and MALDI-TOF-TOF MS, possibly indicating novel and previously uncharacterised sorghum proteins. The results of this study can be used as reference tools by proteomics researchers worldwide as well as a foundation for future studies.

DECLARATION

I declare that **A proteomic analysis of drought and salt stress responsive proteins of different sorghum varieties** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Rudo Ngara May 2009

Signed ----------

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Prof. Bongani K. Ndimba and Prof. Jasper Rees for their guidance throughout the course of the study. Thank you Prof. Ndimba for your invaluable input on the proteomics side of life. My sincere gratitude goes to Prof. Toni Slabas, Dr Bill Simon and their team at Durham University, School of Biological and Biomedical Sciences, for identifying of all the sorghum leaf and secretome proteins by MALDI-TOF and MALDI-TOF-TOF MS. I am also grateful for financial support received from the National Research Foundation as well as the Royal Society of London. My sincere gratitude goes to Dr Mervin Meyer for never saying 'no' in rendering help with the microscopy work. To the lab members of both the Proteomics Research Lab and the Biochem Lab, past and present, Nonku, Ludivine, Joseph, Simpiwe, Majimi James, Claudius, I cannot name you all. Thank you for everything you have shared with me during this learning phase of my life. To my family, husband Kudzayi, son Takudzwa and daughter Ruvimbo, you are all most appreciated for being so patient with me during my studies. Kudzayi, you have been a pillar of strength and immeasurable support during the years. I thank you. To my parents, siblings and the extended family, I thank you all for the roles you have played in my life thus far.

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LIST OF ABBREVIATIONS

AGPase	Adenosine diphosphate glucose pyrophosphatase
ADK	Adenylate kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulfate
ATP	adenosine triphosphate
BLAST	Basic local alignment search tool
BLASTp	Protein to protein BLAST
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CC	Condensed and shrinking cytoplasm
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CF	Culture filtrate
CCOMT	Caffeoyl-CoA *O*-methyltransferase
CW	Cell wall
Da	Dalton
DMSO	dimethyl sulphoxide
DTT	Dithiothreitol Cleland's reagent
ECM	Extracellular matrix
EDC	Even distribution of cytoplasmic contents

EGF	Epidermal growth factor
EST	Expressed sequence tag
GST	Glutathione S transferase
HNL	Hydroxynitrile lyase
Hsc70	Heat shock cognate 70
Hsp70	Heat shock protein 70
IEF	Isoelectric focusing
pI	Isoelectric point
IPG	Immobilised pH gradient
kDa	kilo Dalton
LSU	Large subunit
LEA	Late-embryogenesis abundant protein
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
MALDI-TOF-TOF	Matrix assisted laser desorption/ionisation-time of flight/time of flight tandem mass spectrometry
MASCOT	Matrix Science
MDH	Malate dehydrogenase
MES	2-(N-Morpholino)ethanesulfonic acid
MOWSE	Molecular weight search
MS	Mass spectrometry
MS medium	Murashige and Skoog Basal medium
MSMO	Murashige and Skoog Basal Salt with minimal organics
MS/MS	Tandem mass spectrometry

MSDB	Mass spectrometry protein sequence database
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
MW	Molecular weight
m/z	mass to charge ratio
NAA	1-naphthaleneacetic acid
NCBI	National Center for Biotechnology Information
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenide dinucleotide phosphate
OEC	Oxygen-evolving complex
PAGE	Polyacrylamide gel electrophoresis
PCV	Packed cell volume
PM	Plasma membrane
PMF	Peptide mass fingerprinting
PPase	Pyrophosphatases
PTM	Post translational modification
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RuBisCo	Ribulose-1,5-biphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-biphosphate
SDS	Sodium dodecyl sulfate
SOS	Salt Overly Sensitive
2,4-D	2,4-dichlorophenoxyacetic acid
TBS	Tris-buffered saline

TBST	Tris-buffered saline containing Tween 20
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TOF	Time of flight
TSP	Total soluble protein
1D	One-dimensional
2D	Two-dimensional
2DE	Two-dimensional gel electrophoresis
2D-SDS-PAGE	Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
V	Volts
Vhrs	Volt hours
v/v	volume to volume
w/v	weight to volume
WAK	Wall-associated protein kinase

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CHAPTER 1

Literature Review

1.1 Sorghum

Sorghum [*Sorghum bicolor* (L.) Moench; Figure 1.1] is a drought-tolerant cereal crop (Rosenow *et al.*, 1983), which survives well in hot and dry environmental conditions that are least suitable for other grain crops. In terms of both area planted and production yields, sorghum is ranked the fifth most important cereal in the world after maize, rice, wheat and barley (Doggett, 1988) and second after maize in Africa (Food and Agriculture Organization of the United Nations, 1995; FAOSTAT, 2006). It is mainly used as a food source for humans in Africa and Asia as well as stock feed and a potential source of bio-ethanol in the United States (Sasaki and Antonio, 2009). Apart from its drought tolerant characteristics, sorghum is relatively more salt tolerant than maize (Krishnamurthy *et al.*, 2007), the world's most cultivated cereal. As such, sorghum is not only an important food source in the semi-arid regions worldwide, but also a potential model crop for studying and gaining a better understanding of the molecular mechanisms of drought and salt tolerance in cereals.

(Source: African Crops, 2009)

Figure 1.1: Sorghum crop under cultivation.

1.1.1 Studies on Drought and Salt Stress Responsive Mechanisms of Sorghum

Despite the vast economic potential of sorghum in semi-arid areas, the molecular basis of drought and salt tolerance mechanisms of this crop are still poorly understood. Sorghum's drought tolerance is partly due to biochemical and structural features such as C_4 photosynthesis, a deep root system and a thick waxy cuticle that helps improve the efficiency in water use (Buchanan *et al.*, 2005). Physiological drought tolerance in sorghum is of two types, namely "pre-flowering" and "post-flowering" (Rosenow *et al.*, 1983). Pre-flowering drought tolerance occurs during the early vegetative stages of seedling growth and panicle development. On the other hand, post-flowering drought tolerance is expressed during grain development. Post-flowering drought tolerance is also associated with the stay-green trait, where leaves retain chlorophyll and thus maintain

photosynthetic activities under stress conditions (Sanchez *et al.*, 2002; Harris *et al.*, 2007).

Limited molecular biology studies on sorghum's mechanisms of stress tolerance have been reported. In independent studies, different sorghum genotypes were reported to show a rapid induction and increased accumulation of dehydrin (dehydration-induced protein) mRNA under drought stress conditions (Cheng *et al.*, 1993; Buchanan *et al.*, 2005). The accumulation of dehydrin proteins was also compared between drought-tolerant and drought-susceptible sorghum genotypes under drought stress (Wood and Goldsborough, 1997). Using *in vivo* labelling and immunoblotting analysis both genotypes were found to express a dehydrin protein under water stress, which disappeared upon recovery from the stress. The authors argued that although dehydrins are typical stress responsive proteins, the observed expression patterns between the two sorghum genotypes could not explain phenotypic differences in drought tolerance.

Several plants including sorghum are known to synthesise and accumulate glycine betaine in response to salt and/or water stress. The physiological functions of glycine betaine and other osmolytes are discussed in Section 1.4.1.1.1. Wood and co-workers reported induced expression of betaine aldehyde dehydrogenase and the accumulation of glycine betaine in water-stressed sorghum plants (Wood *et al.*, 1996). Betaine aldehyde dehydrogenases together with choline monooxygenase are enzymes that are involved in a two-step oxidation of choline to glycine betaine via betaine aldehyde (Kotchoni and Bartels, 2003).

A study was also conducted to assess the variability of salt tolerance among 100 sorghum genotypes following 250 mM NaCl treatment (Krishnamurthy *et al.*, 2007). By measuring shoot biomass production after 39 days under salt stress, it was observed that sorghum has a wide genotypic variation in salt tolerance. Therefore, sorghum could be an important crop for use in breeding programmes for the improvement of salt tolerance in other economically important genotypes.

1.2 Moving from Model Plants to Crop Plants

Over the years, most of the knowledge on plant molecular response mechanisms to abiotic stresses has been gained from work using Arabidopsis (*Arabidopsis thaliana*; Zhu, 2000; Denby and Gehring, 2005; Vinocur and Altman, 2005) mainly because of its small and fully sequenced genome (The Arabidopsis Genome Initiative, 2000). On the other hand, rice (*Oryza sativa*) the first cereal crop to be sequenced (International Rice Genome Sequencing Project, 2005) is currently regarded as a model plant amongst the grasses. Complete plant genome sequences are indeed invaluable resource tools for both genomic and proteomics studies as they landmark genes or proteins that are responsive to the stresses under study (Lin *et al.*, 2003; Tester and Bacic, 2005). In addition, genome sequence information is useful for the identification of variations in gene sequence that can be used in various plant breeding strategies (Sasaki and Antonio, 2009).

Nevertheless, even though Arabidopsis provides an excellent model system for studying various plant processes, the use of this plant in salinity and/or drought stress studies has its own limitations. Firstly, Arabidopsis is a typical glycophyte that is not adapted to

either salt or drought stress (Zhu, 2000; Vinocur and Altman, 2005). Secondly, this plant is a dicotyledon (dicot) and agriculturally unimportant (van Wijk, 2001). It is also known that monocotyledons (monocots) and dicots are fundamentally different in their structure, development (Tester and Bacic, 2005) and mechanisms of osmotic adjustment (Flowers and Yeo, 1986). For example, dicot halohytes (salt tolerant plants) are succulent and can tolerate high concentrations of sodium (Na^+) and chloride (Cl^-). On the other hand, monocot halohytes are relatively non-succulent preferring to take up potassium (K^+) more than Na^+ from the environment (Flowers and Yeo, 1986). Therefore, the transfer of physiological knowledge gained using model plants such as Arabidopsis to agriculturally important cereals (Tester and Bacic, 2005) for instance, might not be possible or straight forward (Denby and Gehring, 2005). Although rice is currently providing insight on many plant processes amongst the grasses, the use of rice in studies relating to salt stress is limited by the fact that this crop does not tolerate salt and is the most salt sensitive cereal (Munns and Tester, 2008). It is of much greater value therefore, to gain knowledge from agriculturally crops such as sorghum, which are naturally drought tolerant and moderately salt tolerant. The use of sorghum in comparative proteomic analysis of drought and/or salt stress responsive proteins in encouraged by the recent announcement that the sorghum genome sequencing project (Sorghum Genomic Planning Workshop Participants, 2005) has been completed (Paterson *et al.*, 2009).

1.3 Background on the Effects of Abiotic Stresses on Plants

Plants encounter a wide range of abiotic stresses such as drought, high soil salinity and extremes of temperature, which limit their growth and development (Sachs and Ho,

1986). Under extreme conditions, the plants might even die. For agricultural crops, potential yields may be drastically reduced (Altman, 2003; Tester and Bacic, 2005) thus affecting the world's food supply. Worldwide, abiotic stresses are the primary causes of crop losses (Vinocur and Altman, 2005), reducing the potential yields by more than 50% (Boyer, 1982). By 2030, climatic changes are projected to reduce cereal production in Africa by about 2 to 3% (Food and Agriculture Organization of the United Nations, 2002).

Drought and salinity stresses are most prevalent in semi-arid regions and/or regions that experience low rainfall patterns and thus dependent on irrigation (Wang, Vinocur *et al.*, 2003; Denby and Gehring, 2005). Soil salinization in particular is projected to affect more than 50% of all arable lands by the year 2050 (Wang, Vinocur *et al.*, 2003). With the increase in the world's population coupled with the global climatic changes, there is a growing need to develop food crops that are well adapted to these extreme environmental conditions (Flowers, 2004; Vinocur and Altman, 2005). It is of great importance therefore, to fully understand the underlying plants' tolerance mechanisms to drought and salinity in cereal crops such as sorghum, which are relatively more drought and salt tolerant than maize, the most cultivated cereal. The knowledge gained would be useful for the genetic improvement of stress tolerance of agriculturally important crops in order to improve food security (Sachs and Ho, 1986; Mundree *et al.*, 2002; Seki *et al.*, 2003; Denby and Gehring, 2005).

1.3.1 The Complex Nature of Plant Response Mechanisms to Abiotic Stresses

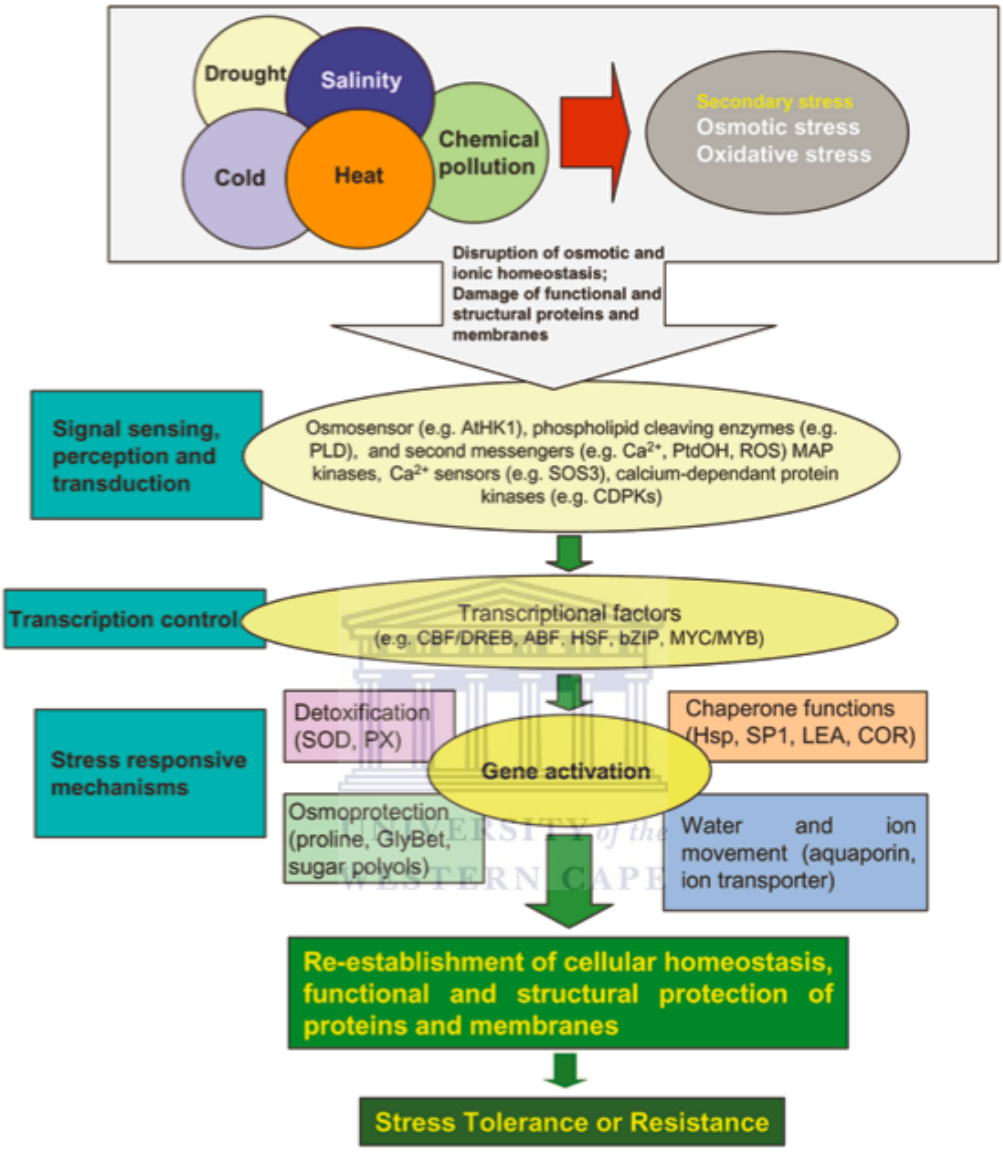
In order to survive, reproduce and complete their life cycles, plants have naturally developed a wide range of adaptive and tolerance mechanisms to abiotic stresses. These mechanisms range from morphological, developmental, biochemical, molecular and cellular changes that occur once the plant is exposed to a specific stress condition. Understanding the nature of these response mechanisms in totality is complicated by several reasons. Firstly, a single stress may occur at multiple stages of the plant's development (Chinnusamy *et al.*, 2004), and thus interacts uniquely with that particular developmental stage. Secondly, more that one stress may simultaneously affect the plant (Chinnusamy *et al.*, 2004) or be separated in time (Knight and Knight, 2001), thus resulting in complex physiological and molecular response (Mittler, 2006). Thirdly, plants under different stresses may share common response mechanisms, perception and signal pathways as protective action against the stresses (Chinnusamy *et al.*, 2004). This being due to the fact that under certain conditions the two stresses cannot be distinguished from one another (Knight and Knight, 2001).

The complexity of studying and understanding plant response mechanisms to abiotic stresses is further compounded by the fact that a primary stress is capable of producing a secondary or even a tertiary stress (Levitt, 1980). In some cases, different primary stresses may produce the same secondary or tertiary stress. For example, primary stresses such as drought, salinity, cold, heat and chemical pollution are interconnected, all producing secondary stresses such as osmotic and oxidative stress (Wang, Vinocur *et al.*, 2003). Therefore, the response to a particular primary stress may result in a complex

network of response mechanisms against effects of all the secondary and tertiary stresses that are induced by a specific primary stress (Levitt, 1980).

Nevertheless, molecular studies on plant responses to abiotic stresses have indicated that gene expression may be altered resulting in an increase, decrease, induction or total suppression of some proteins. Some stress-induced proteins of plants under stress may allow plants to make biochemical and structural adjustments (Sachs and Ho, 1986; Ho and Sachs, 1989; Kasuga *et al.*, 1999), which are necessary for conferring stress tolerance. Abiotic stress inducible genes can thus be classified into two groups (Seki *et al.*, 2003; Shinozaki *et al.*, 2003). The first group encodes proteins that directly protect the plant cells against the stresses such as enzymes involved in the biosynthesis of various osmoprotectants, late-embryogenesis abundant (LEA) proteins, chaperones and detoxification enzymes. The second group encodes for proteins that regulate gene expression and signal transduction in abiotic stress responses such as transcription factors and protein kinases (Seki *et al.*, 2003; Shinozaki *et al.*, 2003).

Figure 1.2 shows a diagrammatic illustration of the complexity of plant response to abiotic stresses. As plants are exposed to a range of abiotic stresses, signals are relayed to the intracellular machinery for the activation of gene expression. As gene expression increases, stress responsive mechanisms are activated thus resulting in the restoration of cellular homeostasis, and the protection and repair of stress damaged proteins and membranes (Wang, Vinocur *et al.*, 2003).



(Source: Wang, Vinocur *et al.*, 2003)

Figure 1.2: The complexity of plant responses to abiotic stresses.

1.4 Salt Stress

Salt stress is defined as the presence of excessive concentrations of soluble salts in a solution and is measured as electrical conductivity (ECe; in deciSiemens/m) at 25°C (Flowers and Yeo, 1986; Hale and Orcutt, 1987). Saline soils have an ECe of at least 4 dS/m, which is equivalent to 40 mM NaCl (Munns and Tester, 2008). Salt stress causes both primary and secondary stresses to plants (Levitt, 1980). The primary stresses induced by salt stress include ionic and osmotic stresses (Hale and Orcutt, 1987; Zhu, 2001) while secondary stresses include ion imbalance, nutrient deficiency (Wyn Jones, 1981; Hurkman, 1990) and oxidative stress (Zhu, 2001). The actual effects of salt stress and the degree of injury to plants vary with plant species, the developmental stage of the plant as well as the severity of the stress and duration of exposure (Levitt, 1980; Munns, 2002). For example, seed germination is generally thought to be much more resistant to salt stress than later stages of seedling growth (Levitt, 1980; Flowers, 2004). However, in sugar beet, the seed germination phase is more susceptible to salt stress than the other phases of growth and development (Hale and Orcutt, 1987). In rice, grain yield is more depressed by salt stress than is vegetative growth (Flowers, 2004). Generally, plants may be classified as halophytes or glycophytes meaning salt tolerant or sensitive respectively (Levitt, 1980).

1.4.1 Physiological Responses of Plants to Primary and Secondary Effects of Salt Stress

1.4.1.1 Osmotic Stress

High concentrations of soluble salts in saline soils lower the osmotic potential of the soil, thus resulting in osmotic stress (Munns, 2002; Sairam and Tyagi, 2004). Under conditions of osmotic stress, plants tend to lose water to the external environment resulting in a decrease in turgor pressure (Munns, 2002; Shao *et al.*, 2007). The loss of turgor pressure in turn affects cellular processes such as cell division and expansion (Zhu, 2001; Xiong and Zhu, 2002), which are essential processes during plant growth and development. To avoid excessive water loss, plants growing under saline conditions decrease their intracellular water potential by osmotic adjustment (Wyn Jones, 1981).

1.4.1.1.1 Osmotic Adjustment

Osmotic adjustment (also termed osmoregulation) of plants under salt stress is achieved by the accumulation of ions or organic solutes (Levitt, 1980). In most halophytes, ions such as Na^+ and Cl^- accumulate from the external environment and are compartmentalised in vacuoles. In cereals, K^+ is selectively retained in the cytoplasm while Na^+ is stored in the vacuoles where it is used as osmoticum for osmotic adjustment (Wyn Jones, 1981). The retention of K^+ in the cytoplasm also balances the osmotic pressure of Na^+ and Cl^- ions in the vacuoles (Munns, 2002). Vacuoles occupy nearly up to 90% of the cell's volume and function in maintaining size, shape as well as storing resources, toxic materials and waste products (Yeo, 1998). This compartmentation

separates toxic ions away from the cytoplasm thus preventing ion toxicity on the cell's metabolic processes as well as accumulating ions for osmotic adjustment in vacuoles (Niu *et al.*, 1995; Hasegawa *et al.*, 2000; Zhu, 2001).

Another mode of osmotic adjustment in plants involves the accumulation of organic solutes (also termed compatible solutes, osmolytes or osmoprotectants) in the cytoplasm and the lumen, matrix or stroma of organelles (Hasegawa *et al.*, 2000) but almost absent in the vacuoles (McNeil *et al.*, 1999). Compatible osmolytes are low molecular weight and highly soluble compounds that are not toxic to the plant's cellular metabolic activities even at high concentrations (McNeil *et al.*, 1999) where Na^+ and Cl^- would be toxic (Yeo, 1998). The types of osmolytes, which accumulate in plants differ depending on plant species but generally include betaines and related compounds (glycine betaine, proline betaine, ß-Ala betaine), polyols and sugars (mannitol, sorbitol, trehalose) and amino acids such as proline (McNeil *et al.*, 1999; Sairam and Tyagi, 2004). For instance, although glycine betaine is generally found to accumulate in the cytoplasm of most plants, Arabidopsis, rice and tobacco are non-accumulators of glycine betaine while mannitol is not usually found in both tobacco and Arabidopsis (Chen and Murata, 2002). On the other hand, soya beans and potatoes lack significant amounts of betaines or other compatible osmolytes (McNeil *et al.*, 1999). Under salt stress conditions, sorghum embryos have been shown to accumulate high levels of proline in comparison with the control (Thakur and Sharma, 2005). Since the increase in proline accumulation was not necessarily due to proteolysis, the authors proposed that proline could be used by

germinating sorghum embryos as an adaptive strategy to prevent germination and seedling development under unfavourable conditions.

Compatible osmolytes can either act as osmotica for osmotic adjustment or as osmoprotectants of cellular molecules under a wide range of abiotic stresses. As osmotica, the accumulation of osmolytes in the cytoplasm facilitates osmotic adjustment by lowering the internal osmotic potential (Flowers and Yeo, 1986; Hasegawa *et al.*, 2000). As osmoprotectants, osmolytes such as glycine betaine help stabilize macromolecules under osmotic stress (Wyn Jones, 1981; Yeo, 1998), while mannitol acts as a reactive oxygen species (ROS) scavenger during oxidative stress (Bohnert and Jensen, 1996) thus preventing damage of cellular structures (Zhu, 2001).

However, the actual mechanism of action of these osmoprotectants in protecting cellular structures during stress conditions is not well known. Xiong and Zhu (2002), state that high concentrations of charged elements in the cytoplasm change the hydration spheres of proteins, thus affecting their conformation and/or charge interaction. On the other hand, organic solutes which act as osmoprotectants, are strong water-structure formers and become excluded from the hydration shells of native proteins (Yeo, 1998). As a result, osmoprotectants help to maintain proteins in their folded states, unlike other solutes such as NaCl and $MgSO_4$, which directly interact with protein surfaces causing proteins to unfold and thus denature (McNeil *et al.*, 1999). The cytoplasmic accumulation of glycine betaine also causes tonoplast (vacuole membrane) Na^+ fluxes resulting in an increase in

Na^+ concentration in the vacuoles (Wyn Jones, 1981) thus protecting cytoplasmic cellular components from Na^+ toxicity (Hasegawa *et al.*, 2000; Zhu, 2001).

1.4.1.2 Ionic Stress

In natural environments, most of the salt stress is due to sodium salts in particular NaCl, the most common salt (Levitt, 1980; Munns and Termaat, 1986). High concentrations of NaCl in soils disturb the ionic balance of Na^+, Cl^-, K^+ and Ca^{2+} (calcium) (Niu *et al.*, 1995). Of these ions, Na^+ and Cl^- are the most important, as they disrupt the metabolic processes of cells both in the cytoplasm and in organelles (Flowers and Yeo, 1986; Hasegawa *et al.*, 2000; Tester and Davenport, 2003). The ability to avoid ionic toxicity therefore relies on amongst other factors, the ability to exclude Na^+ and/or Cl^- from the shoots and to tolerate high levels of these ions in the cytoplasm (Wyn Jones, 1981; Munns and Tester, 2008).

Plants have developed various tolerance mechanisms against ionic effects of salinity but these responses differ with plant species as well as cell types. This results in diverse tolerance mechanisms, which usually occur simultaneously at different levels of organisation within the plant. In general, mechanisms of ion tolerance in whole plant systems include the regulation of ion uptake from the soil, their transportation from the roots to the shoots, compartmentation in vacuoles and/or secretion out of the leaves (Levitt, 1980; Munns and Termaat, 1986; Hale and Orcutt, 1987; Cushman *et al.*, 1990; Yeo, 1998; Munns, 2002; Tester and Davenport, 2003). For some salt sensitive plants, the control of initial salt entry into plants via roots is probably the first possible mechanism

of avoiding ion toxicity. Passive salt absorption is regulated by the roots impermeability to salts even if the plant is growing in a hypersaline environment. In *Phaseolus vulgaris* (common bean), Na^+ is absorbed by the roots but excluded from the shoots by a regulatory mechanism that prevents the translocation of salts from the roots to shoots. Within the roots, salts are stored in the vacuoles (Levitt, 1980).

In cases where the ions have been absorbed into the xylem vessels for translocation to the shoots, another tolerance mechanism should exist in the shoots, which would help minimise the effects of ion toxicity. Leaves are more vulnerable to Na^+ toxicity than roots because the ions are continuously transported unidirectionally via the xylem vessels from the roots to the leaves where they accumulate (Tester and Davenport, 2003). The amount of ions supplied to the leaves varies depending on the xylem ion concentration as well as the rate of transpiration (Flowers and Yeo, 1986). Without adequate and effective means of salt compartmentation in the vacuoles, Na^+ may accumulate in the cytoplasm and cause ion toxicity, necrosis of older leaves and a reduction in plant productivity. Salt concentrations are therefore higher in older leaves than younger ones at any given time (Munns and Termaat, 1986; Munns, 2002; Munns and Tester, 2008).

The exclusion of Na^+ from the cytoplasm as well as organelles and its sequestration in vacuoles via vacuolar Na^+/H^+ antiporters (Cushman *et al.*, 1990; Niu *et al.*, 1995; Zhu, 2001, 2003) is therefore an important mechanism of avoiding Na^+ accumulation and toxicity in the cytosol. The Na^+/H^+ antiporters use the pH gradient generated by P-type H^+-ATPase (for plasma membrane antiporters) or V-type H^+-ATPase or H^+-

pyrophosphatases (PPase; for tonoplast antiporters; Xiong and Zhu, 2002). The activities of these ATPases increase in cells exposed to NaCl stress (Sairam and Tyagi, 2004). Apart from Na^+, other ions that may accumulate in the vacuoles include K^+, Ca^{2+}, Mg^{2+}(magnesium), Cl^-, SO_4^{2-} (sulfate) and NO_3^- (nitrate; Levitt, 1980).

Saline soils are sources of cheap and readily available solutes such as Na^+ and Cl^- for use in osmotic adjustment during salt stress. However, these ions are toxic to cells. To maintain osmoregulatory mechanisms, cells of salt sensitive plants have to expend energy synthesising organic osmolytes (Tester and Davenport, 2003), which stabilize and maintain protein function (Section 1.4.1.1.1). However, dicot halophytes are able to tolerate large concentration of Na^+ and Cl^- for osmotic adjustment while monocot halophytes, which are non-succulent, prefer to take up K^+ rather than Na^+ from saline soils. In the vacuoles, Na^+ acts as osmoticum for osmotic adjustment while potassium and osmolytes are mostly confined to the cytoplasm where they balance out the osmotic pressure of ions in the vacuoles (Munns, 2002). Halophytes can also accumulate large quantities of Na^+ in the shoots (up to 50% of dry weight) without dying (Flowers and Yeo, 1986) or secrete them from leaves onto leaf surfaces via salt glands or bladders (Wyn Jones, 1981; Hasegawa *et al.*, 2000).

1.4.1.3 Nutrient Deficiency

Salinity stress exposes plants to nutrient deficiency due to the competition of ion uptake from the saline soils (Levitt, 1980; Hale and Orcutt, 1987). During conditions of salt stress, there seems to be an over-dominance of nonessential ions over essential ones

(Hale and Orcutt, 1987). Absorption of essential ions is remarkably reduced thus resulting in a deficiency of that nutrient in the plant (Hale and Orcutt, 1987; Sairam and Tyagi, 2004). In addition, as salt stress leads to a reduction in the overall plant growth, reduced root growth may in turn limit the uptake of nutrients (Levitt, 1980).

High concentrations of Na^+ may also disturb the uptake of nutrients by directly interfering with ion transporters in the roots such as K^+ selective ion channels (Wyn Jones, 1981; Niu *et al.*, 1995; Hasegawa *et al.*, 2000). Since K^+ is needed for various intracellular processes such as activation of enzymes (as a co-factor) and protein synthesis where high K^+ are needed for the binding of tRNA to ribosomes (Tester and Davenport, 2003), the deficiency of K^+ in growing cells may affect both enzymatic processes as well as protein synthesis (Wyn Jones, 1981). With over 50 enzymes depending on activation by K^+, high levels of Na^+ or Na^+: K^+ ratios in the cytoplasm may affect the rate of many enzymatically controlled cellular processes (Tester and Davenport, 2003).

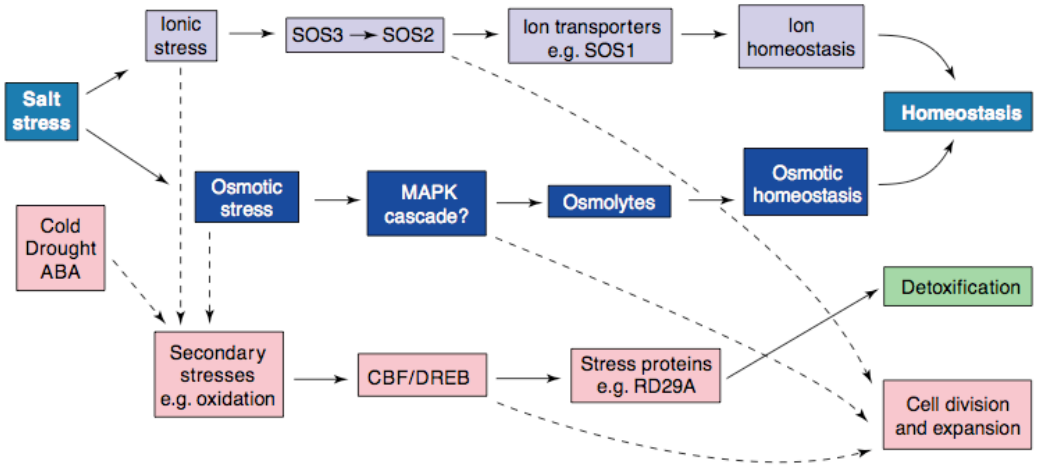
1.4.1.4 Oxidative Stress

In plants, oxidative stress is caused by excessive ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), superoxide radical (O_2^-) and singlet oxygen (O_2^1; Smirnoff, 1993; Mittler, 2002; Sairam and Tyagi, 2004). Under normal growth conditions, ROS are generated by cellular processes such as photorespiration and β-oxidation of fatty acids but their levels increase when plants are exposed to both biotic and abiotic stress conditions (Chen and Murata, 2002; Xiong and Zhu, 2002). Reactive oxygen species may

cause direct damage to cellular components such as membrane lipids, photosystem II complex (Smirnoff, 1993; Chen and Murata, 2002; Mittler, 2002), proteins and DNA (Xiong and Zhu, 2002). To prevent cellular damage, plant eliminate ROS mainly by antioxidant compounds such as glutathione, ascorbic acid, thioredoxin and carotenoids as well as ROS scavenging enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Xiong and Zhu, 2002). The levels and activities of these scavenging enzymes do increase in response to salt stress (reviewed in Section 1.8). Chloroplasts and other photosynthetic cells are prone to oxidative damage mainly because they are sites of high oxygen concentration, contain large amounts of polyunsaturated lipids in the thylakoid membranes and can absorb light and use the energy to form various ROS (Smirnoff, 1993).

1.4.2 Overview of Aspects of Salt Tolerance in Plants

As discussed in Section 1.4.1, salt stress disturbs the osmotic and ionic balance as well as nutrient distribution within plants. Salt stress also causes oxidative damage of proteins and membranes, and a decrease in metabolic activities, which ultimately affects plant growth and development (Zhu, 2001). Therefore, in order to tolerate salt stress and thus maintain cellular activities, plants have to maintain both osmotic and ionic homeostasis as well as growth under the unfavourable conditions. Figure 1.3 illustrates a summary of the mechanisms of salt tolerance in plants (homeostasis, detoxification and cell growth).

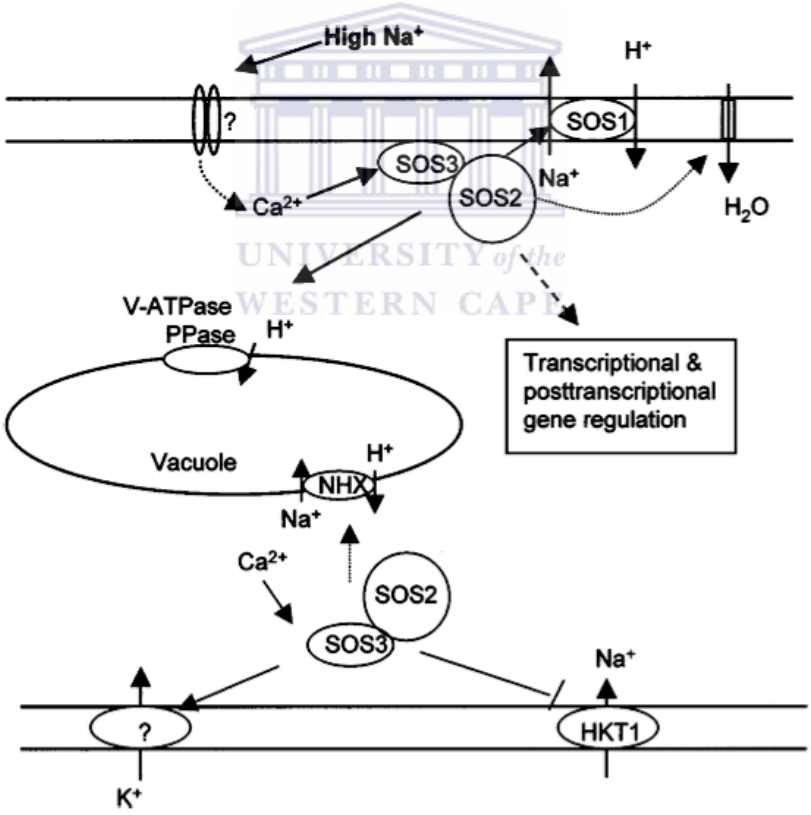


(Source: Zhu, 2001)

Figure 1.3: Summary of three aspects of salt tolerance in plants.

Homeostasis is the keeping constant of internal cellular environments. Ionic homeostasis is largely determined by the activities of ion transporters (Zhu, 2001). In Arabidopsis, the salt overly sensitive (SOS) pathway illustrated in Figure 1.4 is involved in ionic homeostasis (Zhu, 2000, 2001, 2002, 2003). Components of this pathway include; the SOS3 gene, which encodes a calcium-binding protein, the SOS2 gene that encodes a serine/threonine protein kinase and the SOS1 gene that encodes a plasma membrane Na^+/H^+ antiporter (Zhu, 2000, 2001, 2002, 2003). Salt, cold and drought stresses in plants cause an increase in cytosolic Ca^{2+} either due to influxes from the apoplast or cytoplasmic stores (Knight and Knight, 2001; Xiong and Zhu, 2002). Calcium has been reported to have signalling functions through the activation of protein kinases in plants under stress (Bressan *et al.*, 1998; Zhu, 2002). The increase in cytosolic Ca^{2+} is sensed by the SOS3 calcium-binding protein, resulting in the activation of the SOS2 encoded protein kinases. Serine/threonine protein kinases function in protein phosphorylation, one of the most

important post translational modifications (PTMs) in plants under stress (Chitteti and Peng, 2007; Kersten *et al.*, 2009). The SOS3-SOS2 protein kinase complex subsequently controls the expression and activity of the SOS1 plasma membrane Na^+/H^+ antiporters, V-ATPases, PPases and vacuolar Na^+/H^+ antiporters. The plasma membrane Na^+/H^+ antiporters are involved in the transportation of Na^+ efflux out of the cell while the vacuolar Na^+/H^+ antiporters are involved in compartmentation of Na^+ into the vacuoles. On the other hand, V-ATPases and PPases provide the proton motive force for ion transport processes across the tonoplast (Zhu, 2000, 2001, 2002, 2003).



(Source: Zhu, 2002)

Figure 1.4: Regulation of ionic homeostasis by the SOS pathway, as a mechanism of salt tolerance.

Osmotic homeostasis is largely maintained by the synthesis of osmolytes (reviewed in Section 1.4.1.1.1), while oxidative damage of proteins and cellular membranes is reduced by the activities of the antioxidant systems (reviewed in Section 1.4.1.4). As ionic and osmotic homeostasis are maintained and oxidative damage to cellular structures in reduced, plant cell division and expansion ensues thus resulting in continuation of plant growth, although at a reduced rate than under normal conditions.

1.5 Drought Stress

Water is an important requirement for biochemical activities in all forms of life. Drought stress (also referred to as water stress, water deficit or osmotic stress) limits the amount of water available for use in metabolic processes, enzyme activities and photosynthetic processes (Xiong and Zhu, 2002). In extreme cases, drought stress may result in desiccation, which is the loss of most of the protoplasmic free water (Bray, 1997; Mundree *et al.*, 2002; Kotchoni and Bartels, 2003). Desiccation results in cytoplasmic increases in concentrations of toxic ions such as Cl^- and NO_3^-, which inhibit metabolic processes (Mundree *et al.*, 2002). Like the other abiotic stresses, drought stress therefore reduces the potential productivity of crops (Tester and Bacic, 2005). However, the level of reduction varies with the degree of the interaction of the stress with other factors such as genotype, developmental stage, as well as duration and severity of the stress (Clarke and Durley, 1981).

1.5.1 Morphological and Physiological Responses of Plants to Drought Stress

Plants respond to drought stress by a combination of morphological, physiological and molecular changes (Levitt, 1980; Clarke and Durley, 1981; Hale and Orcutt, 1987; Xiong and Zhu, 2002; Altman, 2003). The importance of each one of these responses in conferring resistance or tolerance to drought stress varies with the plant species, genotypes the developmental stage as well as the duration and severity of the stress. Adaptations to drought stress may take the form of avoidance, escape or tolerance (Levitt, 1980; Clarke and Durley, 1981; Hale and Orcutt, 1987; Altman, 2003). Each one of these forms of adaptations has specific morphological and physiological response mechanisms, which help the plants to survive, complete their life cycle and successfully reproduce their offspring.

1.5.1.1 Drought Avoidance

By definition, drought avoidance is when plants avoid water stress through morphological and physiological mechanisms, which help to maintain high water potential during stress (Turner, 1979; Clarke and Durley, 1981; Altman, 2003). This is achieved either by conserving water or by continuously supplying water to tissues under conditions of water stress. Mechanisms of water conservation in plants include; (i) improved water uptake through a deeper, extensive and efficient rooting system; (ii) control of transpiration via reduced leaf surface area, changes in leaf shape and reduction in the size, number and opening of stomata; and (iii) improved water storage in tissues such as stems, leaves and roots (Levitt, 1980; Clarke and Durley, 1981).

Although the control of transpiration via closing of stomata helps plants to conserve water, closed stomata may however limit gaseous exchange between the plant and the atmosphere. For instance, carbon dioxide uptake by the leaf for use in carbon fixation during photosynthesis is reduced. To overcome this problem, some plants have developed metabolic adaptations to help maintain high photosynthetic activities even when water availability is limiting. Crassulecean acid metabolism (CAM; Wyn Jones, 1981) and C_4 photosynthesis (Sage, 1999) help plants to maintain high photosynthetic activities even under limiting conditions. Pineapple and cacti are CAM plants (Wyn Jones, 1981) while maize, sugarcane and sorghum are C_4 plants (Doggett, 1988).

Crassulecean acid metabolising plants absorb carbon dioxide in the dark, use it to synthesise malic acid, which is stored in the cells and eventually decarboxylated during daylight. The released CO_2 is then fixed during photosynthesis (Wyn Jones, 1981). In C_4 plants, carbon fixation occurs in two different types of cells, first in the mesophyll cells and then in bundle sheath cells (Sage, 1999). In the mesophyll cells, CO_2 is used in the carboxylation of phosphoenolpyruvate (PEP) to produce oxaloacetate. This reaction is catalysed by phosphoenolpyruvate carboxylase, an enzyme with high affinity for CO_2. Oxaloacetate is then shuttled to the chloroplast of bundle sheath cells were is it released and re-fixed by RuBisCo in the Calvin Cycle (Sage, 1999). The initial fixation of CO_2 in mesophyll cells helps separate RuBisCo from atmospheric oxygen and thus avoiding photorespiration, a wasteful side reaction of RuBisCo (Sage, 1999). Therefore, in comparison with rice a C_3 plant for instance, sorghum fixes CO_2 more efficiently under

conditions of high temperature, light intensity and water stress (Buchanan *et al.*, 2005; Sasaki and Antonio, 2009).

1.5.1.2 Drought Escape

Drought escape is when plants avoid water stress by growing and completing their life cycles before serious soil and plant water stress develops (Turner, 1979). A typical example would be of desert ephemerals, which grow rapidly, flower and mature before the limited soil moisture is depleted (Levitt, 1980; Clarke and Durley, 1981; Hale and Orcutt, 1987). However, the mechanism of drought escape has no value for cultivated crops because of the short growth cycle, which results in limited biomass production (Altman, 2003).

1.5.1.3 Drought Tolerance

Drought tolerance is the ability of plants to maintain normal physiological functions under stress and is achieved through cellular, biochemical and molecular mechanisms (Turner, 1979; Clarke and Durley, 1981; Hale and Orcutt, 1987; Altman, 2003). During period of water stress, cells lose water and this results in an increase in the cell's solute concentration and a subsequent decrease in turgor pressure (Xiong and Zhu, 2002). As discussed in salt stress (Section 1.4.1.1.1), plants under drought stress maintain turgor via osmotic adjustment largely through the accumulation of compatible solutes (Ingram and Bartels, 1996; Bray, 1997; Wang, Vinocur *et al.*, 2003). Some sorghum varieties have been shown to accumulate glycine betaine (Wood *et al.*, 1996) and soluble sugars

(Newton *et al.*, 1986), which possibly act as compatible solutes for osmotic adjustment under water stress. Plants under drought stress have also been reported to accumulate LEA proteins in vegetative tissues (Ingram and Bartels, 1996; Bray, 1997). These proteins are normally expressed in abundance during the desiccation stage of seed development (Ingram and Bartels, 1996). Although their precise functions in drought tolerance are still not clear, LEA proteins may protect cellular structures from dehydration by (i) acting as a hydration buffer; (ii) sequestrating ions; (iii) maintaining protein and membrane structure; and (iv) possibly renaturing unfolded proteins (Ingram and Bartels, 1996; Bray, 1997; Hong-Bo *et al.*, 2005; Battaglia *et al.*, 2008).

Plants under drought stress may also be subjected to oxidative stress (reviewed in Section 1.4.1.4), which lead to damage of proteins, membranes and DNA (Mundree *et al.*, 2002). Therefore during drought stress, the plant's antioxidant compounds and enzymes increase (reviewed in Section 1.8). In cases where the cellular structures have been damaged beyond repair, enzymes that degrade proteins (proteases) are synthesised (Ingram and Bartels, 1996; Bray, 1997). Protein degradation during conditions of water stress is important in preventing aggregation as well as recycling amino acids for renewed protein synthesis (Xiong and Zhu, 2002). Other drought and salt responsive proteins have been identified in proteomic studies (reviewed in Section 1.8).

1.6 Proteomics

1.6.1 Defining Proteomics

Proteomics is defined as the large-scale analysis of proteins from a particular organism, tissue or cell (Blackstock and Weir, 1999; Pandey and Mann, 2000; van Wijk, 2001), while the proteome is the expressed **prote**in complement of the gen**ome** (Blackstock and Weir, 1999). Unlike the genome that is generally well defined and static over time, the proteome is highly dynamic and constantly changes during development and in responses to both internal and external cues (Heazlewood and Millar, 2003; Speicher, 2004; Komatsu, 2006).

The field of proteomics may be divided in three broad approaches: expression proteomics, cell map proteomics and structural proteomics (Blackstock and Weir, 1999; Ng and Ilag, 2002). Expression proteomics (also termed differential proteomics) is the study of global changes in protein expression. It offers information about specific protein changes in a biological system under specific physiological conditions and in response to various stresses. Cell-map proteomics in the study of protein-protein interactions while structural proteomics is the study of the three-dimensional structure of proteins. This section of the literature review will be limited to expression proteomics in plants.

1.6.2 Why proteomics?

Over the years, several high-throughput transcriptomics technologies, such as differential display, transcript imaging, serial analysis of gene expression (SAGE) and DNA microarrays (Anderson and Seilhamer, 1997; Lockhart and Winzeler, 2000; Zivy and de Vienne, 2000) have been used to measure mRNA expression profiles of different organisms under a range of experimental conditions. Although these technologies provide valuable information about gene expression (Dubey and Grover, 2001), in particular which genes are turned on and when (Abbott, 1999), the techniques do not always provide information about the quality and quantity of the final gene products, the proteins (Zivy and de Vienne, 2000).

Several studies have reported comparative analysis between mRNA and protein abundances of different biological systems using a variety of technologies (Anderson and Seilhamer, 1997; Gygi *et al.*, 1999; Carpentier *et al.*, 2008). Anderson and Seilhamer (1997) measured mRNA and protein abundances of a set of gene products of human liver and obtained a correlation coefficient of 0.48. Gygi *et al*. (1999) reported a comparative analysis of mRNA and protein levels of selected genes of yeast (*Saccharomyces cerevisiae*). When only the lowest abundant proteins were included in that study, a correlation coefficient of between 0.1 and 0.4 was obtained. In correlation data, a value of 1.0 represents a perfect correlation while a value of 0.0 represents no correlation at all (Anderson and Seilhamer, 1997). In a more recent study, Carpentier *et al*. (2008) reported a lack of correlation between mRNA and protein levels in leaf extracts of banana (*Musa acuminata*).

Poor correlation between mRNA and protein levels reported in the above mentioned studies could be attributed in part to the different rates of degradation of individual mRNAs and proteins (Salekdeh *et al.*, 2002b). Furthermore, many proteins undergo PTM such as phosphorylation, glycosylation, or proteolytic processing thus giving rise to several isoforms from a single gene product (Abbott, 1999). Post-translational modifications play important roles in regulating the function, subcellular localization and stability of proteins in a cell (Zivy and de Vienne, 2000; van Wijk, 2001; Kersten *et al.*, 2009). Since it is these post-translationally modified proteins that are functionally active in cellular processes, only the measurement of protein expression itself would thus give a better indication of gene functions at specific physiological states (Dubey and Grover, 2001) as well as insight into subcellular protein localization (van Wijk, 2001). Although several studies have shown that there is generally a poor correlation between mRNA and protein levels, Carpentier *et al*. (2008) reinforces the fact that both transcript and protein measurement techniques such as SAGE and 2D gel electrophoresis (2DE) respectively, are complementary to each other as each technique focuses on a subset of proteins. For example, SAGE techniques are more biased towards low abundant and hydrophobic proteins while 2DE is more biased towards highly abundant and less hydrophobic proteins (Carpentier *et al.*, 2008).

1.6.3 Overview of 2D PAGE Based Proteomics Workflow

Two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) remains the method of choice for separating proteins in complex mixtures (Thiellement *et al.*, 1999; Dunn and Gorg, 2001; Rabilloud, 2002) because of its ability to separate hundreds or thousands

of proteins at a time (Gorg *et al.*, 2000) as well as different protein isoforms (Carpentier *et al.*, 2008). This electrophoresis system separates proteins in two dimensions. In the first dimension, proteins are separated on the basis of their isoelectric point (pI) by isoelectric focusing (IEF). After IEF, proteins are equilibrated in sodium dodecyl sulfate (SDS) containing buffers in preparation for the second dimension as well as to improve protein transfer from the first to the second dimension (Gorg *et al.*, 2000). In the second dimension, the isoelectric focused proteins are separated on the basis on their molecular weight (MW) by SDS-PAGE. Different gel concentrations may be used depending on sizes of the proteins to be resolved. After 2DE, separated proteins are detected in the gels using protein stains and subsequently identified by mass spectrometry (MS) and database searches.

1.6.4 Protein Staining Methods

A number of protein staining methods are available, which allow for the detection and visualisation of protein spots in 2D gels (Gorg and Weiss, 2004; Westermeier, 2005; Hurkman and Tanaka, 2007). The criteria used for selecting which stain to use and when depends on the ease of use, reliability, sensitivity and compatibility with MS (Hurkman and Tanaka, 2007). Widely used stains include Coomassie Brilliant Blue, silver and fluorescent stains.

1.6.4.1 Coomassie Brilliant Blue Stain

Coomassie Brilliant Blue (CBB) is the most commonly used stain in electrophoresis mainly because it is easy to use. Two types of CBB stains, R-250 and G-250 are used for protein staining and both are compatible with MS. Compared to silver and fluorescent stains, both CBB R-250 and G-250 stains are less sensitive requiring at least 100 ng and 30 ng of protein per spot for detection respectively (Westermeier, 2005; Hurkman and Tanaka, 2007).

1.6.4.2 Silver Staining

Silver staining methods are more sensitive than CBB with a detection limit of between 1-10 ng of protein per spot (Hurkman and Tanaka, 2007). However, the use of silver staining in comparative 2D gel analysis is limited by several reasons. Silver staining methods are labour intensive and less reproducible than CBB because staining is terminated subjectively (Gorg and Weiss, 2004). Additionally, methods that use glutaraldehyde and formaldehyde based sensitizers to promote the binding of silver to protein are not compatible with MS. These aldehydes modify and cross link lysine residues, thus reducing the amount of protein extracted from the gel, and also prevents tryptic digestion of protein during sample preparation for MS analysis (Gorg and Weiss, 2004; Hurkman and Tanaka, 2007). To improve compatibility with MS protocols, some silver staining methods have replaced the aldehyde-based sensitizers with sodium thiosulfate (Hurkman and Tanaka, 2007).

1.6.4.3 Fluorescent Stains

Fluorescent dyes can either be used as pre- or post-electrophoretic stains such as cyanine-based dyes (Cydye) and SYPRO Ruby respectively (Patton, 2000; Gorg and Weiss, 2004). Compared to silver stains, fluorescent stains are less sensitive, detecting at least 2-8 ng of protein per spot. However, they have a wider linear dynamic range (Westermeier, 2005) and thus more popular for high throughput quantitative proteomics. All fluorescent dyes are compatible with MS but the major drawback with their use is that they are expensive.

1.6.5 Mass Spectrometry

Mass spectrometry is defined as the accurate mass measurement of charged analytes (Patterson and Aebersold, 2003) and is widely used for the identification of proteins in proteomics. Mass spectrometers measure mass to charge ratios (m/z) of charged molecules. Mass spectrometers have three main components; an ionization source, a mass analyser and a detector (Patterson *et al.*, 2001) and they are named on the basis of their ionization source and mass analysers (Patterson, 2000). Two most commonly used mass spectrometers include the matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and tandem mass spectrometry (MS/MS).

1.6.5.1 Matrix-Assisted Laser Desorption/Ionization Time Of Flight (MALDI-TOF) MS

In MALDI-TOF MS (reviewed in Westermeier, 2005), the ionisation source is the MALDI while the TOF is the mass analyser. Matrix-assisted laser desorption/ionization time of flight mass spectrometry is mainly used for protein identification coupled with peptide mass fingerprinting (PMF). Proteins from gel spots are digested using trypsin and the resultant peptides are mixed with a matrix such as α-cyano-4-hydroxy cinnamic acid or 2,5-dihydroxybenzoic acid (DHB). Matrices are low molecular compounds that form crystals, absorb UV and release a proton in the process (Patterson, 2004). The peptide-matrix mixtures are spotted onto a metal slide, dried and then inserted into a vacuum chamber of the mass spectrometer. When high voltage is applied to the slide, the matrix molecules absorb energy and move from the plate into a gas phase together with the peptides. The generated ions are delivered to the mass analyser (TOF) which measures the time elapsed from acceleration of the charged molecules through a field-free drift region. In general, the smaller lighter ions reach the detector faster than the heavy ones and recorded flight times are used to calculate the mass to charge ratios (Westermeier, 2005).

1.6.5.1.1 Peptide Mass Fingerprinting

The observed peptide masses generated by MALDI-TOF MS are matched with theoretically generated tryptic digests of all expressed protein sequences from the genome sequence databases (Dubey and Grover, 2001), hence the term peptide mass fingerprinting. At least three to four peptides of high mass accuracy are sufficient for

protein identification (Westermeier, 2005). However in some cases, PMF does not yield a positive match. This can be due to one of the following reasons (i) wrong predictions of the open reading frames from the genomic databases; (ii) sequence errors in the genomic databases; (iii) mutations; (iv) PTMs; (v) artifactual modification resulting from sample handling such as oxidation of methionine and (v) incomplete sequence databases, amongst others (Aebersold and Goodlett, 2001; Westermeier, 2005).

1.6.5.2 Tandem Mass Spectrometry

In tandem mass spectrometry (MS/MS; Lin *et al.*, 2003; Patterson and Aebersold, 2003; Westermeier, 2005) peptide ions are selected from the first mass analyser (such as TOF) and passed through a collision cell located between two mass analysers. In the collision cell, peptide ions are further fragmented by high-energy collision with inert gasses such as argon or helium. This is called collision-induced dissociation (CID) and yields fragmented ions, whose masses are subsequently measured by the second mass analyser. The fragmented ions spectra, referred to as MS/MS spectra or CID spectra are matched with fragmented ion masses from the genome sequence databases (Patterson and Aebersold, 2003).

Therefore, unlike MALDI-TOF MS, which only measures peptide masses, tandem mass spectrometry allows for the detection of the amino acid sequences of proteins (Dubey and Grover, 2001). As such, MS/MS is particularly useful for the identification of proteins not identified by a combination of MALDI-TOF MS and PMF, proteins in complex

mixtures as well as proteins from species with incomplete genome sequence information (Aebersold and Goodlett, 2001).

1.7 Plant Proteomics

The field of proteomics is increasingly gaining momentum in plant sciences with several studies having been made and reported on agriculturally important crops (Jorrin *et al.*, 2007; Salekdeh and Komatsu, 2007; Jorrin-Novo *et al.*, 2009). Although there has been major proteomic advances using several other plant species, much of the knowledge gained on plant developmental processes and stress response mechanisms has been gained from work using Arabidopsis and rice (Jorrin *et al.*, 2007; Jorrin-Novo *et al.*, 2009) mainly because of their completed genome sequences, which are publicly available (The Arabidopsis Genome Initiative, 2000; International Rice Genome Sequencing Project, 2005). In proteomics, genome sequences are important resource tools for the identification of proteins. Where fully annotated sequences are not yet available, protein identification can be through similarity searches of homologous proteins in closely related species (Carpentier *et al.*, 2008). Indeed, a proteomic study on maize for instance, a partially sequenced crop, has relied largely on the limited sequence data and homology based protein identification (Porubleva *et al.*, 2001). Alternatively, expressed sequence tags (ESTs), which represent partial gene sequences can also be used (Aebersold and Goodlett, 2001). However, for the other plant species without significant amounts of published genomic DNA or expressed sequence tags (EST) sequences, protein identification success rates are lowered resulting in limited proteomic data being available (Jorrin et al., 2007). Amongst the cereal crops, only rice (International Rice

Genome Sequencing Project, 2005) and recently, sorghum (Paterson *et al.*, 2009) have been fully sequenced to date. These genome sequences offer invaluable tools for the identification of genes and proteins with potential application in plant breeding approaches for both the increase in yield as well as tolerance to both abiotic and biotic stresses (Salekdeh and Komatsu, 2007).

1.7.1 Challenges in Plant Proteomics

In proteomics studies, high-resolution gels with minimal spot streaking and overlap (Thelen and Peck, 2007) are essential to facilitate comparative gel analysis using 2D analysis software (Marengo *et al.*, 2005) and the downstream protein identification via mass spectrometry and database searching. However, obtaining highly resolved gels is somewhat challenging unless factors that affect their resolution are well optimised. These factors include protein extraction and solubilisation procedures, protein separation on both the first and second dimension, as well as protein detection methods used (Rose *et al.*, 2004; Hurkman and Tanaka, 2007).

1.7.1.1 Protein Extraction and Solubilisation Methods

In plants, proteome analysis involves the characterisation of proteins from a variety of materials such as cell suspension cultures, different plant tissues, organs, organelles and sub-cellular compartments. Because of the diversity in the starting materials used and the heterogeneous nature of the composite proteins in terms of MW, pI, hydrophobicity, PTMs and cellular distribution (Jung *et al.*, 2000), there is no universal protein extraction

protocol available that effectively extracts and solubilises all types of proteins (Dunn and Gorg, 2001; Gorg *et al.*, 2004; Rose *et al.*, 2004). Furthermore, in comparison with tissues from other organisms, plant tissues have relatively low protein concentrations as well as high concentration of endogenous proteases and non-protein components, which interfere with downstream protein separation and detection processes (Rose *et al.*, 2004; Carpentier *et al.*, 2005; Westermeier, 2006; Hurkman and Tanaka, 2007; Jorrin-Novo *et al.*, 2009). Non-protein components such as lipids, nucleic acids, polysaccharides, polyphenols, pigments and secondary metabolites that are present in plant cells and tissues interact with proteins during extraction procedures (Carpentier *et al.*, 2005). These interactions cause an increase in the background staining of 2D gels, ultimately reducing their overall resolution and the number of distinctly separated protein spots (Hurkman and Tanaka, 1986). Reduced 2D gel resolution in turn affects effective comparative gel analyses using 2D analysis software and subsequent protein identification by mass spectrometry.

In addition, degradation of proteins by endogenous proteases in plant extracts may result in the accumulation of protein degradation products, which could be erroneously documented as protein changes due to the effects of the physiological conditions under study (des Francs *et al.*, 1985; Dunn and Gorg, 2001; Gorg and Weiss, 2004). Since the ultimate goal of any expression proteomics study is to identify all differentially expressed proteins in a particular biological system (Wilkins *et al.*, 1998; Inagaki and Katsuta, 2004; Patterson, 2004) at a given time, under well defined physiological conditions (Anderson and Anderson, 1998), protein extraction protocols should be able to

effectively extract and solubilise a greater proportion of the proteome with minimal protein degradation, modification and/or non-specific losses (Rose *et al.*, 2004). Therefore, prior to the analysis of any previously uncharacterised proteome, protein extraction and solubilisation procedures have to be optimised and evaluated for their effective extraction capacities while minimising protein degradation (des Francs *et al.*, 1985) as well as the co-extraction of non-protein components (Rabilloud, 1996).

1.7.1.1.1 The TCA/Acetone Extraction Method

Trichloroacetic acid (TCA) in combination with acetone (TCA/acetone) is routinely used for the extraction plant proteins (Wang, Scali *et al.*, 2003; Wang *et al.*, 2006). The low pH of TCA inhibits protein degradation by inactivating most protease activities. Acetone solubilises non-protein components of the extract that might interfere with gel electrophoresis, leaving an acetone insoluble protein precipitate (Wang *et al.*, 2006; Hurkman and Tanaka, 2007). In animal cells, lysosomal proteases are active at low pH and inactivated at high pH (Rabilloud, 1996), and therefore would require a different mechanism for deactivation. Using the TCA/acetone method, acetone-precipitated proteins are subsequently solubilised in a buffer of choice prior to IEF. However, protein losses may be encountered using this method mainly because of incomplete protein precipitation. Even when protein precipitation is nearly complete, some proteins particularly high molecular weight proteins (Carpentier *et al.*, 2005) may not readily resolubilise in solubilization buffers (Gorg and Weiss, 2004). During the analyses of fruit and vegetable proteomes, additional steps of methanol washes of the protein precipitate are included to remove polyphenolic compounds (Wang *et al.*, 2006) that are highly

abundant in such plant materials. Polyphenols can irreversibly complex with proteins during extraction and lead to horizontal streaking in 2D gel profiles and thus a reduction in gel resolution (Westermeier, 2006).

1.7.1.1.2 Sample Solubilisation Methods

Sample solubilisation is a critical step in obtaining highly resolved 2D gels. An effective solubilization buffer should (i) disrupt all non-covalently bound protein complexes and aggregates, (ii) remove interfering substances and (iii) maintain protein components of the extract in solution during 2DE (Dunn and Gorg, 2001). Most solubilisation buffers contain chaotropes (urea or thiourea), detergents (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPS), reducing agents (dithiothreitol; DTT) and ampholytes. Urea is the most commonly used denaturant and helps to avoid protein-protein interactions in extracts. Thiourea is a much stronger denaturant than urea and is used for the solubilisation of hydrophobic membrane proteins (Gorg and Weiss, 2004). However, since thiourea is poorly soluble in water, it is dissolved in urea solutions; urea-thiourea buffers thus greatly improve the solubility of membrane and nuclear proteins (Rabilloud *et al.*, 1997; Rabilloud, 1998; Giavalisco *et al.*, 2003). The zwitterionic detergent CHAPS solubilises membrane proteins, while DTT is used to prevent oxidation steps of proteins (Westermeier, 2005). Although SDS is a powerful solubilizing detergent, its anionic nature makes it unsuitable for IEF (Dunn and Gorg, 2001). Therefore, SDS is only used for pre-solubilisation procedures after which extracts are dialysed against buffers that are compatible with IEF.

1.7.1.2 The Dynamic Range of Protein Expression

The dynamic range of protein expression in biological systems is estimated to be about 10^5 to 10^6 for eukaryotic cells and 10^9 to 10^{10} for serum preparations (Corthals *et al.*, 2000; Rabilloud, 2002; Patterson and Aebersold, 2003). This high dynamic range makes visualisation and quantification of the entire proteome rather impossible (Rabilloud, 2002; Park, 2004; Carpentier *et al.*, 2008). Low copy number proteins with about 10-100 copies per cell such as most signal transduction proteins, regulatory proteins or receptors become masked by the more highly expressed, 'housekeeping' proteins with over 10,000 copies per cell (Blackstock and Weir, 1999; Rose *et al.*, 2004; Xi *et al.*, 2006). Because no PCR equivalent exists for the amplification of proteins (Blackstock and Weir, 1999; Carpentier *et al.*, 2008), low abundant proteins have a higher chance of not being detected in 2D gels. As a result, such proteins are not identified in downstream protein identification procedures (Wilkins *et al.*, 1998).

For example, RuBisCo, makes up to 50% of soluble proteins in some leaf extracts (des Francs *et al.*, 1985) and thus generally prevents the visualisation of moderate to low abundant proteins in leaf total proteomes (Watson *et al.*, 2003). In mammalian cells, actin is the most abundant protein (10^8 copies per cell) while cellular receptors or transcription factors are of relatively lower abundance (100-1,000 copies per cell; Rabilloud, 2002). With the limited loading capacities of immobilised pH gradient (IPG) strips, the bulk of the protein load would therefore be made up of the highly abundant proteins, thus limiting the lower abundant ones from being absorbed by the strips (Kim *et al.*, 2001; Xi *et al.*, 2006). This effectively results in only a fraction of the total proteome being

analysed and identified (Thiellement *et al.*, 2002; Patterson, 2004). Gygi *et al.* (2000) argue that if proteome analysis is to provide comprehensive information about cellular and regulatory processes in a biological system, regulatory proteins, which are typically low abundant should be detected in 2D gels and identified.

1.7.1.2.1 Improving the Coverage of the Proteome

Patterson (2004) estimates that approximately 25% of an expected proteome is detected on 2D gels. To improve on the coverage of the proteome as well as reduce its complexity, biological or biochemical pre-fractionation procedures (Rose *et al.*, 2004) and/or the use of overlapping narrow range IPG strips in the first dimension (Gygi *et al.*, 1999; Gygi *et al.*, 2000) may be employed prior to 2D analysis. Biological pre-fractionation steps involve the targeting of specific subsets of the total proteome such as organelles or sub-cellular compartments (Corthals *et al.*, 2000; Jung *et al.*, 2000; Dreger, 2003).

Biochemical fractionation procedures such as liquid chromatography (Lilley *et al.*, 2002), affinity chromatography (Lee and Lee, 2004), free flow electrophoresis (Righetti *et al.*, 2005), or sequential protein precipitation (Kim *et al.*, 2001) are also used for the enrichment of different components of the proteome into separate fractions prior to 2D analysis. Kim *et al*. (2001) extracted rice leaf proteins first in Mg/Nonidet P-40 buffer followed by polyethylene glycol (PEG) fractionation. The PEG fractionation was performed in three phases; 10% PEG, 10-20% PEG and the final supernatant being precipitated with acetone. Using this protocol, high amounts of RuBisCo were enriched for in the 20% PEG precipitate fraction. Western blotting analysis of the different extracts

using antibodies raised against known signal transduction components such as G-alpha protein, ADP ribosylation factor, small GTP binding protein and the 14-3-3 protein illustrated that even these low abundant gene products could be detected more in the different PEG pellet and supernatant fractions than in the total extract (Kim *et al.*, 2001). Therefore, the enrichment step of RuBisCo in one fraction resulted in the improved detection of low abundant proteins such as signal transduction components in other fractions and thus improved the total percentage of proteome coverage.

Although pre-fractionation steps reduce complexity of the proteome under study, it also creates problems; namely (i) an increase in the number of protein fractions for downstream analysis; and (ii) an increase in the probability of non-specific protein losses during multiple handling procedures (Giavalisco *et al.*, 2003; Rose *et al.*, 2004). To reduce the degree of non-specific protein losses, pre-fractionation processes with fewer steps should be used (Corthals *et al.*, 2000).

1.8 Application of Proteomics in Studying Plant Stress Responses Mechanisms

Proteomic tools using 2DE and MS are useful for the separation, visualisation and identification of stress responsive proteins in plants (Thiellement *et al.*, 1999; Zivy and de Vienne, 2000; Thiellement *et al.*, 2002). In addition, expressional proteomics between plant genotypes/cultivars of varying tolerance to stresses has a potential of linking expressed proteins to specific phenotypic traits (Salekdeh *et al.*, 2002a). The stress responsive proteins may either show qualitative (positional shift, present/absent) or quantitative expressional changes between the unstressed and stressed experimental

groups (Salekdeh *et al.*, 2002a; Thiellement *et al.*, 2002). Positive identification of these proteins lead to the discovery of expressed genes that play a role in stress tolerance (Salekdeh *et al.*, 2002a). Therefore, plant stress proteomics has the potential of identifying possible candidate genes that can be used for the genetic improvement of plants against stresses (Cushman and Bohnert, 2000). Table 1.1 lists some of the targeted candidate gene for use in genetic engineering of plants for stress tolerance.

However, not all genes and/or proteins that are induced under a specific stress condition contribute to stress tolerance (Zhu, 2000). The induced expression of other proteins may be an indication of stress damage. For example, increased degradation products of the large subunit of RuBisCo have been identified in proteomic studies of rice under cold stress (Komatsu *et al.*, 1999; Yan *et al.*, 2006) as well as sugar beet (Hajheidari *et al.*, 2005), rice (Salekdeh *et al.*, 2002a) and maritime pine seedlings (Costa *et al.*, 1998) under water stress. Degradation of RuBisCo might be due to protease-dependent and/or protease-independent mechanisms (Salekdeh and Komatsu, 2007). In wheat chloroplast exposed to light and oxidative stress, the large subunit of RuBisCo was degraded via a protease-independent mechanism that involved damage by hydroxyl radical ROS (Ishida *et al.*, 1999). For this reason, after the proteomic analysis and identification of stress responsive proteins using 2DE and MS, the precise function and level of contribution to stress tolerance ought to be ascertained using reverse genetics (Salekdeh *et al.*, 2002a).

Table 1.1: Examples of candidate genes for use in genetic engineering of plants for stress tolerance.

Class of target	Examples	Possible mode of action
Osmoprotectants	Proline; glycine betaine; mannitol; sorbitol; sucrose; trehalose; fructan	Osmotic adjustment; Protein/membrane protection; ROS scavenging
ROS scavengers	Catalase; GST; glutathione peroxidase; ascorbate peroxidase;	Detoxification of ROS
Stress proteins	LEA proteins	Protein/membrane protection; water binding properties; ion sequestration
Heat shock proteins	Several heat shock proteins responsive to different abiotic stresses	Reversal/prevention of protein unfolding
Ion/proton transporters	High affinity K^+ transporters; H^+ ATPases; Na^+/H^+ antiporters	K^+/Na^+ uptake and transport; establishment of proton gradients; removal of toxic ions from the cytoplasm and organelles
Signalling components	Protein kinases; Protein phosphatases; Ca^{2+} sensors	Ca^{2+} sensors; Phosphorylation mediated signal transduction
Control of transcription	Transcription factors	Upregulation/activation of transcription

(adapted from Cushman and Bohnert, 2000)

1.8.1 Plant Proteome Response to Salt and Drought Stress

Due to the dynamic nature of the proteome (Heazlewood and Millar, 2003; Speicher, 2004; Komatsu, 2006), proteomic studies have incorporated a wide range of experimental designs in order to capture the spatial and temporal expression changes in proteome responses under stress. These studies have included the use of different plant species, genotypes, types and levels of stress, sampling times, organs, tissues, organelles and/or subcellular compartments (Salekdeh and Komatsu, 2007; Vincent and Zivy, 2007). As previously discussed in Section 1.3, responses of plants to stresses are relatively complex, involving signal perception, transduction and gene expression which ultimately contribute to stress tolerance. In addition, salt and drought stresses exert a common element of osmotic stress to the plants and therefore may share common stress sensory mechanisms, a concept broadly referred to as cross-talk signalling systems (Knight and Knight, 2001; Chinnusamy *et al.*, 2004). Proteomic technologies are able to dissect the different components of such systems in different stress response mechanisms. Outlined below are examples of different experimental designs that have been used for studying proteome changes in response to either salt or drought stresses.

Chourey and co-workers studied protein changes in shoots of rice seedlings following germination and growth on 100 mM NaCl for 14 days (Chourey *et al.*, 2003). Four salt-induced LEA proteins were identified, accumulating during salt stress but were subsequently degraded during recovery from the stress. Late-embryogenesis abundant proteins (reviewed in Section 1.5.1.3) are known stress responsive proteins that are believed to have protein or membrane protective functions and/or water binding functions

in plants under stress (Ingram and Bartels, 1996; Bray, 1997; Hong-Bo *et al.*, 2005; Battaglia *et al.*, 2008).

Zang and Komatsu (2007) investigated proteome changes in the leaf sheath of rice seedlings in response to osmotic stress. Two-week old seedlings were treated with 400 mM mannitol for 48 hrs. Of the 327 protein spots that were reproducibly expressed, a total of 12 and 3 spots were upregulated and downregulated respectively. Amongst the upregulated proteins included GST, a lipid transfer protein, glyoxalase I and other unidentified proteins. All the downregulated proteins were chaperone proteins (a calreticulin, heat shock protein and a dnak-type molecular chaperone). Glutathione S-transferase and heat shock proteins are common stress responsive proteins that are expressed in plants under a variety of stresses (Wang *et al.*, 2004; Salekdeh and Komatsu, 2007). However, it is not clear why the three chaperone proteins were downregulated in rice seedlings under mannitol treatment (Zang and Komatsu, 2007).

Other proteomic studies have focused on expression changes of phosphoproteomes following salt stress. Chitteti and Peng (2007), studied changes in the phosphoproteome of 20-day old rice roots exposed to 150 mM NaCl for 10 or 24 hrs. Differential protein expression was measured by a 2-fold change. Using Pro-Q Diamond stain, a phosphoprotein fluorescent stain, 20 and 18 proteins were upregulated and downregulated respectively. Seventeen of the 20 upregulated proteins and 11 of the 18 downregulated were positively identified. Examples of upregulated proteins included GST, a mannose binding rice lectin, Hsp70 and a salt induced protein. Down regulated

proteins included a protein kinase, ATP synthase beta chain and glyceraldehyde 3-phosphate dehydrogenase. The authors state that although all the 17 upregulated proteins are known to be responsive to salt stress, some had not been identified in any proteomic studies of rice under salt stress. These observations suggested that Pro-Q Diamond stain is useful for the identification of phosphorylated proteins under salt stress.

Salinity stress induces osmotic and ionic effects resulting in a two-phase growth response (Munns, 1993, 2002; Munns and Tester, 2008). In the first phase, a decrease in plant growth is attributed to osmotic stress due to an increase in salts in the external environment. As the duration of salt stress treatment increases, ions accumulate in the shoots of whole plants leading to ionic toxicity and possibly death of the plant. Based on this rationale, proteome expression changes between salt sensitive and salt-resistant wheat genotypes in the first phase of salt stress were investigated (Saqib *et al.*, 2006). At 10 days after exposure to the salt treatment, more that 50% of the proteins changed in expression between the control and salt treatments groups of both genotypes. However, there was no significant difference in genotype specific changes in the first phase. Saqib and co-workers concluded that no adaptations at the protein level occurred within the first phase of salt stress in the wheat genotypes used in the study.

A comparative proteome analysis in rice root following salt stress was also carried out between two cultivars with contrasting tolerance to salt stress (Salekdeh *et al.*, 2002a). Using a hydroponic system, rice plants were subjected to incremental salt treatment using 50 mM and 100 mM NaCl at 14 days and 21 days respectively. The seedlings were

harvested at 28 days and root tissue proteins were extracted. Of the proteins analysed by 2DE, only a few showed qualitative changes, while many showed quantitative changes in expression. Five spots showing quantitative changes in expression were analysed by MALDI-TOF MS and electrospray ionization tandem quadrupole mass spectrometry (ESI-Q MS/MS). This resulted in the identification of three proteins; an ASR1-like protein, ascorbate peroxidase and caffeoyl-CoA *O*-methyltransferase (CCOMT). The ASR1-like protein, showed a 3-fold increase in the salt resistant cultivar and not in the sensitive one. This protein is known to be stress responsive, possibly playing a role in the protection of DNA during stress. Interestingly, the expression of ascorbate peroxidase, an ROS scavenging enzyme, increased in both cultivars following stress. This indicated that both cultivars respond to oxidative stress using common mechanisms. The expression of CCOMT was remarkably higher in the resistant cultivar than the sensitive one. Caffeoyl-CoA *O*-methyltransferase plays a role in lignification, a processes that may protect plants from salt stress by impeding passive Na^+ rich waterflow from the medium into the plant cell via the apoplastic route. Collectively, the observed difference in the expression patterns of the three proteins might indicate that the salt resistant rice cultivar was better protected against salt stress by the constitutively expressed proteins (Vincent and Zivy, 2007).

1.8.2 Summary of Commonly Expressed Salt and Drought Stress Responsive Proteins

Because of the variations in experimental designs of stress proteomics studies (as outlined in Section 1.8.1), differences in the identities and expression patterns

(qualitative/quantitative) of stress responsive proteins are inevitable. Nevertheless, a review of published literature shows that the expression patterns of some protein spots are consistently increased following salt and/or drought stress in comparison with the control (Table 1.2). Some of these proteins would be good candidates for the genetic engineering of plants against salt and drought stress.

1.8.2 Cereal Proteomics

Recent reviews on plant and crop proteomics (Jorrin *et al.*, 2007; Salekdeh and Komatsu, 2007; Jorrin-Novo *et al.*, 2009) have indicated that amongst the cereal crops, rice, wheat and maize have received considerable proteomic analyses in response to both salt and drought stresses. Rice, the first cereal to have a fully sequenced genome (International rice genome sequencing project, 2005), and currently regarded as the model plant amongst the grasses tops the list. However rice, which naturally grows in flooded fields (paddies), is the most salt sensitive crop amongst the cereals (Munns and Tester, 2008). As an alternative, sorghum a naturally drought tolerant crop (Rosenow *et al.*, 1983) that is relatively more salt tolerant than maize and exhibits wide genotypic diversity in salt tolerance (Krishnamurthy *et al.*, 2007) could possibly provide more insight into the molecular basis of drought and salt tolerance amongst the cereals. Furthermore, comparative studies of salt and drought stress responsive proteins between sorghum genotypes of contrasting tolerance to these abiotic stresses could possibly yield valuable information on the identities of expressed proteins and ultimately expressed genes. The latter could be used as targets for the genetic improvement of cereals (Cushman and Bohnert, 2000).

Table 1.2: Examples of proteins that are upregulated in response to salt and/or drought stresses.

Protein	Plant species/tissue	Stress	References
LEA proteins	Maize/leaf	Drought	Riccardi *et al.*, 1998
	Soybean/hypocotyl/root	Salt	Aghaei *et al.*, 2009
	Rice/leaf	Salt	Chourey *et al.*, 2003
ATP synthase beta subunit	Rice/leaf	Salt	Parker *et al.*, 2006
	Foxtail millet/seedlings	Salt	Veeranagamallaiah *et al.*, 2008
	Potato/shoot	Salt	Aghaei *et al.*, 2008
Heat shock proteins	Sugar beet/leaf	Drought	Hajheidari *et al.*, 2005
	Maritime pine/needle	Drought	Costa *et al.*, 1998
	Potato/shoot	Salt	Aghaei *et al.*, 2008
ROS detoxifying enzymes	Rice/root	Salt	Salekdeh *et al.*, 2002a
	Maritime pine/needle	Drought	Costa *et al.*, 1998
	Rice/leaf	Salt	Parker *et al.*, 2006
	Sugar beet/leaf	Drought	Hajheidari *et al.*, 2005
	Rice/root	Salt	Chitteti and Peng, 2007
	Rice/leaf sheath	Drought	Zang and Komatsu, 2007
RuBisCo activase	Rice/leaf	Salt	Parker *et al.*, 2006
	Rice/leaf	Drought	Salekdeh *et al.*, 2002a, 2002b

RuBisCo LSU (fragment)	Maritime pine/needle	Drought	Costa *et al.*, 1998
	Sugar beet/leaf	Drought	Hajheidari *et al.*, 2005
	Rice/leaf	Drought	Salekdeh *et al.*, 2002a, 2002b
Caffeoyl CoA-*O*-methyltrasferase	Maritime pine/needle	Drought	Costa *et al.*, 1998
	Foxtail millet/seedlings	Salt	Veeranagamallaiah *et al.*, 2008
	Rice/root	Salt	Salekdeh *et al.*, 2002a

ROS detoxifying enzmes include any one of the following: Cu/Zn superoxide dismutase, ascorbate peroxidase, glutathione S-transferase and/or glutathione peroxidase.

1.9 Aims of this Research

The aim of this research project was to work on both descriptive and differential expressional proteomics of sorghum, using two plant systems: sorghum cell suspension cultures and whole plants. In proteomic studies, cell suspension cultures offer advantages such as the supply of large amounts of highly reproducible experimental material while whole plant systems offer tissue specific protein expression profiles. For descriptive proteomics, the main objective was to extract, separate, identify and map proteins that are expressed in (i) the secretome of sorghum cell suspension cultures and (ii) the leaf tissue of two sorghum varieties (AS6 and MN1618). In differential expressional proteomics, the main objectives were to (i) identify salt (NaCl) and hyperosmotic (sorbitol) stress responsive proteins in the secretome of cell suspension cultures and (ii) compare salt

stress responsive proteins of two sorghum varieties (AS6 and MN1618), which have different levels of salt tolerance. The proteome expression data obtained in this study would be used as a pioneering sorghum reference tool for use in cereal proteomics. In addition, the identification of salt and hyperosmotic stress responsive proteins would lead to the identification of expressed sorghum genes that could possibly be used as candidate targets in genetic engineering approaches for the development of both drought and salt stress tolerant crops in future studies.

CHAPTER 2

Materials and Methods

2.1 General Chemicals and Suppliers

All chemicals used in the study, their suppliers and respective catalogue numbers are listed alphabetically in Table 2.1.

Table 2.1: List of chemicals used in the study.

Chemical	Supplier	Catalogue No.
Acetone	Merck	SAAR1022040LC
Acetonitrile	Merck	1.00030.2500
40% Acrylamide/Bis Solution, 37.5:1(2.6% C)	BIO-RAD	161-0148
Agar bacteriological	Merck	HG000BX1.500
Agarose D-1 LE	Whitehead Scientific	H101119
Ammonium hydrogen bicarbonate	Merck	1.01131.5000
Ammonium persulfate (APS)	Sigma	A3678
100x Bio-Lyte 3/10 Ampholyte	BIO-RAD	163-2094
BIO-RAD Protein assay dye reagent concentrate	BIO-RAD	500-0006
Bovine Serum Albumin (BSA) Fraction V	Roche	10 735 078 001
Bromophenol blue sodium salt	Sigma	B5525

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Sigma	C3023
Coomassie® brilliant blue (CBB) R-250	BIO-RAD	161-0400
2,4-Dichlorophenoxyacetic acid (2,4-D)	Sigma	D7299
Dithiothreitol (DTT) Cleland's reagent	Fermentas	R0861
Ethanol 99.9%	Kimix	-
Evans Blue	Sigma	E2129
Glacial acetic acid	Merck	SAAR1021020LC
Glycerol	Merck	267 65 20 LC
Glycine	BIO-RAD	161-0724
Hydrochloric acid	Merck	306 30 40 LP
Iodoacetamide	BIO-RAD	163-2109
2-(N-Morpholino)ethanesulfonic acid (MES) hydrate	Sigma	M2933
Methanol	Merck	SAAR4164080LC
Methylthiazolyldiphenyl-tetrazolium bromide (MTT)	Sigma	M5655
Mineral oil (PlusOne DryStrip Cover Fluid)	GE Healthcare	17-1335-01
Miracloth	Merck	475855
Murashige and Skoog Basal medium (MS)	Sigma	M5519
Murashige and Skoog Basal Salt with minimal organics (MSMO)	Sigma	M6899

1-Naphthaleneacetic acid (NAA)	Sigma	N0640
PageRuler™ unstained protein ladder	Fermentas	SM0661
Ponceau S	Fluka	81460
Potassium hydroxide pellets	Merck	1.05033.0500
Propan-2-ol (isopropanol)	Merck	SAAR5075040LC
Sodium chloride	Merck	582 23 00 EM
Sodium dodecyl sulfate (SDS)	BIO-RAD	161-0302
Sodium hydroxide	Merck	1.06498.0500
Sodium hypochlorite solution (12%)	Kimix	-
Sucrose	Merck	SAAR5881500EM
D-Sorbitol	Sigma	S-8143
N,N,N',N'-Tetramethylethylenediamine (TEMED)	BIO-RAD	161-0801
Thiourea	Sigma	T8656
Trichloroacetic acid (TCA)	Merck	611 05 00 EM
Trifluoroacetic acid (TFA)	Merck	8.08260.0100
Tris(hydroxymethyl)-aminomethane	BIO-RAD	161-0719
Trypsin	Promega	V5111
TWEEN® 20	Merck	8.22184.05000
Urea	Sigma	U0631

2.2 General Stock Solutions and Buffers

80% acetone: 80% (v/v) acetone in distilled water.

0.5% agarose sealing solution: 0.5% (w/v) agarose prepared in 1X SDS-PAGE running buffer with a tint of bromophenol blue.

10% APS: 10% (w/v) APS in distilled water. The solution was freshly prepared before use.

0.5% blocking solution: 0.5% (w/v) Elite fat free instant milk powder in TBS

1% blocking solution: 1% (w/v) Elite fat free instant milk powder in TBS.

Bradford reagent: 1 part BIO-RAD Protein Assay dye reagent concentrate diluted with 4 parts distilled water.

5 mg/ml BSA stock solution: 5 mg/ml BSA in urea buffer.

1.25% (w/v) CBB stock solution: 1.25% (w/v) CBB R-250 in distilled water.

CBB staining solution I: 50 ml of 1.25% (w/v) CBB stock solution, 10% (v/v) glacial acetic acid and 25% (v/v) propan-2-ol in distilled water.

CBB staining solution II: 6.25 ml of 1.25% (w/v) CBB stock solution, 10% (v/v) glacial acetic acid and 10% (v/v) propan-2-ol in distilled water.

CBB staining solution III: 6.25 ml of 1.25% (w/v) CBB stock solution and 10% (v/v) glacial acetic acid in distilled water.

Destaining solution: 10% (v/v) acetic acid and 1% (v/v) glycerol in distilled water.

Displacing solution: 0.375 M Tris-HCl, pH 8.8, 50% (v/v) glycerol with a tint of bromophenol blue.

50% DTT: 50% (w/v) DTT in urea buffer.

Evans blue stock solution: 0.5% (w/v) in distilled water.

Equilibration base buffer: 6 M urea, 2% SDS, 0.05 M Tris-HCl, pH 8.8 and 20% (v/v) glycerol in distilled water.

70% ethanol: 70% (v/v) ethanol in distilled water.

Fixing solution: 40% (v/v) ethanol and 10% (v/v) acetic acid in distilled water.

0.1 M HCl: 0.1 M HCl in distilled water.

5 mM MES: 5mM 2-(N-Morpholino)ethanesulfonic acid (MES) in distilled water.

5 mg/ml MTT stock solution: 5 mg/ml MTT in distilled water.

5 M NaCl: 5 M NaCl in distilled water.

Seed sterilization solution: absolute commercial bleach containing 0.1% (v/v) Tween 20.

2X SDS sample loading buffer: 60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 200 mM DTT, 0.025% (w/v) bromophenol blue.

1X SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine containing 0.1% (w/v) SDS.

5 M sorbitol: 5 M sorbitol in distilled water.

10% TCA: 10% (w/v) TCA in acetone.

Tris-buffered saline (TBS): 50 mM Tris and 150 mM NaCl, pH 7.5.

TBST: TBS containing 0.1% (v/v) Tween 20.

Transfer buffer: 25 mM Tris, 192 mM glycine and 20% (v/v) methanol in distilled water.

0.5 M Tris-HCl, pH 6.8: 0.5 M Tris in distilled water adjusted to pH 6.8 with concentrated HCl.

1.5 M Tris- HCl, pH 8.8: 1.5 M Tris in distilled water adjusted to pH 8.8 with concentrated HCl.

Urea buffer 9 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The solution was kept at -20ºC.

2.3 Plant Tissue Culture Media and Plant Growth Hormones

All plant tissue culture media and plant growth hormones were supplied by Sigma and prepared as follows:

3 mg/ml 2,4-D: 3 mg 2,4-dichlorophenoxyacetic acid (2,4-D) dissolved in 250 µl of absolute ethanol and made up to 1 ml with distilled water.

2.5 mg/ml NAA: 2.5 mg 1-naphthaleneacetic acid (NAA) dissolved in 250 µl of 1 M NaOH and made up to 1 ml with distilled water.

Sorghum callus initiation medium: 4.4 g/l Murashige and Skoog Basal Salt with minimal organics (MSMO) medium (Murashige and Skoog, 1962), 3% (w/v) sucrose, 3 mg/l 2,4-D, 2.5 mg/l NAA and 0.8% (w/v) agar adjusted to pH 5.8 using 1 M NaOH.

Sorghum cell suspension culture medium: 4.4 g/l Murashige and Skoog Basal Salt with minimal organics (MSMO) supplemented with 3% (w/v) sucrose, 3 mg/l 2,4-D, 2.5 mg/l NAA adjusted to pH 5.8 using 1 M NaOH.

Sorghum seed germination medium: 2.2 g/l Murashige and Skoog basal media (MS) medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose, 5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) and 0.8% (w/v) agar adjusted to pH 5.8 using 2M KOH.

Sorghum seed growth medium: 4.4 g/l Murashige and Skoog basal media (MS) medium, 3% (w/v) sucrose, 5 mM MES and 0.8% (w/v) agar adjusted to pH 5.8 using 2M KOH.

The plant tissue culture media were autoclaved at 121°C for 20 min using a High Pressure Steam Autoclave HL-340 (Gemmy Industrial Corp, Taiwan).

2.4 Plant Material

White sorghum seeds were purchased from Agricol, Brackenfell, South Africa. Thirteen other sorghum seed varieties were obtained from Dr Pangirai Tongoona, University of KwaZulu-Natal, Pietermaritzburg, South Africa. These varieties were: AS1; AS4; AS6; AS7; AS12; AS19; MN1500; MN1618; MN4320; MN1435; MN1812; SAR16 and SAR29.

2.5 Plant Tissue Culture

2.5.1 Seed Decontamination

Sorghum seeds were surface decontaminated by briefly immersing them in 70% (v/v) ethanol for 1 min followed by absolute commercial bleach (12% sodium hypochlorite solution) for 20 min with intermittent shaking. The decontamination solution was discarded and the seeds were rinsed three times with sterile distilled water. Decontaminated seeds were air-dried on filter paper and plated on appropriate seed germination or growth media as described in Sections 2.5.2 and 2.6.2 respectively.

2.5.2 Callus Initiation and Maintenance in Culture

White sorghum seeds (Section 2.4) were used for the establishment of callus and sorghum cell suspension cultures. Surface decontaminated sorghum seeds (Section 2.5.1) were plated on sorghum seed germination medium [2.2 g/l Murashige and Skoog basal media (MS) medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose, 5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) and 0.8% (w/v) agar, pH 5.8] and incubated in the dark at 25°C for three to four days. Shoots of three to four day old sorghum seedlings were cut into pieces of approximately 5 mm in length and used as explants for the initiation of callus. The explants were plated on 10 cm diameter tissue culture dishes (Techno Plastic Products, Switzerland) containing sorghum callus initiation medium [4.4 g/l Murashige and Skoog Basal Salt with minimal organics (MSMO) medium supplemented with 3% (w/v) sucrose, 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2.5 mg/l 1-naphthaleneacetic acid (NAA) and 0.8%

(w/v) agar, pH 5.8] and incubated in the dark at 25°C. Callus induction and growth was visually assessed over a four to five week period. Only the soft, easily breakable (friable) callus masses were maintained in culture by subculturing four to five week old callus onto fresh callus initiation medium. Well-established friable callus masses were used for the initiation of sorghum cell suspension cultures (Section 2.5.3).

2.5.3 Establishment of Sorghum Cell Suspension Cultures

Four to five week old friable callus masses (Section 2.5.2) were used to initiate cell suspension cultures. Three large clumps (approximately 4.5 g total wet weight) of actively growing friable callus masses were placed in 250 ml Erlenmeyer flasks containing 50 ml of sorghum cell suspension culture medium medium [4.4 g/l MSMO supplemented with 3% (w/v) sucrose, 3 mg/l 2,4-D, 2.5 mg/l NAA, pH 5.8]. The flasks were incubated on a horizontal incubator-shaker (Labcon, Roodepoort, South Africa) under dark conditions at 25°C with agitation at 130 rpm. After one week, 10 ml of fresh cell suspension culture medium was added to each cell culture. The flasks were further incubated for another week until the cells developed into a thick suspension. Cells were subcultured every 14 days by transferring 40 ml of the cell culture into 250 ml Erlenmeyer flasks containing 60 ml of fresh medium.

2.5.4 Measurement of Growth Parameters of the Sorghum Cell Suspension Cultures

2.5.4.1 Growth Curve Measurements

The growth of sorghum cell suspension cultures was estimated over 18 days using the packed cell volume (PCV) method as previously described (Ndimba, 2001). Three independently established cell culture lines were used as biological replicates. Established cell cultures were subcultured into fresh cell suspension culture medium as described in Section 2.5.3. Immediately after subculturing (Day 0), each flask was gently shaken and 1 ml aliquot of the cell culture was placed in a graduated 1.5 ml microcentrifuge tube. Sampling was repeated three times from each flask at each sampling time point (technical replicates). The cells were left to settle at the bottom of the tube for 5 min on a flat bench top. After the incubation period, the upper level of the settled cells was marked with a fine marker pen. Packed cell volume was estimated as illustrated in Figure 2.1.

$$\text{PCV (\%)} = \frac{\text{Volume of packed cells}}{\text{Total volume of sample}} \times 100$$

Figure 2.1: Estimation of packed cell volume (PCV) of sorghum cell suspension cultures.

The PCV readings were taken every two days starting from Day 0 until Day 18 when the suspension cultures became too dense for consistent and reproducible sampling using a 1 ml pipette. The readings were taken exactly at the same time of the day throughout the sampling days so as to accurately document the growth rate over time. The average PCV value of the three independently established sorghum cell culture lines was used to plot a growth curve over time.

2.5.4.2 Cell Viability Test using Evans Blue

The viability of the sorghum cell suspension cultures was determined using Evans blue as previously described (Baker and Mock, 1994). Three independently established cell culture lines (biological replicates) were subcultured into fresh medium as described in Section 2.5.3. Immediately after subculturing (Day 0), each flask was gently shaken and a 1 ml aliquot of the cell suspension was taken. The medium was discarded leaving packed cells. Approximately 20 mg of the cells (wet weight) was mixed with 180 µl of distilled water and 20 µl of 0.5% (w/v) Evans blue solution. The cells were stained for 15 min with gentle shaking at room temperature. The staining procedure was repeated three times from each flask at each time point (technical replicates). After incubation, stained cells were centrifuged at 2,300 x *g* for 5 min. The supernatant was discarded and the cell pellet was washed three times in 1 ml of distilled water by centrifugation at 2,300 x *g* for 5 min per wash. After washing, 1.2 ml of 1% (w/v) SDS in 50% (v/v) methanol was added to the cells. The cells were ground using a plastic pestle and incubated at 50°C for 6 hrs. After incubation, the homogenate was centrifuged at 2,300 x *g* for 5 min and the released Evans blue stain was collected in the supernatant fraction. The optical density of

the released stain was measured spectrophotometrically at 600 nm on a Milton Roy Spectronic GENESYS 5 Spectrophotometer (Spectronic Analytical Instruments, Leeds, UK) using 1% (w/v) SDS in 50% (v/v) methanol as a blank solution.

To determine total cell death (0% viability), sorghum suspension cultured cells were deliberately killed by heating at 95°C for 10 min on Dry Block Heater (FMH Instruments). The deliberately killed cells were stained with Evans blue and absorbed stain was quantified in parallel with unboiled cells as described above. Cell viability was estimated as shown in Figure 2.2. The viability of sorghum cells at each sampling time point was taken as an average of three biological replicates. The data was used to plot a bar graph of cell viability (%) over time.

$$\textbf{Cell viability (\%)} = \frac{A_B - A_{UB}}{A_B} \times 100$$

where $\mathbf{A_B}$ is the absorbance of stain released from boiled cells; $\mathbf{A_{UB}}$ is the absorbance of stain released from unboiled cells.

Figure 2.2: Estimation of cell viability using the Evans blue method.

2.5.4.3 Conductivity and pH Measurements of the Culture Medium

Conductivity and pH measurements of the sorghum cell suspension culture medium were measured simultaneously using a Portable Multimeter MM40 (Crison Instruments, SA, Allella, Barcelona, Spain). Prior to use, the multimeter was calibrated according to the manufacturer's instructions. Established sorghum cell cultures were subcultured into fresh cell suspension culture media as described in Section 2.5.3. One millilitre of the cell suspension was sampled every second day beginning at Day 0 until Day 19 for the pH and conductivity measurements. The measurements were taken for three independently established sorghum cell suspension culture lines (biological replicates) at each sampling time point. The average pH and conductivity readings of the three biological replicates were used to plot graphs showing changes in pH and conductivity of the culture medium over time.

2.6 Stress Treatment Experiments

2.6.1 Sorghum Cell Suspension Cultures

2.6.1.1 Salt and Osmotic Treatment of the Sorghum Cell Suspension Culture System

Established sorghum cell suspension cultures were subcultured into fresh medium as described in Section 2.5.3. Individual 250 ml Erlenmeyer flasks containing 100 ml of 10-days old cell cultures were either treated with 4 ml of 5 M NaCl or 8 ml of 5 M sorbitol to simulate salt (200 mM NaCl) or hyperosmotic stress (400 mM sorbitol) respectively. Eight millilitres of sterile distilled water was added to the control flasks. Both treated and untreated cell suspension cultures were incubated for 6 hrs under dark conditions at 25°C

on a horizontal incubator-shaker (Labcon) with agitation at 130 rpm. Treatment procedures were repeated on three independently established cell suspension culture lines for each treatment (biological replicates). After the 6 hr incubation period, cells were either sampled for estimation of cell viability (Section 2.6.1.3) or harvested for protein extraction procedures (Section 2.7).

2.6.1.2 Harvesting of Cell Suspension Cultures

Salt and hyperosmotic stress treated as well as control 10-day old cell suspension cultures (Section 2.6.1.1) were separated from the culture media by filtering over four layers of Miracloth (Merck, Darmstadt, Germany). The culture medium now termed culture filtrate (CF) was collected for CF protein extraction (Section 2.7.2). On the other hand, the cells were washed once by filtration with 10 ml sterile distilled water. The cells were collected, flash frozen in liquid nitrogen and stored at -20°C until use in protein extraction procedures (Section 2.7.1).

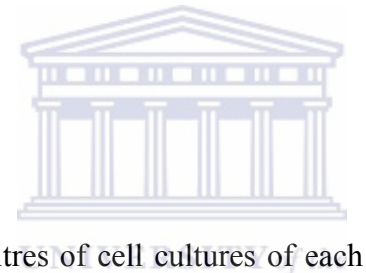
2.6.1.3 Estimation of Cell Viability Following Stress Treatment

A hundred millilitres of a ten-day old sorghum cell suspension culture was subdivided into three subcultures of 30 ml each. The resultant three subcultures were either treated with water (control), 200 mM NaCl (salt stress) or 400 mM sorbitol (hyperosmotic stress) as described in Section 2.6.1.1. Three such independent experiments were carried out thus representing three biological replicates for each stress treatment group. The viability

of cell cultures following each respective treatment was estimated using both Evans Blue and 3-[4,5-Dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) tests.

2.6.1.3.1 The Evans Blue Test

Sampling of cell cultures was carried out at 0, 6 and 24 hrs for all three biological replicates of each treatment group (control, salt or hyperosmotic stress). For the technical replicates, three sub-samples of each stress treatment per time point were included in the analyses. Evans blue staining and quantification was performed as described in Section 2.5.4.2.



2.6.1.3.2. The MTT Test

One hundred and fifty microlitres of cell cultures of each treatment group (control, salt and sorbitol stress) was sampled into 1.5 ml microcentrifuge tubes at 0, 6 and 24 hrs. For all three biological replicates, sampling was repeated three times from each flask per treatment group (technical replicates). To each sample, 50 μl of a 5 mg/ml MTT stock solution (prepared in water) was added. The microcentrifuge tubes were incubated with opened lids for 30 minutes with gentle shaking in a fume hood. After the incubation period, the cells were left to settle on a flat bench top. The resultant supernatant was discarded without disrupting the cell pellet. A volume of 1 ml dimethyl sulphoxide (DMSO) was added to all tubes before incubating for a further 10 minutes with gentle shaking. The MTT treated cells were left to settle for 5 min after which the supernatant was collected for absorbance measurements at 490 nm against a DMSO blank solution.

Cell viability of each cell culture treatment was graphically represented as average absorbance reading of the biological replicates per each sampling time (0, 6 and 24 hrs).

2.6.2 Sorghum Whole Plant Systems

2.6.2.1 Screening Sorghum Seed Varieties for Salt Tolerance

The 13 sorghum seed varieties (Section 2.4) were screened for salt tolerance by germinating them in sorghum seed growth medium supplemented with 100 mM NaCl (salt stress) or without NaCl (control) for 14 days as described in Section 2.6.2.2. After 14 days in culture, the seedlings were harvested and shoot length was measured for at least 10 control and 10 salt stressed seedlings per sorghum variety. Percentage reduction in shoot length was calculated per seed variety. Basing on these results, two varieties AS6 (salt sensitive) and MN1618 (salt tolerant) were selected for use in comparative proteomic analysis of salt stress responsive proteins.

2.6.2.2 Salt Stress Treatment of Whole Plant Systems

For each sorghum variety, eight surface decontaminated seeds (Section 2.5.1) were plated in 175 ml capacity plant tissue culture vessels (Sigma-Aldrich, Saint Louis, MO., USA) containing 50 ml of sorghum seed growth media [4.4 g/l MS medium, 3% (w/v) sucrose, 5 mM MES and 0.8% (w/v) agar, pH 5.8] supplemented with either 100 mM NaCl (salt treatment) or without NaCl (control). At least four such vessels were prepared for each seed variety per treatment group. The vessels were incubated at 25ºC under a 16 hr light/8 hr dark regime for 14 days. At 14 days post plating, sorghum seedlings were

carefully removed from the solid media avoiding damaging any plant parts in the process and placed on paper towel. Leaf, sheath and root tissues were excised from the seedlings and immediately flash frozen in liquid nitrogen. The different tissues were separately stored at -20°C until use in protein extraction procedure (Section 2.7.3).

2.7 Protein extraction

2.7.1 Sorghum cell suspension culture total soluble protein (TSP)

Approximately 2 g (wet weight) of liquid nitrogen frozen cells (Section 2.6.1.2) were finely ground using a pestle and mortar. The ground cells were transferred to a sterile 15 ml Falcon tube and precipitated with 10 ml of 10% (v/v) TCA in acetone. The mixture was centrifuged at 13,400 x *g* for 10 min at room temperature. The supernatant was discarded and the pellet was washed three times with 10 ml of ice-cold 80% (v/v) acetone by centrifuging at 13,400 x *g* for 10 min per wash. The pellet was then air dried at room temperature for 5 min and re-suspended 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. The homogenate was centrifuged at 15,700 x *g* for 10 min to remove the cell debri in the pellet fraction. Total soluble proteins (TSP) were collected in the supernatant fraction and stored at -20°C until use in protein quantification (Section 2.8) and electrophoresis (Section 2.9) procedures.

2.7.2 Sorghum Cell Suspension Culture filtrate (CF)

The spent sorghum cell suspension culture medium was collected after filtering suspension cell cultures through four layers of Miracloth (Merck). The filtered culture medium was centrifuged at 2,500 x *g* for 10 min to collect cell free culture fitrate (CF) in the supernatant fraction. The CF proteins were precipitated in 80% (v/v) acetone for at least 1 hr at -20°C. After precipitation, the samples were centrifuged at 15,700 x *g* for 10 min. The supernatant was discarded and the protein precipitate pellet was washed three times using ice-cold 80% (v/v) acetone by centrifuging at 15,700 x *g* for 10 min per wash. The acetone washed pellet was air dried at room temperature for about 5 min and re-suspended in 2 ml of urea buffer for at least 1 hr with vigorous vortexing at room temperature. The solubilised protein mixture was centrifuged at 5,700 x *g* for 10 min. The supernatant containing CF soluble protein was collected and stored at -20°C until use in protein quantification (Section 2.8) and electrophoresis (Section 2.9) procedures.

2.7.3 Protein Extraction from Whole Plant Systems

Leaf, sheath and root tissues (Section 2.6.2.2) were separately ground in liquid nitrogen using pestle and mortar and precipitated with 10% (w/v) TCA. Leaf and sheath samples were from an average of 10, 14-day old plants, while the root extracts were from an average of 20, 14-day old plants. The homogenate was centrifuged at 13,400 x *g* for 10 min at room temperature. The supernatant was discarded and the pellet was washed three times with 1.5 ml of ice-cold 80% (v/v) acetone by centrifuging at 13,400 x *g* for 10 min for each wash. The pellet was air dried for 5 min at room temperature and resuspended in 1 ml of urea buffer for at least 1 hr with vigorous vortexing at room temperature. The

homogenate was centrifuged at 15,700 x *g* for 10 min. The supernatant containing soluble protein was collected and stored at -20°C until use in protein quantification (Section 2.8) and electrophoresis (Section 2.9) procedures.

2.8 Protein Quantification

The concentration of all protein extracts was determined using a modified Bradford Assay (Bradford, 1976), as previously described by Ndimba *et al.* (2003). Bovine serum albumin (BSA) standards were prepared in duplicate from a 5 mg/ml BSA stock solution in 2 ml plastic cuvettes as indicated in Table 2.2. Protein extracts were also prepared in duplicate in 2 ml plastic cuvettes by mixing 5 µl of unknown protein sample with 5 µl of urea buffer, 10 µl of 0.1 M HCl and 80 µl of distilled water. The Bradford reagent (BIO-RAD, Hercules, CA, USA) was diluted five times with distilled water prior to use. A volume of 900 µl of the diluted Bradford reagent was added to all standards and protein extracts, mixed and incubated for 5 min at room temperature. Absorbance was measured at 595 nm on a Milton Roy Spectronic GENESYS 5 Spectrophotometer (Spectronic Analytical Instruments) using the 0 mg/ml BSA standard as a blank solution. The standards were used to plot a standard curve from which concentrations of all the unknown protein extract samples were extrapolated.

Table 2.2: Preparation of BSA protein standards for protein quantification.

BSA final concentration (mg/ml)	BSA 5mg/ml stock solution (µl)	Urea Buffer (µl)	0.1 M HCl (µl)	Distilled water (µl)
0*	0	10	10	80
0.005	1	9	10	80
0.01	2	8	10	80
0.02	4	6	10	80
0.04	8	2	10	80
0.05	10	0	10	80

* blank solution

2.9 One-Dimensional (1D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein extracts were separated on 1D SDS-PAGE as previously described (Laemmli, 1970). This system is composed of two types of gels: a stacking gel and a resolving gel. The stacking gel has large pores that allow proteins to concentrate into thin zones before they enter the resolving gel. In the stacking gel, protein mobility depends on the net protein charge rather than MW. The resolving gel is composed of smaller pore sizes and proteins in this zone are separated on the basis of MW (Westermeier, 2005).

One dimensional (1D) gels were cast on 10.1 cm (width) x 8.3 cm (height) spacer glass plates (BIO-RAD) mounted with 1 mm thick spacers using the Mini-PROTEAN® 3 Electrophoresis Cell gel casting system (BIO-RAD). Resolving gels of 12% (v/v) were prepared from a 40% Acrylamide/Bis stock solution 37.5:1 (2.6% C) (BIO-RAD) using a 1.5 M Tris-HCl gel buffer, pH 8.8 as indicated in Table 2.3. Five millilitres of the prepared resolving gel mixture was poured into cast plates, overlaid with 1 ml of 100% isopropanol and left to polymerise for 30 min at room temperature. After polymerisation, the isopropanol overlay was rinsed off with distilled water and gel surfaces were blotted dry with filter paper. Stacking gels of 5% (v/v) were prepared from 40% Acrylamide/Bis stock solution 37.5:1 (2.6% C) (BIO-RAD) using 0.5 M Tris-HCl gel buffer, pH 6.8 as indicated in Table 2.3. One millilitre of the prepared stacking gel was poured over the resolving gel. Immediately thereafter, 1 mm thick 10- or 15-well combs (BIO-RAD) were placed in the stacking gels to form sample-loading wells.

Protein samples were mixed at a ratio of 1:1 with 2X SDS sample loading buffer [60 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 200 mM DTT, 0.025% (w/v) bromophenol blue] and denatured by heating on a Dry Block Heater (FMH Instruments) at 95 °C for 5 min. Samples were pulse centrifuged before loading onto the gels. Total protein quantities of 10 µg and 20 µg per well were typically loaded onto 15- and 10-welled gels respectively. Electrophoresis was carried out in 1X SDS-PAGE running buffer [25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS] on a Mini-PROTEAN® 3 Electrophoresis Cell (BIO-RAD) using a PowerPac™ Universal Power supply (BIO-

RAD). The run was initiated at 100 V for the first 30 min and then 120 V until the bromophenol blue migration-tracking dye reached the bottom of the gel plates. The gels were removed from the gels plates, stained with CBB and destained as described in Section 2.11.

Table 2.3: Preparation of resolving and stacking gels for SDS-PAGE.

	12% Resolving gel buffer	**5% Stacking gel buffer**
Distilled water	4.3 ml	1.48 ml
40% Acrylamide/Bis stock solution 37:5:1 (2.6% C)	3 ml	0.25 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	-
0.5 M Tris-HCl, pH 6.8	-	0.25 ml
10% SDS	0.1 ml	0.02 ml
10% APS	0.1 ml	0.02 ml
TEMED	6 ul	2 μl
Total volume	**10 ml**	**2 ml**

2.10 Two-Dimensional (2D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.10.1 Protein Load for 2D Gels

The amount of protein extracts applied on 2D gels varied with sample complexity as well as the length and pH range of immobilised pH gradient (IPG) strips used for IEF.

2.10.2 Rehydration of 7cm IPG Strips

Protein extracts (100-150 µg) were mixed with 0.8% (v/v) DTT, 0.2% (v/v) ampholytes (BIO-RAD), a tiny pinch of bromophenol blue and made up to a final volume of 125 µl using urea buffer. The samples were mixed by vortexing for 5 min, pulse centrifuged and then placed in individual channels of an Immobiline™ Dry Strip Reswelling Tray (GE Healthcare, Amersham, UK). Linear, 7 cm ReadyStrip™ IPG strips of pH range 3-10 or pH range 4-7 (BIO-RAD) were carefully placed on top of the sample, gel side being directly in contact with the sample, avoiding trapping any air bubbles in the process. The strips were then covered with mineral oil (PlusOne DryStrip Cover Fluid; GE Healthcare) to prevent sample evaporation during the rehydration process and left to passively rehydrate to their original gel thickness of 0.5 mm for at least 15 hrs at room temperature.

2.10.3 Rehydration of 18 cm IPG Strips

Protein extracts (400-800 µg) were mixed with 0.8% (v/v) DTT, 0.2% (v/v) ampholytes (BIO-RAD), a tiny pinch of bromophenol blue and made up to a final volume of 315 µl using urea buffer. The samples were mixed by vortexing for 5 min, pulse centrifuged and

used to passively rehydrate linear 18 cm ReadyStrip™ IPG strips (BIO-RAD) of pH range 4-7 as described in Section 2.10.2 for at least 20 hrs at room temperature.

2.10.4 First Dimension IEF of IPG Strips

After rehydration, IPG strips were briefly rinsed with distilled water to remove unabsorbed protein sample and carefully blotted with moist filter paper. The strips were then placed gel side up on the focusing platform of an Ettan™ IPGphor II™ (GE Healthcare). Distilled water moistened wicks were placed at the extreme ends of both the anodic and cathodic ends of the IPG strips to collect excess salts and impurities from the sample during focusing. The IPG strips were then covered with mineral oil to avoid sample evaporation and carbon dioxide absorption during focusing. Isoelectric focusing was performed in a three phase stepwise programme at 20°C as indicated in Tables 2.4 and 2.5 for 7 cm and 18 cm IPG strips respectively.

After IEF, 18 cm IPG strips were equilibrated (Section 2.10.5) in preparation for the second dimension (Section 2.10.7), while 7 cm IPG strips were either equilibrated (Section 2.10) straight away or stored in 15 ml Falcon tubes at -20°C until use in the second dimension SDS-PAGE (Section 2.10.6).

Table 2.4: Isoelectric focusing programme for 7 cm IPG strips.

Step	Volts (V)	Duration (hrs)/Volt hours (Vhrs)
1	250	0.15 hr
2	4 000	1 hr
3	4 000	12 000 Vhrs

Table 2.5: Isoelectric focusing programme for 18 cm IPG strips.

Step	Volts (V)	Duration (hrs)/Volt hours (Vhrs)
1	250	0.15 hr
2	8 000	2 hrs
3	8 000	60 000 Vhrs

2.10.5 Equilibration of IPG Strips

After IEF, IPG strips were equilibrated in SDS-containing buffers in order to solubilise focused proteins and allow SDS binding prior to second dimension SDS-PAGE. The focused IPG strips were incubated gel side up in reswelling tray channels containing 2.5 ml equilibration buffer [6 M urea, 2% (w/v) SDS, 50 mM Tris/HCl, pH 8.8 and 20% (v/v) glycerol], firstly containing 2% (w/v) DTT for 15 min followed by 2.5% (w/v) iodoacetamide for another 15 min with gentle agitation at room temperature. After equilibration, the isoelectric focused proteins were ready for separation on second-dimension SDS-PAGE of either mini (Section 2.10.6) or large format gels (Section 2.10.7).

2.10.6 Second Dimension SDS-PAGE of Mini Format Gels

Mini format 2D SDS-PAGE gels were cast on 10.1 cm (width) x 8.3 cm (height) spacer glass plates (BIO-RAD) mounted with 1 mm thick spacers using the Mini-PROTEAN® 3 Multi-Casting Chamber (BIO-RAD) which accommodates twelve 1.0 mm glass plates. Hundred millilitres of a 12% (v/v) resolving gel solution was prepared as described in Table 2.3. The resolving gel solution was poured into the cast plates according to the instruction manual and each gel was overlaid with 1 ml of 100% isopropanol. The gels were left to polymerise for 1 hr at room temperature. After polymerisation, the isopropanol overlay was rinsed off with distilled water and gel surfaces were blotted dry with filter paper.

Equilibrated 7 cm IPG strips (Section 2.10.5) were gently rinsed with 1X SDS-PAGE running buffer and placed on top of mini format 12% SDS-PAGE resolving gels with the plastic backing against the spacer plate. Three microlitres of PageRuler™ unstained protein ladder (Fermentas Life Sciences, Ontario, Canada) were spotted on small pieces of filter paper, air-dried and placed at the anodic side of each IPG strip. The IPG strips were then overlaid with 1 ml of 0.5% (w/v) molten agarose prepared in 1X SDS-PAGE running buffer containing a tint of bromophenol blue, which was used as a migration tracking dye during electrophoresis. Electrophoresis was carried out using the Mini-PROTEAN® 3 Dodeca™ cell (BIO-RAD) at 100 V during the first 30 min and then at 150 V until the bromophenol dye reached the bottom of the glass plates. After the second dimension, gels were stained with CBB (Section 2.11).

2.10.7 Second Dimension SDS-PAGE of Large Format Gels

Large format 2D SDS-PAGE gels were cast on 27.6 cm (width) x 21.6 cm (height) spacer glass plates (GE Healthcare) mounted with 1.0 mm thick spacers using the Ettan™ DALTtwelve Gel Caster (GE Healthcare) which accommodates up to fourteen 1.0 mm glass plates. The gel caster was assembled according to the Ettan™ DALTtwelve Gel Caster instruction manual (GE Healthcare). The balance chamber was filled with 100 ml of displacing solution [0.375 M Tris-HCl, pH 8.8, 50% (v/v) glycerol with a tint of bromophenol blue]. One litre of a 12% (v/v) resolving gel solution was prepared as described in Table 2.3 and poured into the cast gel plates according to the Ettan™ DALTtwelve Gel Caster instruction manual (GE Healthcare). Each gel was overlaid with 3 ml of 100% isopropanol and left to polymerise for at least 1 hr at room temperature. After polymerisation, the isopropanol overlay was rinsed off with distilled water and gels were stored overnight in a moist chamber at 4ºC.

Equilibrated 18 cm IPG strips (Section 2.10.5) were gently rinsed in 1X SDS-PAGE running buffer and placed on top of large format 12% (v/v) SDS-PAGE resolving gels with the plastic backing against the spacer plate. Five microlitres of PageRuler™ unstained protein ladder (Fermentas Life Sciences) were spotted on a small pieces of filter paper, air-dried and placed at the anodic side of each IPG strip. The IPG strips were then overlaid with 3 ml of 0.5% (w/v) molten agarose. The large format second dimension gels were electrophoresed on an Ettan™ DALTtwelve System (GE Healthcare), initially at 5 W per gel for 30 minutes and then at 17 W per gel (at a constant temperature of 25°C) until the bromophenol blue migration tracking dye reached the

bottom of the gel plates as described by Ndimba *et al*. (2005). After the second dimension, gels were stained with CBB (Section 2.11).

2.11 Coomassie Brilliant Blue Staining in SDS-PAGE Gels

Proteins separated by either 1D (Section 2.9) or 2D (Section 2.10) SDS-PAGE were routinely detected using a modified CBB R-250 staining protocol that uses three sequential staining steps. After electrophoresis, the gels were dismounted from the gel plate assembly and immersed firstly in CBB staining solution I [0.025% (w/v) CBB R-250, 10% (v/v) glacial acetic acid and 25% (v/v) propan-2-ol], heated for 1 min in a microwave at maximum power and incubated for 30 min with shaking at room temperature. The CBB staining solution I was discarded and the staining process was repeated using CBB staining solution II [0.003% (w/v) CBB R-250, 10% (v/v) glacial acetic acid and 10% (v/v) propan-2-ol] and finally CBB staining solution III [0.003% (w/v) CBB R-250 and 10% (v/v) glacial acetic acid] for 30 min in each stain as described above. After staining, the gels were immersed in destaining solution [10% (v/v) acetic acid and 1% (v/v) glycerol] with shaking at room temperature until the protein bands or protein spots (in 1D or 2D gels, respectively) were visibly distinct against a clear background. The gels were imaged using a Molecular Imager PharosFX Plus System (BIO-RAD).

2.12 Comparative Analysis of 2D SDS-PAGE Gels

Comparative analysis of 2D SDS-PAGE gels within defined experiments was done using the PDQuest™ Advanced 2D Analysis Software version 8.0.1 build 055 (BIO-RAD). Two-dimensional gels were initially imaged using the Molecular Imager PharosFX Plus System (BIO-RAD) and then analysed according to the PDQuest™ Advanced 2D Analysis Software user manual (BIO-RAD). All experiments analysed with this software had three biological replicates per treatment group. The gels were normalised using the local regression model to compensate for gel to gel differences in spot quantities due to non-expression related variations. Prior to differential protein expression analysis across treatment groups of each experiment, spots were manually edited using the group consensus tool to obtain spot expression consensus in all three biological replicates per treatment group. Differentially expressed protein spots were either qualitative (present/absent spots), quantitative (showing at least a 2-fold expression change) and/or Student's *t*-test (95% significance level) significant spots. Protein spots of interest were either manually picked using pipette tips or automatically using the ExQuest™ spot cutter (BIO-RAD) for identification using mass spectrometry and database searching (Section 2.14).

2.13 Western Blotting for Heat Shock Protein 70 (Hsp70)

2.13.1 Transfer of Protein from 1D and 2D SDS-PAGE Gels onto PVDF Membrane

Proteins samples separated by either 1D (Section 2.9) or 2D (Section 2.10) SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P PVDF

membrane, GE Healthcare) as previously described (Towbin *et al.*, 1979), using a Mini Trans-Blot® Electrophoresis Transfer Cell (BIO-RAD). Prior to protein transfer, electrophoresed 1D or 2D SDS-PAGE gels were pre-equilibrated in cold transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol] for at least 15 min with shaking at room temperature. The PVDF membranes were pre-wet in 100% (v/v) methanol for 10 seconds, washed three times in distilled water and equilibrated in cold transfer buffer for at least 15 min with shaking at room temperature prior to transfer.

The gel-membrane sandwich was prepared on the gel holder cassette according to the Mini Trans-Blot® Electrophoresis Transfer Cell instruction manual (BIO-RAD). Electrophoretic transfer of proteins was performed at 36 V, overnight at 4°C with constant stirring of the transfer buffer to maintain even buffer temperature and ion distribution in the tank. A Bio-Ice Cooling unit (BIO-RAD) previously frozen with water was used as a cooling system during protein transfer.

2.13.2 Immunoprobing of PVDF Membrane with Antibodies

After protein transfer, the membrane was stained for total protein with Ponceau S stain for 10 min with gentle agitation at room temperature. The relative positions of the protein markers on the membrane were marked using a scalpel blade. The membrane was rinsed in several changes of distilled water to remove residual Ponceau S stain and washed once in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5) for 10 min before blocking in blocking solution [1% (w/v) Elite fat free instant milk powder in TBS] for 1 hr. The membrane was then incubated with the primary antibody [human HeLa cells anti-

Hsp70/Hsc70 monoclonal antibody raised in mouse (Stressgen Bioreagents Corp., Victoria, Canada)] diluted 1:2,500 in 0.5% (w/v) blocking solution for 1 hr. The membrane was washed three times with TBST [TBS containing 0.1% (v/v) Tween 20] for 10 min per wash. The membrane was then washed with 0.5% (v/v) blocking solution for 10 min and incubated with the secondary antibody [goat anti-mouse IgG (H & L) horseradish peroxidase conjugate (Invitrogen Corp., Carlsbad, CA, USA)] diluted 1:1,000 in 0.5% (w/v) blocking solution for 1 hr.

2.13.3 Immunodetection of Proteins using a Chemiluminescent Substrate

After the secondary antibody incubation, the membrane was washed three times in TBST for 15 min per wash. Heat shock proteins were detected using a SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's instructions. The Amersham Hyperfilm ECL (GE Healthcare) was exposed and automatically developed using the Curix 60 (Agfa- Gevaert, N.V., Mortsel, Belgium).

2.14 Mass Spectrometry

2.14.1 Protein Identification using MALDI-TOF MS

Coomassie Brilliant Blue stained gels were imaged using the Molecular Imager PharosFX Plus System (BIO-RAD) and the experimental MW and pIs of proteins of interest were estimated from the gels. Protein spots were either picked manually with pipette tips or automatically using ExQuest™ spot cutter (BIO-RAD) and transferred into

sterile microcentrifuge tubes. Gel pieces were then destained twice for 30 min using 50 mM ammonium bicarbonate and acetonitrile at a ratio of 1:1 with occasional vortexing. The gel pieces were dehydrated with 100 µl acetonitrile for 5 min, and then completely desiccated using the 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 hrs at 37°C. The digested proteins were then stored at 4°C until further analysis.

One microlitre from each digested protein sample was mixed with the same volume of α-cyna-hydroxy-cinnamic acid (matrix) and spotted onto a MALDI target plate for analysis by MALDI-TOF MS using a Voyager DE Pro Biospectrometry workstation (Applied Biosystems, Forster City, CA, USA) to generate peptide mass fingerprints. The MALDI-TOF was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential with optimized parameters. Close external calibration was employed using the Sequazyme calibration mixture II, containing angiotensin I, ACTH (1-17 clip), ACTH/18-39 clip and ACTH (18-39 clip) and bovine insulin (Applied Biosystems). This calibration method provided mass accuracy of 100 to 200 ppm across the mass range 900 to 5 000 Da. Peptide spectra of accumulated 1,200 shots each were automatically processed for baseline correction, noise removal and peak deisotoping. The threshold was manually adjusted to 2-8% base peak intensity. All searches were performed against the National Center for Biotechnology Information (NCBI) and Mass spectrometry protein sequence database (MSDB) using MASCOT

(http://www.matrixscience.com/search_form_select.html. Candidate protein matches with molecular weight search (MOWSE) scores higher than 66 ($p<0.05$) were considered as positive identifications. In addition, positive protein assignments required at least a 10% sequence coverage. In cases where more that one protein satisfied the above mentioned threshold criteria, the entry with the highest MOWSE score was considered as a probable positive identification.

2.14.2 Protein Identification using MALDI-TOF-TOF MS

Protein spots for identification by MALDI-TOF-TOF MS were sent to Durham University, School of Biological and Biomedical Sciences and were identified by Prof. Toni Slabas, Dr Bill Simon and their team.

2.14.2.1 Tryptic Digestion of Protein Bands

Tryptic digestion of protein spots was performed using a ProGest robot (Genonic Solutions, Huntingdon, Cambridgeshire, UK) programmed with the long trypsin digestion method as described below. Protein spots were excised from the gel using ExQuest^{TM} spot cutter (BIO-RAD) and transferred to the wells of a 96 well microtitre plate. This plate is designed with microscopic holes at the bottom of the wells, which allow for positive displacement of liquids during reagent changes on the robot. Gel pieces were first equilibrated in 50 µl of 50 mM ammonium bicarbonate and proteins were reductively alkylated with 10 mM DTT and 100 mM iodoacetamide. The protein spots were then destained and gel plugs desiccated with acetonitrile.

Gel plugs were rehydrated with 50 mM ammonium bicarbonate containing 5% (w/v) trypsin and the proteins were digested for 12 hrs at 37°C. Resulting peptides were extracted from the gel plugs with 2 X 25 µl washes of 50% acetonitrile, 0.1% (v/v) TFA. Peptide extracts were freeze dried and then re-suspended in 10 µl of 0.1% formic acid. A saturated solution of α-cyano-4-hydroxy-cinnamic acid (matrix) was prepared in 50% acetonitrile, 0.1% TFA, 10 mM ammonium acetate. For each sample, 1 µl of the matrix solution was spotted on the MALDI target immediately followed by 1 µl of protein sample into the matrix spot. The sample/matrix droplet was allowed to slowly air dry.

2.14.2.2 MALDI-TOF-TOF Analyses

Matrix-assisted laser desorption/ionization time of flight-time of flight (MALDI-TOF-TOF) analysis was performed on a 4800 Proteomic analyser (Applied Biosystems) using the following protocol. Firstly, MALDI-TOF MS analysis was performed on all the target spots using automated data acquisition and processing under the control of Applied Biosystems 4000 series Explorer software (version 3.5) using reflector mode, a mass range of 700-4000 m/z, 1000 total laser shots per spectrum and a laser intensity of 3300 V. Following acquisition, the TOF-MS spectra were noise corrected, peak de-isotoped and internally calibrated using the trypsin autolysis peaks 842.500 and 2211.100 m/z. The 10 most abundant precursor ions from each spectrum were then selected by the software for fragmentation and MS-MS analysis using a 1 kV CID fragmentation method collecting 4000 laser shots per spectra with a laser intensity of 3800 over the mass range.

Peak lists of ion masses were generated by GPS Explorer software version 3.6 (Applied Biosystems) from the calibrated and de-isotoped MS and MS-MS spectra for each sample. Combined lists of the MS and MS-MS data were used for database searching with MASCOT version 2.2 (Matrix Science) against all entries in the NCBI database. Database search parameters used were; digestion enzyme trypsin, single missed cleavage allowed, variable modification of carboxymethyl cysteine and oxidised methionine, precursor mass tolerance of 50 ppm and fragmentation ion tolerance of 0.2 Da. The MASCOT outputs were generated for each sample. Candidate protein matches with MOWSE scores higher than 76 ($p<0.05$) were considered as positive identifications.

CHAPTER 3

Establishment of a Sorghum Cell Suspension Culture System for Use in Proteomics Studies

3.1 Introduction

Part of the data included in this chapter has been published in Ngara, R., Rees, J. and Ndimba, B.K. (2008). Establishment of sorghum cell suspension culture system for proteomics studies. African Journal of Biotechnology, 7 (6), 744-749. A copy of the publication is attached in Appendix I. Included below is a more comprehensive account of the work.

Cell suspension cultures are a homogenous group of rapidly growing undifferentiated cells that are grown in liquid culture media (Evans *et al.*, 2003). Cell suspension cultures are initiated from callus, which is an unorganised mass of undifferentiated cells. In nature, callus is formed as a plant's protective mechanism to seal off damaged plant tissue. Consequently, in plant tissue culture, callus can be induced from various parts of the plant that are grown on solid culture media with appropriate combinations of plant hormonal supplements (Collin and Edwards, 1998; Evans *et al.*, 2003).

There are several advantages of using cell suspension culture systems in proteomics studies. Firstly, in comparison with whole plant systems, which have a relatively long growth cycle as well as complex tissue specific proteomes, cells in suspension have a shorter life cycle and remain undifferentiated. Therefore, cell suspension cultures provide

for a continuous supply of homogenous experimental units. Secondly, cell suspension cultures are grown under well-defined conditions and this ensures reproducibility within and between experiments (Ndimba *et al.*, 2005). Thirdly, cell suspension cultures can be easily manipulated in feeding experiments when studying plant responses to both biotic and abiotic stress and are thus useful experimental material in such studies (Laukens *et al.*, 2007).

After cell suspension cultures have been initiated and established from callus, it is important to assess their growth characteristics. Parameters that are routinely used to evaluate the growth of the cultured cells include measurement of cell growth, cell viability, and changes in the pH and conductivity of the culture medium (Collin and Edwards, 1998; Evans *et al.*, 2003). The growth curve of suspension cultures can be monitored by measuring wet or dry weight, packed cell volume (PCV) and/or cell numbers (Evans *et al.*, 2003). Generally, the growth cycle of cell suspension cultures can take anything between 1 and 3 weeks, showing three basic growth phases; a lag phase, an exponential phase and a stationary phase. However, the actual length of each phase differs with plant species, growth conditions and the initial cell density used for the purpose of subculturing (Collin and Edwards, 1998). Cell viability is an important growth parameter to monitor during the growth of cell suspension cultures as it gives information on the metabolic state of the cells. Cell viability can be evaluated using stains or by quantifying the activity of cells using enzymatic-based assays.

This chapter describes the establishment of sorghum callus, the initiation and maintenance of sorghum cell suspension cultures and the evaluation of their potential for application in proteomics studies.

3.2 Initiation and Maintenance of Sorghum Callus and Cell Suspension Cultures

Sorghum callus was successfully initiated from three-four day old shoot explants cultured on MS medium supplemented with 2,4-D and NAA (Section 2.5.2). Visual assessment of callus growth under alternate 16 hr light/8 hr dark regime versus a 24 hr dark regime showed that the callus grew better under continuous dark conditions. Thereafter, all callus incubations were carried out under dark conditions. Figure 3.1 shows sorghum callus cultures at different stages of growth. Figure 3.1A shows the sizes of the callus masses that were used for the purposes of subculturing and maintenance of the callus in culture. After 4-5 weeks of incubation under dark conditions, the callus masses grew into large cell masses as shown in Figure 3.1B. About three large clumps (approximately 4.5 g total wet weight) of 4-5 week old actively growing friable callus (Figure 3.1B) were used to initiate cells in suspension as described in Section 2.5.3. Only friable, light-coloured callus masses were used for the maintenance of callus in culture and for the initiation of cell suspension cultures. Figure 3.2 shows three different lines of sorghum cell suspension cultures (biological replicates) at about 14 days post subculture, showing consistency in the cell mass.

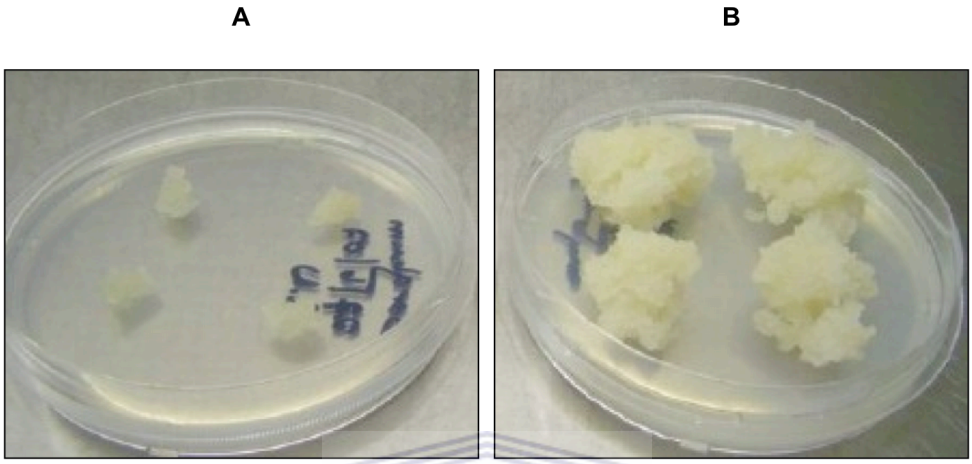


Figure 3.1: Sorghum callus cultured on MS medium under dark conditions.

The medium was supplemented with 3 mg/l of 2,4-D and 2.5 mg/l of NAA. (A) Callus masses used for subculturing and maintenance of callus. (B) Friable callus masses after a 4-5 week incubation period used for initiation of sorghum cell suspension cultures.

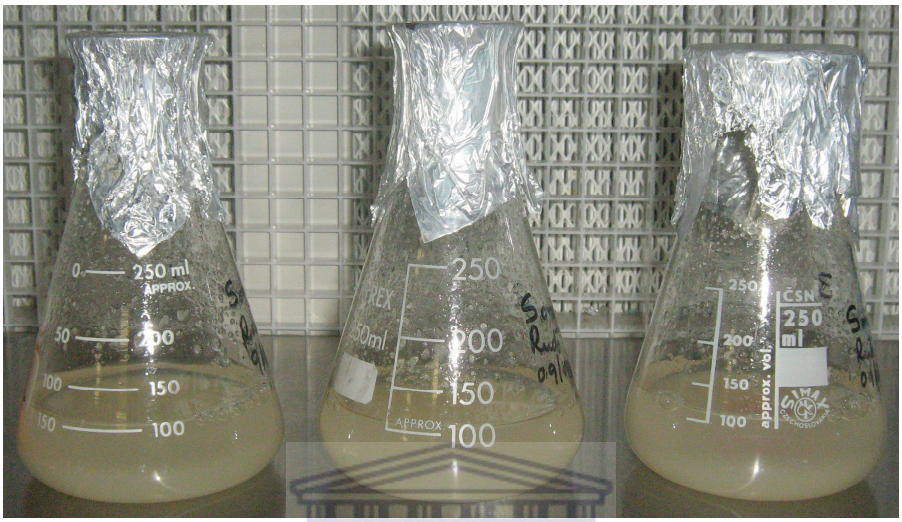


Figure 3.2: Sorghum cell suspension cultures from three independently established lines at day 14-post subculture.

3.3 Monitoring the Growth of Sorghum Cell Suspension Cultures

3.3.1 Packed Cell Volume Measurements

The growth of sorghum cell suspension cultures was monitored using the packed cell volume (PCV) method as described in Section 2.5.4.1. The PCV measurements from three independently established cell culture lines (biological experiments) were used to establish a growth curve over time. Figure 3.3 shows a sigmoidal growth curve of sorghum cell suspension cultures over an 18-day period. The growth curve is distinctly divided into 3 phases; a lag phase, an exponential phase and a stationary phase. The lag phase, which begins on the day of subculture until about day 8 is characterised by a very slow increase in PCV. After day 8, the PCV increases exponentially until about day 14 thus representing the exponential phase Thereafter, the cells go through a stationary phase where the growth rate of cells increase but at a slower rate. Due to an increase in cell density, PCV measurements were terminated at day 18 as it became difficult to sample consistent and reproducible 1 ml aliquots of the cell cultures using a 1 000 µl pipette.

The structure of sorghum cells was also analysed under the microscope. Figure 3.4 illustrates microscopic pictures of sorghum cell suspension cultures taken using a light microscope at a magnification of 10X. From the microscopic pictures, cell clusters and aggregates of various sizes were observed (indicated by the white arrows). Individual cells were also observed having a dense cytoplasm with numerous mobile cytoplasmic vesicles (indicated by black arrows). The mobile cytoplasmic vesicles could possibly indicate cytoplasmic streaming which is associated with high metabolic activities in cells.

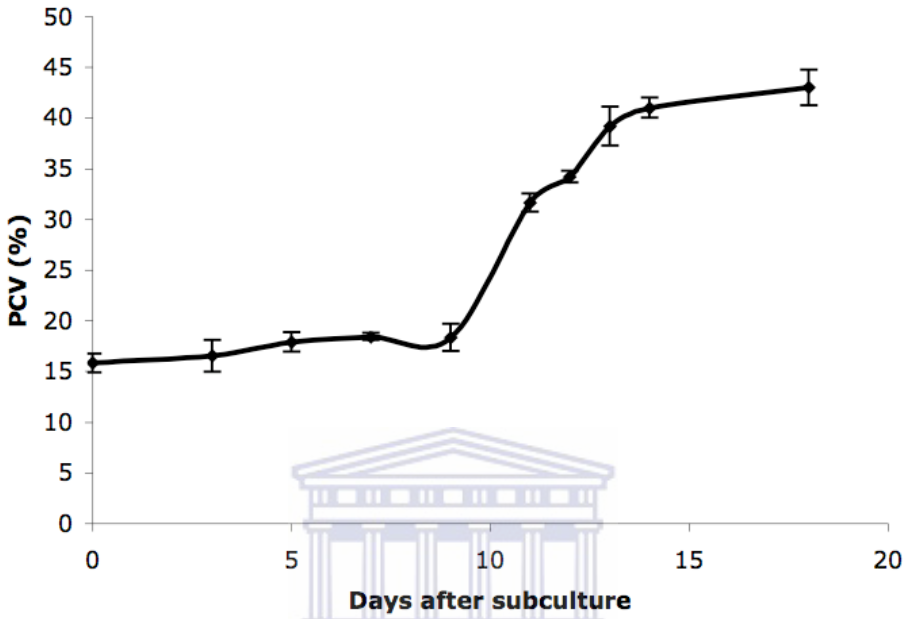


Figure 3.3: The growth curve of sorghum cell suspension cultures.

The growth of sorghum cell suspension cultures was plotted using packed cell volume (PCV) readings over an 18-day period. Each PCV reading is an average of three independently established sorghum cell suspension culture lines. Vertical bars indicate standard error.

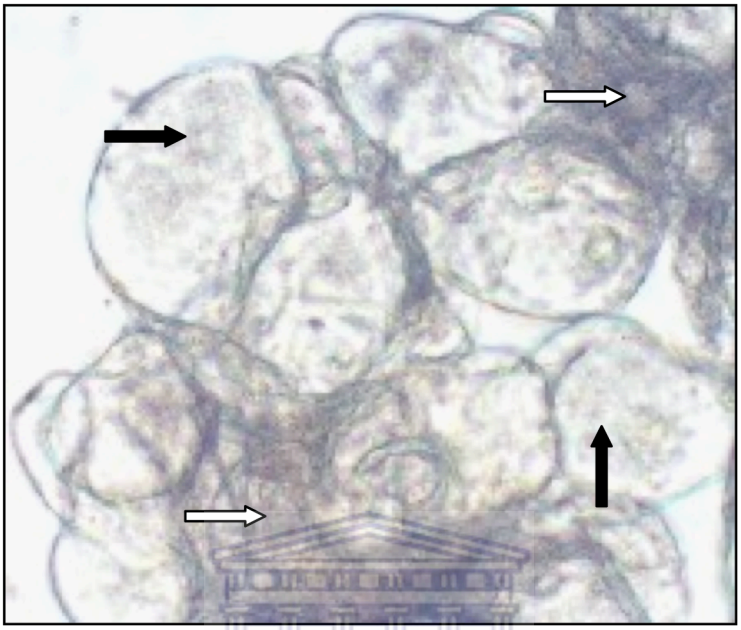


Figure 3.4: Microscopic analysis of sorghum cell suspension cultures.

The microscopic picture was taken using a Nikon Inverted Light Microscope fitted with a Leica Firecam digital camera at a magnification of 10X. From the picture, cell clusters and aggregates (indicated by white arrows) and individual cells with numerous mobile cytoplasmic vesicles were observed (indicated by black arrows).

3.3.2 Measurement of Cell Viability

The viability of the sorghum cell suspension cultures was estimated using Evans blue stain as described in Section 2.5.4.2. The principle of this viability stain is that live cells are selectively permeable to molecules from the external environment. However, when cells die, their membranes become damaged and thus lose the ability to selectively exclude specific molecules from the interior of the cell. As such, Evans blue stain, which is normally excluded from living cells can therefore stain contents of dead cells (Baker and Mock, 1994). The amount of stain that is absorbed by the dead cells can be released from cell debri using a detergent and quantified spectrophotometrically. Figure 3.5 shows cell viability (%) of sorghum cell cultures from day 0 to day 20-post subculture. It was observed that sorghum cultured cells maintained cell viability of over 60% throughout the 20-day period, with the highest cell viability values of approximately 76% being observed on days 0, 12 and 20.

3.3.3 Measurement of pH and Conductivity of the Culture Medium

The pH and conductivity of the sorghum cell suspension culture medium was taken using a pH meter as described in Section 2.5.4.3. The pH of cell suspension culture medium changes due to oxidation as well as the uptake and secretion of substances by the growing cells (Beyl, 1999). On the other hand, the conductivity of the medium also changes due to the uptake of electrolytes (such as Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-) and their subsequent utilization as nutrients by cells during growth (Evans *et al.*, 2003). The average values of pH and conductivity of the culture medium of three independently established cell suspension culture lines (biological replicates) were used to plot graphs.

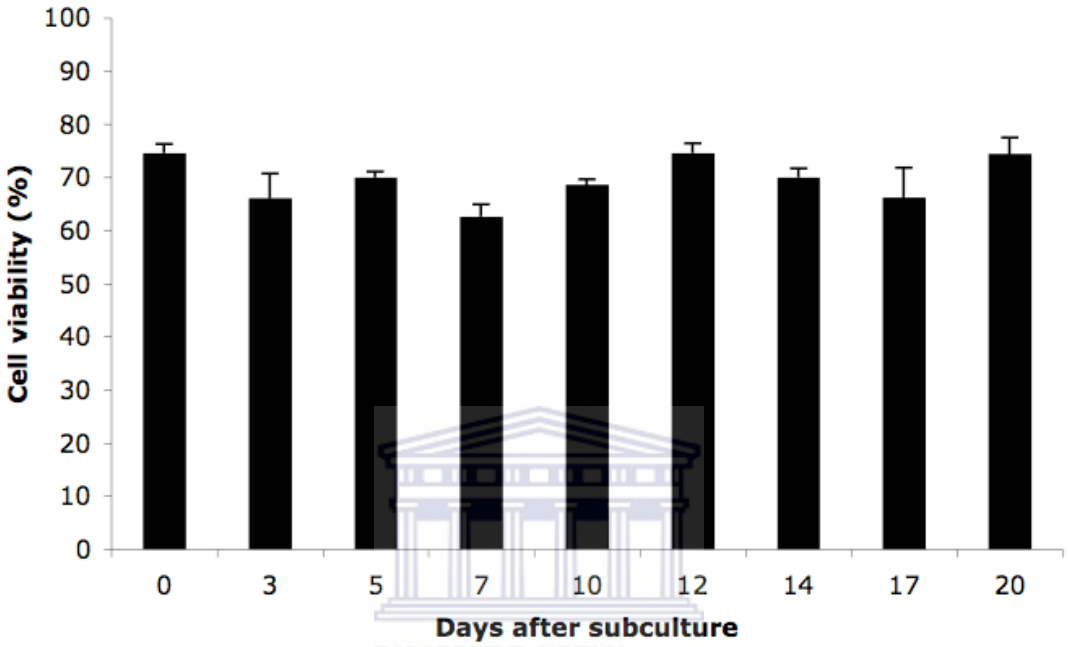


Figure 3.5: Cell viability of sorghum cell suspension cultures estimated using Evans blue over a 20-day period in culture**.**

Viability results at each time point are an average of three experiments from three independently established sorghum cell suspension culture lines. Each experiment had two replicates. Vertical bars indicate standard error.

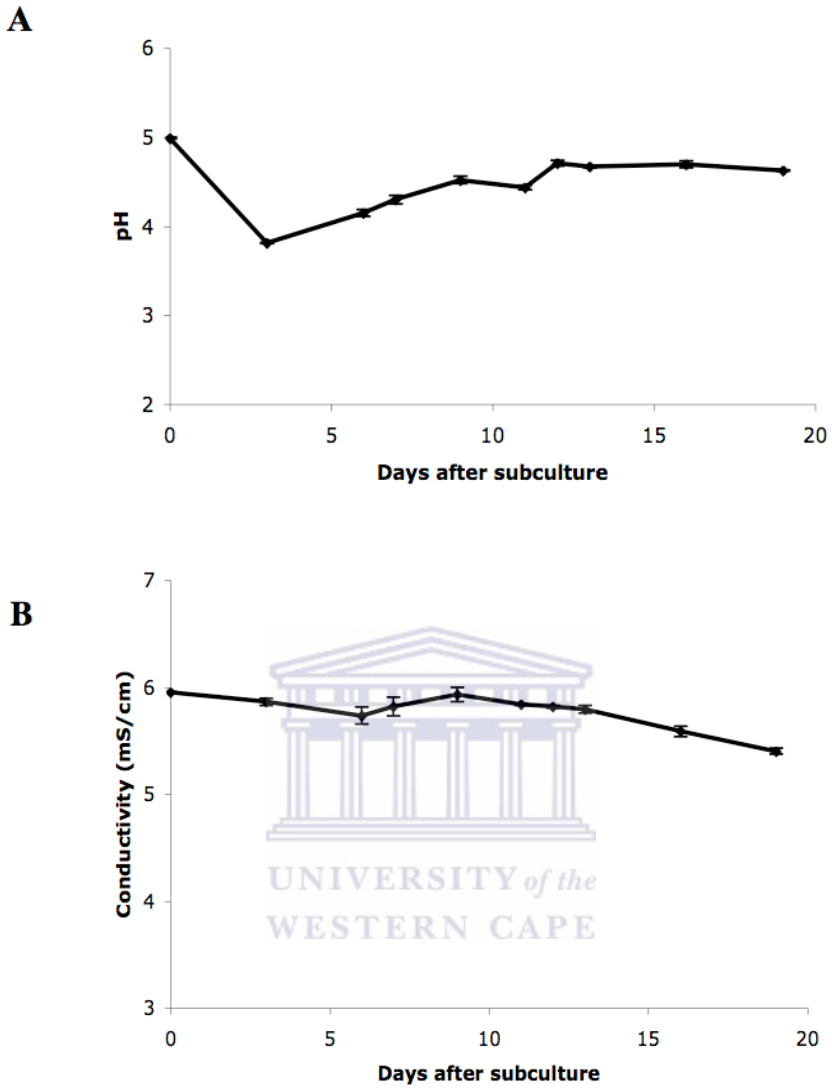


Figure 3.6: pH and conductivity measurements of the culture medium of sorghum cell suspensions over a 20-day period in culture.

(A) pH and (B) conductivity measurements. The measurements are an average of three independent experiments. Vertical bars indicate standard error.

(Figure 3.6). Figure 3.6A shows changes in pH of the culture medium over a 19-day period. After 3 days in culture, the pH of the medium decreased from pH 5 to pH 3.8. Thereafter, the pH gradually increased to pH 4.7 on day 12. After day 12, the pH was maintained at an average 4.7 until day 19. The conductivity of the cell suspension culture medium is shown in Figure 3.6B. The conductivity gradually decreased from about 6 mS/cm on the day of subculture to about 5.4 mS/cm on day 19 possibly indicating a gradual uptake of nutrients by from the medium by cells during growth.

3.4 The Two Major Proteomes of Sorghum Cell Suspension Cultures

The ultimate objective of this chapter was to establish a viable sorghum cell suspension culture system, which could be used in proteomics studies to study and gain an understanding of sorghum physiology in response to salinity and osmotic stresses. Established sorghum cell suspension cultures (Section 2.5.3) were harvested at day 10 post subculture. The sorghum suspension cells were separated from culture medium by filtering over 4 layers of Miracloth (Section 2.6.1.2). To prevent contamination of the cells with secreted proteins present in the culture medium, the filtered cells were washed once with distilled water. The cells were then extracted of total soluble protein (TSP) as described in Section 2.7.1. Proteins present in the culture medium (culture filtrate; CF) were precipitated out of solution using 80% acetone and resolubilised in urea buffer as described in Section 2.7.2. It was observed that not all precipitated CF proteins could completely re-solubilised in the urea buffer. Increasing the amount of solubilisation buffer did not necessarily increase the total amount of protein that was resolubilised. The

protein contents of all extracts were quantified using the Bradford assay as described in Section 2.8.

One-dimensional (1D) gel electrophoresis was carried out to check the quality of the extracts prior to 2D gel electrophoresis as described in Section 2.9. Figure 3.7 shows the 1D SDS-PAGE analysis of the sorghum cell suspension culture TSP and CF samples. Lane M shows the molecular weight marker. Lanes 1, 3 and 5 represents the TSP, while lanes 2, 4 and 6 represent the CF protein extracts. The gel illustrates that the TSP and CF display different protein expression profiles. The TSP is composed of numerous proteins covering a wider MW range of between 10-200 kDa. On the other hand, the CF is composed of a smaller number of proteins with molecular weight range of between 25-100 kDa.

3.4.1 Two-Dimensional (2D) Gel Electrophoresis of Sorghum Cell Suspension Culture TSP and CF Proteins

The TSP and CF protein extracts were separately subjected to 2D SDS-PAGE analysis (Section 2.10) on IPG strips of different lengths and pH ranges depending on the nature, composition and complexity of the protein mixture. The protein loads also differed between the two proteomes because of variations in protein complexity. The TSP profile was better resolved on large format 18 cm strips, pH range 4-7 while the CF was resolved on 7 cm IPG strips pH range 3-10 and pH range 4-7. Figure 3.8 shows the 2D profile of sorghum cell suspension culture TSP when separated on 18 cm IPG strips, pH range 4-7. Figure 3.8A shows TSP profile using a protein load of 400 µg while Figure 3.8B shows

the TSP profile using a protein load of 800 µg. Doubling TSP protein load on the 18 cm IPG strips from 400 µg to 800 µg resulted in an increased intensity of CBB stained protein spots without causing any spot streaking due to protein overloading. Overall, the sorghum cell suspension culture TSP is composed of numerous low abundant protein spots, which are evenly distributed within the pH range 4-7.

Due to the less complex nature of sorghum CF fractions, mini gels (7 cm) were used to resolve the CF proteome. Figure 3.9 illustrates the 2D protein expression profile of the CF proteome. Figure 3.9A shows the whole CF proteome resolved on 7 cm IPG strips, pH range 3-10 while Figure 3.9B shows the CF proteome on 7 cm IPG strips pH 4-7. Although the total CF fraction could be resolved on the broad range IPG strips, pH range 3-10 (Figure 3.9A), narrowing down the pH range to 4-7, resulted in an increase in spot resolution (Figure 3.9B). Protein spots, which were observed to be either closely migrating or co-migrating on IPG strips pH range 3-10, became relatively well separated on IPG strips pH 4-7. Protein spot clusters 1, 2 and 3 illustrates some of the gel regions that became better resolved when the proteome was separated on IPG strips, pH range 4-7 compared to when separated on pH 3-10. However, in some regions of the gel such as protein cluster 4, protein spots remained better resolved on IPG strips pH 3-10 than on pH 4-7. Since it was observed that the two IPG strips (pH range 3-10 and 4-7) had differential spot resolving advantages when used on the CF proteome, these two pH ranges were subsequently used for the mapping exercise of the secreted proteins (Chapter 5).

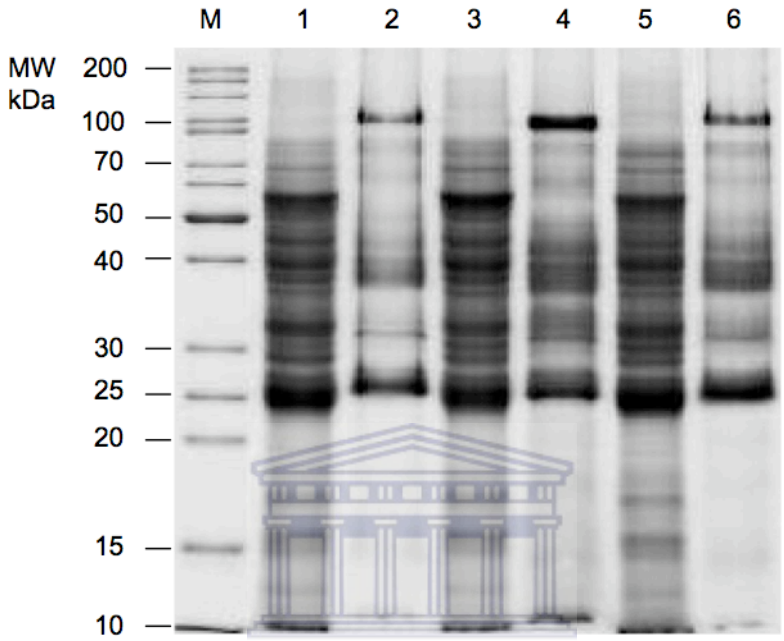


Figure 3.7: Comparative 1D SDS-PAGE analysis of sorghum cell suspension culture total soluble protein and culture filtrate proteomes.

Approximately 20 µg of protein was loaded onto a 12% SDS-PAGE and stained with CBB. Lane M is the molecular weight marker. Lanes 1, 3 and 5 are total soluble protein; and Lanes 2, 4 and 6 are culture filtrate samples from three independent experiments.

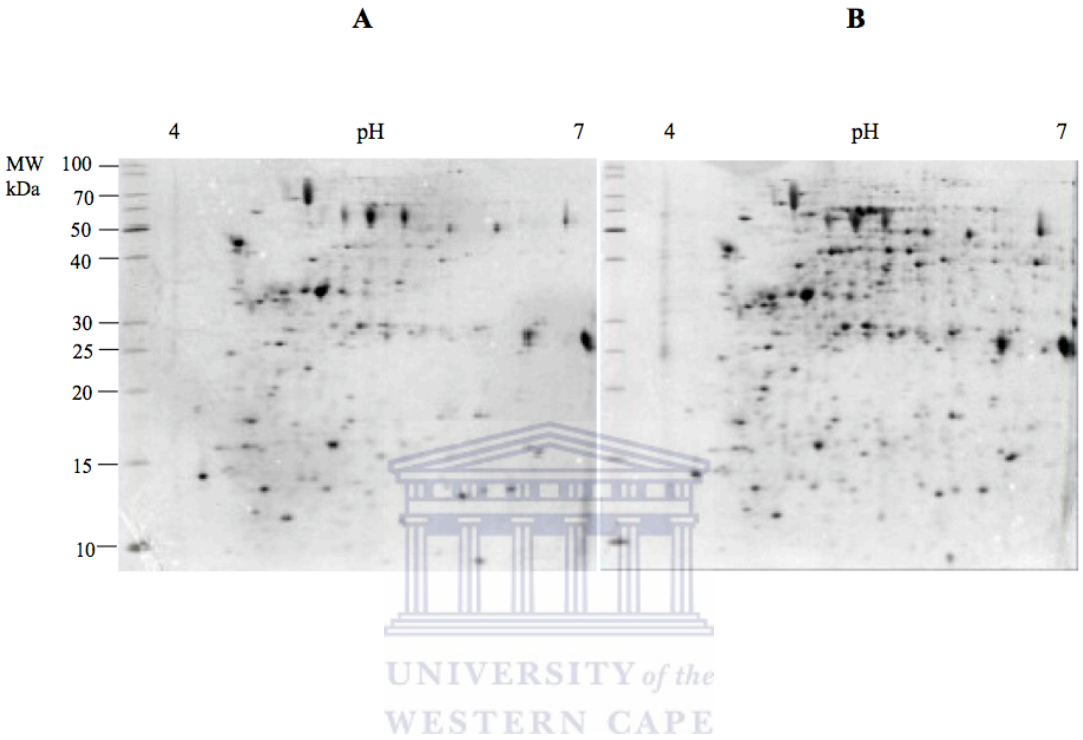


Figure 3.8: Two-dimensional gel electrophoresis of sorghum cell suspension culture total soluble protein.

Proteins were separated by IEF using 18 cm linear IPG strips, pH range 4-7 and by 12% SDS PAGE and stained with CBB. (A) 400 μg and (B) 800 μg of total soluble proteins were loaded onto the IPG strips.

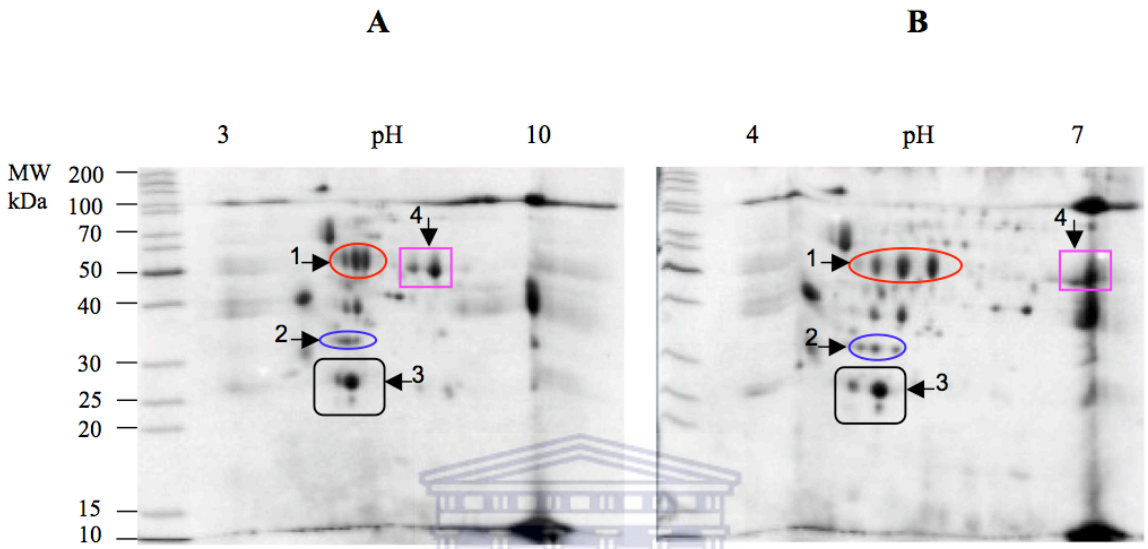


Figure 3.9: Two-dimensional gel electrophoresis of sorghum cell suspension culture filtrate proteins.

Culture filtrate proteins (100 µg) were separated by IEF on 7 cm linear IPG strips and by 12% SDS-PAGE and stained with CBB. (A) pH range 3-10 and (B) pH range 4-7. Highlighted protein clusters 1, 2 and 3 show examples of protein spots, which were closely migrating on IPG strip pH range 3-10 but whose resolution improved on IPG strips pH range 4-7. Protein cluster 4 illustrates a region where spots were better resolved on IPG strips pH range 4-7 rather than IPG strips pH range 3-10.

3.5 Discussion

Sorghum callus was successfully initiated from young shoot explant material cultured on MS medium supplemented with 2,4-D and NAA. All callus incubations were carried out under dark conditions for 4-5 weeks. Light-coloured friable callus masses consisting of soft easily separable cells were maintained in culture by transferring approximately 3-4 mm callus masses (Figure 3.1A) onto fresh media every 4-5 weeks. Suspension culture cells were also established from the friable callus masses (Figure 3.2).

The growth of sorghum cell suspension cultures was estimated using the PCV method over an 18-day period. The growth curve showed three distinct phases: a lag phase from day 0-8, an exponential phase from day 8-14 and a stationary phase from day 14-18 (Figure 3.3). However, as the cell density of the suspension cultures increased with time in culture, it became increasingly difficult to sample consistent and reproducible 1 ml aliquots of the cells. This was probably due to the cell clusters that formed in the suspension cultures. Although these cell clusters could be fairly distributed when agitated, the cells settled quickly in flasks resulting in inconsistencies in sampling. For this reason, PCV measurements where terminated at day 18. Microscopic analysis of the cultured cells also confirmed the presence of cell clusters and aggregates of various sizes (Figure 3.4). Individual cells were also observed under the light microscope having numerous mobile cytoplasmic vesicles, possibly indicating cytoplasmic streaming, which is associated with high metabolic activities in cells.

The viability of sorghum cell suspension cultures was estimated using the Evans blue staining method. It was observed that sorghum cell cultures maintained a viability of over 60% throughout the 20-day period (Figure 3.5). Days 0, 12 and 20 had the highest cell viability measurements of 76%. The pH measurements of the culture medium showed a drastic decline from pH 5 to pH 3.8 between day 0 and day 3 respectively (Figure 3.6). This characteristic drop in pH is known to occur in plant cell suspension cultures (Evans *et al.*, 2003) and had been reported between day 0 and day 4 in the growth cycle of *Psychotria carthagenensis* (Lopes *et al.*, 2000). This possibly indicates a time during which cells adapted to their new culture environment. During this period, cells differentially absorbed nutrients from the medium and/or secreted other substances into the culture medium (Beyl, 1999). For example, the drastic drop in pH in the early days of culture may indicate proton production during metabolic activities in the cell and their transportation into the ECM. In addition, cells may rapidly absorb nutrients such as phosphates, which act as buffering substances in the culture medium (Evans *et al.*, 2003). From day 3, the pH of the media gradually increased to pH 4.7 on day 12 and was maintained at that level until day 19.

Electrical conductivity (ECe), a measure of the amount of soluble salts in a solution (Flowers and Yeo, 1986; Hale and Orcutt, 1987) was also determined. As such, a decrease in conductivity measurements of the culture medium indicates a depletion of nutrients from the medium by the growing cells (Evans *et al.*, 2003). In sorghum cell suspension cultures, the conductivity of the medium gradually declined from the day of

subculture until day 19 (Figure 3.6B), possibly indicating gradual nutrient absorption from the medium by the growing cells for use in various metabolic processes.

The sorghum cell suspension culture system established here is an invaluable system, which provides for undifferentiated cell mass that can be subjected to various experimental conditions. Two proteomes, the TSP and CF were extracted from the cells and culture medium respectively. Both 1D (Figure 3.7) and 2D SDS-PAGE of the TSP (Figure 3.8) and CF (Figure 3.9) protein extracts showed that these two proteomes displayed different protein expression profiles. Due to differences in the nature, composition and complexity of the TSP and CF proteomes, IPG strips of different lengths and pH ranges as well as different protein loads were used so as to obtain well-resolved 2D gel profiles. The TSP, which consisted of numerous protein spots was better resolved on large format, 18 cm strips, pH range of 4-7 (Figure 3.8). The less complex CF protein extract was well resolved on mini gels (7cm) without any spot overlaps. Furthermore, it was observed that IPG strips of pH range 3-10 and 4- 7 had differential spot resolving advantages in separating the CF extracts (Figure 3.9A and B). As a result, both pH ranges were used in subsequent 2D SDS-PAGE analysis of the CF as described in Chapters 5 and 7.

The observed differences in 2D proteome expression profiles of the fractions (TSP and CF) reinforces the fact that different proteomes, even if from the same plant system, are dynamic and heterogeneous, being composed of a complex mixture of proteins with different chemical and functional properties. The suspension culture system here

described provides for two sets of proteomes, which although different in complexity play distinct but equally vital roles in maintaining biological processes in plant cells. The mapping and analysis of stress responsive proteins in these two compartments of the suspension culture system will provide some insight into the biological processes that occur in the cytoplasm as well as in the ECM of sorghum cells. Out of these two proteomes, part of this study (Chapters 5 and 7) will focus mainly on the CF rather than the TSP because of the less complex nature of the CF (in terms of overall protein numbers). The CF proteins (secretome) were identified by a combination of mass spectrometry and genomic database searches (Chapter 5) thus providing the first sorghum secretome map that landmarks proteins expressed in this compartment. Furthermore, the CF was also used in both salt and sorbitol stress treatment experiments in order to study protein changes that occur in response to these abiotic stresses (Chapter 7). There is no doubt therefore, that the culture system described here would provide invaluable experimental material for use in descriptive and expressional proteomics in response to both biotic and abiotic stress responses.

CHAPTER 4

The Establishment of 2DE Proteome Reference Maps for Sorghum Soluble Proteins

4.1 Introduction

Over the past decade, 2DE and MS based proteomic approaches have been applied in plant sciences to study various biological processes during plant growth and development. Comparative studies on protein expression within and between species, at specific developmental stages and in response to biotic and/or abiotic stresses have also been investigated. In proteomics studies, both cell suspension cultures and whole plant systems are used as experimental materials. Although suspension cultures are useful sources of homogenous material that can be easily manipulated (as reviewed in Section 3.1), they remain an artificial system (Isaacson and Rose, 2006). In contrast, whole plant systems allow for the integration of tissue specific proteome profiling and hence in depth analysis of biological processes simultaneously at work in different locations of the plant. Therefore, comparative studies using both types of experimental systems would provide comprehensive protein expression profiles of a given species under defined conditions.

Several proteome reference maps have been developed for cell suspension cultures and different tissues from a range of agriculturally important plants (Porubleva *et al.*, 2001; Koller *et al.*, 2002; Hochholdinger *et al.*, 2004; Mechin *et al.*, 2004; Albertin *et al.*, 2009). Proteome maps give information about the nature (MW and pI) and abundance of expressed proteins. For cell suspension cultures, these maps provide a generalised view

of expressed proteins and thus gene function in the plant species of origin. In contrast, tissue specific proteome maps give specialised protein expression profiles depending on the major biological functions of the tissue under investigation. Some studies have generated proteome maps for single tissues such as the leaf (Porubleva *et al.*, 2001), endosperm (Mechin *et al.*, 2004) and root (Hochholdinger *et al.*, 2004) of maize (*Zea mays*). Others have conducted comparative proteome mapping of different tissues within a single plant species. For example, barrel medic (*Medicago truncatula*) reference maps have been established for leaves, stems, roots, flowers and seed pods (Watson *et al.*, 2003). Rice (*Oryza sativa*) proteome reference maps have also been developed for leaves, roots and seeds (Koller *et al.*, 2002). More recently, Albertin *et al*. (2009) published a comparative proteome study of leaf, stem and root tissues of rapeseed (*Brassica napus*). When fully annotated, these maps provide invaluable tools for land marking expressed proteins under specific physiological states as well as in response to stress conditions (Thelen, 2007). Furthermore, inter-tissue proteome comparisons are possible. These comparative studies not only provide insight into tissue specific proteome expression but also common house keeping and/or ubiquitous proteins and hence common metabolic pathways in a biological system (Watson *et al.*, 2003; Albertin *et al.*, 2009).

Despite the economic potential of sorghum in the semi-arid regions of the world and the promising technique of proteomic approaches in understanding plant biological systems, to our knowledge, no global proteomics studies on sorghum have been reported to date. Therefore, the objectives of this chapter are to (i) optimise the extraction of total soluble proteins of leaf, sheath and root tissues of two seed varieties, AS6 and MN1618, (ii) to

establish the proteome profiles of the tissues by 2DE, (iii) to work towards the establishment of a sorghum leaf proteome reference map by a combination of 2DE and mass spectrometry (MALDI-TOF MS and MALDI-TOF-TOF MS) and (iv) to compare protein expression profiles of leaf tissues between AS6 and MN1618 sorghum varieties. The two sorghum varieties AS6 and MN1618 were chosen on the basis of their differences in salt tolerance as observed in the experiment on screening of sorghum varieties for salt tolerance (Chapter 6; Section 6.2).

4.2 Protein Extraction from Different Tissues of Sorghum Seedlings

Two sorghum varieties, AS6 and MN1618, were used as sources of plant material. The sorghum seeds were surface decontaminated and germinated on MS media as described in Sections 2.5.1 and 2.6.2.2 respectively. At 14 days post plating, sorghum seedlings were harvested. Three different plant tissues (leaf, sheath and roots) were carefully excised from the seedlings. To minimise protein degradation, the plant material was immediately flash frozen in liquid nitrogen and stored at -20°C until needed for protein extraction procedures. The different tissues were separately extracted of composite proteins using the TCA/acetone method as described in Section 2.7.3. Leaf and sheath protein extracts were prepared from an average of 10, 14-day old sorghum seedlings. To bulk up root material for protein extraction, root protein extracts were prepared from at least 20, 14- day old sorghum seedlings. Extracted proteins were quantified using the Bradford assay as described in Section 2.8.

4.3 One Dimensional Protein Profiles of the Sorghum Leaf, Sheath and Root Tissues

One-dimensional (1D) gel electrophoresis (Section 2.9) was carried out to evaluate the quality and loading quantities of the protein extracts prior to 2D gel electrophoresis. Figure 4.1 shows CBB stained 1D profiles of leaf, sheath and root proteomes of the two sorghum varieties. Figure 4.1 A: AS 6 leaf; B: MN 1618 leaf; C: AS 6 sheath; D: MN 1618 sheath; E: AS6 and MN1618 root proteomes. Lanes M shows the molecular weight markers. Lanes 1-4 shows protein profiles from four independent biological replicate extractions for the leaf and sheath tissues. Each lane was loaded with approximately 10 µg of total protein of the respective tissue extract. It was observed that the quality of the leaf, sheath and root protein extracts were good, showing no visible signs of streaking and protein degradations. The biological replicates (Lane 1-4) within an experiment (Figure 4.1A-D) also showed high similarity in terms of protein expression, abundance and banding patterns. This suggests that protein preparation was reproducible between independent extractions. When comparing protein loading across the four biological replicates (Lanes 1-4) within the leaf and sheath experiments for both sorghum varieties, (Figure 4.1A-D), it was observed that protein load was relatively uniform. Figure 4.1E illustrates 1D protein profiles of root tissue. Lanes R1 and R2 represents root tissue from AS6 and MN 1618 sorghum varieties respectively. Both lanes were loaded with 10 µg of total protein. When compared to the leaf and sheath proteomes (Figure 4.1A-D), the root proteome profiles of both seed types (Figure 4.1E) were composed of more low abundant proteomes. However, protein extracts from all three tissues, leaf, sheath and root covered the MW range of between 10 and 110 kDa.

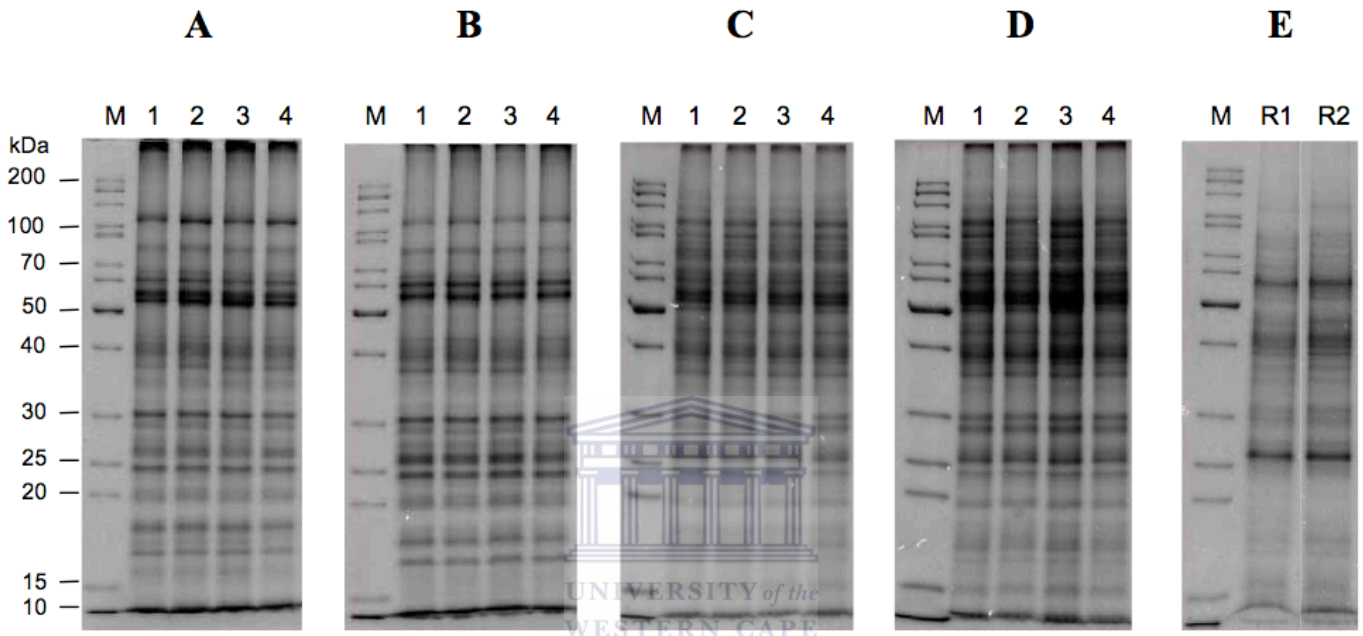


Figure 4.1: One-dimensional SDS-PAGE analysis of soluble protein of leaf, sheath and root tissues of AS6 and MN1618 sorghum varieties.

Approximately 10 µg of soluble protein of each tissue was loaded onto 12% SDS-PAGE gels. (A) AS6 leaf, (B) MN1618 leaf, (C) AS6 sheath, (D) MN1618 sheath and (E) AS6 and MN1618 roots samples. Lane M is the molecular weight marker. Lanes 1-4 represent independent biological replicate protein extracts for the leaf and sheath samples (A-D). Lanes R1 and R2 represent root samples from AS6 and MN1618 sorghum varieties respectively.

4.4 Two Dimensional Protein Profiles of the Sorghum Leaf, Sheath and Root Tissues

Three of the four biological replicates of protein extracts from leaf and sheath tissues (Lanes 1-4, Figure 4.1A-D) were randomly selected for further separation using 2DE analysis as described in Section 2.10. Due to the limitations in protein quantities for both AS6 and MN 1618 root extracts, only one composite proteome gel was prepared for each of the two sorghum varieties. Protein loading on the 2D gels also varied between tissues depending on the overall complexity and abundance of proteins per tissue type. For sheath samples, 150 µg of total protein was used to load the IPG strips, while for both the leaf and root samples, a protein load of 100 µg was used. For all tissues, mini, 7 cm gels using IPG strips of pH range 4-7 were used. The pH range 4-7 was used because most of the soluble proteins fell within that pH range. It was observed that spot resolution and abundance between three biological replicate gels of each of the leaf and sheath extracts was uniform. This indicates that 2DE was reproducible between different samples within an experiment. Figures 4.2 and 4.3 illustrate representative 2D gels of leaf, sheath and root tissues of AS6 and MN1618 sorghum varieties respectively. Figure 4.2 A, B, C, shows the leaf, sheath and root proteomes of AS 6 sorghum variety. Figure 4.3 A, B, C, represent the leaf, sheath and root proteomes of MN1618 sorghum variety. In general, it was observed that the sheath shared common spots with both the leaf and root. However, protein expression between the leaf and root was remarkably different. As observed on the 1D gels (Figure 4.1), the 2D proteome profiles of all three tissues were shown to cover the MW range between 10-110 kDa

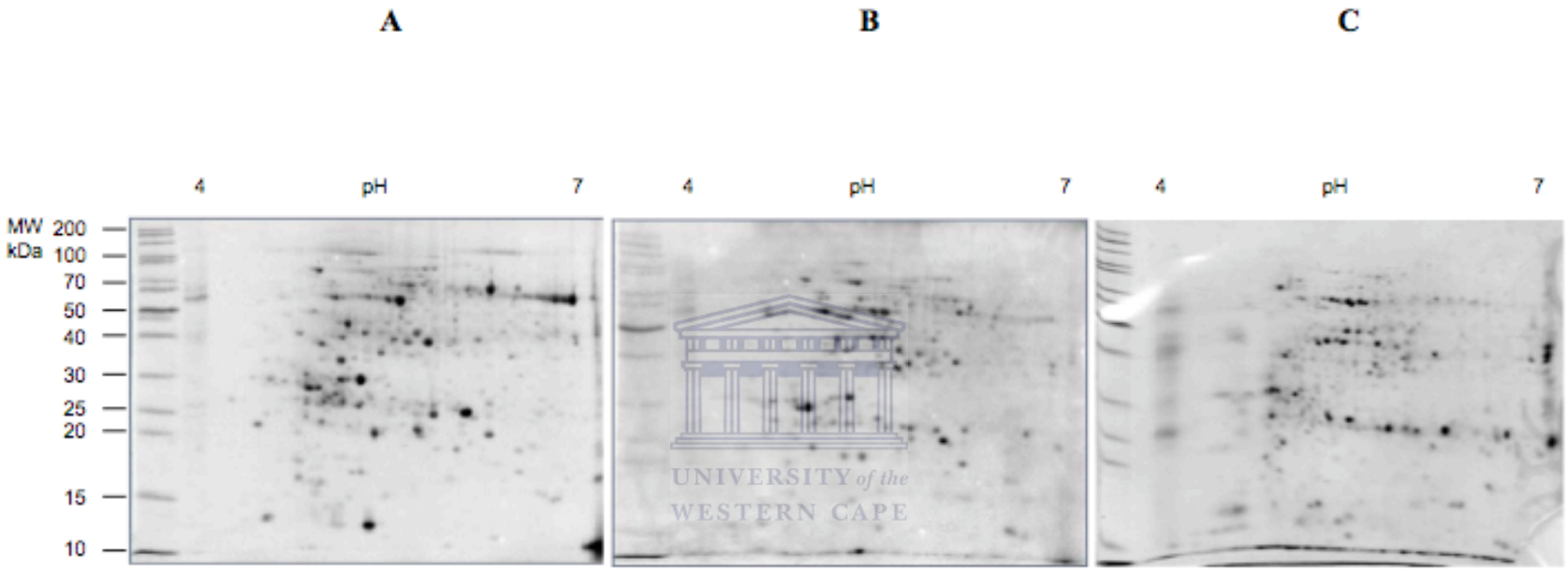


Figure 4.2: Representative CBB stained 2D gels of leaf, sheath and root tissues of AS6 sorghum variety.

Protein extracts were separated in the first dimension by IEF using 7 cm linear IPG strips, pH range 4-7; and 12 % SDS PAGE gels in the second dimension. (A) Leaf (100 µg); (B) sheath (150 µg) and (C) Root (100 µg).

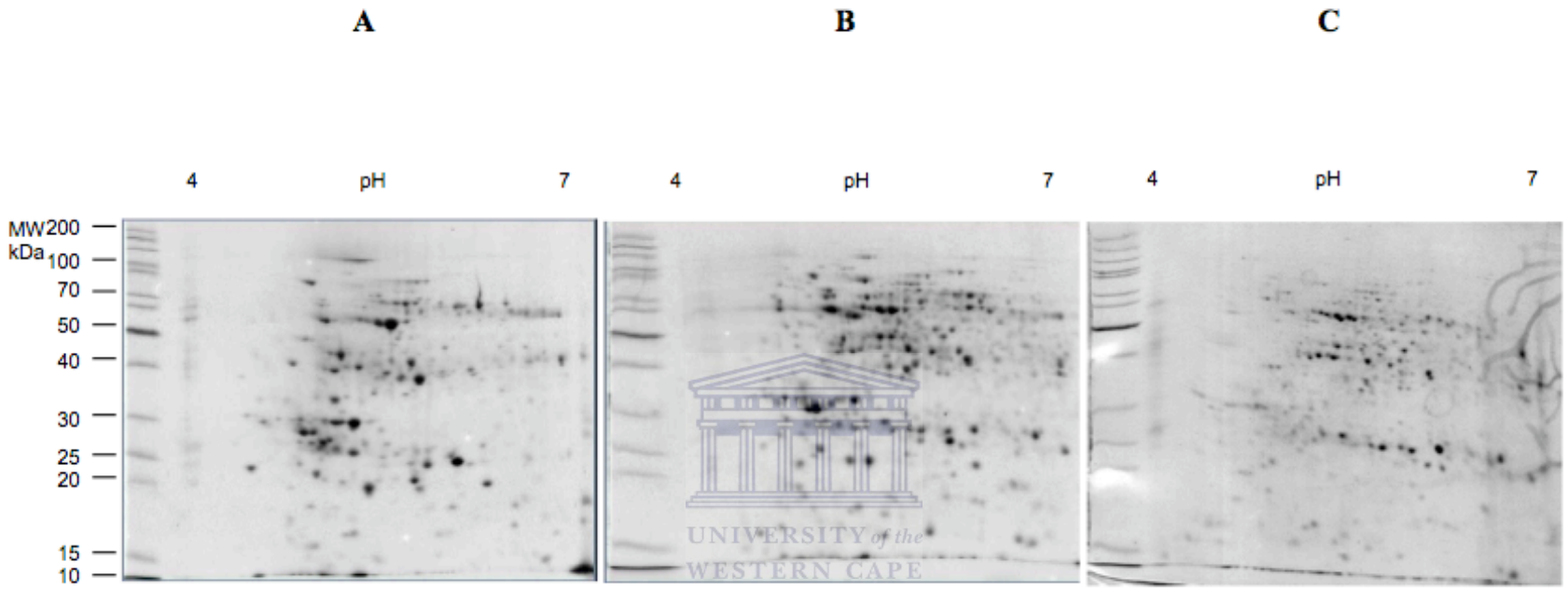


Figure 4.3: Representative CBB stained 2D gels of leaf, sheath and root tissues of MN1618 sorghum variety.

Protein extracts were separated in the first dimension by IEF using 7 cm linear IPG strips, pH range 4-7; and 12 % SDS PAGE gels in the second dimension. (A) Leaf (100 µg); (B) sheath (150 µg) and (C) Root (100 µg).

4.5 Establishment of a Sorghum Leaf Proteome Map

One of the main objectives of this Chapter was to work towards the establishment of a sorghum leaf proteome map. To achieve this goal, MN1618 leaf proteome was used in the mapping exercise. Of the 171 reproducible CBB stainable leaf protein spots observed amongst three biological replicate extractions of MN1618 (Section 4.6.1), a total of 40 well resolved protein spots of varying degrees of abundance and MW were selected for identification using a combination of MS and database searches. The selected protein spots (spots 1-40; Figure 4.4) were picked from CBB stained gels using the ExQuest^{TM} spot cutter (BIO-RAD). Protein contained in the picked gel plugs was trypsinised and peptide digests were analysed using MALDI-TOF MS (Section 2.14.1) and MALDI-TOF-TOF MS (Section 2.14.2). The resultant peptide mass fingerprints and well as fragmented ion spectra generated for each proteins by MALDI-TOF and MALDI-TOF-TOF MS respectively were used in sequence database searching to find their protein identities. Since the sorghum genome sequence is not yet publicly available in full, database searches were carried out against all entries in the NCBI database using MASCOT version 2.2. Only spot identities with the highest MOWSE score equal to or greater than 76 ($p<0.05$) were regarded as significant protein matches.

Mass spectrometry using a combination of MALDI-TOF and MALDI-TOF-TOF MS, and genomic database searches of the trypsinised 40 protein spots resulted in the positive identification of 28 protein spots (70% successful identification of the total number of picked spots for MS identification). The remaining 12 spots did not match any protein identity with the acceptable MOWSE score values.

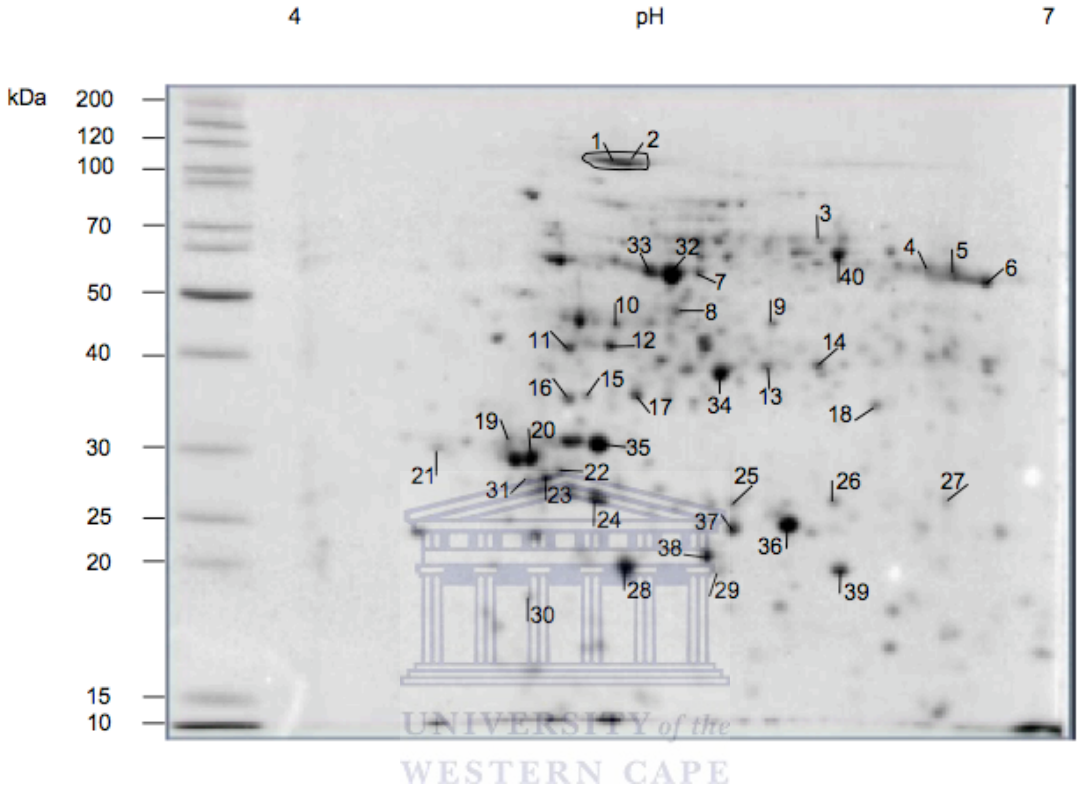


Figure 4.4: A representative CBB stained 2D gel of the sorghum MN1618 leaf proteome showing spots picked for mass spectrometry analysis.

About 100 µg of the leaf protein extract was separated in the first dimension by IEF using 7 cm linear IPG strips, pH range 4-7; and 12 % SDS PAGE gels in the second dimension Numbered spots (1-40) were selected for identification using a combination of MALDI-TOF MS, MALDI-TOF-TOF MS and database searches.

Table 4.1 gives a summary of the identified protein spots, their best match identities, plant species of origin and corresponding NCBI accession numbers. Both the theoretical and experimental molecular weight (MW)/isoelectric points (pIs) of the proteins are also shown in Table 4.1 to give a comparative analysis of the two ranges of data. The positively identified protein spots matched the identities of proteins from a wide range of plants possibly showing the high degree of conserved genes and gene products within higher plants. Furthermore, these identified spots were of varying degrees of protein abundance as indicated on CBB stained gels. On the other hand, unidentified proteins (spots 3; 4; 15; 16; 19; 22; 23; 25; 27; 30; 31 and 38; Figure 4.4; Table 4.1) were of varying degrees of abundance, MW and pI values.

4.5.1 Proteins Identified in Multiple Spots

It was observed that seven classes of proteins were represented in multiple spots on the 2D gels (Figure 4.4; Table 4.1). These are pyruvate phosphate dikinase (spots 1 and 2); RuBisCo (spots 5 and 6); malate dehydrogenase (spots 10 and 14); ferrodoxin-NADP oxidoreductase (spots 17 and 18); adenosine diphosphate glucose pyrophosphate (spot 29 and 39); chloroplastic ATP synthase proteins (spots 7; 32; 33; 13; 40) and hydroxynitrile lyase (spots 20 and 28). The multiple protein-spotting pattern observed in this sorghum leaf proteome can be loosely classified into four groups. Group one consists of protein spots with the same NCBI accession number, migrating at the same MW but different pIs. Examples of proteins in this group include (i) pyruvate phosphate dikinase (spots 1 and 2; accession AAP23874; Table 4.1) which migrated at the same MW of approximately 110 kDa but different pIs of 5.2 and 5.3 respectively; and (ii) adenosine

Table 4.1: List of sorghum leaf proteins identified by a combination of MALDI-TOF MS, MALDI-TOF-TOF MS and database searching.

Spot[a)]	Best Protein Match	Plant Species	Accession No. [b)]	MOWSE score [c)]	Theo. MW/pI [d)]	Exp. MW/pI [e)]	Location [f)]
	Carbohydrate Metabolism						
1	Pyruvate phosphate dikinase	*Sorghum bicolor*	AAP23874	291	102/5.7	110/5 .2	chloroplast
2	Pyruvate phosphate dikinase	*Sorghum bicolor*	AAP23874	201	102/5.7	110/5.3	chloroplast
5	RuBisCO, large subunit	*Stachys byzantina*	AAM33283	120	50/6.9	53/6.5	chloroplast
6	RuBisCO, large subunit	*Anemopsis californica*	AAF14707	238	51/6.6	51/6.6	chloroplast
9	Cytosolic 3-phosphoglycerate kinase	*Zea mays*	AAO32644	250	32/4.7	45/5.8	cytoplasm
10	Chloroplastic NADP-dependent malate dehydrogenase	*Sorghum bicolor*	7MDH_D	121	41/4.9	45/5.2	chloroplast
14	cytoplasmic NAD-dependent malate dehydrogenase	*Zea mays*	AAB64290	151	36/6.0	39/6.0	cytoplasm
11	Sedoheptulose-1,7-biphosphate precursor	*Oryza sativa*	AA022559	143	42/5.9	42/5.0	chloroplast

12	Phosphoribulokinase, chloroplast precursor	*Mesembryanthemum crystallium*	P27774	159	44/6.4	42/5.2	chloroplast
17	Putative ferrodoxin- NADP oxidoreductase	*Oryza sativa*	NP_910234	261	40/8.7	36/5.3	chloroplast
18	Ferrodoxin- NADP oxidoreductase	*Zea mays*	1GAW_ B	108	39/7.3	35/6.2	chloroplast
29	Adenosine diphosphate glucose pyrophosphatase	*Triticum asetivum*	CAC85479	137	22/6.1	20/5.6	apoplast
39	Adenosine diphosphate glucose pyrophosphatase	*Triticum asetivum*	CAC85479	143	22/6.1	20/6.1	apoplast
34	Fructose 1,6-biphosphate aldolase precursor	*Avena sativa*	AAF74202	269	42/9.2	39/5.6	chloroplast
35	Oxygen evolving enhancer protein 1 precursor	*Bruguiera gymnorrhia*	BAA96365	268	35/6.7	30/5.1	chloroplast
36	Photosystem II oxygen-evolving complex protein 2 (fragment)	*Arabidopsis thaliana*	PA0013	112	1.4/10.2	39/5.9	chloroplast
37	Chlorophyll a/b-binding protein type III precursor	*Lycopersicon esculentum*	1609235A	114	29/9.1	38/5.6	chloroplast

	Proton Transport						
7	ATP synthase subunit beta, chloroplastic	*Sorghum bicolor*	1711264A	314	54/5.1	55/5.5	chloroplast
32	ATP synthase subunit beta, chloroplastic	*Sorghum bicolor*	1711264A	650	54/5.1	55/5.4	chloroplast
33	ATP synthase subunit beta, chloroplastic	*Sorghum bicolor*	1711264A	213	54/5.1	55/5.3	chloroplast
13	ATP synthase gamma chain 1, chloroplast	*Oryza sativa*	XP_478377	118	40/8.5	39/5.8	chloroplast
40	ATP synthase CF1 alpha chain	*Oryza sativa*	AAP54723	455	56/6.1	57/6.0	chloroplast
	Protein Synthesis						
8	Translational elongation factor Tu	*Oryza sativa*	XP_466527	277	50/6.6	47/5.4	chloroplast
21	Nucleic acid- binding protein- maize	*Zea mays*		82	33/nd	30/4.5	n.d
	Hydrolytic enzymes						
20	Hydroxynitrile lyase	*Sorghum bicolor*	1GXS_C	260	30/4.5	30/4.9	cytoplasm
28	Hydroxynitrile lyase	*Sorghum bicolor*	1GXS_C	285	18/4.5	20/5.1	cytoplasm

	Nucleic Acid Metabolism						
24	Putative adenylate kinase, chloroplast	*Oryza sativa*	XP_47972 1	126	32/8.1	27/5.1	chloroplast
	Detoxifying Enzymes						
26	Glutathione transferase (EC 2.5.1.18) I	*Zea mays*	P12653	108	24/7.5	26/6.0	cytoplasm
	Spots with no significant matches 3; 4; 15; 16; 19; 22; 23; 25; 27; 30; 31; 38.						

[a] Spot number as indicated on the 2D gel images (Figure 4.4).

[b] Accession number in the National Center for Biotechnology Information (NCBI) database.

[c] Combined MOWSE score for MALDI-TOF and MALDI-TOF-TOF MS.

[d] Theoretical MW and pI were calculated on the mature peptide using the programme on http://isoelectric.ovh.org.

[e] Experimental MW and pI were estimated from the 2D gels shown in Figure 4.4.

[f] Subcellular location of the proteins as predited by TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP/; Predotar version 1.03 (http://urgi.versailles.inra.fr/predotar/predotar.html and literature sources.

nd: not determined.

diphosphate glucose pyrophosphate spots 29 and 39 (accession CAC85479) both migrated at the 20 kDa but different pIs of 5.6 and 6.1 respectively. These protein spots are most likely to represent post translationally modified peptides and thus isoforms of each enzyme.

Group two consists of proteins with the same accession number, but different MW as well as pIs. The hydroxynitrile lyase protein (spots 20 and 28; accession 1GXS_C; Table 4.1) could be classified in this group. These spots migrated at 30 and 20 kDa and had pI values of 4.9 and 5.1 respectively. According to Wajant and Mundry, *S. bicolor* hydroxynitrile lyase is composed of two different subunits α and β with molecular sizes of 33 and 22 kDa respectively (Wajant and Mundry, 1993). These subunits occur as αβ dimers (MW= 55 kDa) while the active enzyme is a heterotetramer ($\alpha_2\beta_2$; MW range 95-105 kDa). Therefore, the two hydroxynitrile lyase spots observed in this study could probably represent α and β subunits of the enzyme. Hydroxynitrile lyases are also documented to have pI ranges of between 4 and 5.5 (Poulton, 1990), a characteristic that is consistent with the results obtained in this study (Table 4.1).

Group three consists of spots that match the same protein name but of different accessions, MWs and pIs. Examples of Group three proteins include malate dehydrogenase (spots 10 and 14) and ferrodoxin-NADP oxidoreductase (spots 17 and 18). These peptides are most likely to represent isoforms from a multigene family. For example, the malate dehydrogenase protein spots represent isoforms of different subcellular locations, co-enzyme specifictity and therefore are likely to be involved in

different metabolic functions in the cell. Spot 10 represents a chloroplastic NADP-dependent malate dehydrogenase while spot 14 represent that of a cytoplamic specific NAD-dependent malate dehydrogenase (Table 4.1).

Group four consists of spots with a combination of parameters from the other three groups but are known to be part of protein-protein complexes. Examples are (i) the chloroplastic ATP synthase proteins (spots 7; 32; 33; 13 and 40; Table 4.1). These spots corresponded to the alpha (spot 40), beta (spots 7; 32 and 33) and gamma (spot 13) subunits of the chloroplastic ATP synthase complex; and (ii) RuBisCo large subunits (spots 5 and 6; Table 4.1). The identification of component subunits of protein complexes has also been reported in previous studies. Albertin and co-workers identified six spots corresponding to the alpha, beta, delta and gamma subunits of the chloroplastic ATP synthase complex in *B. napus* stem tissue (Albertin *et al.*, 2009). In the same study, both the small and large subunits of RuBisCo were identified in stem tissue, while alpha and beta subunits of tubulin were detected in both the stem and root tissues.

4.5.2 Putative Functional Classification of the Identified Leaf Proteins

After the identification of expressed proteins in a particular tissue, it is important to establish their functions. Knowledge on protein function would lead to the identification of cellular processes at work. As such, the main metabolic pathways and biological functions of the tissue under study can be elucidated. The putative functions of the identified sorghum leaf proteins (Table 4.1) were assessed by a combination of similarity

searches on the Arabidopsis database (http://www.arabidopsis.org), Universal Protein Sequence database (http://www.uniprot.org) and literature sources.

Using the bioinformatics tools stated above and literature sources, all the 28 positively identified protein spots (Table 4.1) were successfully classified into six broad functional categories; carbohydrate metabolism, proton transport, protein synthesis, hydrolytic enzymes, nucleotide metabolism and detoxifying enzymes. The functional categories and proteins in each respective class are listed in Table 4.1 while a graphical representation of the distribution of proteins in each class is illustrated in Figure 4.5. A description of proteins and their functions in each of the functional categories is given below.

4.5.2.1 Carbohydrate Metabolism

The majority of the identified sorghum leaf proteins (60.7%, Figure 4.5) had a function in carbohydrate metabolism. This observation is consistent with results obtained in other leaf proteomics studies of maize (Porubleva *et al.*, 2001), barrel medic (Watson *et al.*, 2003), rice (Nozu *et al.*, 2006) and pea (Schiltz *et al.*, 2004). In this functional category, many biological processes and metabolic pathways including the light reaction of photosynthesis, the Calvin cycle, starch biosynthesis, glycolysis and malate/oxaloacetate shuttle system among others were represented. The high representation of these metabolic pathways in the leaf tissue of seedlings demonstrates their important contribution in the growth and development of plants as well as the primary function of plant leaves. A detailed description of the putative functions of proteins in each subclass of carbohydrate metabolism is give below.

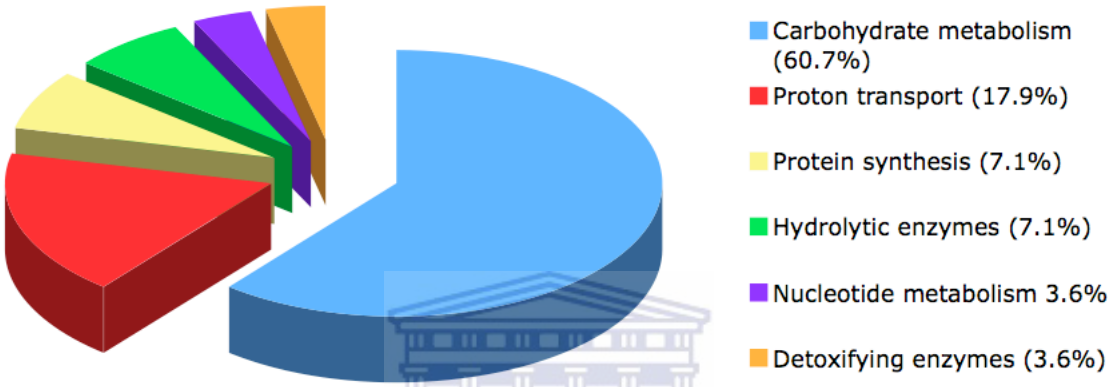


Figure 4.5: Functional distribution of the MALDI-TOF and MALDI-TOF-TOF MS identified sorghum leaf proteins.

Numbers indicated in brackets represent the proportion of proteins within each functional category expressed as a percentage of the 28 MALDI-TOF MS positively identified protein spots.

4.5.2.1.1 The Light Reactions of Photosynthesis

Five proteins that are directly involved in light reactions of photosynthesis were identified (Figure 4.4; Table 4.1). These include the chlorophyll a/b binding protein type II precursor (spot 37), the oxygen evolving enhancer protein 1 precursor (spot 35), the photosystem II oxygen evolving complex protein 2 (spot 36) and ferrodoxin NADP-oxidoreductase (spots 17 and 18).

Chlorophyll binding proteins are synthesised as precursor molecules in the cytoplasm and imported into the chloroplast where they are inserted in the thylakoid membranes (Bassi *et al.*, 1997). They have several functions including light harvesting, energy dissipation and pigment storage. As components of the light harvesting complexes in plants, the primary functions of chlorophyll a/b binding proteins is the absorption of light and the transfer of the excitation energy to the photochemical reaction centres (Bassi *et al.*, 1997; Ganeteg *et al.*, 2001). In some cases, plants are exposed to higher light intensities than used in photosynthesis. Therefore, to prevent photoinhibition and damage to the photosynthetic machinery, excess energy is then dissipated by these light-harvesting proteins. In addition, chlorophyll a/b binding proteins are believed to have a function in pigment storage (Bassi *et al.*, 1997).

The light energy absorbed by chlorophyll a/b binding proteins (Spot 37; Table 4.1) is used to drive the light dependent oxidation of water, releasing molecular oxygen. Hydrogen ions are also released in the process, creating a transmembrane chemiosmotic potential that is utilised by ATP synthases during ATP synthesis (Section 4.5.2.2).

Photolysis of water occurs in the oxygen-evolving complex (OEC) of Photosystem II (PS II) reaction centres (McEvoy and Brudvig, 2006; Sproviero *et al.*, 2007; Raymond and Blankenship, 2008). The OEC is composed of four manganese ions, calcium and possibly chloride ions, which are bound to extrinsic proteins (McEvoy and Brudvig, 2006). Two of these extrinsic OEC proteins were identified in this study; the Photosystem II oxygen evolving complex protein 2 spot 36; Table 4.1) and an oxygen evolving enhancer protein precursor (spot 35; Table 4.1). Photosystem II OEC proteins are involved in retaining calcium and chloride ions, two inorganic cofactors for the water-splitting reaction (Ifuku *et al.*, 2005). The oxygen evolving enhancer protein is believed to have a dual function; (i) optimising the manganese cluster during photolysis and (ii) protecting the reaction centre proteins from damage by oxygen radical formed in light (Heide *et al.*, 2004).

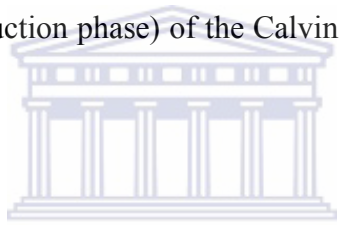
Two isoforms of ferrodoxin-NADP oxidoreductases (spots 17 and 18; Table 4.1), with MW of approximately 36 and 35 kDa respectively were identified in the sorghum leaf proteome. The MW range of these spots is consistent with the average 35 kDa MW of the plant reductases (Arakaki *et al.*, 1997). In plants, this enzyme exists in two different forms; photosynthetic and heterotrophic forms, which are encoded for by different genes and may be associated with different metabolic pathways (Gummadova *et al.*, 2007). In this study, only the photosynthetic isoforms were identified. Ferrodoxin-NADP oxidoreductases catalyse the reversible electron transfer between one electron carrier systems (ferrodoxin) and the two-electron carrying NADP(H) (Thomas *et al.*, 2006). In chloroplasts, the main physiological function of this enzyme is to catalyse the final step

of the photosynthetic electron transport, providing NADPH, which is then utilised in the carbon fixation step of the Calvin cycle (Arakaki *et al.*, 1997).

4.5.2.1.2 The Calvin Cycle

The Calvin cycle (also termed the reductive pentose phosphate pathway) is a metabolic pathway that produced pentose sugars (Heldt, 1997). The cycle is characterised by three phases; the carboxylation, reduction and regeneration phases. Some proteins involved in all the three phases were identified in this study. Two spots (5 and 6; Figure 4.4; Table 4.1) representing the RuBisCO large subunits were identified. RuBisCO is a multimeric enzyme with two subunits; large (50-55 kDa) and small (12-18 kDa; Andersson and Backlund, 2008). In this study, the RuBisCO proteins were observed as forming a train of spots on the basic side of the gel (pIs 6.5 and 6.6) with MW of approximately 53 and 51 kDa respectively. This observation is consistent with results from other proteomic studies. In the pea leaf proteome, Schiltz *et al.* (2004) observed that RuBisCO proteins formed an abundant train of spots between pH 6-7, at approximately 50 kDa. Similarly in the maize leaf proteome, several RuBisCO large subunits were also identified between pH 6-7 and MW of approximately 50-56 kDa (Porubleva *et al.*, 2001). However, contrary to reports that RuBisCO may contribute to the low quality of 2D gels and also obscure the resolution of other relatively low abundant proteins (Watson *et al.*, 2003), in our study, the expression of the large subunit of RuBisCo did not seem to compromise the quality of our sorghum leaf 2DE gel images using 7 cm, IPG strips pH range 4-7.

Functionally, RuBisCO proteins catalyse carbon fixation (carboxylation) reactions in the Calvin cycle of photosynthetic plants. In this process, ribulose 1,5-bisphosphate (RuBP), a 5-carbon compound serves as an acceptor molecule for CO_2 to form an unstable 6-carbon compound. The 6-carbon intermediate compound immediately breaks down, forming two molecules of 3-phosphoglycerate (3PGA; Kellogg and Juliano, 1997; Tabita *et al.*, 2007; Andersson and Backlund, 2008). The end product of this carboxylation reaction, 3PGA, is phosphorylated by ATP to form 1,3-biphosphoglycerate and ADP. This reaction in catalysed by a cytosolic 3-phosphoglycerate kinase also identified in this study as protein spot 9 (Figure 4.4; Table 4.1). The above reaction is one of the two that occur in the second phase (reduction phase) of the Calvin cycle (Heldt, 1997; Macdonald and Buchanan, 1997).



In the third phase, RuBP molecules are regenerated to allow the first carbon fixation step to occur. The regeneration phase is characterised by a series of enzymatic reactions that convert triose phosphate to RuBP (Heldt, 1997; Macdonald and Buchanan, 1997). Some of enzymes involved in the intermediate steps of this phase; a sedoheptulose-1,7-biphosphate precursor (spot 11; Table 4.1) and fructose 1,6-biphosphate aldolase precursor (spot 34; Table 4.1) were identified in this study. Together with others, these two enzymes catalyse reactions, which ultimately result in the formation of ribulose-5-phosphate. The ribulose-5-phosphate is then phosphorylated to form RuBP by phosphoribulokinase, an enzyme that was also identified in this study (spot 12; Figure 4.1; Table 4.1). To complete the cycle, RuBP is then used as a substrate by RuBisCo in

the first phase of carbon fixation. Some of the triose phosphate produced in the Calvin cycle is used for sucrose and starch biosynthesis (Raines, 2003; Tamoi *et al.*, 2005).

4.5.2.1.3 Starch Biosynthesis

Starch is an important storage polysaccharide in plants, providing an energy source for various metabolic processes (Kruger, 1997). Starch synthesis involve three enzymes; adenosine diphosphate glucose pyrophosphatase (AGPase), a starch synthase and a branching enzyme (Martin and Smith, 1995; Preiss, 1997; Guan and Keeling, 1998). Only one of these three enzymes was identified in the sorghum leaf proteome. Two protein spots (29 and 39: Figure 4.4; Table 4.1) representing an AGPase were identified, both migrating at the same MW of approximately 20 kDa but having different pIs of 5.6 and 6.1 respectively. Plant AGPases are tetrameric in structure, being composed of two different subunits, which are products of different genes. The small and large subunits have a subunit MW range of 50-54 kDa and 51-60 kDa respectively (Preiss, 1997). However, although the experimental and theoretical MWs of the two AGPases spots were in close range, 20 kDa versus 22 kDa respectively for both spots (Spots 29 and 39; Table 4.1), these values were almost half of those published for general plant AGPases. This observation could possibly indicates (i) the high similarity in protein sequences between the two grasses (wheat and sorghum) and (ii) differences in amino acid sequences and MW between AGPases of grasses and other plant AGPases.

AGPases catalyse the formation of ADP-glucose and inorganic pyrophosphate from ATP and glucose-1-phosphate (Boehlein *et al.*, 2005). The end product of this reaction, ADP-

glucose is a precursor for starch synthesis (Tetlow *et al.*, 2003). Starch synthase then transfers the glucose from ADP-glucose to the nonreducing end of a growing acceptor chain thus elongating the α-1,4 glucan chains. In the third step, the starch branching enzyme then cleaves an elongated α-1,4 glucan chain simultaneously transferring it to an acceptor chain to form α-1,6 linkages (Martin and Smith, 1995; Preiss, 1997; Guan and Keeling, 1998).

4.5.2.1.4 Malate/Oxaloacetate Shuttling System

Two isoforms of malate dehydrogenase (MDH) were identified in the sorghum leaf proteome. Spot 10 represent a chloroplast NADP-dependent MDH (EC 1.1.1.82) with an approximate experimental MW of 45 kDa and pI of 5.2 (Figure 4.4; Table 4.1). The second isoform in spot 14 represents a cytoplasmic NAD-dependent MDH (EC 1.1.1.37) with an approximate experimental MW of 39 kDa and pI of 6 (Figure 4.1; Table 4.1). Plant cells are known to contain multiple isoforms of MDHs, which differ in co-enzyme specificity, subcellular localisation and biological function (Minarik *et al.*, 2002; Ding and Ma, 2004). In plants, five different classes of MDHs are present; (i) chloroplast NADP-dependent MDH; (ii) mitochondrial NAD-dependent MDH; (iii) microbody NAD-dependent MDH; (iv) chloroplast NAD-dependent MDH and (v) cytosolic NAD-dependent MDH (Ding and Ma, 2004). These enzymes occur as homodimers, with subunit MW ranging between 32-37 kDa for the NAD-dependent MDH and 42-43 for the NADP dependent MDHs (Ding and Ma, 2004). The identification of these two isoforms of MDH with different subcellular localisation and co-enzyme specificity in sorghum leaf tissue reinforces this notion.

The experimental and theoretical MW ranges of 45/41 kDa and 39/36 kDa for spots 10 and 14 respectively (Figure 4.4; Table 4.1) are in close range. This possibly indicates the high degree of similarities between the amino acid sequences of the proteins identified in this study and those found in the plant databases. Furthermore, the experimental MW of spot 10, a chloroplastic NADP-dependent MDH (MW= 45 kDa; Table 4.1) is in close agreement with the average MW range (42-43 kDa) for chloroplastic NADP-dependent MDH. Similarly, the experimental MW of spot 14, a cytoplasmic NAD-dependent MDH (MW= 39 kDa; Table 4.1) is in close agreement with the average MW range (32-37 kDa) for cytoplasmic NAD-dependent MDH. With respect to the pIs of the two proteins, experimental and theoretical values also compare well for both spots. For example, the pI of spot 14 (pI= 5.2; Table 4.1), a cytoplasmic NAD-dependent MDH is comparable with that of a stromal NAD-dependent MDH isoform (pI= 5.3) isolated from chloroplast of spinach leaves (Cvetic *et al.*, 2008).

Generally, MDHs catalyse the interconversion of oxaloacetate and malate using the NAD/NADP coenzyme system (Goward and Nicholls, 1994; Minarik *et al.*, 2002). However, different isoforms in different subcellular locations are thought to have different functions. For example, the chloroplastic NADP-dependent MDH forms part of a malate valve system (Scheibe, 2004), which converts excess NADPH into malate and transports in from the chloroplast into the cytosol (Fridlyand *et al.*, 1998). Therefore, it is highly probable that the chloroplastic NADP-dependent MDH identified in this study might have a function in balancing reducing equivalents between the cytosol and the chloroplast stroma. In C_4 plants such as sorghum and maize, this chloroplastic NADP-

dependent MDH isoform may have an additional role in the synthesis of malate, which is transported into the chloroplast of bundle sheath cells and takes part in carbon fixation (Ding and Ma, 2004).

On the other hand, cytoplasmic NAD-dependent MDH isoforms are less well characterised with limited structural and functional information being known. Nevertheless, a cytoplasmic NAD-dependent MDHs was isolated from wheat (TaMDH; (Ding and Ma, 2004). Both mRNA and protein expression studies showed that this MDH was expressed in leaves, stems and roots. The TaMDH had a MW of approximately 40 kDa, which corresponds very well with 39 kDa for protein spot 14 (Figure 4.4; Table 4.1), observed in the current study. Since the cytoplasmic NAD-dependent MDH isoforms were shown to be present in different plants tissues (Ding and Ma, 2004), they are proposed to have house keeping functions in plant metabolism. However, their actual physiological functions and mechanism of action are yet to be elucidated.

4.5.2.2 Proton Transport

The second major functional category (17.9%) identified in this study related to proteins associated with the transportation of protons across the chloroplastic thylakoid membranes. A total of five spots (spots 7, 13, 32, 33 and 40; Figure 4.4; Table 4.1) representing various subunits of the chloroplastic, ATP synthase complex were identified. The relative abundances of the subunits also varied as observed in Figure 4.4. Various subunit components of this complex have also been identified in the proteomes of rapeseed stem (Albertin *et al.*, 2009) and maize leaf (Porubleva *et al.*, 2001). Structurally,

chloroplastic ATP synthases have two major components; an extrinsic CF_1, which synthesises ATP and the membrane bound CF_0 that translocates protons across the thylakoid membrane. The CF_1 has five subunits: alpha, beta, gamma, delta and epsilon while CF_0 has three main subunits: a, b and c (McCarty, 1992). The five protein spots identified in this study represent three of CF_1 subunits; alpha, beta and gamma with experimental MW of 57, 55 and 39 kDa respectively (Figure 4.1; Table 4.1). These estimated MW ranges are highly comparable to the theoretical estimates of 56, 54, and 40 kDa (Table 4.1) and 55, 54 and 36 kDa (McCarty, 1992) for alpha, beta and gamma subunits respectively. The main physiological function of the chloroplastic ATP synthase is to produce ATP from ADP in the presence of a proton gradient across the thylakoid membrane (McCarty, 1992; von Ballmoos and Dimroth, 2007). This proton gradient is created during the light reactions of photosynthesis as described in section 4.5.2.1.1. The ATP then drives a wide variety of energy consuming cellular processes such as the Calvin cycle (Section 4.5.2.1.2) during plant cell growth and development.

4.5.2.3 Protein Synthesis

Two proteins (spots 8 and 21; Figure 4.4; Table 4.1) represented the protein synthesis group thus constituting 7.1% of all the identified proteins. These proteins were the translational elongation factor Tu (spot 8) and the nucleic acid-binding protein (spot 21). Protein synthesis is important in providing the cells with the needed proteins and enzymes which participate in many biological processed within the cell. During protein synthesis, elongation factor Tu binds aminoacyl-tRNAs and guanosine tri-phosphate (GTP) to form a complex, which then associates with a ribosome that is complexed to

messenger RNA and transfer RNA (Harris *et al.*, 1994; Kang *et al.*, 1998). In this way, polypeptides are elongated during synthesis.

4.5.2.4 Nucleotide Metabolism

The nucleotide metabolism functional class was represented by one protein, a putative adenylate kinase (spot 24; Figure 4.4; Table 4.1) thus constituting about 3.6% of all the identified proteins. Adenylate kinases (ADK) are small monomeric enzymes of approximately 21-27 kDa in size (Schiltz *et al.*, 1994). The enzymes catalyse the reversible formation of ADP by the transfer of one phosphate group from ATP to adenosine monophosphate (AMP; Lange *et al.*, 2008). As such, ADK is considered to be an important enzyme in energy metabolism as well as in maintaining the equilibrium of adenylates (ADP, ATP and AMP) *in vivo* (Carrari *et al.*, 2005; Igamberdiev and Kleczkowski, 2006). In maize, a C_4 plant, ADK is important for efficient CO_2 fixation in the C_4 cycle by removing and recycling AMP produced in the pyruvate phosphate dikinase reaction (Schiltz *et al.*, 1994). This enzyme could possibly have a similar function in sorghum, another C_4 plant.

4.5.2.5 Hydrolytic Enzymes

The hydroxynitrile lyase (HNL) spots (20 and 28) were classified as hydrolytic enzyme (7.1%). These enzymes catalyse the cleavage of cyanogenic glycosides into aldehydes or ketones and hydrogen cyanide (Lauble *et al.*, 2002; Purkarthofer *et al.*, 2007). Hydrogen cyanide is toxic, and its production (cyanogenesis) is initiated by tissue damage (White *et*

al., 1998). In plants, cyanogenesis acts as a defense mechanism against herbivores and microbial attack as well as alternative nitrogen source for amino acid synthesis by young seedlings (Hickel *et al.*, 1996; Fetcher, 2004). In all cyanogenic plants, such as sorghum and cassava, cyanogenic glucosides (substrate) and the corresponding enzymes are located in different cellular compartments (Morant *et al.*, 2008). This spatial separation of the substrate and enzyme helps prevent cyanogenesis until the tissue is damaged (Poulton, 1990; Vetter, 2000). In sorghum, cyanogenic glucosides are located in the vacuoles of epidermal cells while the HNL are located in the cytoplasm of mesophyll cells (Hickel *et al.*, 1996).

4.5.2.6 Detoxifying Enzymes

Glutathione S transferase (GST; EC 2.5.1.18) I (spot 26) was identified in this study. Plant GSTs are mainly cytosolic enzymes (Dixon *et al.*, 2002), occurring either as homo- or heterodimers, each with a MW range of between 25-27 kDa (Edwards *et al.*, 2000). Therefore, it is highly probable that the 26 kDa GST protein (spot 26, Figure 4.4; Table 4.1) identified in this study could represent a subunit of the enzyme. Functionally, GSTs are glutathione-dependent detoxifying enzymes, which conjugate glutathione to a wide range of natural products, environmental toxins (such as herbicides) as well as products of oxidative stress. The glutathione conjugates are then transported to the vacuoles for further metabolism into a range of sulphur-containing metabolites (Edwards *et al.*, 2000). The observation of a GST protein spot in our sorghum leaf proteome provided evidence that these enzymes are present in plant cells even under non-stress conditions. However, their precise functions in normal cellular processes are still not clearly understood. It is

proposed that GSTs may have a function in detoxifying endogenous products of oxidative damage such as membrane lipid peroxides and products of oxidative DNA damage. Glutathione S transferases might also act as nonenzymatic carrier proteins (ligandins), which bind and transport plant hormones such as indoleacetic acid (Marrs, 1996).

4.5.3 Subcellular Localization of the Identified Sorghum Leaf Proteins

Knowledge of the protein localization into subcellular compartments is important as it helps clarify protein function and mechanisms of action (Kumar *et al.*, 2002; van Wijk, 2004). Subcellular localizations of the identified sorghum leaf proteins were predicted using a combination of TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson *et al.*, 2007), Predotar version 1.0 (http://urgi.versailles.inra.fr/predotar/predotar.html; Small *et al.*, 2004) and literature sources. The subcellular localisation of each of the positively identified proteins is given in Table 4.1 while a graphical representation of the total number of proteins in each subcellular location is shown in Figure 4.6.

Sorghum leaf proteins identified in this study were predicted to be localised in the chloroplast (20 spots; 71.4%), followed by the cytoplasm (5 spots; 17.9%), apoplast (2 spots; 7.1%), and unknown location (1 spot; 3.6%). These results are consistent with the observation that the most prominent functional category amongst the identified sorghum leaf proteins (Figure 4.4; Table 4.1) was carbohydrate metabolism and photosynthesis related processes (Figure 4.5; Table 4.1). In green plants such as sorghum, photosynthesis

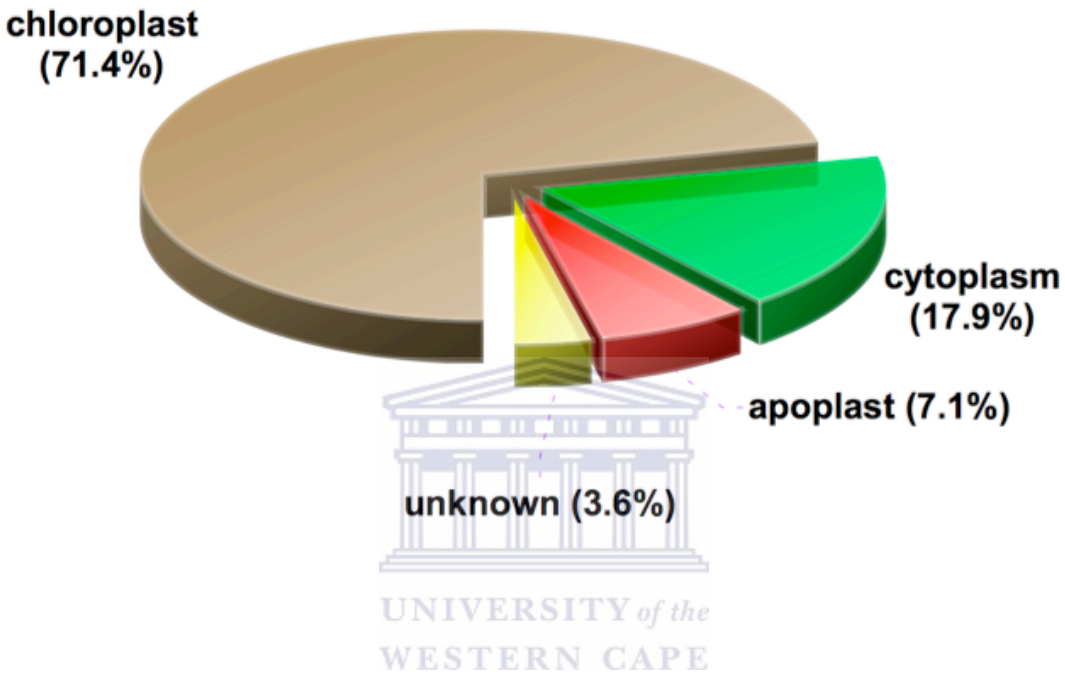


Figure 4.6: Subcellular localisation results of the identified sorghum leaf proteins.

Subcellular localizations of the identified sorghum leaf proteins were predicted using a combination of TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson *et al.*, 2007), Predotar version 1.03 (http://urgi.versailles.inra.fr/predotar/predotar.html; Small *et al.*, 2004) and literature sources. Numbers indicated in brackets represent the proportion of proteins within each subcellular compartment expressed as a percentage of the 28 spots that were positively identified by a combination of MALDI-TOF and MALDI-TOF-TOF MS and database searches.

and carbohydrate metabolic pathways occur primarily in photosynthetic organelles called chloroplasts (van Wijk, 2004). Therefore, the dominance of chloroplast as the main subcellular location of sorghum leaf proteins in this study correlates well with the functional classification results (Table 4.1; Figure 4.5).

The chloroplast itself has different compartments; (i) the outer and inner envelop membranes that surround the organelle, (ii) the soluble stroma, (iii) the thylakoid membrane and (iv) the thylakoid lumen (van Wijk, 2004). Each of these compartments has a different subset of proteins or subproteomes. Collectively, the chloroplast has a diverse population of proteins, which are either soluble and membrane associated. The representation of these two broad classes of proteins in any proteomic study also differs depending on the extraction procedures utilised in the experiment. Membrane proteins are usually poorly represented because of their hydrophobic nature and thus low solubility in most 2DE extraction and solubilisation buffers (Molloy, 2000; Santoni *et al.*, 2000).

To determine the sub-organellar locations of the 20-chloroplast predicted proteins (Table 4.1), searches were done on the Plant Proteome Database (PPDB; http://ppdb.tc.cornell.edu/; Sun *et al.*, 2009) using *A. thaliana* homologs. Table 4.2 gives a summary of the chloroplast subproteome protein identities and their locations (stroma versus membrane). The membrane proteins were further divided into peripheral or integral proteins depending on their interaction with the membrane (Friso *et al.*, 2004). From the results, it was observed that both the stromal and membrane proteins were represented in equal proportions. Of the 10 membrane-associated proteins, only one, the

chlorophyll a/b binding protein (spot 37; Table 4.2) was an integral protein while the other nine were associated with either the lumenal or stromal side of the thylakoid membrane.

Table 4.2: List of stromal and membrane associated sorghum leaf chloroplast proteins.

Protein name	Spot [a)]	Stroma	Membrane	
			Integral	Peripheral
Pyruvate phosphate dikinase	1 & 2	+		
RuBisCo	5 & 6	+		
Chloroplastic NADP-dependent malate dehydrogenase	10	+		
Sedoheptulose-1,7-biphosphate precursor	11	+		
Phosphoribulikinase, chloroplastic precursor	12	+		
Ferrodoxin-NADP oxidoreductase	17 & 18			+
Fructose 1,6-biphosphate aldolase precursor	34	+		
Oxygen evolving enhancer protein 1 precursor	35			+
PSII oxygen evolving complex protein 2	36			+
Chlorophyll a/b-binding proteins type III precursor	37		+	
ATP synthase	7, 32, 33, 13 & 40			+
Translational elongation factor Tu	8	+		
Putatative adenylate kinase, chloroplast	24	+		

[a)] Spot number as indicated on the 2D gel image (Figure 4.4).

+ Indicates subcellular localisation of proteins.

4.6 Comparative Analysis of AS6 and MN1618 Sorghum Leaf Proteomes

One of the objectives of this Chapter was to perform comparative expression proteomics between leaf proteomes of two sorghum varieties, AS6 and MN1618. Proteomics technologies using a combination of 2DE, comparative spot analysis using softwares packages and the subsequent protein identification by MS has been used over the years to study protein expression between plant mutants and/or varieties. Although the genome of the two sorghum varieties, AS6 and MN 1618, might be broadly similar, gene expression and subsequently protein expression might to some extent differ during plant growth and development.

4.6.1 Total Number Of Reproducible Leaf Spots Of Each Sorghum Variety

To test the above-mentioned hypothesis, a comparative analysis of the leaf proteomes of the two varieties was performed using PDQuest™ Advanced 2D Analysis Software version 8.0.1 build 055 (BIO-RAD) as described in Section 2.12. Three CBB stained biological replicate gels per sorghum variety were used in the analysis. Table 4.3 gives a summary of PDQuest™ analysis of leaf protein expression between the two sorghum varieties. It was observed that an approximate number of 191 and 171 CBB stained leaf protein spots were reproducibly expressed amongst the three biological replicates of AS6 and MN1618 leaf respectively. Of these, 167 were common to both sorghum varieties albeit with varying degrees of abundances. The observation of a large proportion of commonly expressed proteins between AS6 and MN1618 indicates that the genome and consequently the gene and protein expression of the two varieties are broadly similar.

Some of these common proteins might include house keeping proteins that function in general metabolic processes for the maintenance of plant growth and development.

4.6.2 Analysis Sets for Comparative Proteomics Between the Sorghum Varieties

4.6.2.1 Qualitative Protein Spots

Approximately 24 and 4 protein spots were exclusively expressed in AS6 and MN1618 respectively. Of these 28 sorghum variety specific proteins spots, only two (spots 30 and 31; expressed in MN1618) were represented amongst the 40 spots (Figure 4.4; Table 4.1) that were picked for tryptic digestion, MALDI-TOF MS and peptide mass fingerprinting. For both spots, no protein identities were significantly matched to the protein spots (Table 4.1). Nevertheless, these qualitative variations in spot expression possibly indicate variety specific proteins in the two sorghum varieties.

4.6.2.2 Quantitative Protein Spots

Of the 167 common proteins, a total of 20 spots showed quantitative variation in expression level by at least a 2-fold margin (≥ 2-fold) between the two sorghum varieties (Table 4.3). These were equally distributed between AS6 and MN1618 sorghum varieties. Amongst the 40 leaf spots that were picked for MALDI-TOF analysis (Figure 4.4), only five (spots 4; 5; 6; 17 and 23) were members of the quantitative analysis set between AS6 and MN1616 (Table 4.3). Of these five spots, only 3 were positively identified by a combination of MALDI-TOF and MALDI-TOF-TOF MS (spot 5 and 6 RuBisCo large subunit; spot 17-putative ferrodoxin-NADP oxidoreductase) and all were

more abundant in AS6 seed type than MN1618. The other two spots (spots 4 and 23; Figure 4.4; Table 4.1) were not significantly matched to any proteins on the plant databases. Nevertheless, the quantitative difference in protein expression between the two varieties could possibly indicate differential interaction of the genome with the environment.

4.6.2.3 Student's *t*-test significant protein spots

Using the Student's *t*-test analysis at a 95% significance level, the expression pattern of 99 protein spots was shown to be significantly different between the two sorghum varieties. Of these, 46 (46.5%) and 53 (53.5%) were more abundant in AS6 and MN1618 sorghum varieties respectively. A total of 10 of these 99 spots were amongst the 40 protein spots that were picked for MALDI-TOF analysis. These are spots (8; 11; 12; 17; 18; 22; 23; 30; 31; and 40; Figure 4.4; Table 4.1). Of these 10 spots, only six (spot 8; 11; 12; 17; 18 and 40) were positively identified (Table 4.1).

Table 4.3: Summary of comparative proteomics between leaf proteomes of AS6 and MN1618 sorghum varieties.

Sorghum variety	Total spots [a)]	Analysis set		
		Qualitative [b)]	Quantitative [c)]	Student's *t*-test [d)]
AS6	191	24	10	46
MN1618	171	4	10	53
Total		**28**	**20**	**99**

[a)] Total number of reproducible spots amongst three independent biological replicate gels of each sorghum variety.

[b)] Variety specific spots.

[c)] Spots showing at least a 2-fold expression change between the two sorghum varieties.

[d)] Differentially expressed spots between the sorghum varieties as determined by the Student's *t*-test at a 95% significance level.

4.6.3 Examples of Protein Expression Patterns Between the Sorghum Varieties

As described in Sections 4.6.1 and 4.6.2, the protein expression patterns observed between AS6 and MN 1618 leaf proteomes can be loosely categorised into five groups. Figure 4.7 illustrates examples of each of these classes. In Figure 4.7, all three biological replicate gels per variety are included so as to illustrate reproducibility of protein expression between experiments. Figure 4.7A: uniform protein expression between the two sorghum varieties; B: increased expression in AS 6 relative to MN1618; C: increased expression in MN1618 relative to AS6; D: exclusively present in AS6; and E: exclusively present in MN1618. The numbered arrows indicate the respective protein spots under comparative analysis. The spot numbers are consistent with those shown in the annotated sorghum leaf proteome gel (Figure 4.4) while protein X was not amongst the 40 spots that were picked for tryptic digestion and mass spectrometry due its low abundance on 2D gels.

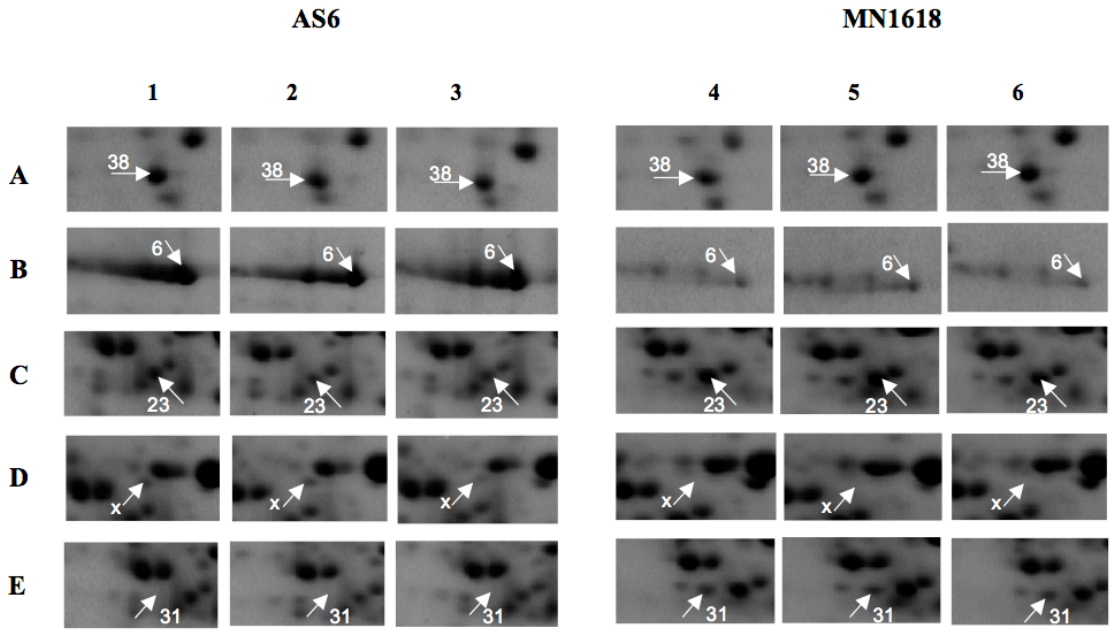


Figure 4.7: Examples of differential protein expression patterns between AS6 and MN1618 sorghum varieties.

Zoomed in sections of three biological replicates of AS6 (gel panels 1-3) and MN1618 (gel panels 4-6) per expression pattern are shown. (A) Uniform protein expression between the two sorghum varieties. (B) Increased expression in AS6 relative to MN1618. (C) Increased expression in MN1618 relative to AS6. (D) Exclusively present in AS6. (E) Exclusively present in MN1618. The numbered arrows indicate the respective protein spots under comparative analysis. Spot numbers are consistent with those shown in the annotated sorghum leaf proteome gel (Figure 4.4; Table 4.1) while protein X was not amongst the 40 spots that were picked for tryptic digestion and mass spectrometry due its low abundance on 2D gels.

4.7 Discussion

The extraction of total protein from the leaf, sheath and root tissues of AS6 and MN1618 sorghum varieties was optimised using the TCA/acetone method. Plant extracts are known to be rich in proteases as well as non-protein components, which affect the overall 2D gel profiles and spot resolution. An effective protein extraction protocol should be able to reduce protein degradation by endogenous proteases as well as remove non-protein components of plant extracts, which co-extract with proteins. The use of the TCA/acetone method (reviewed in Section 17.1.1.1) for the extraction of plant proteins has two main advantages. Firstly, the low pH of TCA inactivates most proteases and thus reduces protein degradation. Secondly, acetone washes solubilise non-protein components of plant extracts, which when present would interfere with the spot resolution (Wang *et al.*, 2006; Hurkman and Tanaka, 2007).

The 1D protein extracts of the sorghum leaf, sheath and root tissues (Figure 4.1A-E) showed that the extracts were of high quality showing no signs of streaking and protein degradation. This indicates that the TCA/acetone method used for protein extraction was effective for both reducing protein degradation as well as the removing interfering non-protein components present in plant tissues. The 1D protein profiles between the biological replicates (Lanes 1-4; Figure 4.1) of the leaf and sheath extracts showed high similarities in terms of protein expression, abundance and banding patterns. These observations suggest that protein preparation was reproducible between independent extractions. Furthermore, the protein loading between biological replicates within experiments was also uniform. Reproducible protein extraction and uniform protein

loading both within and between experiments is important in comparative proteomics for setting a baseline for comparative protein expression analysis. Non-reproducible extractions and inconsistent protein loads in 2DE may introduce artefactual spots that might be erroneously regarded as differential protein expression during comparative gel analysis. It was observed that the sorghum root tissue has limited amount of total protein (Section 4.2) and this limited the number of biological replicates of the tissue. This characteristic of low total protein quantities in root tissue has also been documented in proteomics of soybean root hair (Brechenmacher *et al.*, 2009).

The 2D gel profiles for all the three tissue extracts (leaf, sheath and root) was limited to pH range 4-7 and MW range 10-110 kDa. Protein separation by 2D gel electrophoresis is known to have physical limitation in separating proteins, where proteins of extreme pIs and MW are excluded from the 2D profiles (Lin *et al.*, 2003; Jain *et al.*, 2008). The limited MW range of 2D electrophoresed spots has also been reported in other studies of the proteome of maize leaves (Porubleva *et al.*, 2001), and cell suspension cultures (Lei *et al.*, 2005) as well as different tissues (Watson *et al.*, 2003) of barrel medic, amongst others. The visualisation of high MW proteins in 2D gels may be limited by the reduced protein uptake of the current commercially available IPG strips with a 4% total acrylamide concentration (Candiano *et al.*, 2002). Since the ultimate goal of any proteomics study is to characterise the total proteome of a cellular compartment, cell type, tissue or organ, the visualisation of partial proteomes due to the physical limitations of the 2D gel based separation system remains a challenge in plant proteomics.

Two-dimensional gel electrophoresis only allows for the separation and visualisation of proteins within a sample under study but does not identify proteins. Sorghum leaf protein where then identified by a combination of MALDI-TOF MS, MALDI-TOF-TOF MS and database searching. An organism's genome sequence is thus useful for providing essential information linking genes to gene product (Lin *et al.*, 2003). For sorghum, the completion of the genome-sequencing project (Sorghum Genomic Planning Workshop Participants, 2005) had only been recently published (Paterson *et al.*, 2009). Therefore until recently, the identification of sorghum proteins has relied largely on homology searches against sequence information from other green plants as well as sorghum ESTs. Out of the 40 spots that were picked for mass spectrometry analysis (spots 1-40; Table 4.4), only 28 were positively identified by database searches (Table 4.1). This gave a 70% success rate in protein identification. This high identification success rate of the sorghum leaf proteins can be attributed to the high number of conserved proteins in higher plants. These results are in agreement with previous report on the proteomic analysis of maize leaves were protein identification was limited to the partial genome sequence data of maize and homology–based protein identification against conserved proteins in higher plants, *Streptophyta* (Porubleva *et al.*, 2001). More recently, Brechenmacher *et al.* (2009) reported a 67% success rate on protein identification using the partially available soybean genome sequence data and homology-based protein matching against other plant species. Furthermore, the high success rate in the positive identification of sorghum proteins even without extensive publicly available genome sequence data can be attributed to the use of MALDI-TOF-TOF MS. As reviewed in Section 1.6.5.2, tandem mass spectrometry has an advantage of detecting amino acid

sequences of proteins (Dubey and Grover, 2001) and is thus particularly useful for the identification of proteins of organisms with partial genome information (Aebersold and Goodlett, 2001).

Of the 40 spots that were picked for MS analysis (Figure 4.1; Table 4.1), 12 were unidentified (Table 4.1). These unidentified protein spots exhibited varying degrees of protein abundances (Figure 4.4). For this reason, the non-identification of these spots might be due to two main reasons; (i) For some of the low abundant proteins, the protein quantities present in the picked gel plugs might be a limiting factor for positive identification. (ii) For others such as protein spots 23 and 38 (Figure 4.4; Table 4.1) whose abundances were relatively high after CBB staining, their identification might be limited by the unavailability of full sorghum genome sequence data. Spot 23 and 38 might therefore represent sorghum specific proteins, which are not common amongst other grasses or higher plants.

A total of seven proteins were observed to be present in multiple spots within the 2D gels (Table 4.1; Section 4.5.1). The existence of proteins in multiple spots on 2D gels has been reported in other proteomic studies of maize leaves (Porubleva *et al.*, 2001), different tissues of barrel medic (Watson *et al.*, 2003) and rapeseed (Albertin *et al.*, 2009) amongst others. Multiple spots for a single protein may be due to several reasons such as PTMs, the presence of dimeric and monomeric forms of proteins on the same gel, presence of proteolytic degradation by endogenous proteases, presence of different

protein isoforms from the multigene families as well as the chemical modification of proteins during sample preparation (Albertin *et al.*, 2009).

The identified sorghum leaf proteins were grouped into six broad functional categories (Section 4.5.2; Table 4.1). These functional groups remain putative until the actual functions of the proteins are determined experimentally. The main functional categories and the proportion of protein (Figure 4.5) in these classes are; carbohydrate metabolism (60.7%), proton transport (17.9%), protein synthesis (7.1%), hydrolytic enzymes (7.1%), nucleotide metabolism (3.6%) and detoxifying enzymes (3.6%). The identification of carbohydrate metabolism as the main functional class of the identified proteins is in line with the primary function of the leaf in autotrophs such as sorghum. Within the carbohydrate metabolism functional category, several proteins related to the light reactions of photosynthesis, Calvin cycle, starch biosynthesis, malate/oxalate shuttling system among others were identified. These observations reinforce the importance of protein functional classification in elucidating the primary function of the plant tissue under study. The subcellular localisation results of the identified proteins showed that the majority of the proteins were localised in the chloroplast (71.4%; Table 4.1; Figure 4.6). Once again, these results correlate well with the functional classification of the identified proteins. The main function of a green plant leaf is to harvest light energy, convert it into chemical energy as well as its storage through the process of photosynthesis. Since the main photosynthetic organelle in plants is the chloroplast, the identification of the chloroplast as the main subcellular localisation of the identified sorghum leaf proteins is expected.

Interestingly, of the 28 positively identified proteins (Table 4.1), 10 were associated with the thylakoid membranes (Table 4.2); one being an integral protein while the rest were peripheral proteins. The under representation of integral protein in proteomic studies is has been reported by other authors (Porubleva *et al.*, 2001; Albertin *et al.*, 2009; Brechenmacher *et al.*, 2009) and still remains as one of the limitations of 2D gel electrophoresis (Lin *et al.*, 2003; Rose *et al.*, 2004). Membrane proteins are usually poorly represented because of their hydrophobic nature and thus low solubility in most 2DE extraction and solubilisation buffers (Molloy, 2000; Santoni *et al.*, 2000). To extract, separate and visualise integral proteins, more specialised extraction protocols using organic solvents would be necessary (Friso *et al.*, 2004).

The partial sorghum leaf proteome map established in this study (Figure 4.4; Table 4.1) was used to land mark proteins in a comparative proteomic analysis of two sorghum varieties (AS6 and MN1618; Section 4.6). It was observed that the two sorghum varieties shared about 167 common leaf protein spots (Section 4.6.1). These protein spots might represent common house keeping and/or ubiquitous proteins that are found in sorghum (Albertin *et al.*, 2009). However, both sorghum varieties also exhibited qualitative spot expression patterns (Table 4.3; Figure 4.7). The visualisation of variety specific proteins at a particular developmental stage and their subsequence identification by MS analysis might lead to a wealth of information on the differences in growth and development of varieties in a species. Apart from the qualitative differences in protein expression, about 20 spots were observed to have quantitative differences in expression by at least 2-fold (Table 4.3). Examples of these are putative ferrodoxin-NADP oxidreductase (spot 17;

Figure 4.4) and RuBisCO proteins (spots 4 and 6; Figure 4.4). Both these spots are involved in photosynthetic processes. However, the actual significance of this ≥ 2-fold change in expression between the two varieties would need to be studied further. Using the Student's *t*-tests at 95% significance level, the expression of 99 proteins was also observed to be statistically different between the two sorghum varieties (Table 4.3). Some of these proteins include sedoheptulose-1,7-biphosphate precursor (spot 11), phosphoribulokinase, chloroplast precursor (spot 12), ferrodoxin- NADP oxidoreductase (spots 17 and 18) and ATP synthase CF1 alpha chain (spot 40; Table 4.1).

In conclusion, the following objectives were achieved in this Chapter; (i) the extraction of sorghum leaf, sheath and root tissue proteins was optimised using the TCA/acetone extraction method. This resulted in reproducible, high quality 2D gel profiles of all the tissues (leaf, sheath and root) analysed in this study. (ii) Of the 40 protein spots that were picked for analysis by mass spectrometry (MALDI-TOF and MALDI-TOF-TOF MS) and database searching, 28 (70%) were positively identified. This high protein identification success rate could be attributed to the high number of conserved genes and gene products in higher plants as well as the added advantage of using MALDI-TOF-TOF MS in the identification of proteins from organisms with partial genome sequence information. (iii) Both functional classification and subcellular localisation of the identified proteins were important parameters in elucidating the main metabolic function that are operational in sorghum leaf. (iv) This work provided the first attempt working towards the mapping of sorghum leaf proteome. Proteome reference maps are important tools in land marking proteins both in comparative studies between plant varieties as well as in stress response

studies. This partial reference map has been utilised in the identification of differentially expressed proteins between two sorghum varieties, AS6 and MN1618. In addition, the proteome map would be used in the identification of salt stress responsive proteins identified in Chapter 6.

CHAPTER 5

Identification of Proteins Secreted into the Culture Medium of Sorghum Cell Suspension Cultures Using a Combination of 2DE and Mass Spectrometry

5.1 Introduction

Of the two major proteomes of the sorghum cell suspension culture system described in Chapter 3 (TSP and CF; Section 3.4), only the CF is further characterised in this study. This is because of its relatively less complex nature in terms of overall spot numbers observed in the proteome when compared to the TSP (Figures 3.8 and 3.9).

Plant cells secrete proteins into the extracellular matrix (ECM) and these proteins are collectively known as the secretome (Greenbaum *et al.*, 2001; Hathout, 2007). The secretome and other components of the ECM play vital roles in cell-cell interaction, growth and development (Knox, 1995; Pennell, 1998; Brownlee, 2002) and defense mechanisms upon wounding and pathogenic attack (Showalter, 1993). However, the actual composition and function of the ECM varies with plant species, cell types, developmental stages as well as responses to both abiotic and biotic stresses (Ye and Varner, 1991).

Proteomic approaches have been used to study the secretome of different plant species such as tobacco (*Nicotina tabacum*; Okushima *et al.*, 2000; Dani *et al.*, 2005), rice (*Oryza sativa*; Zhang *et al.*, 2009); chickpea (*Cicer arietinum* L.; Bhushan *et al.*, 2007) or

Arabidopsis (*Arabidopsis thaliana*; Charmont *et al.*, 2005; Ndimba *et al.*, 2005; Oh *et al.*, 2005). In these studies, source of secreted proteins included the apoplastic fluids of whole plant systems, and/or the culture medium of either cells in suspension or hydroponically grown seedlings. Like most proteomic studies, secretomics aims at isolating ECM fractions with minimal or no contamination from other cellular compartments, and to identify the secreted proteins by mass spectrometry (MS). Lack of contamination of the secretome with cytoplasmic proteins can be confirmed by the determination of enzyme activities of cytosolic marker proteins. Alternatively, the presence of the cytosolic contaminants can be evaluated by western blotting analysis of the secreted protein fractions using antibodies of known cytosolic and/or organellar marker proteins (Chivasa *et al.*, 2002; Oh *et al.*, 2005).

Although there has been sound progress in the accumulation of data from the secretome analysis of various plant species to date, to our knowledge no work has been reported on the expression profiling of the secretome of sorghum cell suspension cultures. Therefore, the main objective of this chapter is to isolate, separate, visualise and identify the entire sorghum cell suspension culture secretome. The identification of these proteins would help in the generation of a sorghum secretome map that would provide invaluable knowledge towards the understanding of sorghum physiology.

5.2 Isolation of Secreted Proteins from Sorghum Cell Suspension Cultures

Sorghum cell suspension cultures were established and maintained in culture as described in Section 2.5.3. To isolate proteins secreted into the culture medium (also termed culture

filtrate proteins; CF), the medium was first separated from cells by filtration through Miracloth™ (Section 2.7.2). The CF proteins were then recovered by acetone precipitation, resolubilised in urea buffer (Section 2.7.2) and quantified using the Bradford assay (Section 2.8). Hundred micrograms of secreted CF proteins was separated on 2D SDS-PAGE (Section 2.10). Figure 5.1 shows the 2D profile of secreted sorghum CF proteins after CBB staining. Approximately 25 protein spots with varying degrees of abundance were visualised on 2D gels after staining with CBB. As previously discussed in Section 3.4.1, CF proteins were separated on IPG strips of two pH ranges, pH 4-7 (Figure 5.1A) and pH 3-10 (Figure 5.1B). The MWs of the CF proteins ranged between 25 and 100 kDa, the majority of which were acidic, having pIs between the range of 4 and 6. (Figure 5.1A). Numbered arrows indicate protein spots that were picked for identification by mass spectrometry (Section 5.3).

5.3 Identification of Secreted CF Proteins by a Combination of MALDI-TOF and MALDI-TOF-TOF MS

Out of the observed 25 CBB stained CF protein spots, 15 were selected for identification by a combination of MALDI-TOF and MALDI-TOF-TOF MS, and database searches. Spot selection was on the basis of high abundance and good resolution on the IPG strip pH ranges used. Selected protein spots are numbered 1-9 and 10-15 on Figures 5.1A and Figure 5.1B respectively. These protein spots of interest were picked from the CBB stained gels using the ExQuest^{TM} spot cutter (BIO-RAD). Protein contained in the picked gel plugs was trypsinised and peptide digests were analysed using MALDI-TOF and MALDI-TOF-TOF MS as described in Section 2.14.2. The resultant peptide mass

fingerprints and/amino acid sequences generated for each protein by MALDI-TOF MS and MALDI-TOF-TOF MS respectively were used in sequence database searching to find their protein identities. Since the sorghum genome sequence in not yet publicly available in full, database searches were carried out against all entries in the NCBI database. Mass spectrometry analysis and database searches of the trypsinised 15 protein spots resulted in the positive identification of 14. Table 5.1 gives a summary of the identified protein spots, their best match identities, plant species of origin and corresponding NCBI accession numbers. Both the theoretical and experimental MW/pIs of the proteins are also shown in Table 5.1 to give a comparative analysis of the two ranges of data. The identified proteins matched those from other grasses such as rice (*Oryza sativa*), maize (*Zea mays*) and barley (*Hordeum vulgare*). The protein identities represented four different classes of proteins namely; peroxidases (spots 1-7), alpha-galactosidases (spots 8-9), oxalate oxidases (spots 11-12) and germin proteins (spots 13-15). However, spot number 10 remained unidentified after database searching.

It was observed that all four classes of proteins identified were represented in multiple spots on the 2D gel (Figure 5.1; Table 5.1). For example, protein spots 2, 3 and 4 (Figure 5.1A) are well-resolved protein spots with the same experimental MW of approximately 50 kDa but different experimental pI values of 5.3, 5.5 and 5.8 respectively (Table 5.1). The peptide mass fingerprints and/or amino acid sequences of all three protein spots matched the identity of a peroxidase (*Zea mays*; NCBI accession AAS75402) with high MOWSE scores of 210, 205 and 350 respectively. Similarly, protein spots 6 and 7 (Figure 5.1A) have the same MW of approximately 36.0 kDa but different pI values of

5.3 and 5.6 respectively (Table 5.1). The peptide mass fingerprints and/or amino acid sequences of both protein spots matched the identity of a rice (*Oryza sativa*) putative peroxidase (NCBI accession NP_908705) with MOWSE scores of 77 and 79 respectively. Furthermore, protein spots 1 and 5 (Figure 5.1A) had different experimental MW and pIs (60 kDa/5.1; 39 kDa/4.6 respectively; Table 5.1). However, the mass fingerprints and/or amino acid seqeunces of the peptide digests from both spots matched the identities of peroxidases, albeit with different NCBI accessions of NP_908708 and AAC49818 respectively. Protein spots 1-7 therefore represent different peroxidase isoenzymes.

Spots 8 and 9 (Figure 5.1) had the same experimental MW of 40 kDa but different pIs of 6.1 and 6.3 respectively. However, both protein spots matched the identity of rice alpha-galactosidase (NCBI accession 1UAS_A). For this reason, spots 8 and 9 are regarded as isoforms of the same protein. Interestingly, spots 11-15 formed a horizontal train of well-resolved spots with the same MW of approximately 26.5 kDa but differing in spot abundance and pIs. (Figure 5.1B). These spots matched the identities of two classes of proteins; oxalate oxidases (spots 11-12) and germin proteins (spots 13-15) as shown in Table 5.1. Spots 11 and 12 (pIs of 6.4 and 6.8 respectively) represented isoforms of a putative oxalate oxidase (NCBI accession XP_469351) while spots 13 and 14 (pIs of 7.8 and 8.5 respectively) represented isoforms of a germin E protein (NCBI accession AAG00429). Spot 15 with a pI of 9.2 also matched the identity of a germin protein type 1 (NCBI accession XP_480453). Altogether, the 14 positively identified sorghum CF

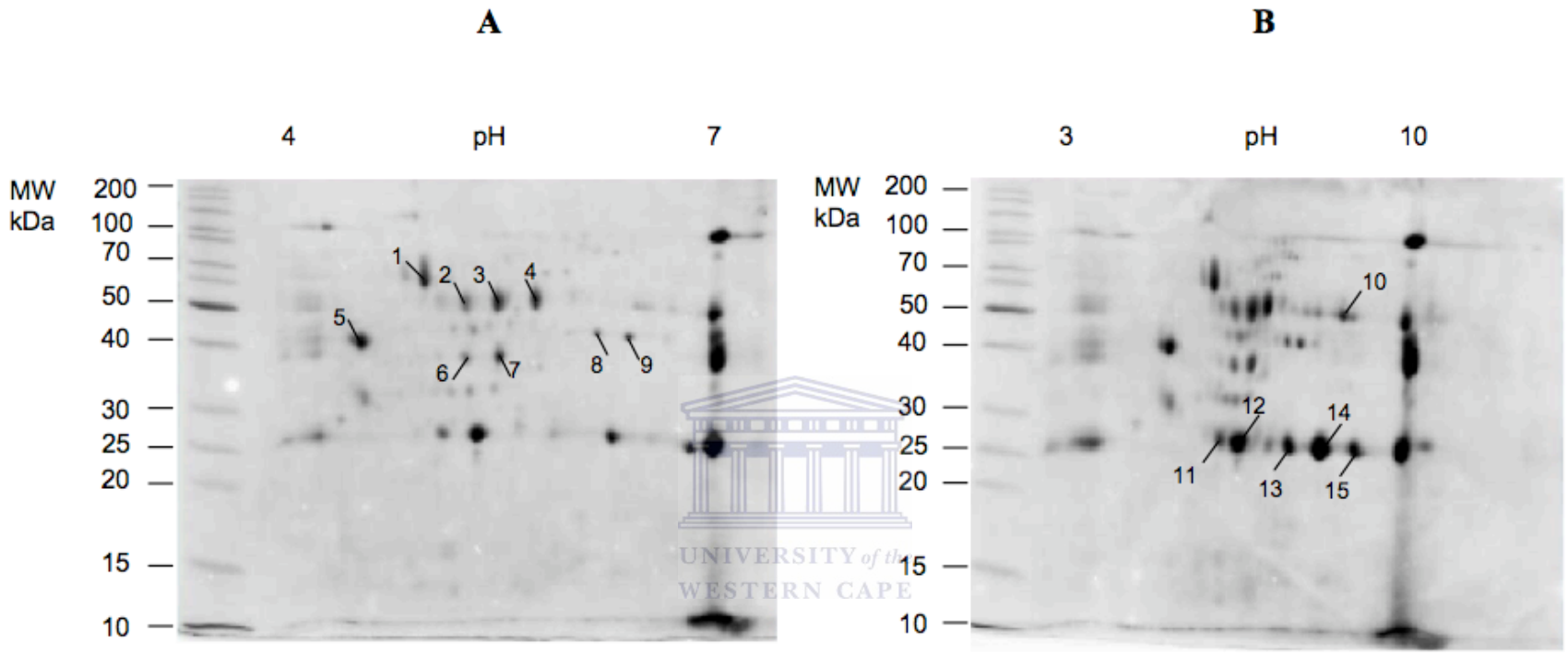


Figure 5.1: CBB stained 2D gels of sorghum culture filtrate proteins analysed by a combination of MALDI-TOF MS, MALDI-TOF-TOF MS and database searching.

Approximately 100 µg of culture filtrate proteins were each separated on IPG strips of (A) pH range 4-7 and (B) pH range 3-10. Numbered spots were selected for identification by a combination of MALDI-TOF MS, MALDI-TOF-TOF MS and database searches.

Table 5.1: List of identified sorghum culture filtrate proteins using a combination of MALDI-TOF MS, MALDI-TOF-TOF MS and database searching.

Spot [a)]	Best Match Protein	Species	Accession No. [b)]	MOWSE score [c)]	Theoretical [d)] Mass (kDa)	pI	Experimental [e)] Mass (kDa)	pI
1	Putative peroxidase	*Oryza sativa*	NP_908708	84	34.6	5.3	60.0	5.1
2	Peroxidase	*Zea mays*	AAS75402	210	36.1	5.7	50.0	5.3
3	Peroxidase	*Zea mays*	AAS75402	205	36.1	5.7	50.0	5.5
4	Peroxidase	*Zea mays*	AAS75402	350	36.1	5.7	50.0	5.8
5	Peroxidase	*Oryza sativa*	AAC49818	113	30.5	6.1	39.0	4.6
6	Putative peroxidase	*Oryza sativa*	NP_908705	77	35.2	6.0	36.0	5.3
7	Putative peroxidase	*Oryza sativa*	NP_908705	79	35.2	6.0	36.0	5.6
8	Alpha-galactosidase	*Oryza sativa*	1UAS_A	80	40.0	5.9	40.0	6.1
9	Alpha-galactosidase	*Oryza sativa*	1UAS_A	86	40.0	5.9	40.0	6.3
10	No Match						50	8.8
11	Putative oxalate oxidase	*Oryza sativa*	XP_469351	192	20.9	5.6	26.5	6.4
12	Putative oxalate oxidase	*Oryza sativa*	XP_469351	197	20.9	5.6	26.5	6.8
13	Germin E	*Hordeum vulgare*	AAG00429	79	20.3	7.7	26.5	7.8

14	Germin E	*Hordeum vulgare*	AAG00429	108	20.3	7.7	26.5	8.5
15	Germin protein type 1	*Oryza sativa*	XP_480453	104	22.2	8.2	26.5	9.2

[a] Spot number as indicated on the 2D gel images (Figure 5.1).

[b] Accession number in the National Center for Biotechnology Information (NCBI) database.

[c] Combined MOWSE score for MALDI-TOF and MALDI-TOF-TOF MS.

[d] Theoretical mass and pI were calculated on the mature peptide using the programme on http://isoelectric.ovh.org.

[e] Experimental mass and pI were estimated from the 2D gels shown in Figure 5.1.

proteins represented four different classes of protein (peroxidases, alpha galactosidases, oxalate oxidases and germins) with a great diversity of isoforms and/or isoenzymes.

5.4 Identification of Classical Extracellular Matrix Proteins

Generally, classical extracellular matrix proteins have a signal peptide at their N-termini, which targets them for the secretory pathway via the endoplasmic reticulum (ER) in eukaryotes (Nielsen *et al.*, 1997). The N-terminal signal peptide is approximately 15-30 amino acids long and is cleaved off during protein translocation across the membrane. Signal peptides are composed of three structural regions: a positively charged N-terminal region (n-region), followed by a hydrophobic region (h-region) in the middle and a polar c-region at the C terminal end. For cleavage to occur correctly, the amino acid residues at positions -3 and -1 relative to the cleavage sites, should be small and neutral, and are highly conserved among proteins (Nielsen *et al.*, 1997; Emanuelsson *et al.*, 2007; Guo *et al.*, 2008). Since the signal peptide is cleaved off during protein secretion, its presence in the pre-protein can be predicted by bioinformatics tools (Jamet *et al.*, 2006). To verify if the identified sorghum CF proteins were indeed destined for the ECM, we used the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/), to investigate for the presence of a predicted cleavable N-terminal signal peptide domain (Bendtsen *et al.*, 2004; Guo *et al.*, 2008). With the exception of two proteins, alpha-galactosidase (spots 8 and 9) and germin E (spots 13 and 14), the predictions on the presence and location of signal peptide cleavage sites of all the other CF proteins were carried out using full protein sequences obtained from the NCBI database. The signal peptide prediction results are summarised in Table 5.2.

Table 5.2: Summary of signal peptide predictions of sorghum culture filtrate proteins using the SignalP prediction server.

Protein class	Spots [a]	Accession No. [b]	Signal peptide [c]	Cleavage site [d]
Peroxidases	1	NP_908708	1-27 (0.999)	27 and 28: SRG-QL
	2, 3 & 4	AAS75402	1-21 (0.983)	21 and 22: ATA-AC
	5	AAC49818	1-25 (1.000)	25 and 26: ASA-QL
	6 & 7	NP_908705	1-19 (1.000)	19 and 20: ALA-CS
Alpha-galactosidases	8 & 9	1UAS_A	1-33 (0.998)*	33 and 34: AAA-LG
Oxalate oxidases	11 & 12	XP_469351	1-29 (1.000)	29 and 30: TDA-DP
Germin proteins	13 & 14	AAG00429	-	-
	15	XP_480453	1-23 (1.000)	23 and 24: AIA-FD

[a)] Spot number as indicated on the 2D gel images (Figure 5.1).

[b)] Accession number in the National Center for Biotechnology Information (NCBI) database.

[c)] Length of signal peptide predicted using the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/). Both the neutral networks (NNs) and the hidden Markov models (HMMs) were used (Emanuelsson *et al.*, 2007). Numbers in brackets indicate the probability of having a signal peptide.

[d)] Most likely cleavage site position and corresponding amino acid sequence.

* Signal peptide predicted on the full protein sequence, accession number Q9FXT4 on the Swiss-prot database.

- No signal peptide predicted using SignalP because the published amino acid sequence of AAG00429 is incomplete. However, the same protein is a known apoplastic protein (Wu *et al.*, 2000).

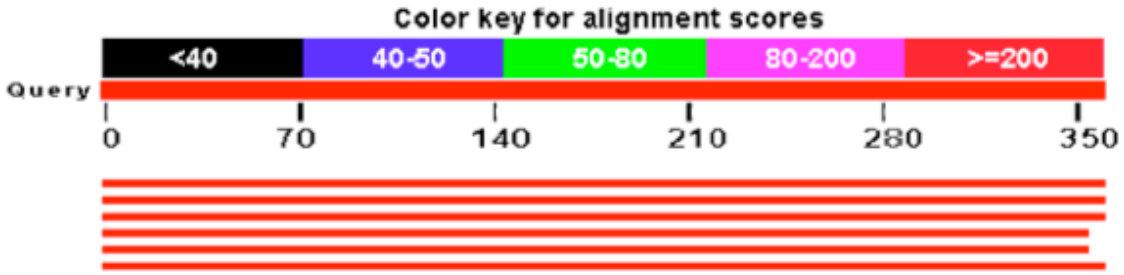
The mass fingerprints and/or amino acid seqeunces of protein spots 8 and 9 matched the identity of a fragment of alpha-galactosidase (NCBI accession 1UAS_A; Chain A, Crystal structure of rice alpha-glactosidase; Table 5.1). Since the published protein sequence of 1UAS_A is a partial sequence (Fujimoto *et al.*, 2003) of the alpha galactosidase pro-protein (Kim *et al.*, 2002) the sequence could not be used for the signal peptide prediction analysis. For this reason, protein BLAST searches were carried out using NCBI BLASTp 2.2 programme to obtain a full sequence of the rice alpha galactosidase or similar identities of this protein. Searches were restricted to the non-redundant protein sequences. Figure 5.2 illustrates part of the BLASTp results showing only the first seven positive hits.

The protein with the highest similarity to IUAS_A on the BLASTp search results was of a 417 amino acid long, full alpha galactosidase protein (Swiss-prot accession: Q9FXT4; Figure 5.2A and B). These two proteins showed a 100% sequence alignment homology

between the entire 362 amino acid sequence of 1UAS_A and the region between 56 - 417 amino acids of Q9FXT4 (Figure 5.2C). The latter region constitutes the 362-amino acid mature alpha-galactosidase, while the 1-55 amino acid region is the signal sequence (Kim *et al.*, 2002). A schematic representation of this alignment is shown in Figure 5.3. Consequently, signal peptide predictions of spots 8 and 9 were carried out using the full protein sequence of alpha galactosidase Q9FXT4. Using this sequence, a cleavable signal peptide was predicted on the rice alpha galactosidase protein with the most probable cleavage site between positions 33 and 34: AAA-LG (Table 5.2).

Signal peptide predictions of the two germin E proteins (spots 13 and 14) did not yield a positive prediction result for a cleavable signal peptide using the SignalP prediction server. This could be due to the fact that the published sequence of NCBI is a partial sequence of the protein. However, the same protein sequence was published as a known apoplastic protein (Wu *et al.*, 2000). For this reason, we considered the germin E proteins to be *bona fide* secretory proteins.

A



B

Sequences producing significant alignments:	Score (Bits)	E Value
sp|Q9FXT4.1|AGAL_ORYSJ RecName: Full=Alpha-galactosidase; Alt...	759	0.0
ref|NP_001064939.1| Os10g0493600 [Oryza sativa (japonica cult...	758	0.0
pdb|1UAS|A Chain A, Crystal Structure Of Rice Alpha-Galactosi...	755	0.0
gb|EEC67231.1| hypothetical protein OsI_34152 [Oryza sativa I...	747	0.0
gb|EAZ16532.1| hypothetical protein OsJ_030741 [Oryza sativa ...	687	0.0
gb|ACF88364.1| unknown [Zea mays]	684	0.0
gb|ACF80887.1| unknown [Zea mays]	683	0.0

C

gb|ABB47819.1| Alpha-galactosidase precursor, putative, expressed [Oryza sativ
(japonica cultivar-group)]
Length=417

Score = 759 bits (1960), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 362/362 (100%), Positives = 362/362 (100%), Gaps = 0/362 (0%)

```
Query  1    FENGLGRTPQMGWNSWNHFYCGINEQIIRETADALVNTGLAKLGYQYVNIDDCWAEYSRD  60
            FENGLGRTPQMGWNSWNHFYCGINEQIIRETADALVNTGLAKLGYQYVNIDDCWAEYSRD
Sbjct  56   FENGLGRTPQMGWNSWNHFYCGINEQIIRETADALVNTGLAKLGYQYVNIDDCWAEYSRD  115

Query  61   SQGNFVPNRQTFPSGIKALADYVHAKGLKLGIYSDAGSQTCSNKMPGSLDHEEQDVKTFA  120
            SQGNFVPNRQTFPSGIKALADYVHAKGLKLGIYSDAGSQTCSNKMPGSLDHEEQDVKTFA
Sbjct  116  SQGNFVPNRQTFPSGIKALADYVHAKGLKLGIYSDAGSQTCSNKMPGSLDHEEQDVKTFA  175

Query  121  SWGVDYLKYDNCNDAGRSVMERYTRMSNAMKTYGKNIFFSLCEWGKENPATWAGRMGNSW  180
            SWGVDYLKYDNCNDAGRSVMERYTRMSNAMKTYGKNIFFSLCEWGKENPATWAGRMGNSW
Sbjct  176  SWGVDYLKYDNCNDAGRSVMERYTRMSNAMKTYGKNIFFSLCEWGKENPATWAGRMGNSW  235

Query  181  RTTGDIADNWGSMTSRADENDQWAAYAGPGGWNDPDMLEVGNGGMSEAEYRSHFSIWALA  240
            RTTGDIADNWGSMTSRADENDQWAAYAGPGGWNDPDMLEVGNGGMSEAEYRSHFSIWALA
Sbjct  236  RTTGDIADNWGSMTSRADENDQWAAYAGPGGWNDPDMLEVGNGGMSEAEYRSHFSIWALA  295

Query  241  KAPLLIGCDVRSMSQQTKNILSNSEVIAVNQDSLGVQGKKVQSDNGLEVWAGPLSNNRKA  300
            KAPLLIGCDVRSMSQQTKNILSNSEVIAVNQDSLGVQGKKVQSDNGLEVWAGPLSNNRKA
Sbjct  296  KAPLLIGCDVRSMSQQTKNILSNSEVIAVNQDSLGVQGKKVQSDNGLEVWAGPLSNNRKA  355

Query  301  VVLWNRQSYQATITAHWSNIGLAGSVAVTARDLWAHSSFAAQGQISASVAPHDCKMYVLT  360
            VVLWNRQSYQATITAHWSNIGLAGSVAVTARDLWAHSSFAAQGQISASVAPHDCKMYVLT
Sbjct  356  VVLWNRQSYQATITAHWSNIGLAGSVAVTARDLWAHSSFAAQGQISASVAPHDCKMYVLT  415

Query  361  PN  362
            PN
Sbjct  416  PN  417
```

Figure 5.2: BLASTp results generated for the 1UAS_A alpha-galactosidase sequence.

(A) Graphical representation of BLASTp protein sequence alignment results of 1UAS_A (Query) and only seven of the top best matches. (B) List of sequences of only the seven top best matches producing significant alignment to 1UAS_A. (C) Amino acid sequence alignment of Query (1UAS_A) and the subject (Q9FXT4). The two proteins show a 100% amino acid sequence homology between the entire 362 sequence of 1UAS_A and the region 56-417 amino acids of Q9FXT4.

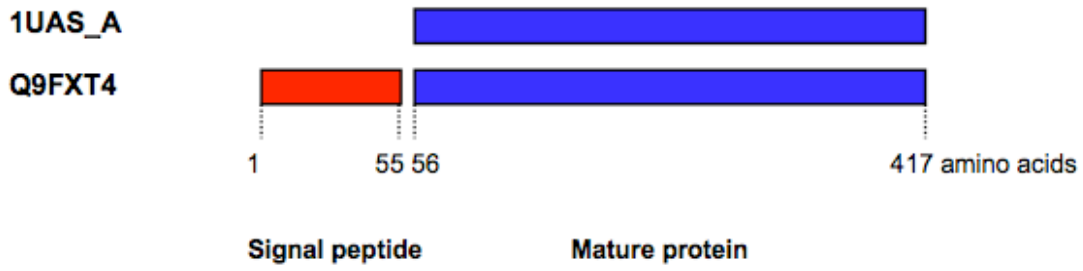


Figure 5.3: Schematic representation of the amino acid sequence alignment of 1UAS_A and Q9FXT4 alpha galactosidase proteins.

1UAS_A is homologous to the 56-417 amino acid region of Q9FXT4. The signal peptide shown in this Figure is as reported by Kim *et al*. (2002).

5.5 The General Distribution and Putative Functional Classification of the Identified Sorghum CF Proteins

The general distribution of the identified sorghum CF proteins is illustrated in Figure 5.4. The number of proteins present in each class is indicated in brackets.

5.5.1 Peroxidases

Figure 5.4 illustrates that 50% of the identified sorghum CF proteins were peroxidases. Peroxidases thus formed the largest group of proteins identified in the secretome of our sorghum cell suspension cultures system (spots 1-7; Figure 5.1; Table 5.1). Extracellular peroxidases have also been identified in other proteomics studies, being secreted by tobacco BY-2 cell suspension cultures (Okushima *et al.*, 2000), *A. thaliana* seedlings (Charmont *et al.*, 2005) and *A. thaliana* cell suspension cultures in response to fungal elicitors (Ndimba *et al.*, 2003). In plants, extracellular peroxidases are bound to cell wall polymers by ionic or covalent bonds (Minibaeva and Gordon, 2003) and can be released into the apoplastic fluids of plant cells or secreted into the culture medium of cell suspension cultures or hydroponically grown seedlings. Therefore, the peroxidases identified in this study can be regarded as *bona fide* residents of the ECM. Furthermore, these heme-containing glycoproteins are encoded for by a large multigene family and thus exist as isoenzymes in individual plant species. The isoenzymes have variable amino acid sequences and show a diverse expression profile (Hiraga *et al.*, 2001). This well-documented existence of a diverse population of peroxidase isoenzymes in literature

further reinforces our observation that, of the 14 identified CF proteins, 50% were peroxidases with a diverse MW and pI range.

Functionally, peroxidases are known to catalyse the oxidoreduction between H_2O_2 and a variety of organic and inorganic substances (Chittoor *et al.*, 1997; Hiraga *et al.*, 2001). Plant peroxidases are thus involved in a number of normal physiological functions including cell wall metabolism (lignification and suberization), auxin metabolism and defense mechanisms against wounding and pathogenic attack (Hiraga *et al.*, 2001).

5.5.2. Germins and Oxalate Oxidases

Germins and oxalate oxidases are discussed together because of their common functional features. Collectively, these two classes of proteins form the second largest protein group identified (36%) in the current study, with a total of three and two isoforms of each protein type respectively (Figure 5.4). Various isoforms of germin proteins have also been identified in other proteomics studies. They were identified in both the apoplastic fluids of *A. thaliana* rosettes (Boudart *et al.*, 2005) and secreted proteins from *A. thaliana* seedlings grown in liquid culture (Charmont *et al.*, 2005). Furthermore, a total of 4 germin-like proteins were found to dominate the apoplastic proteins of rice (*O. sativa*) leaves (Haslam *et al.*, 2003) a result, which is consistent with the findings of the current study. Germins were first discovered in germinating wheat embryos and therefore can be regarded as protein markers of early plant development. Germins are apoplastic, multimetric glycosylated proteins, which are resistant to both heat and protease activities (Lane *et al.*, 1993). These proteins have been shown to have both oxalate oxidase (Lane,

1994) and superoxide dismutase activity (Woo *et al.*, 2000) and therefore, implicated in defense roles against pathogenic attack (Borderies *et al.*, 2003).

Oxalate oxidases and germins with oxalate oxidase activity degrade oxalate in the presence of dioxygen to form CO_2 and H_2O_2 (Lane, 1994). The H_2O_2 produced may (i) be used in the peroxidase-catalysed cross linking reactions of the cell wall thereby initiating and terminating the expansion of the cell wall; (ii) function as a powerful oxidizing agent for the direct destruction of pathogens; and/or (iii) act as a signal transduction molecule during pathogenic infection (Lane, 1994; Low and Merida, 1996). Therefore, both germin and oxalate oxidase proteins may have roles in cell wall biosynthesis and defense mechanisms (Hurkman and Tanaka, 1996) as well as other roles in plant growth and development.

5.5.3 Alpha-galactosidases

Only two isoforms of alpha-galactosidases (spot 8 and 9; Figure 5.1; Table 5.1) were identified in this study. This class of protein thus constituted about 14% of the identified sorghum CF proteins. Alpha-galactosidases are *bona fide* extracellular matrix proteins (Boudart *et al.*, 2005) and have also been identified in the apoplast proteome of tobacco leaf (Dani *et al.*, 2005). They catalyse the hydrolysis of α-1,6-linked galactosyl residues from galacto-oligosaccharides and polymeric galacto-(gluco)mannas (Fujimoto *et al.*, 2003). As such, they are classified into glycoside hydrolase family, a group of proteins that is involved in the modification of cell walls.

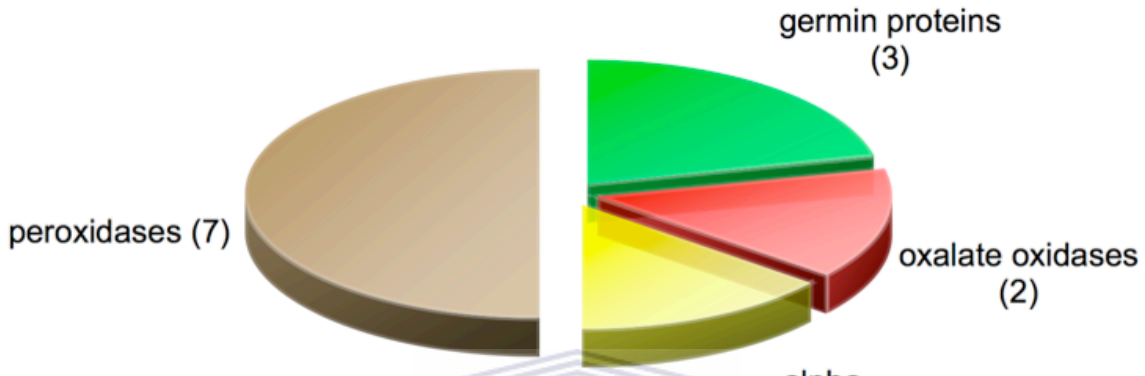


Figure 5.4: Distribution of the four classes of proteins identified in the sorghum culture filtrate fraction.

The identified proteins included peroxidases, germins, oxalate oxidases and alpha galactosidases. The number of proteins present in each class in indicated in brackets.

5.6 Discussion

Proteins secreted in the culture medium of sorghum cell suspension cultures were separated by 2DE. About 25 CBB stained proteins with a diversity of MWs, pIs and abundance were visualised on the gels. The majority of the proteins were limited to the MW and pI ranges of 25-100kD and 4-6 respectively. The dominance of acidic proteins in the secreted proteome fractions of plants has been reported in other proteomic studies. About 50% of proteins secreted into the culture medium of *A. thaliana* seedlings had acidic pIs (Charmont et al., 2005). Furthermore, Jamet *et al*. (2006) reported that the majority of secreted proteins found in the culture medium of cell suspensions or hydroponically grown seedlings are acidic, with pI ranges of between 2 and 6. These reports are in agreement with observations in the current study.

Out of the 25 observed CBB stained protein spots, 15 highly abundant and well-resolved spots (Figure 5.1) were selected for MS analysis using a combination of MALDI-TOF and MALDI-TOF-TOF MS. Of these 25 protein spots, 14 were positively identified (Table 5.1). The identified proteins matched the identities of peroxidases, alpha-galactosidases, oxalate oxidases and germin proteins from other grasses. This observation demonstrates the close phylogenetic relationship between sorghum and other grasses such as rice, maize and barley. Nevertheless, the observed differences between the theoretical and experimental MW and pI values of the respective protein spots (Table 5.1) further indicates the species to species variations in the amino acid sequences of the respective proteins.

Of the identified proteins, 12 were predicted to have N-terminal signal peptides (SignalP predictions; Table 5.2), while germin E protein (spots 13 and 14) was previously reported as a secretory protein (Wu *et al.*, 2000). Signal peptides target proteins to the secretory pathway. Therefore the positive signal peptide predictions in proteins secreted in the culture medium, an extracellular compartment of suspension-cultured cells, reinforces the fact that these CF proteins are *bona fide* secretory proteins. SignalP predictions therefore provided bioinformatics evidence that the identified proteins were indeed destined for the ECM. In addition, the absence of any unexpected cytoplasmic proteins in the CF protein preparations further validates the reliability of our experimental system.

All four classes of identified proteins were represented in multiple spots on the 2D gels (Table 5.1), an observation that has been reported in other studies (Chivasa *et al.*, 2002; Ndimba *et al.*, 2005; Oh *et al.*, 2005). As previously discussed in Section 4.7, the representation of the same protein in multiple spots may indicate, amongst others; (i) the existence of different forms of PTMs and/or (ii) amino acid sequence differences present in isoenzymes encoded for by multigene families (Albertin *et al.*, 2009). Functionally, the identified proteins exhibit a variety of putative biological roles. These range from cell wall metabolism, defense mechanisms against pathogenic attack and/or signal transduction processes. The identification of numerous oxidoreductases (peroxidases) and defense proteins (germins and oxalate oxidases) even at resting conditions may be expected in artificial systems such as cell suspension cultures (Isaacson and Rose, 2006). It is known that the cell suspension cultures are non-physiological systems which exert both mechanical stress to the cells during continuous agitation and oxidative stress that

occurs in liquid culture medium (Jamet *et al.*, 2006). These stress conditions produce ROS such as H_2O_2, which would be used by peroxidases in a range of oxidation-reduction enzymatic reactions.

However, not all classes of the expected secretory proteins were identified in this study. This may be due to limitations of the 2D-PAGE proteomics approaches. These limitations relate to (i) protein solubility, (ii) protein abundance and (iii) the under representation of proteins with extreme MW and pIs. Firstly, secretomics offers technical challenges relating to protein solubility as some proteins are embedded in an insoluble polysaccharide matrix (Rose *et al.*, 2004). The solubility of these proteins is thus drastically reduced. As a result, only a partial fraction of the secretome is represented on the 2D gels for further analysis by mass spectrometry and database searches. This reasoning could possibly explain the low total number of visible CBB stained proteins (25 protein spots) observed on the whole sorghum secretome in this study. Secondly, since only the highly abundant protein spots were selected for MS analysis, low abundant spots and/or poorly resolved ones are not represented here. Thirdly, it was observed that the majority of the proteins fell in the MW and pI ranges of 25-100 kDa and 4-6 respectively. This suggests that not all proteins with extreme MW and pI outside these ranges were presented in this study.

Since the ultimate goal of any proteomics study is to fully characterise the total proteome of a biological system at a specific time, under well-defined physiological conditions (Anderson and Anderson, 1998; Patterson, 2004) the mapping of this sorghum secretome

proteome has to be improved. This can be achieved firstly by improving protein solubility prior to 2DE analysis. Secondly, during 2D gel separation, different narrow range IPG strips covering the whole pH range of 3-10 should be used. Thirdly, protein loading per strip should be increased. All these technical adjustments would contribute to improving the separation and/or the abundance of protein spots observed on the 2D gels and ultimately resulting in improved coverage of the sorghum proteome.

Nevertheless, this current study has provided insight into the identities of proteins secreted in the culture medium of sorghum cell suspensions. It is known that subcellular localisation of proteins is an important clue to its function. Additionally, secreted proteins are involved in a variety of functions involved in the normal physiological and defense related processes. Therefore, this sorghum secretome map and it changes following various stimuli would provides vital information towards the understanding of the growth and development of sorghum. As such, this secretome map would be used to land mark salt (NaCl) and/or hyperosmotic (sorbitol) stress responsive proteins secreted in the culture medium of sorghum cell suspension cultures (Chapter 7).

CHAPTER 6

Identification of Salt Stress Responsive Proteins of the Leaf Tissues of Various Sorghum Varieties

6.1 Introduction

High soil salinity (reviewed in Section 1.4) affects plant growth and development by inducing hyperosmotic stress, ion toxicity, nutrient deficiency and oxidative stress. It is more prone in the semi-arid/arid areas and those that depend of irrigation water for supplementing erratic rainfall. Agriculturally important cereals such as rice and maize are sensitive to salt (Mahajan and Tuteja, 2005; Munns and Tester, 2008) and thus show reduced productivities when cultivated in highly saline soils. With the increasing soil salinization, which is projected to affect more than 50% of all arable lands by the year 2050 (Wang, Vinocur *et al.*, 2003) as well as the growing world population, there is increasing need to develop crops that are well adapted to salt stress.

Proteomic approaches have been used to study salt stress responsive protein expression in agriculturally important crops such as rice (*Oryza sativa*; Parker *et al.*, 2006), potato (*Solanum tuberosum*; Aghaei *et al.*, 2008) and foxtail millet (*Setaria italica* L. cv Prasad; Veeranagamallaiah *et al.*, 2008) amongst others. These studies provide information on the identities of proteins whose abundances increase or decrease following salt stress. Consequently, biological processes and metabolic pathways that are involved in salt tolerance or those that are affected by salt stress are identified. This is in line with the knowledge that salt stress like all abiotic stresses causes changes in gene expression

(Hasegawa *et al.*, 2000; Seki *et al.*, 2003), which ultimately affects the expression of gene products, the proteins. Despite developments in the availability and improved accessibility of proteomic technologies worldwide, no proteomics related work has been done on sorghum outside of our research group.

Sorghum is considered to be moderately tolerant to salt, being particularly more tolerant than maize (Krishnamurthy *et al.*, 2007), the world's most cultivated crop (FAOSTAT, 2006). 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 (Krishnamurthy *et al.*, 2007) transcriptome changes in response to dehydration and high salinity (Buchanan *et al.*, 2005), the evaluation of growth parameters, ion accumulation (Netondo *et al.*, 2004) and soluble carbohydrate contents (Almodares, Hadi, and Ahmadpour, 2008; Almodares, Hadi, and Dosti, 2008), 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 (reviewed in Section 1.4.1.1.1), there is need to identify the specific proteins that contribute to salt tolerance mechanisms. It is these proteins that are functionally active in cellular processes and therefore, measurement of protein expression would thus provide a better indication of gene functions under salt stress.

The objectives of this chapter are to (i) isolate, separate, visualise and identify salt stress responsive proteins in the leaf tissue of sorghum, (ii) compare protein expression profile of salt responsive protein in two sorghum varieties; AS6 and MN1618.

6.2 Screening of Sorghum Seed Varieties for Salt Tolerance

Thirteen sorghum varieties (Section 2.4) were screened for relative tolerance to salt stress. The objective was to obtain two seed types, one relatively salt sensitive and the other relatively more salt tolerant for use in subsequent comparative proteomic analysis of salt stress responsive proteins (Section 6.3). Prior to the seed screening experiment, the level of salt stress to be used in the study was determined. Surface decontaminated (Section 2.5.1) white sorghum seeds purchased from Agricol (Section 2.4) were germinated on solid MS sorghum seed growth medium (Section 2.3) supplemented with 0 mM (control), 50, 100, 150 and 200 mM NaCl for 14 days (Section 2.6.2.1). Figure 6.1 illustrates the effects of 0-200 mM NaCl on the growth of white sorghum seeds at 10 days post plating. It was observed that salt stress levels of 150 and 200 mM NaCl drastically reduced seed germination and growth of the seedlings. For this reason, 100 mM NaCl was selected as the concentration for use in the subsequent salt treatment experiments of the whole plant system.

The thirteen sorghum seed varieties (Section 2.4) were screened for salt tolerance by germinating them on solid MS sorghum seed growth medium supplemented with 100 mM NaCl (salt stress) or without NaCl (control) for 14 days (Section 2.6.2.1). Effect of salt stress on the growth of seed varieties was presented as percentage reduction in shoot

growth at 14 days post plating. Of these 13 sorghum varieties, a total of seven (AS4, AS17, MN1500, MN4320, MN1812, SAR16 and SAR29) showed very poor or no germination even under control conditions. This possibly indicated reduced viability of the respective seeds. The other six seed varieties (AS1, AS6, AS7, AS12, MN1618 and MN1435) showed varying degrees of salt tolerance as shown in Figure 6.2. From these six varieties, AS6 (salt sensitive) and MN1618 (salt tolerant) were selected on the basis of contrasting levels of tolerance to 100 mM NaCl. From the three relatively salt tolerant seed types (AS12, MN1618 and MN1435), MN1618 was chosen based on the amount of seed material that was available to cover all protein extraction procedures for the entire study. Therefore, sorghum varieties AS6 and MN1618 were used as experimental material for comparative proteomic analysis of salt stress responsive proteins of sorghum whole plant systems (Section 6.3).

6.3 Salt Stress Treatment and Protein Extraction from the Leaf, Sheath and Root Tissues of Sorghum Seedlings

Two sorghum varieties, AS6 and MN1618, were used as sources of plant material (Section 6.2). The sorghum seeds were surface decontaminated (Section 2.5.1) and germinated on MS sorghum seed germination medium supplemented with 100 NaCl (salt stress) or without NaCl (control) as described in Section 2.6.2.2. At 14 days post plating, sorghum seedlings were harvested and leaf, sheath and root tissues were carefully excised and stored at -20°C. Soluble protein was extracted and quantified using the Bradford assay as described in Section 2.8.

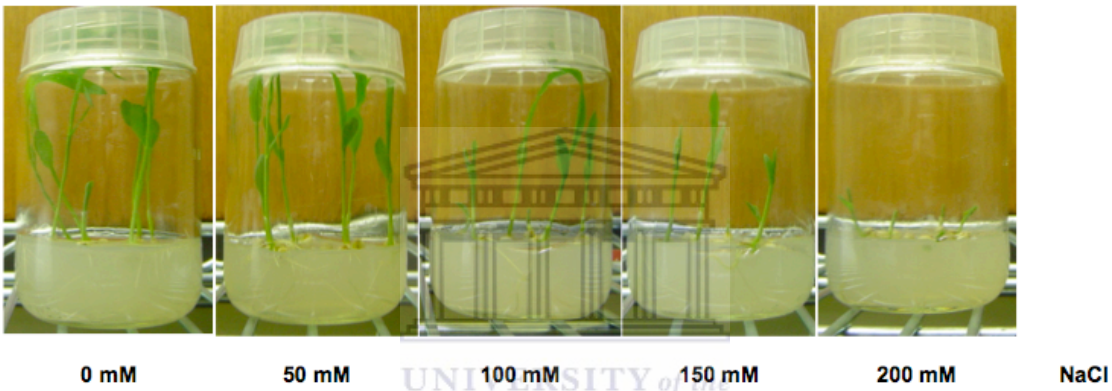


Figure 6.1: Effect of different concentrations of NaCl on the germination and growth of sorghum.

White sorghum seeds purchased from Agricol were surface decontaminated and germinated on MS medium supplemented with 0-200 mM NaCl for a total of 14 days. The picture was taken at 10 days post plating.

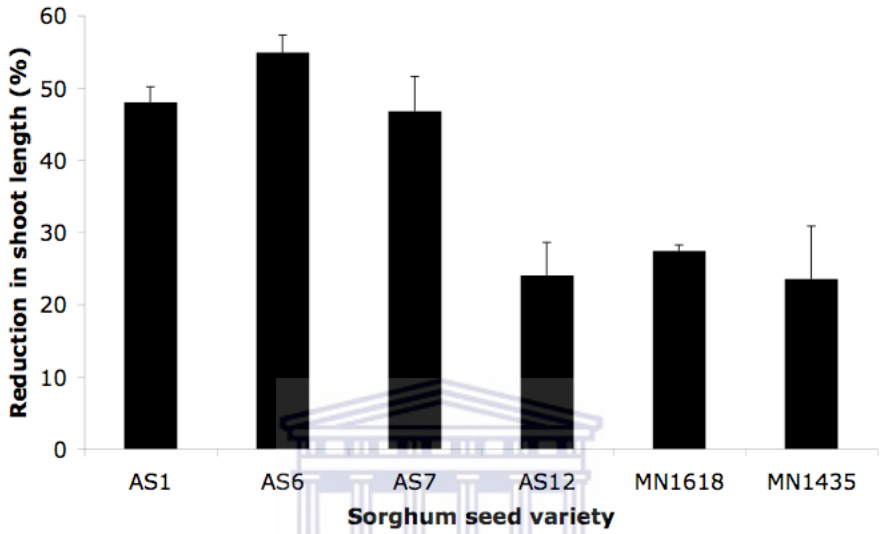


Figure 6.2: Effect of 100 mM NaCl on shoot growth of various sorghum varieties.

Thirteen sorghum seed varieties were surface decontaminated and germinated on MS medium supplemented with 100 mM NaCl (salt stress) or without NaCl (control). At 14 days post plating, seedlings were harvested and shoot length measured for an average of 10 seedlings per treatment group per sorghum variety. The effect of salt stress on each sorghum variety was presented as percentage reduction in shoot length. Of these varieties, AS6 (salt sensitive) and MN1618 (salt tolerant) were chosen as experimental material for use in a proteomic analysis of salt stress responsive proteins of sorghum whole plant systems. Vertical bars indicate standard error of the mean of percentage reduction in shoot length.

6.3.1 One Dimensional Protein Profiles of Sorghum Leaf, Sheath and Root Tissues Following Salt Stress

One dimensional (1D) gel electrophoresis (Section 2.8) was carried out to evaluate the quality and loading quantities of the protein extracts prior to 2D gel electrophoresis. Figure 6.3 illustrates CBB stained 1D profiles of control and salt treated leaf and sheath samples from both sorghum varieties. Figure 6.3 A: AS 6 leaf; B: MN 1618 leaf; C: AS 6 sheath; D: MN 1618 sheath. Lanes M shows the molecular weight markers. Lanes 1-4 and 5-8 represent protein profiles from four independent biological replicate extractions for the control and salt treated tissue samples respectively. Each lane was loaded with approximately 10 µg of soluble protein of each respective tissue extract. It was observed that the protein expression, abundance and loading across the biological replicates was relatively uniform in both the leaf (Figure 6.3A and B) and sheath (Figure 6.3C and D) extracts as previously observed and discussed in Section 4.3. This suggests that protein preparation was reproducible between independent extractions. Figure 6.4 shows CBB stained 1D profiles of the root extracts from both sorghum varieties. Lanes M shows the molecular weight markers. Lanes R1: AS6 root, 0 mM NaCl; R2: AS6 root, 100 mM NaCl; R3: MN1618 root, 0 mM NaCl; R4 MN1618 root 100 mM NaCl. Each lane was loaded with approximately 20 µg of soluble root protein. It was observed that the protein extracts were of high quality, showing no streaking.

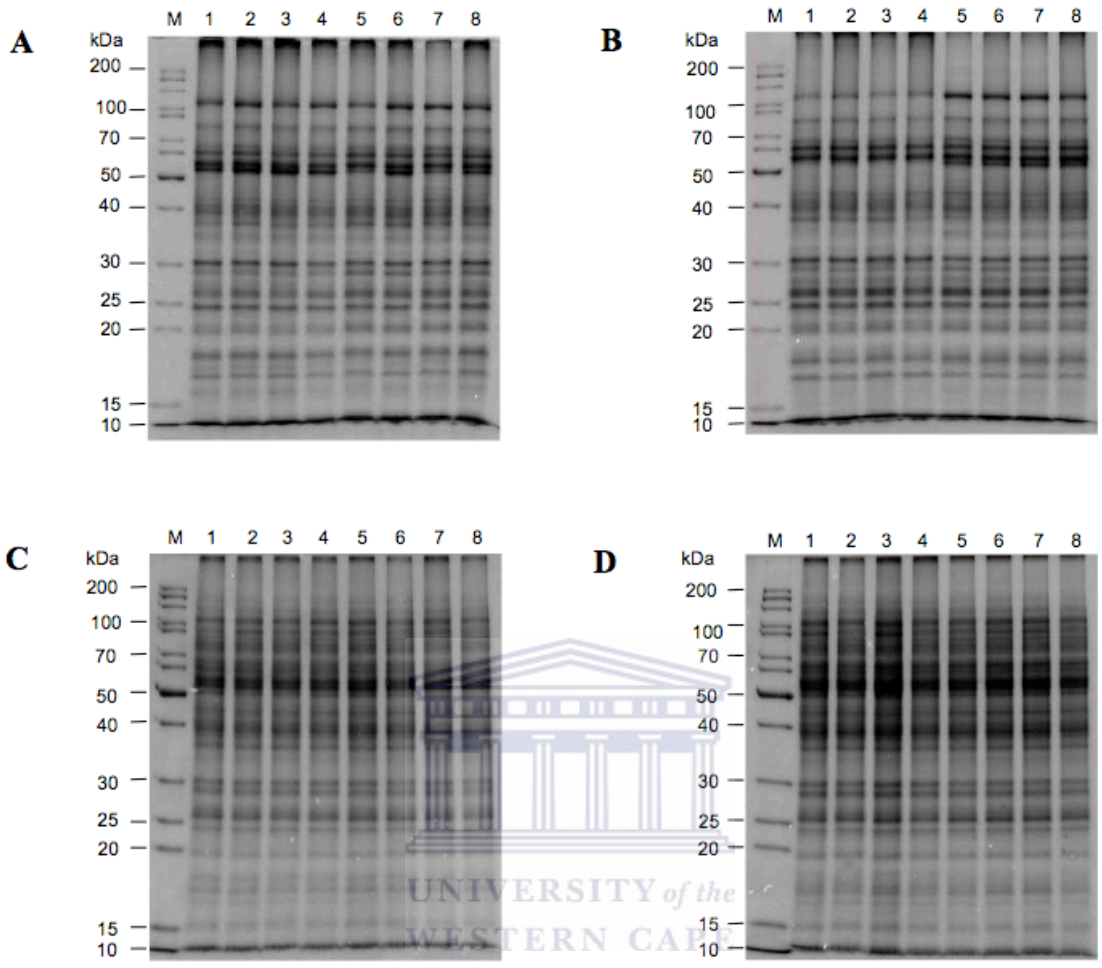


Figure 6.3: One-dimensional gel electrophoresis of protein extracts of control and salt treated leaf and sheath tissues of AS6 and MN1618 sorghum varieties.

Approximately 10 µg of soluble protein of each tissue extract was loaded onto 12% SDS-PAGE gel and stained with CBB. (A) AS6 leaf, (B) MN1618 leaf, (C) AS6 sheath and (D) MN1618 sheath. Lane M is the molecular weight marker. Lanes 1-4 (A-D) and Lanes 5-8 (A-D) represent independent biological replicate protein extracts of the leaf and sheath samples under control (0 Mm NaCl) and salt treatment (100 mM NaCl) respectively.

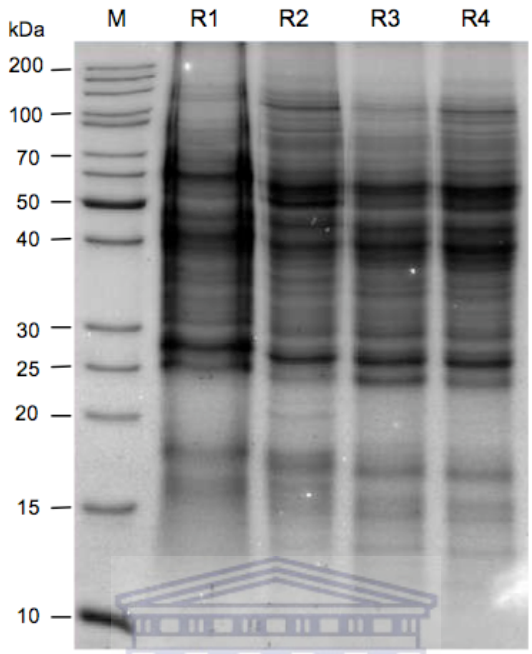


Figure 6.4: One-dimensional gel electrophoresis of control and salt treated root protein extracts of AS6 and MN1618 sorghum varieties.

Approximately 20 µg of soluble protein of each root extract was loaded onto 12% SDS-PAGE gel and stained with CBB. Lanes R1 and R3: control (0 mM NaCl) root extracts of AS6 and MN1618 sorghum varieties respectively. Lanes R2 and R4: salt treated (100 mM NaCl) root extracts of AS6 and MN1618 sorghum varieties respectively.

6.3.2 Heat Shock Protein 70 Expression Pattern in Sorghum Leaf, Sheath and Root Following Salt Stress

The main objective of this Chapter was to identify salt stress responsive proteins in leaf tissues of two different sorghum varieties (AS6 and MN1618). To determine if 100 mM NaCl treatment used in this study was capable of inducing hypersalinity stress responses at the protein level, we investigated the expression of Hsp70, a known stress responsive protein (Wang *et al.*, 2004; Ndimba *et al.*, 2005) in all three tissues. Western blotting techniques using the human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody were used to detect the expression of Hsp70 in the different tissues as described in Section 2.13.

6.3.2.1 Heat Shock Protein 70 Western Blotting on 1D Protein Profiles of Leaf, Sheath and Root Tissues

Three biological replicates of each of the control (0 mM NaCl) and salt (100 mM NaCl) treated leaf and sheath samples of both AS6 and MN1618 sorghum varieties were used. For the root protein samples, only one extract per treatment group per sorghum variety was used. Figure 6.5 shows the 1D profile of Hsp70 expression pattern between the leaf, sheath and root tissues of the two sorghum varieties. Both the western blotting results and the corresponding CBB stained gel panels are shown. Figure 6.5A: leaf; B**:** sheath; C: root tissues for both AS6 and MN1618 sorghum varieties. Lanes M shows the molecular weight markers. Figure 6.5A: Lanes 1-3 and 4-6 represent AS6 control and salt treated leaf extracts respectively; Lanes 7-9 and 10-12 represent MN1618 control and salt treated leaf extracts respectively. Figure 6.5B: Lanes 1-3 and 4-6 represent AS6 control and salt

treated sheath extracts respectively; Lanes 7-9 and 10-12 represent MN1618 control and salt treated sheath extracts respectively. Figure 6.5C: Lanes R1 and R2 represent AS6 control and salt treated root extracts respectively. Lane R3 and R4 represent MN1618 control and salt treated root extracts respectively. About 10 μg of soluble protein was loaded per well for each of the leaf and sheath samples (Figure 6.5A and B) while 20 μg of soluble protein was loaded for each of the root samples (Figure 6.5C).

For all the three tissues, it was observed that only one protein band with an approximate relative MW of 77 was reactive to the Hsp70 antibodies used in this study. Under control conditions, basal levels of Hsp70 were detected in all three tissues (leaf, sheath and root) for both AS6 and MN1618 sorghum varieties. However, these initial abundance levels of Hsp70 differed between the sorghum varieties. For the leaf tissue (Figure 6.5A), under control conditions AS6 samples (Lane 1-3) showed higher basal levels of Hsp70 compared to the MN1618 samples (Lanes 7-9). After treatment with 100 mM NaCl, the abundance levels of Hsp70 increased in the leaf tissues of both sorghum varieties. This expression pattern resulted in approximately the same levels of salt stress induced Hsp70 in both AS6 leaf samples (Figure 6.5A: Lanes 4-6) and MN1618 leaf samples (Figure 6.5A; Lanes 10-12). The Hsp70 expression pattern observed for the sheath samples (control and 100 mM NaCl; Figure 6.5B) was consistent with the leaf results for both sorghum varieties. However, the root tissue (Figure 6.5C) showed a different Hsp70 expression pattern from that observed in either the leaf or sheath tissues. Under control conditions (Figure 6.5C Lanes R1 and R3 for AS6 and MN1618 respectively), both sorghum varieties had very negligible root basal levels of Hsp70.

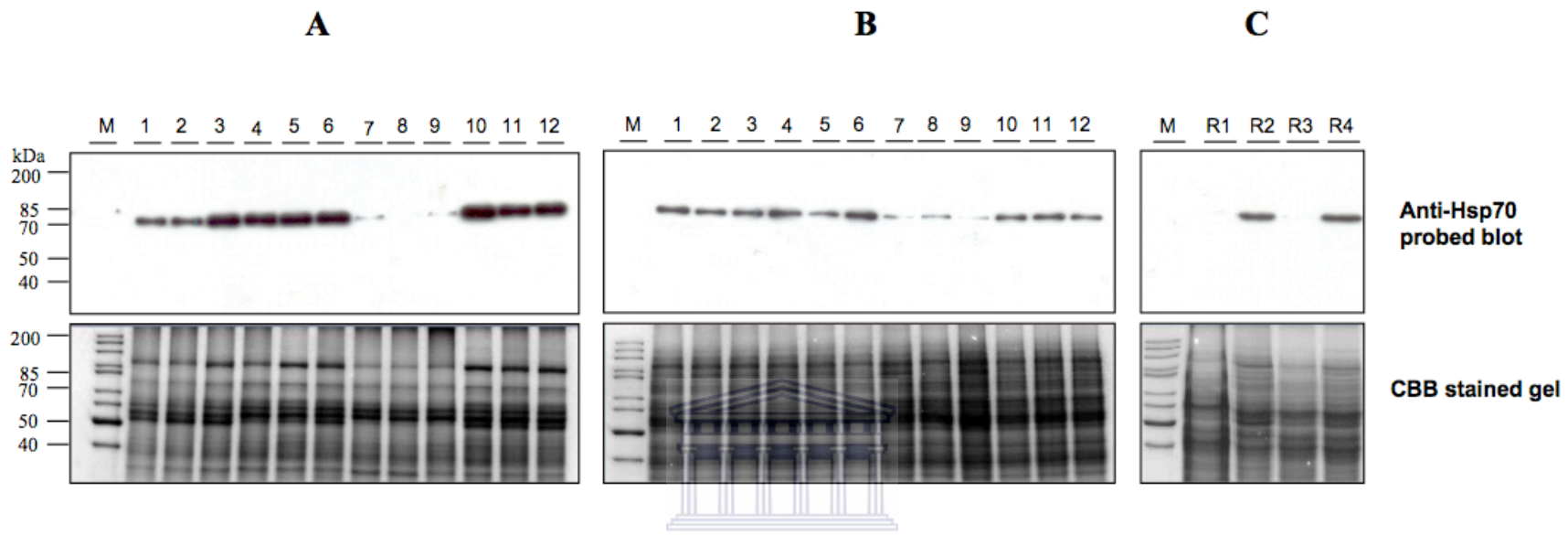


Figure 6.5: Western blotting analysis of Hsp70 expression patterns on 1D profiles of control and salt treated sorghum leaf, sheath and root protein extracts.

Both the immunoblot and the corresponding CBB stained gel panels are shown. (A) leaf, (B) sheath and (C) root protein extracts of both sorghum varieties. Lane M is the molecular weight marker. (A) Lanes 1-3 and 4-6 represent AS6 control and salt treated leaf extracts respectively; Lanes 7-9 and 10-12 represent MN1618 control and salt treated leaf extracts respectively. (B) Lanes 1-3 and 4-6 represent AS6 control and salt treated sheath extracts respectively; Lanes 7-9 and 10-12 represent MN1618 control and salt treated sheath extracts respectively. (C) Lanes R1 and R2 represent AS6 control and salt treated root extracts respectively; Lane R3 and R4 represent MN1618 control and salt treated root extracts respectively.

However, following salt treatment, both AS6 (Lane R2) and MN1618 (Lane R4) root samples exhibited higher Hsp70 abundances compared with the controls. The levels of root Hsp70 before (Lanes R1; R3) and after (R2; R4) salt treatment was relatively similar between the two sorghum varieties. This similar pattern of Hsp70 response in root of both varieties possibly indicates that both root systems perceive and respond to salt stress in a similar manner and to the same extent.

6.3.2.2 Heat Shock Protein 70 Western Blotting on 2D Protein Profiles of MN1618 Sorghum Sheath

After having had detected increased levels of Hsp70 following salt stress treatment on 1D gels of all three tissues for both sorghum varieties (Figure 6.5), Hsp70 Western blotting analysis was conducted on 2D gels. The objective of this experiment was to resolve the Hsp70 protein(s) present in sorghum and confirm their identities using MALDI-TOF MS and database searches. The MN1618 sheath sample was randomly selected for this exercise. Two-dimensional gel electrophoresis of sheath samples was carried out on 7 cm strips, pH range 4-7 as described on Section 2.10. About 150 μg of protein was loaded onto the IPG strips for both the control and salt treated samples. Western blotting analysis of Hsp70 was carried out as described in Section 2.13.

Figure 6.6 shows western blotting results of Hsp70 expression pattern on 2D profile of MN1618 sheath samples. Figure 6.6A: MN1618 control sheath; B: MN1618 salt treated sheath. Both the western blot result and the corresponding CBB stained gel are shown. As previously observed on the 1D western blot results (Figure 6.5), Hsp70 abundance levels

on 2D gels also increased following salt treatment (Figure 6.6). The Hsp70 immunological reactive region on the 2D gel consisted of three closely migrating spots at a MW of approximately 77 kDa (spots S1; S2; S3; Figure 6.6). The pIs of these protein spots ranged between 5.3-5.5. The three spots were picked from CBB stained gels for protein identification using MALDI-TOF MS and database searches (Section 2.14.1), resulting in the positive identification of two spots, S1 and S2. Proteins with MOWSE scores greater than 66 ($p<0.05$) were considered to be significant matches. Table 6.1 gives a summary of the identified Hsp70 spots, their best match identities, plant species of origin and corresponding NCBI accession numbers. Both the theoretical and experimental MWs and pIs of the proteins are also shown in Table 6.1 to give a comparative analysis of the two ranges of data. Spot S1 and S2 (Figure 6.6) matched the identities of a 70 kDa heat shock cognate protein 2 (NCBI accession AAS57913) and putative Hsp70 (NCBI accession NP_001044757) respectively. Spot S3 did not match any protein with a significant MOWSE score. Collectively, the western blotting results and the positive identification of spots S1 and S2 as heat shock cognate protein and Hsp70 respectively (Table 6.1) gave supporting evidence that our salt stress treatment experiment does indeed induce known salt stress proteins in different sorghum tissues.

6.3.3 The Biological Significance of Hsp70 Salinity Induced Expression

Heat shock protein 70s, are encoded for by a multi-gene family (Sung, Kaplan *et al.*, 2001; Sung, Vierling *et al.*, 2001). Their expression patterns respond to a wide range of abiotic factors such as heat, cold, drought, salinity and oxidative stress (Wang *et al.*,

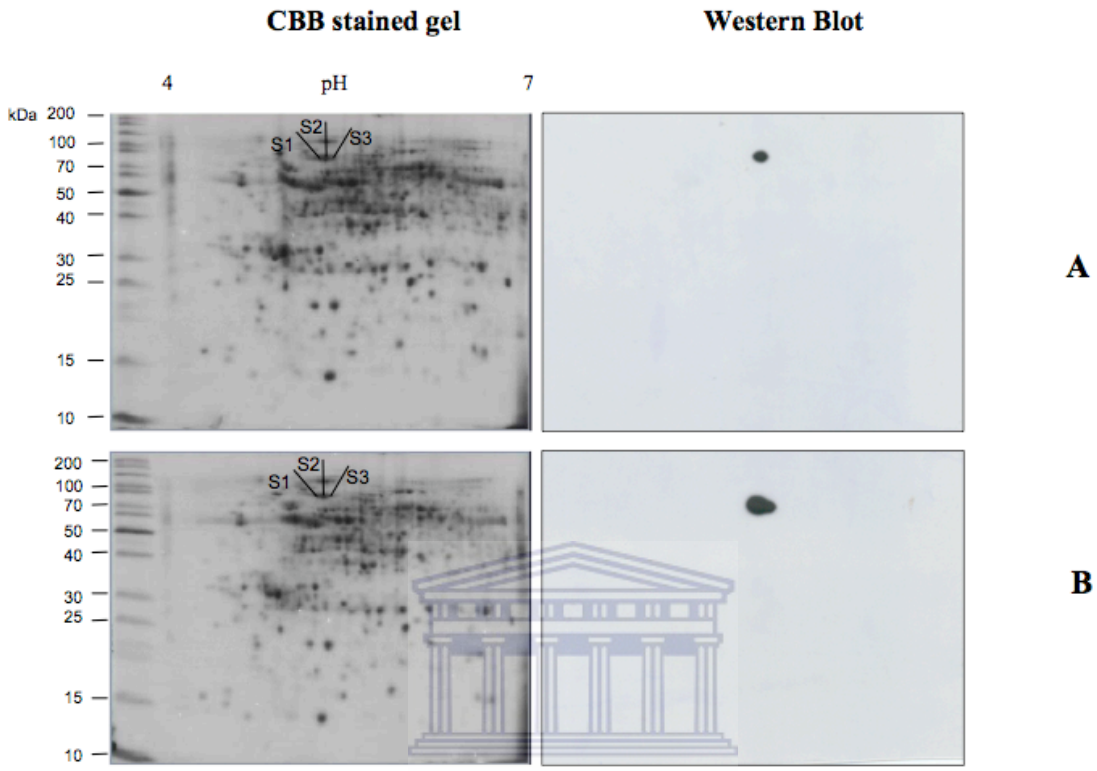


Figure 6.6: Western blotting analysis of Hsp70 expression pattern on 2D profiles of control and salt treated sorghum sheath protein extracts.

Both the western blotting results and the corresponding CBB stained gels are shown. (A) 2D profile of the control sheath protein extract. (B) 2D profile of the salt treated sheath protein extract. The Hsp70 immuno-reactive spots numbered spots S1-S3 were picked for identification using MALDI-TOF MS and database searching.

Table 6.1: Identification of sorghum sheath Hsp70 immuno-reactive spots using MALDI-TOF MS and database searching.

Spot[a)]	Best Protein Match	Species	Accession No. [b)]	MOWSE score	Theoretical MW/pI [c)]	Experimental MW/pI [d)]
S1	70 kDa hsc protein 2	*Vigna radiata*	AAS57913	69	71/ 5.14	77/ 5.3
S2	Putative Hsp70	*Oryza sativa*	NP_001044757	68	71/ 5.1	77/ 5.4
S3	No significant match					77/ 5.5

[a)] Spot number as indicated on the 2D gel images (Figure 6.6).

[b)] Accession number in the National Center for Biotechnology Information (NCBI) database.

[c)] Theoretical MW and pI were obtained from the MASCOT search results.

[d)] Experimental MW and pI were estimated from the 2D gels shown in Figure 6.6.

2004). Plant Hsp70 have identifiable motifs at their C terminus that target them to different subcellular compartments (Guy and Li, 1998). As such, Hsp70 are localised in the cytosol, chloroplast, mitochondria and the endoplasmic reticulum (Kiang and Tsokos, 1998; Sung, Kaplan *et al.*, 2001; Wang *et al.*, 2004). Structurally, Hsp70 consists of a highly conserved N-terminal ATPase domain of approximately 44 kDa and a C-terminal peptide-binding domain of approximately 25 kDa (Miernyk, 1997; Bukau and Horwich, 1998; Kiang and Tsokos, 1998; Wang *et al.*, 2004). Two different forms of this protein exist; the constitutively expressed heat shock cognate 70 (Hsc70) and the stress induced Hsp70, (Miernyk, 1997; Sung, Kaplan *et al.*, 2001; Wang *et al.*, 2004). The constitutively expressed Hsc70s play a role in the folding of *de novo* synthesised proteins, the import and translocation of precursor proteins, as well as targeting damaged proteins for degradation (Sung, Kaplan *et al.*, 2001; Wang *et al.*, 2004). On the other hand, stress induced Hsp70s prevent the aggregation of stress denatured proteins and also facilitate the refolding of proteins to restore their native biological functions (Sung, Kaplan *et al.*, 2001; Wang *et al.*, 2004). As such, Hsp70s are broadly known as molecular chaperones (Miernyk, 1997).

The two heat shock proteins identified in this study (spots S1 and S2; Figure 6.6; Table 6.1) were both cytosolic proteins. Although functional studies on cytosolic heat shock proteins are limited, they are known to be involved in cellular processes such as protein folding, refolding denatured proteins, preventing protein aggregation and also facilitating protein importation processes (Sung, Kaplan *et al.*, 2001). Therefore, the two salt inducible heat shock protein identified in this study (S1 and S2; Table 6.1) might be

involved in chaperone activities that help salt stress denatured proteins to refold as well as to prevent protein aggregation and thus maintain biological activity of protein during salt stress conditions.

6.4 Comparative Analysis of Leaf Salt Stress Responsive Proteins of Two Sorghum Varieties

The main objective of this Chapter was to identify and compare salt stress responsive proteins between the leaf proteomes of AS6 and MN1618 sorghum varieties. Two-dimensional gel electrophoresis of leaf samples was carried out on 7 cm strips, pH range 4-7 as described on Section 2.10. About 100 μg of protein was loaded onto the IPG strips for both the control and salt treated samples. The gels were scanned and imaged using a Molecular Imager PharosFX Plus System (BIO-RAD). Comparative analysis of the leaf salt stress responsive proteins between the two sorghum varieties was performed using PDQuest^{TM} Advanced 2D Analysis Software version 8.0.1 build 055 (BIO-RAD) as described in Section 2.12. Three CBB stained biological replicate gels were used for each treatment group, control (0 mM NaCl) and salt stress (100 mM NaCl) treatment for each sorghum variety. The inclusion of biological replicates in comparative proteomic studies is important for reducing the chances of detecting non-reproducible protein expression differences (artefacts) between experiments. For this reason, all spots included in this analysis were reproducibly expressed amongst the three biological replicates of each treatment group (control and salt treatment) per sorghum variety (AS6 and MN1618).

Figures 6.7 and 6.8 show the PDQuest^{TM} analysis gels for AS6 and MN1618 sorghum leaf experiments respectively. In each experiment, three biological replicate gels are shown per treatment group as well as a master gel that was automatically created by the analysis software. The master gel of each experiment includes all spots that are reproducibly present in all three biological replicate gels per treatment group for both treatment groups. For both experiments, the 2D protein profiles were reproducible between the gels within each treatments group. The 2D gels were of high quality and resolution with no apparent streaking being observed on the gels.

The total number of reproducible CBB stained spots per treatment group (control and salt treatment), per sorghum variety (AS6 and MN1618) are shown in Table 6.2. It was observed that the AS6 leaf proteome had 213 while MN1618 had 183 reproducible spots amongst the three biological replicates of the control treatment group (Table 6.2). This trend of relatively higher spots numbers in AS6 compared to AS6 under control condition is consistent with the reported results on the sorghum leaf proteome mapping exercise (Chapter 4; Table 4.3). However, following salt treatment, about 22 spots (approximately 10%) completely disappeared in AS6 leaf proteome resulting in 191 spots being reproducibly expressed between the three biological replicates of AS6 leaf salt treatment group (Table 6.2). On the other hand, of a total 24, equating to 13% new proteins were induced following salt treatment in the MN1618 leaf proteome (Table 6.2).

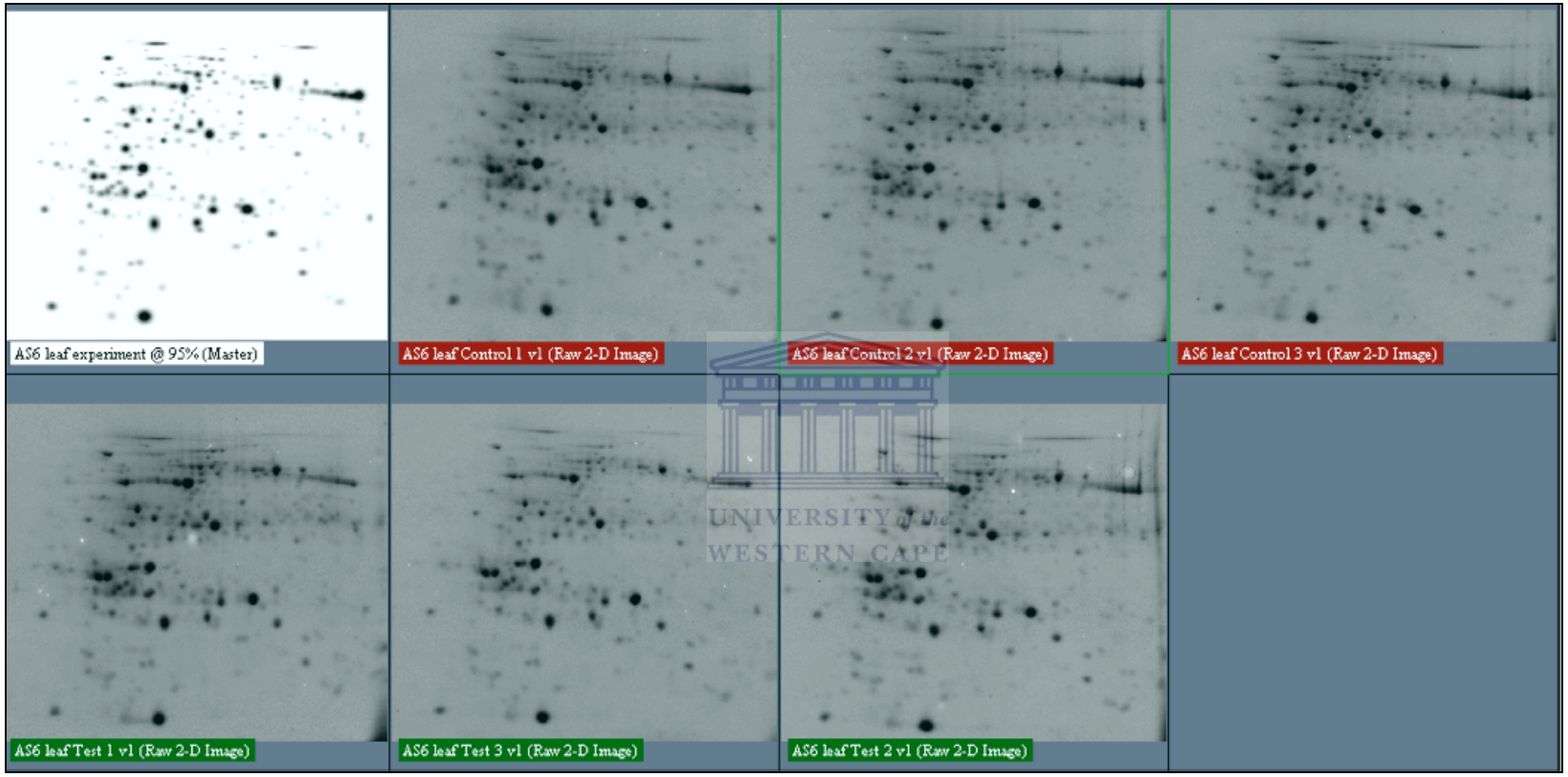


Figure 6.7: PDQuest™ analysis gels of the AS6 sorghum leaf proteome.

Three biological replicates for each treatment group 0 mM NaCl (Control) and 100 mM NaCl (Test) are shown. The master gel was automatically created by the analysis software and it contains all spots that are reproducibly expressed in each of the treatment groups.

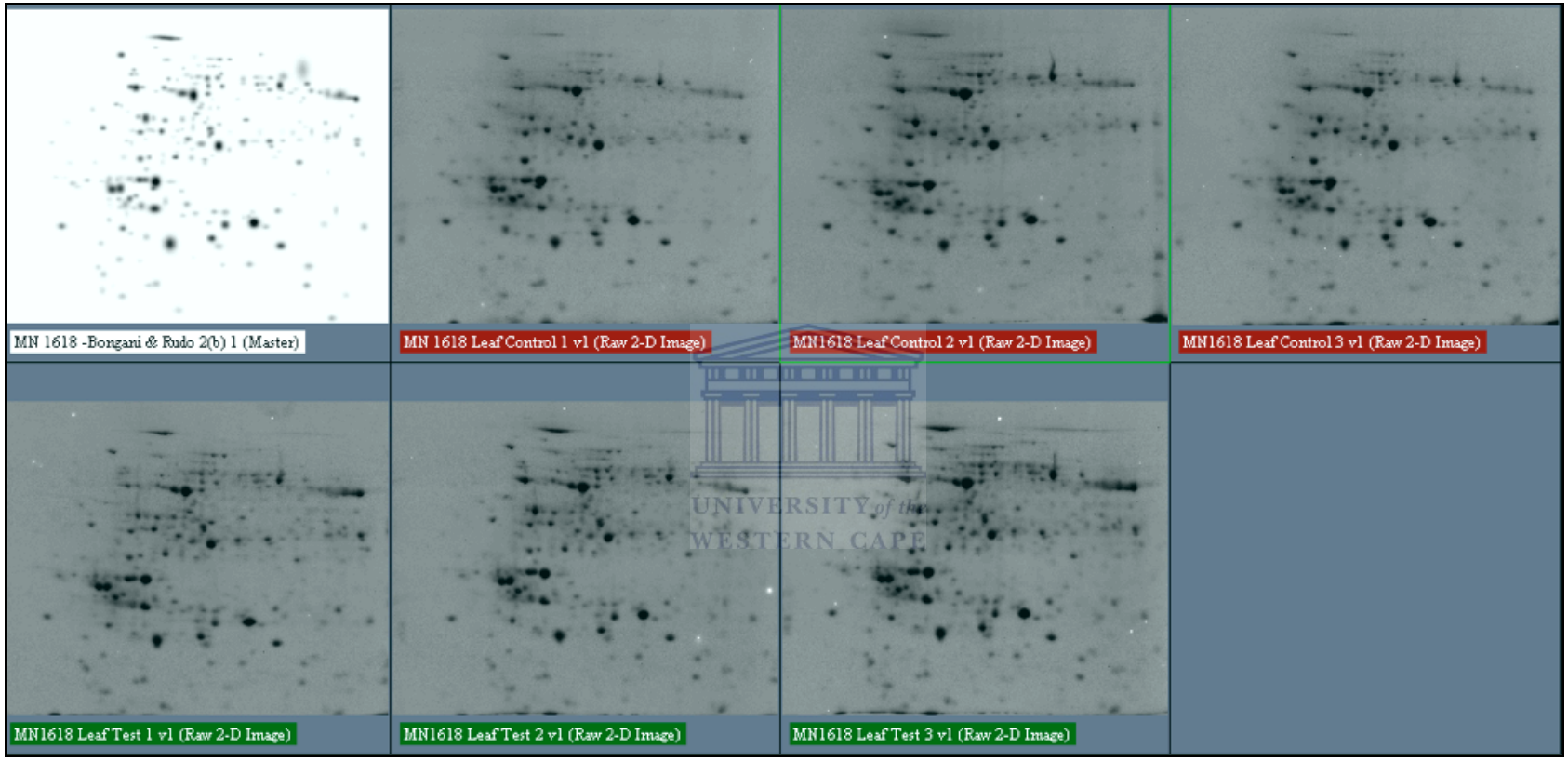


Figure 6.8: PDQuest™ analysis gels of the MN1618 sorghum leaf proteome.

Three biological replicates for each treatment group 0 mM NaCl (Control) and 100 mM NaCl (Test) are shown. The master gel was automatically created by the analysis software and it contains all spots that are reproducibly expressed in each of the treatment groups.

To summarise the differential protein expression patterns between the control and salt treatment groups of AS6 and MN1618 sorghum varieties, qualitative, quantitative and statistical analysis sets were created using the PDQuest^{TM} software (BIO-RAD). A description of the specific parameters used to define each analysis set and the total number of proteins in each set per sorghum variety is described below. Table 6.2 gives a summary of PDQuest^{TM} analysis of the leaf salt stress responsive proteins between the two sorghum varieties while a graphical representation of the data is shown in Figure 6.9.

6.4.1 Analysis Sets for Leaf Salt Stress Responsive Proteins of the Two Sorghum Varieties

6.4.1.1 Qualitative Differential Protein Expression Analysis Set

In this experiment, qualitative spots are defined as spots that were detected in either the control or treatment groups but not in both. Therefore, qualitative spots can either be (i) spots that were induced by salt stress and are thus only present in the salt treatment group; or (ii) spots that completely disappeared after stress treatment and are therefore only present in the control treatment group. The total number of qualitative spots observed following salt stress in AS6 and MN1618 sorghum leaf proteomes were 22 and 24 respectively (Table 6.2; Figure 6.9A). For AS6 variety, all 22 proteins were completely absent after stress treatment. For the MN1618 variety, all 24 spots were induced by salt stress and thus only present in the salt treatment group. This remarkable contrast in qualitative protein expression between the control and salt treatment groups of the two sorghum varieties might have an implication in the overall performance of the varieties under salt stress conditions. The proteins that disappeared following stress

treatment might be components of metabolic processes that are highly sensitive to salt stress. On the contrary, protein spots that were induced following salt stress might represent components of salt stress responsive mechanisms, which contribute to salt tolerance.

6.4.1.2 Quantitative Differential Protein Expression Analysis Set

In this experiment, quantitative spots refer to those with at least a 2-fold expression change (≥ 2-fold) either up- or down- regulated after salt treatment but also meeting the student t-test conditions of statistical significance. It is well accepted that quantitative variations in protein abundance will always exist between gel members within and/or between experiments. These variations may be attributed to either technical errors or variations during the 2DE experimental work-flow or biological variation in the starting plant material, which is not linked to the experimental treatment (Choe and Lee, 2003; Marengo *et al.*, 2005; Parker *et al.*, 2006). To offset these sources of non-reproducible errors and variations in 2D spot profiles, three independent biological replicate gels were included in this analysis as indicated in Figures 6.7 and 6.8. Furthermore, all quantitative spots considered were those with at least a 2-fold abundance change. High fold thresholds used to detect quantitative variations increase the confidence intervals and therefore are appropriate for detecting *bona fide* biological differences in protein expression (Choe and Lee, 2003; Marengo *et al.*, 2005; Parker *et al.*, 2006).

A total of 11 and 29 spots showed at least a 2-fold change in abundance between the control and salt treatment groups of AS6 and MN1618 sorghum leaf proteomes

respectively (Table 6.2; Figure 6.9B). Of these, for AS6 sorghum variety, the abundances of four spots increased, while those of seven, decreased following salt stress treatment. Of the 29 quantitative spots of MN1618 leaf proteome, the abundances of 12 spots increased, while those of 17 decreased following salt treatment (Table 6.2; Figure 6.9B). Altogether, these quantitative changes in protein abundance following stress treatment highlight the protein responsive proteins of sorghum. As observed with the qualitative spots (Section 6.4.1.1; Figure 6.9A), protein spots with increased quantitative abundances following salt stress might have a function in salt tolerance mechanisms, while those with a decrease in abundance might illustrate some of the metabolic pathways that are affected by salt stress.

6.4.1.3 Student's *t*-Test Analysis of Protein Expression after Salt Stress

Comparative analysis of differential protein expression between the control and salt treatment groups of AS6 and MN1618 was also evaluated using the Student's *t*-test at 95% significance level. Using this statistical analysis test, the abundances of 28 AS6 leaf spots and 93 MN1618 leaf spots were shown to be significantly different between the control and salt treatment groups of each respective variety (Table 6.2; Figure 6.9C). Of these, the abundance of 7 spots increased in AS6 while those of 14 spots were decreased (Figure 6.9C). Of the 93 differentially expressed spots in MN1618, the abundances of 51 spots increased, while those of 42 decreased following salt treatment. As observed in both the qualitative and quantitative analysis sets of salt stress responsive proteins, MN1618 showed a higher proportion of salt stress responsive proteins than AS6 sorghum variety. These observations possibly suggest that MN1618 is generally more responsive

to salt at 100 mM NaCl than AS6 sorghum variety. Furthermore, since MN1618 has a greater proportion of protein spots that have increased abundances following salt stress compared to AS6, the former variety could possibly be more tolerant to salt stress than AS6. This confirms the salt screening results, which indicated that MN1618 was relatively more tolerant to 100 mM NaCl than AS6 (Section 6.2; Figure 6.2).

Table 6.2: Summary of comparative proteomics of salt stress responsive leaf proteins between AS6 and MN1618 sorghum varieties.

Sorghum variety	**Total spots** [a]		**Qualitative spots** [b]	**Quantitative spots** [c]	**Student's *t*-test significant spots** [d]
	Control	**100 mM NaCl**			
AS6	213	191	22 [0 induced; 22 down]	11 [4 up; 7 down]	28 [7 up; 14 down]
MN1618	183	207	24 [24 induced; 0 down]	29 [12 up; 17 down]	98 [51 up; 42 down]

[a] Total number of reproducible spots amongst three independent biological replicate gels of each treatment group per sorghum variety.

[b] Number of spots that are only present in either the control or salt treatment groups.

[c] Spots showing at least a 2-fold expression change following salt treatment.

[d] Differentially expressed spots as determined by the Student's *t*-test at 95% significance level.

induced: spots only present in the salt treatment group.

up: spots with increased abundances following salt stress.

down: spots with decreased abundances following salt stress.

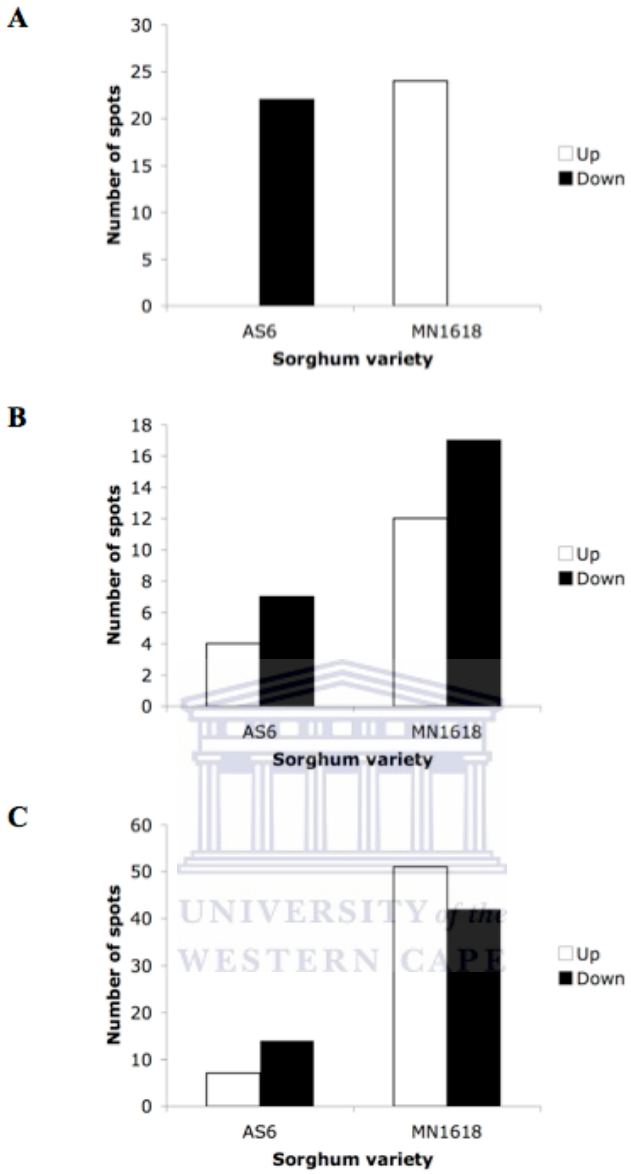


Figure 6.9: A graphical representation of analysis sets of salt stress responsive proteins of AS6 and MN1618 sorghum varieties.

(A) Qualitative spots of each sorghum variety. (B) Quantitative spots showing at least a 2-fold change in abundance between the control and salt treatment groups of each sorghum variety. (C) Student's *t*-test significant spots (at 95% significance level) showing differential expression profiles between the control and salt treatment groups of each sorghum variety. up: spots with increased expression/abundances following salt stress. down: spots with decreased expression/abundances following salt stress.

6.5 Identification of Sorghum Leaf Salt Stress Responsive Proteins Using the Established Sorghum Leaf Proteome Map

As previously discussed in Section 4.1, proteome reference maps provide invaluable tools for land marking differential protein expression patterns under specific physiological states as well as in response to stress conditions (Thelen, 2007). As such, this section describes the application of the established sorghum leaf proteome map (Chapter 4; Figure 4.4; Table 4.1) in identifying salt stress responsive proteins of two sorghum varieties, AS6 and MN1618. Figure 6.10 shows a combination of salt stress responsive and non-responsive spots of both sorghum varieties amongst the 40 spots that were initially picked for MALDI-TOF and MALDI-TOF-TOF MS analysis as a "proof of concept" of the planned mapping exercise (Section 4.5; spots 1-40; Figure 4.4; Table 4.1). Spot numbers highlighted in red are responsive while those in black are non-responsive to salt stress. Table 6.3 summarises the number of salt stress responsive and non-responsive protein spots between the AS6 and MN1618 sorghum varieties from the 40 spots that were picked for the mapping exercise. A graphical representation of this data is given in Figure 6.11B. This data is a subsection of the total number of responsive and non-salt stress responsive proteins in the two varieties (Figure 6.1A). The responsive spots are a union of all analysis sets shown in Table 6.2. Of these 40 spots, a total of 30 (75%) and 10 (25%) were collectively responsive and non-responsive to salt stress respectively, amongst the two sorghum varieties. Of the responsive spots, the abundances of seven and 30 spots changed between the control and salt treatment groups of AS6 and MN1618 sorghum varieties respectively (Table 6.3). Of the seven salt stress responsive leaf proteins of AS6, the abundances of two increased while those of five decreased

following salt stress. For MN1618, out of the 30 salt stress responsive spots, the abundances of 19 spots increased while those of 11 spots decreased following salt stress.

Table 6.4 summarises the effect of 100 mM NaCl treatment on the expression of each one of the 40 spots (spots 1-40; Figure 4.4; Table 4.1; Figure 6.10) used in the mapping exercise. The abundances of the 40 spots (Spots 1-40; Table 6.4) are defined by either an upregulation (up) or downregulation (down) or no change (-) in the normalised spot volume following salt treatment. The differential expression patterns of all up- or downregulated spots of each sorghum variety are correlated with the type of analysis set used to determine the respective differential expression pattern; [c)] qualitative, [d)] quantitative or [e)] Student's *t*-test. The specifications of the parameters used to define each of the analysis sets are defined in Section 6.4.1.

It should be noted that six protein spots (spots 5; 6; 20; 26; 16 and 19) were responsive to salt in both AS6 and MN1618 (Table 6.4). Of these only two, spots 20 and 26 representing a hydroxynitrile lyase and glutathione S transferase respectively has increased abundances in both AS6 and MN1618 (Table 6.4). Spots 16 and 19, which currently does not match any protein on the database (Table 4.1) had reduced abundances in both sorghum varieties (Table 6.4) following salt stress. Interestingly, the abundances of spots 5 and 6, both representing the large subunit of RuBisCo (Table 4.1), were decreased in AS6 but increased in MN1618 (Table 6.4) following salt stress.

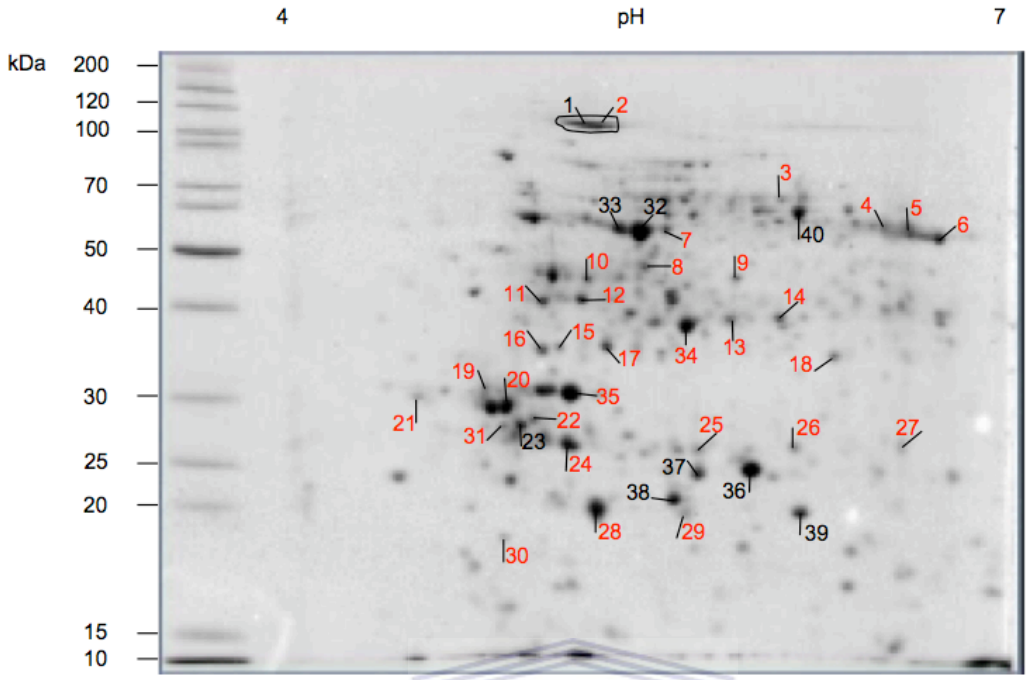


Figure 6.10: The partial sorghum leaf proteome map landmarking salt stress responsive and non-responsive protein spots in the two sorghum varieties.

Spots numbers marked in red indicate salt stress responsive spots while those in black are non-responsive.

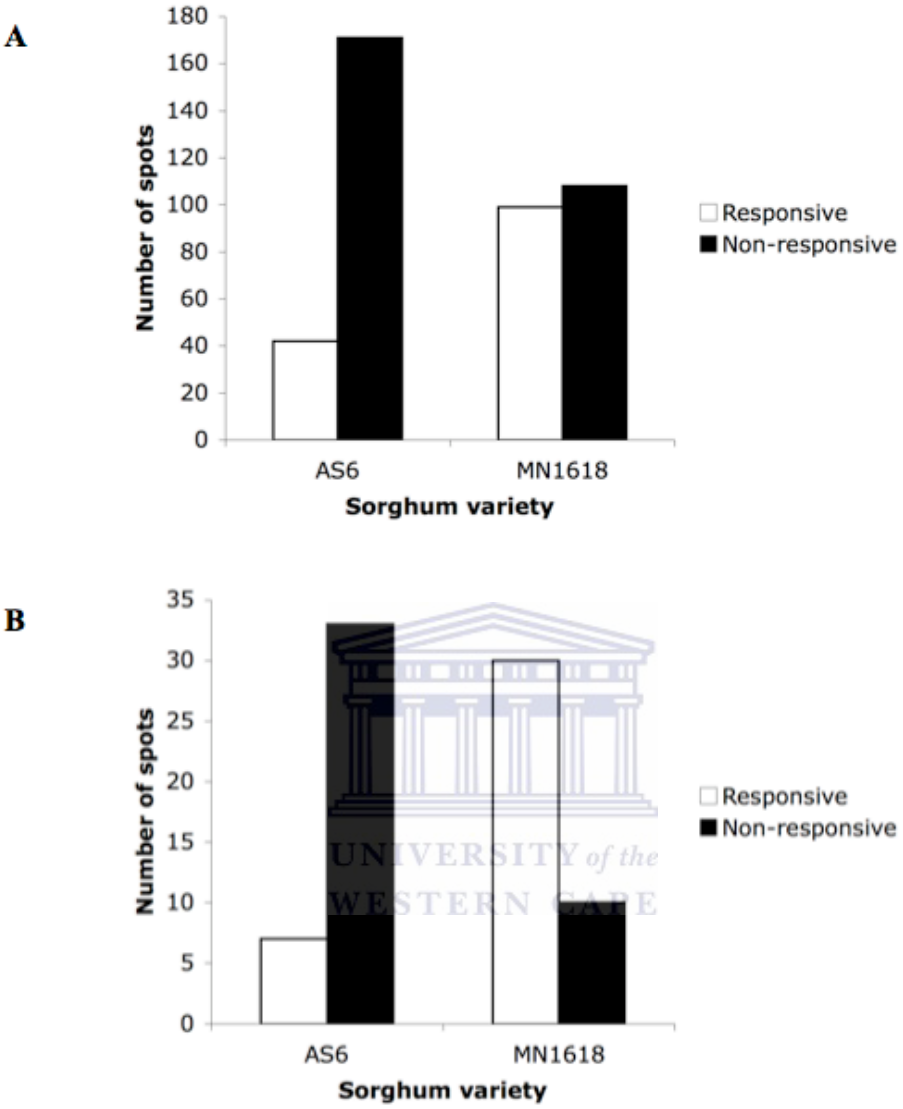


Figure 6.11: A graphical representation of salt stress responsive and non-responsive leaf protein spots of AS6 and MN1618 sorghum varieties.

(A) Total number of responsive and non–responsive spots per seed variety. (B) Subset of responsive and non-responsive spots from of the 40 spots that were picked for the mapping exercise.

The significance of these different protein expression patterns following salt stress are discussed in Section 6.6 below.

Table 6.3: Summary of salt stress responsive and non-responsive leaf protein spots of AS6 and MN1618 sorghum varieties amongst the 40 spots used in the mapping exercise.

Sorghum variety	Responsive spots	Non-responsive spots	Total
AS6	7 (2 up; 5 down)	33	40
MN1618	30 (19 up; 11 down)	10	40

Table 6.4: Effect of salt stress on the MALDI-TOF MS identified protein spots in the two sorghum varieties.

Spot[a)]	Best Protein Match	Accession No[b)]	Sorghum variety	
			AS6	MN1618
	Carbohydrate metabolism			
1	Pyruvate phosphate dikinase	AAP23874	-	-
2	Pyruvate phosphate dikinase	AAP23874	-	up[d; e)]
5	RuBisCO, large subunit	AAM33283	down[d)]	up[d; e)]
6	RuBisCO, large subunit	AAF14707	down[d)]	up[d; e)]
9	Cytosolic 3-phosphoglycerate kinase	AAO32644	-	up[e)]
10	Chloroplastic NADP-dependent malate dehydrogenase	7MDH_D	-	up[d; e)]
14	cytoplasmic NAD-dependent malate dehydrogenase	AAB64290	-	up[e)]
11	Sedoheptulose-1,7-biphosphate precursor	AA022559		down[e)]
12	Phosphoribulokinase, chloroplast precursor	P27774		down[e)]
17	Putative ferrodoxin- NADP oxidoreductase	NP_910234	-	up[d; e)]
18	Ferrodoxin- NADP oxidoreductase	1GAW_ B	-	up[d; e)]
29	Adenosine diphosphate glucose pyrophosphatase	CAC85479	-	down[e)]
39	Adenosine diphosphate glucose pyrophosphatase	CAC85479	-	-
34	Fructose 1,6-biphosphate aldolase precursor	AAF74202	-	down[e)]

35	Oxygen evolving enhancer protein 1 precursor	BAA96365	-	down [e]
36	Photosystem II oxygen-evolving complex protein 2 (fragment)	PA0013	-	-
37	Chlorophyll a/b-binding protein type III precursor	1609235A	-	-
	Proton transport			
7	ATP synthase subunit beta, chloroplastic	1711264A	-	up [e]
32	ATP synthase subunit beta, chloroplastic	1711264A	-	-
33	ATP synthase subunit beta, chloroplastic	1711264A	-	-
13	ATP synthase gamma chain 1, chloroplast	XP_478377	-	up [d]
40	ATP synthase CF1 alpha chain	AAP54723	-	-
	Protein synthesis			
8	Translational elongation factor Tu	XP_466527	-	up [e]
21	Nucleic acid- binding protein- maize		-	down [e]
	Hydrolytic enzymes			
20	Hydroxynitrile lyase	1GXS_C	up [e]	up [e]
28	Hydroxynitrile lyase	1GXS_C	-	up [e]
	Nucleic acid metabolism			
24	Putative adenylate kinase, chloroplast	XP_479721	-	up [e]
	Detoxifying enzymes			
26	Glutathione transferase (EC 2.5.1.18) I	P12653	up [e]	up [e]

	Spots with no significant matches		
3		*	up [c; e)]
4		down [d)]	-
15		-	up [d; e)]
16		down [d; e)]	down [e)]
19		down [e)]	down [d)]
22		-	down [e)]
23		-	-
25		-	up [e)]
27		*	up [c)]
30		-	down [e)]
31		*	down [d; e)]
38		-	-

[a)] Spot number as indicated on the 2D gel image (Figure 6.10).

[b)] Accession number in the National Center for Biotechnology Information (NCBI) database.

[c)] Spots only present in either the control or salt treatment group.

[d)] Spot showing at least a 2-fold expression change following salt treatment.

[e)] Differential expression as determined by the Student's *t*-test at 95% significance level.

* Spot not present either in the control or salt treatment groups of AS6 (MN1618 specific spot).

up: increased abundance following salt stress.

down: decrease in abundance following salt stress.

-: no significant change in abundance between the control and salt treatment group.

6.6 Biological Significance of the Differential Protein Expression Following Salt Treatment Between the Two Sorghum Varieties

In order to speculate on the physiological implications of the observed differential protein expression patterns on salt tolerance and/or susceptibility between the two sorghum varieties (Table 6.4), the stress responsive proteins were discussed within each functional group as previously described in Chapter 4; Table 4.1.

6.6.1 Carbohydrate Metabolism

Of the 17 protein spots identified to have putative functions in carbohydrate metabolism (Table 4.1; Section 4.5.2.1), a total of 2 and 13 were responsive to salt stress in AS6 and MN1618 sorghum varieties respectively (Table 6.4). Three of the spots that are involved the light reactions of photosynthesis, namely, the oxygen evolving enhancer (OEE) protein 1 precursor (spot 35) and two ferrodoxin-NADP oxidoreductases (spots 17 and 18) were responsive to salt stress only in the MN1618 sorghum variety. As outlined in Table 6.4, the abundance of OEE protein 1 precursor spot decreased following salt stress, while those of the two ferrodoxin-NADP oxidoreductases increased. The reduced levels of OEE protein 1 precursors has also been reported in the proteomic analysis of potato under salt stress (Aghaei *et al.*, 2008) and in holm oak (*Quercus ilex*) under drought stress (Echevarria-Zomeno *et al.*, 2009). As previously discussed in Section 4.5.2.1.1, OEE proteins form part of the extrinsic proteins of the OEC. These proteins have functions in optimising the manganese cluster during photolysis as well as protecting the reaction centre proteins from damage by oxygen radicals formed in light conditions (Heide *et al.*, 2004). Although the actual cause of the observed reduction in the levels of

OEE in this study is not clear, it might either be due to down regulation of expression or increase degradation of the protein following stress treatment (Echevarria-Zomeno *et al.*, 2009). Consequently, reduced levels of this protein might contribute to decrease efficiencies in the rate overall rate of the water oxidation process. On the other hand, the increased levels of the two ferrodoxin-NADP oxidoreductases that are involved in catalysing the final step of the photosynthetic electron transport (as discussed in Section 4.5.2.1.1) may result in increased levels of NADPH for use in the Calvin cycle (Arakaki *et al.*, 1997).

A total of six spots that are involved in the carboxylation, reduction and regeneration phases of the Calvin cycle (as discussed in Section 4.5.2.1.2) were responsive to stress (Table 6.4). These are spot 5 and 6 (RuBisCo large subunit); spot 9 (cytosolic 3-phosphoglycerate kinase); spot 11 (sedoheptulose- 1,7-biphosphate precursor); spot 12 (phosphoribulokinase, chloroplast precursor) and spot 34 (fructose 1,6-biphosphate aldolase precursor). The RuBisCo large subunit spots (spots 5 and 6; Table 6.4) were responsive in both sorghum varieties but in a contrasting manner. Both spots had reduced abundances in AS6 but increased levels in MN1618 following salt treatment (Table 6.4). The amount of RuBisCo in plant leaves is controlled by the rate of synthesis and degradation (Parry *et al.*, 2002). The reduced abundance levels of RuBisCo in AS6 leaf proteomes following salt stress might therefore be due to decreased levels of protein synthesis or increased degradation of this protein. Increased degradation of RuBisCo has been reported in the proteomic analysis of potato under salt stress (Aghaei *et al.*, 2008),

sugar beet under drought stress (Hajheidari *et al.*, 2005), rice under chilling stress (Yan *et al.*, 2006), and maritime pine seedling under water stress (Costa *et al.*, 1998).

However, the physiological significance of the observed contrasting responses of RuBisCo large subunits to salt stress between the two sorghum varieties in not so clear; but it might have implications on the different rate of carbon fixation in the two varieties under stress conditions. As previously discussed in Section 4.5.2.1.2, RuBisCo proteins are involved in the carbon fixation phase of the Calvin cycle (Heldt, 1997). As such, a reduction in the expression/abundance levels of this protein as observed in AS6, could possibly result in decreased carboxylation efficiencies and thus a subsequent reduction in the levels of triose phosphate, an end product of the Calvin cycle. Since some of the triose phosphate produced in the Calvin cycle is used for sucrose and starch biosynthesis (Raines, 2003; Tamoi *et al.*, 2005), it is highly probable that these two latter processes would be affected thus causing reduced energy stores as well as plant growth and development.

On the other hand, the increased levels of the RuBisCo and the cytosolic 3-phosphoglycerate kinase protein spots in MN1618 sorghum variety following salt stress (Table 6.4) might indicate the variety's need for increased carbon fixation as well as carbohydrates for use in energy consuming metabolic pathways. However, the above speculation is complicated by the observation that all three proteins previously identified as being involved in the regeneration phase (sedoheptulose-1,7-biphosphate precursor, spot 11; phosphoribulokinase, chloroplast precursor; spot 12; fructose 1,6-biphosphate

aldolase precursor; spot 34) of the same cycle (discussed in Section 4.5.2.1.2) had reduced abundances following salt stress (Table 6.4). This observation of different protein expression patterns between proteins involved in different reactions of the same pathway may point out to the differential effects of salt stress at different phases of a pathway together with their regulatory systems. The significances of other salt stress responsive proteins with putative function in carbohydrate metabolic functions are discussed in Section 6.6.5 below.

6.6.2 Proton Transport

Among the five chloroplastic, ATP synthase subunits that were identified in the sorghum leaf proteome map exercise (spots 7; 13; 32; 33; 40; Figure 4.4; Table 4.1), only two (spots 7; 13; Figure 6.10; Table 6.4) were responsive to salt stress in MN1618 and none in AS6 sorghum varieties. Both spots (spot 7 and 13) were upregulated following salt stress in MN1618, a trend that has also been reported in the proteomic analysis of potato shoots (Aghaei *et al.*, 2008), Arabidopsis cell suspension cultures (Ndimba *et al.*, 2005) and rice leaf lamina (Parker *et al.*, 2006) under salt stress. The ATP synthase complex (reviewed in Section 4.5.2.2) produces ATP from ADP in the presence of a proton gradient across the thylakoid membrane (McCarty, 1992; von Ballmoos and Dimroth, 2007). The upregulation of the beta and gamma subunits of the chloroplastic ATP synthase observed in this study might indicate the need for increased levels of ATP for use under stress conditions.

The ATP produced by ATP synthase may then be transported from the chloroplastic stroma to support increased activities of H^+-ATPases (Parker *et al.*, 2006) either at the plasma membrane or vacuoles during salt stress. Both the plasma membrane (P-type H^+-ATPase) and vacuolar (V-type H^+-ATPase or H^+-pyrophosphatases) H^+-ATPases are important in creating a pH gradient across their respective membranes (Xiong and Zhu, 2002). This proton gradient is then used by Na^+/H^+ antiporters during the transportation of Na^+ form the cytoplasm into the vacuole (Cushman *et al.*, 1990; Niu *et al.*, 1995; Zhu, 2001); thus preventing the accumulation and toxic effects of Na^+ to various metabolic processes during salt stress. Additionally, the increase amounts of ATP produced by ATP synthase complex may be used by stress protein such as heat shock proteins in their chaperone activities. Heat shock protein 70s for instance, have an ATPase domain; and it is believed that ATP hydrolysis is an important process in facilitating the chaperone activity of these stress proteins (Miernyk, 1997). However, it is still not clear why only two of the five ATP synthase subunits spot had elevated abundances following salt stress. It is possible though that these different expression changes between subunits of a single complex might reflect to some extent the different functions and the regulatory mechanisms of each subunit under stress conditions.

6.6.3 Hydrolytic Enzymes

The abundances of two hydroxynitrile lyase proteins (spots 20 and 28; Figure 6.10; Table 6.4) were both increased in MN1618 variety, while only one (spots 20) had increased in AS6. Hydroxynitrile lyases (discussed in Section 4.5.2.5) are hydrolytic enzymes, which play a role in cyanogenesis, a defense mechanism of plants against herbivores and

microbial attack (Hickel *et al.*, 1996; Fechter and Griengl, 2004). The increased expression of this enzyme under salt stress has not been reported before and therefore its role in salinity stress tolerance is still not characterised. However, since hydroxynitrile lyases also play a role in providing alternative nitrogen source for amino acids in young seedlings (Hickel *et al.*, 1996; Fechter and Griengl, 2004), its is highly probable that these proteins might be fulfilling the same function in these two sorghum variety under salt stress. Furthermore, sorghum hydroxynitrile lyases have been shown to be structurally similar to serine carboxypeptidase (Wajant *et al.*, 1994) and thus might have a function in proteolysis, processing and recycling redundant polypeptides (Ndimba *et al.*, 2005) and thereby participating in protein turnover processes (Liu *et al.*, 2008). In addition, it was reported that Arabidopsis plants overexpressing a serine carboxypeptidase gene showed increased tolerance to oxidative stress (Liu *et al.*, 2008). With the knowledge that salt stress induces oxidative stress as a secondary stress, sorghum might be utilising the serine carboxypeptidase activity of hydroxynitrile lyases for oxidative stress tolerance. For this reason, it is possible that the hydroxynitrile lyase protein spots whose abundances increase following salt stress in the current study might also function in oxidative stress tolerance mechanisms.

6.6.4 Detoxifying Enzymes

The abundance of glutathione S tranferase (GST; spot 26; Figure 6.10; Table 6.4) was increased in both sorghum varieties after salt treatment. Increased levels of GSTs following salt stress have also been reported in Arabidopsis (Ndimba *et al.*, 2005; Jiang *et al.*, 2007) and sorghum (Jogeswar *et al.*, 2006) following salt stress. Salt stress induces

oxidative stress that is characterised by accumulation of ROS, which are toxic to cells, damaging membranes and macromolecules (discussed in Section 1.4.1.4). Plants under salt stress have developed antioxidant enzymes such as GSTs, superoxide dismutase, catalase, ascorbate peroxidase (Xiong and Zhu, 2002; Wang, Vinocur *et al.*, 2003; Hajheidari *et al.*, 2005; Ndimba *et al.*, 2005; Parker *et al.*, 2006), which scavenge for and eliminate these ROS. Glutathione S tranferase (reviewed in Section 4.5.2.6) is a glutathione-dependent detoxifying enzyme, which conjugates glutathione to a wide range of natural products, environmental toxins as well as products of oxidative stress (Edwards *et al.*, 2000). Increased levels and activity of antioxidant enzymes is thus believed to be a salt tolerance mechanism in plants (Wang, Vinocur *et al.*, 2003; Jogeswar *et al.*, 2006). Therefore, the increased levels of GST (spot 26; Table 6.4) under salinity stress in the current study indicates that both AS6 and MN1618 sorghum varieties utilise this enzyme in the cell detoxification system for scavenging excess ROS during oxidative stress.

6.6.5 Other Salt Stress Responsive Proteins

Other salt stress responsive proteins observed in this study (Table 6.4) included malate dehydrogenases (spots 10 and 14); translational elongation factor Tu (spot 8); the nucleic acid-binding protein (spot 21), the chloroplastic adenylate kinase (spot 24) and several unidentified proteins (spots 3; 4; 15; 16; 19; 22; 25; 27; 30 and 31). Of these spots, the malate dehydrogenases, translational elongation factor Tu, chloroplastic adenylate kinase, and three of the unidentified protein (spots 3; 25 and 27) had increased abundances following salt stress in sorghum MN1618 but no significant changes were observed in AS6 sorghum variety. Translational elongation factors are involved in protein synthesis,

and increased abundances of this protein during salt stress probably reflects the plant's need for increased protein synthesis. Salt stress caused protein damage and degradation either due to the oxidative damage or proteolytic activities. In order to replenish the denatured proteins, increased level of protein synthesis may ensue. Increased levels of translational elongation factors following salt stress have also been reported in Arabidopsis cell suspension cultures (Ndimba *et al.*, 2005).

The abundance levels of adenylate kinase protein (spot 24) were increased following salt stress in MN1618 sorghum variety. As discussed in Section 4.5.2.4, this enzyme catalysis the reversible formation of ADP by transferring a phosphate group from ATP to AMP (Lange *et al.*, 2008); and therefore functions in energy metabolism and in maintaining the equilibrium of ATP, ADP and AMP i*n vivo* (Carrari *et al.*, 2005; Igamberdiev and Kleczkowski, 2006). As such, the observed increase in the abundance of adenylate kinase in MN1618 might indicate the need for increased energy supply during salt stress.

Amongst the unidentified salt stress responsive proteins, a total of five (spots 4; 16; 19; 22; 30 and 31;) had reduced abundances in AS6 and MN1618 sorghum varieties combined (Table 6.4). Of these, the abundances of spots 16 and 19 were reduced in both varieties, spot 4 only in AS6 while spots 22, 30 and 31 in MN1618 (Table 6.4). Spots 15, 25 and 27 had increased abundances following salt stress and thus possibly indicating novel uncharacterised proteins that are involved in salt tolerance.

6.7 Example of Salt Stress Responsive Protein Expression Patterns Between the Sorghum Varieties

Examples of the protein expression patterns observed between the control and salt treatment groups of both sorghum varieties are given in Figure 6.12. Only one representative gel from each treatment groups per sorghum variety is shown. The numbered arrows indicate the respective protein spots under comparative analysis. The spot numbers are consistent with those shown in Figure 6.10 and Table 6.4.

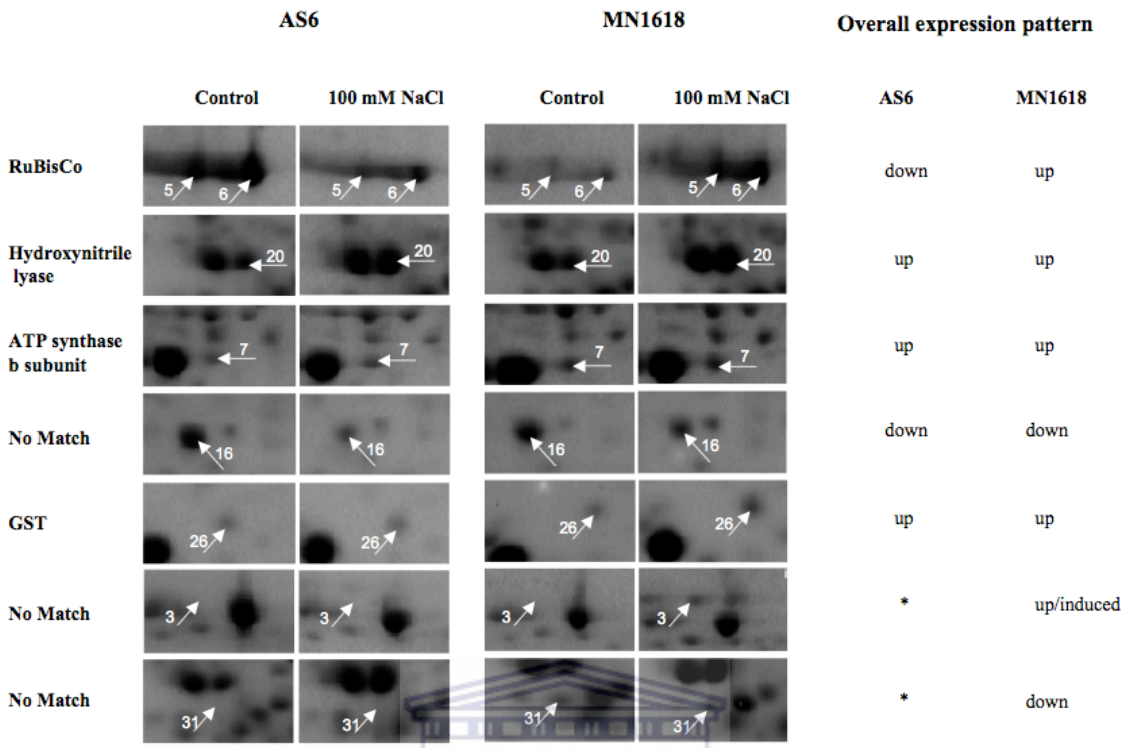


Figure 6.12: Examples of representative gels showing salt stress responsive proteins of the two sorghum varieties.

Gel panels show zoomed in sections of one control and one 100 mM NaCl treatment gel of each of the two sorghum varieties (AS6 and MN1618). The overall expression pattern is from three biological replicates per sorghum variety. up: increased expression/abundance following salt stress. down: decreased expression/abundance following salt stress. up/induced: only present following salt stress.*: spots absent in both control and salt treatment groups of AS6 sorghum variety. The numbered arrows indicate the respective protein spots under comparative analysis. The spot numbers are consistent with those shown in Table 6.4. Protein names are also included as identified in the mapping exercise (Table 4.1).

6.8: Discussion

Comparative proteomics of salt stress responsive proteins of two sorghum varieties, AS6 (salt sensitive) and MN1618 (salt tolerant) was studied. Sorghum seedlings were germinated on MS media without supplementary salt (control; 0 mM NaCl) or containing 100 mM NaCl (salt treatment). At 14 days post plating, the seedlings were harvested; leaf, sheath and root tissues were excised and extracted of soluble protein using the TCA/acetone method. One dimensional gel electrophoresis of the leaf and sheath (Figure 6.3), and root (Figure 6.4) tissues showed that all protein extracts were of high quality, showing no signs of protein degradation and streaking. Furthermore, protein extraction between independent biological replicates of control and salt treated leaf and sheath tissues of both sorghum varieties showed high levels of reproducibility between independent extractions (Figure 6.3A-D).

To determine if 100 mM NaCl treatments of the sorghum varieties were high enough to induce any salt stress responsive protein expression, we evaluated the differential protein expression of Hsp70, between the control and salt treatment groups of all tissues using western blotting techniques. Heat shock protein 70 is a known stress responsive protein (Ndimba *et al.*, 2005), and is expressed in response to abiotic stresses such as heat, cold, drought, salinity and oxidative stress (Wang *et al.*, 2004). In this study, it was observed that sorghum leaf, sheath and root tissues had basal levels of Hsp70 (approximately MW=77 kDa) prior to salt stress albeit in at varying levels (Figure 6.5). Following salt stress treatment using 100 mM NaCl, Hsp70 abundance levels increased in all three tissues for both sorghum varieties. In order to resolve the Hsp70 protein(s) and confirm

their identities using MALDI-TOF MS and database searches, Hsp70 western blotting was performed on 2D gel profile of one of the tissues. The sheath was randomly selected for this exercise, resulting in the immuno-detection of three Hsp70 spots (S1, S2 and S3; Figure 6.6) of MW 77 kDa and pIs of 5.3, 5.4 and 5.5 respectively. Matrix-assisted laser desorption/ionization-time of flight analysis of the three immuno-detected Hsp70 spots resulted in the positive identification of two (spots S1 and S2; Table 6.1). Spots S1 and S2 matched the identities of a 70 kDa heat shock cognate protein 2 and a putative Hsp70, respectively. Spot S3 did not match any proteins in the plant database possibly indicating the need for a complete sorghum genomic database for the identification of all sorghum proteins, which are not homologous to proteins from other plant species. Nevertheless, both western blotting (Figures 6.5 and 6.6) and MALDI-TOF MS results (Table 6.1) gave consistent and complementary evidence that our salt treatment experiment using 100 mM NaCl does indeed induce salt stress responsive protein expression in the two sorghum varieties. For this reason, we proceeded to use the sorghum plant material to study other differential protein expression patterns in sorghum leaf following salt stress treatment with 100 mM NaCl.

Comparative analysis of leaf salt stress responsive proteins of the two sorghum varieties (AS6 and MN1618) was performed using PDQuest^{TM} software (BIO-RAD). Three CBB stained biological replicate gels of each treatment group (0 mM NaCl and 100 mM NaCl) per sorghum variety were used (Figures 6.7 and 6.8). The inclusion of biological replicates is important for eliminating non-reproducible protein expression differences between experiments. In order to summarise salt stress responsive protein spots between

the AS6 and MN1618 sorghum varieties (Figure 6.11A), qualitative, quantitative and statistical analysis sets were performed between the control and salt treatment groups of each variety. Following salt stress, 22 and 24 qualitative spots (Table 6.2; Figure 6.9A) were observed in AS6 and MN1618 sorghum varieties respectively. For AS6, all 22 spots disappeared from the salt treatment gel groups. For MN1618, all 24 spots were induced by salt and thus only appeared in the salt treatment groups. This overall contrast in salt stress response proteins between the varieties may indicate that AS6 is more susceptible to salt stress than MN1618.

Apart from the qualitative changes in protein expression, expression of some proteins changed with at least a 2-fold change (quantitative spots) following salt treatment in both varieties. The use of high threshold values is particularly important in comparative proteomics between different plant genotype, varieties or cultivars that have been subjected to the same level of stress as they help indicate outstanding differences in expression. For AS6 sorghum variety, a total of 11 spots showed at least a 2-fold change in expression following salt stress. Of these, the abundances of four spots increased while that of seven decreased (Figure 6.9B). Of the 29 quantitative spot of MN1618 leaf proteome, the abundances of 12 spots increased while that of 17 decreased following salt stress (Table 6.2; Figure 6.9B). Using the Student's *t*-test analysis at 95% significance level, at total of 28 and 93 spots were differentially expressed between the control and salt treatment groups of AS6 and MN1618 respectively (Table 6.2; Figure 6.9C).

As observed in all three analysis sets (qualitative; quantitative; Student's *t*-test), the abundance of different proteins following salt stress either increased, decreased, totally disappeared or were induced in the salt treatment gels. These results are consistent with the reports that salt stress alters gene expression (Hasegawa *et al.*, 2000), which ultimately results in the increase, decrease, induction or total suppression of some proteins (Sachs and Ho, 1986; Ho and Sachs, 1989). Stress-induced proteins are believed to allow the plants to make biochemical and structural adjustments (Sachs and Ho, 1986; Ho and Sachs, 1989), which are necessary for conferring stress tolerance. Abiotic stress inducible genes may be classified into two groups. The first group encodes proteins that directly protect the plant cells against the stresses such as enzymes involved in the biosynthesis of various osmoprotectants, chaperones and detoxification enzymes. The second group encodes for proteins that regulate gene expression and signal transduction in abiotic stress responses such as transcription factors and protein kinases (Seki *et al.*, 2003; Shinozaki *et al.*, 2003).

The observation that MN1618 sorghum variety had more salt stress inducible protein spots compared to AS6 (Figure 6.9A) probably indicated that it expresses more gene and gene products, which are directly involved in salt tolerance. Furthermore, it was observed that AS6 sorghum variety has a greater number of spots that disappeared following salt stress (Figure 6.9A) thus possibly an indication towards the underlying reason for its susceptibility to salt stress. These results are also consistent with results of our salt tolerance screening experiment of various sorghum varieties (Figure 6.2), which showed that AS6 was more susceptible to salt stress than MN1618 at 100 mM NaCl. The

MALDI-TOF MS identification of these differentially expressed proteins of the two sorghum varieties would provide clues on genes and metabolic pathways that are susceptible and/or those that contribute to salt tolerance mechanisms. Salt stress inducible genes and gene products in sorghum could possibly help us towards gaining a better understanding of molecular responses of sorghum to salinity stress. Furthermore, since sorghum is both more drought tolerant (Rosenow *et al.*, 1983) and moderately more salt tolerant that maize (Krishnamurthy *et al.*, 2007) for instance, it is a potential cereal crop for the dry and moderately saline environments.

The established partial sorghum leaf proteome reference map (Chapter 4; Section 4.5) was used to land mark some of the salt stress responsive proteins of both sorghum varieties (Table 6.4). Of the 40 spots that were initially picked for MALDI-TOF MS analysis in the mapping exercise (Figure 6.10) a total of 7 and 30 were responsive to salt in AS6 and MN1618 sorghum variety respectively (Figure 6.11B). Of these only six spots were common to both varieties (Table 6.4). The salt stress responsive protein had functions in carbohydrate metabolism, proton transport, hydrolytic enzymes, detoxifying enzymes and protein synthesis (Table 6.4). The observed differences in protein expression between controls and salt treatment groups of the two sorghum varieties illustrates the differential salt stress responses of the varieties towards the same level of stress. The results obtained in this chapter also confirm those reported by Krishnamurthy *et al*. (2007) that sorghum has a large genetic variation in salt tolerance amongst the different varieties. Therefore, proteomic studies that investigate salt stress responsive proteins of different varieties would help to establish the underlying tolerance

mechanisms of sorghum to salt stress. This is particularly important in identifying candidate genes for the genetic improvement of crops to salt stress.

CHAPTER 7

Identification of a Salt and Hyperosmotic Stress Responsive Protein in Sorghum Secretome

7. 1 Introduction

As previously discussed in Chapter 5, Section 5.1, the secretome consists of proteins that are secreted into the extracellular matrix (ECM; Greenbaum *et al*., 2001; Hathout, 2007). These proteins are not only involved in normal physiological processes during plant cell growth and development but also respond to abiotic and biotic stresses. Some components of the ECM such as wall-associated kinases are believed to act as signalling components (Brownlee, 2002) which link the cell's external and internal environments (Zhang *et al.*, 2009). Although a lot of studies have been reported on proteomic analysis of salt and/or sorbitol stress response of either cytosolic or cell wall proteins, there is still limited understanding of what happens to the secretome. Only a few proteomics based studies have been reported on the effect of salt stress in the apoplast/secretome composition (Dani *et al.*, 2005; Zhang *et al.*, 2009).

Dani and co-workers studied protein changes in the tobacco leaf apoplast following 100 mM NaCl treatment for 20 days (Dani *et al.*, 2005). Amongst some of the proteins whose abundances increased included alpha-galactosidase, acidic chitinase, germin like protein and lipid transfer proteins; while those of cell wall peroxidases decreased. In another proteomic study on salt stress responsive proteins in rice root apoplast, Zhang and co-workers, identified peroxidases, glucanase, thioredoxin, rab5B and a putative

pathogenesis-related proteins that responded to salt stress (Zhang *et al.*, 2009). Collectively these studies show that the apoplast contains a diverse group of proteins, which could be participating in response mechanisms such as salt stress adaptation.

The objectives of this chapter were to isolate, separate and identify sorghum secretome proteins that are responsive to either salt (200 mM NaCl) and/or hyperosmotic (400 mM sorbitol) stress treatment.

7.2 Salt and Osmotic Stress Treatment of Sorghum Cell Suspension Cultures Cells

Sorghum cell suspension cultures were treated with either 200 mM NaCl or 400 mM sorbitol to simulate salt and hyperosmotic stress respectively, as described in Section 2.6.1.1. Control cell suspension cultures were spiked with sterile distilled water, the volume of which was equivalent to that used in sorbitol treatments. To determine if the above treatment conditions were capable of inducing the expected salt and/or hyperosmotic stress responses in the cells, the following parameters were monitored after treatment; (i) cell viability; (ii) morphological changes in cell structure; and (iii) the pH changes in extracellular culture medium.

7.2.1 Cell Viability

The viability of sorghum cell suspension cultures without treatment (control) and following either salt or hyperosmotic stress treatments were monitored at 0, 6 and 24 hrs after exposure. Zero hours denote sampling time soon after application of each respective

stress. Both the Evans Blue and the MTT tests for cell viability were used as described in Section 2.6.1.3. The different time points were chosen so as to determine the most appropriate time for harvesting cell cultures for the proteomic analysis of resultant changes in response to the respective stress conditions. Figure 7.1 shows a graphical representation of cell viability results of the sorghum suspension cultures in the control, 200 mM NaCl and 400 mM sorbitol treated samples.

Evans blue stains dead cells and the rationale of using it to estimate the viability of cell cultures in described in Section 3.3.2. Using the Evans Blue test for cell viability (Figure 7.1A), at 0 hrs, the control samples had the highest cell viability of 60%, while that of the NaCl and sorbitol treated cells was both at approximately 30%. At 6 hrs into the treatment course, the control and sorbitol treated cells maintained cell viability as at 0 hrs (60% and 30% respectively), while that of NaCl treated cells further decrease to about 17%. At 24 hrs, the control cells had a cell viability of about 61%, while that of the NaCl treated cells increased to about 30%. The sorbitol treated cells however, showed a dramatic increase in cell viability to about 56%.

The MTT test for cell viability measures metabolic activity of living cells. Live cells use mitochondrial dehydrogenase to cleave the tetrazolium ring of MTT resulting in the formation of purple MTT formazan crystals, which are insoluble in cells (Fernandez-da Silva and Menendez-Yuffa, 2006). The formazan crystals are then solubilised in solvents such as DMSO or isopropanol and the purple colouration is quantified spectrophotometrically. Absorbance readings are therefore proportional to the level of

viability of the cell cultures. Using the MTT test (Figure 7.1B), at 0 hrs, both the control and sorbitol treated cells had similar levels of cell viability with absorbance reading of about 0.17 while the NaCl treated samples had a slightly lower readings of about 0.16. At 6 hrs, cell viability of all three samples; control, NaCl and sorbitol treatments decreased as compared to those obtained at 0 hrs. The NaCl treated samples had the lowest cell viability as indicated by an absorbance reading of 0.09, followed by the sorbitol and control samples with values of 0.12 and 0.14 respectively. At 24 hrs, both the control and NaCl treatment maintained the same level of cell viability at observed at 6 hrs. However the sorbitol treated cells had an increase in cell viability up to the same level at that observed in the control samples (Figure 7.1B).

Overall, it was observed that the viability of sorghum cell cultures treated with either 200 mM NaCl or 400 mM sorbitol for 6 hrs decreased when compared to the controls (Figure 7.1A and B). These observations may possibly be due to a combination of factors such as decreased cell growth, increased cell death as well as a decrease in metabolic activities of the cells following stress treatment. Of particular interest is the observation that the viability of sorbitol treated cells dramatically increases at 24 hrs (Figure 7.1A and B for both Evan blue and MTT tests respectively), almost approximating that of the control samples. This possibly indicates that 400 mM sorbitol induces sub-lethal hyperosmotic stress to our experimental cells. Since the cell culture viability increased between the 6 to 24 hr time points, this showed that at 6 hrs, both the salt and sorbitol treatments retarded cell growth but did not completely kill the cells. Therefore, the 6 hr time point was

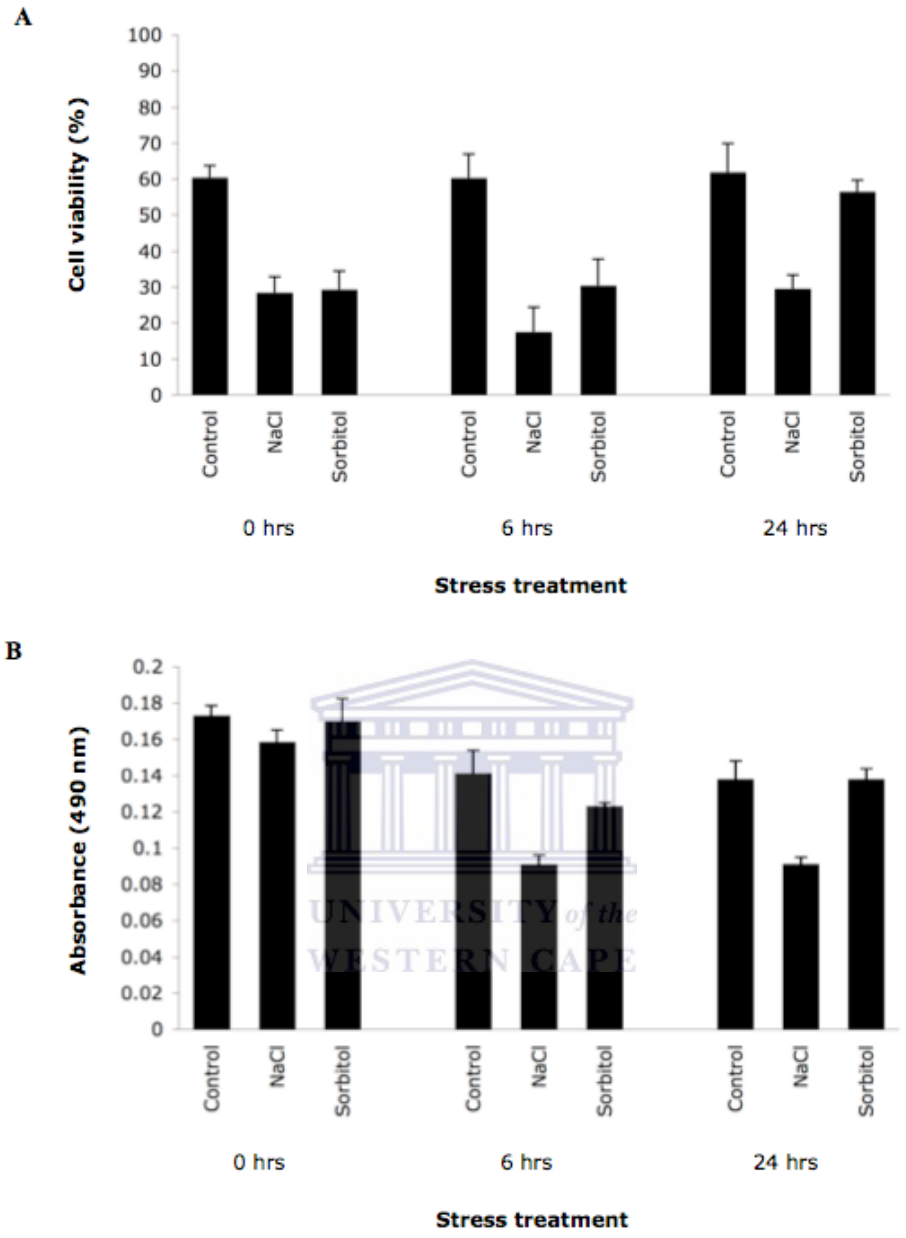


Figure 7.1: Viability of sorghum cell suspension cultures following stress treatments.

Ten-day old cell cultures were treated with water (control), 200 mM NaCl (salt stress) or 400 mM sorbitol (hyperosmotic stress). Cells for viability estimation with (A) Evans blue test and (B) MTT test were taken at 0 hrs (soon after introduction of stress), and following 6 and 24 hrs in each respective stress condition. Vertical bars indicate standard error from three independent experiments.

selected as a suitable time point for assessing stress induced changes in the sorghum secretome (Section 7.4).

7.2.2 Microscopic Analysis of Cell Structure

Morphological changes of sorghum suspension cells following 6 hrs of stress treatment (200 mM NaCl or 400 mM sorbitol) and without treatment (control) were observed under a Nikon Inverted light microscope at a magnification of 10X. Figure 7.2 illustrates the microscopic pictures of (A) control, (B) salt and (C) osmotic stress treated sorghum suspension cells after 6 hrs of incubation with each respective treatment. These results show that cell morphology changed after exposure to either 200 mM NaCl (Figure 7.2B) or 400 mM sorbitol (Figure 7.2C) in comparison with the control (Figure 7.2A). After 6 hours of incubation, control cells had an elongate structure, with a consistent and even distribution of cytoplasmic contents (EDC; Figure 7.2A). However, following salt treatment with 200 mM NaCl, the cells were generally smaller in size, globular in shape and their interior had a characteristic circular or dense structure in the centre of the cell possibly an indication of condensed and shrinking cytoplasm (CC; Figure 7.2B). Following sorbitol stress treatment, cells remained elongate although they seemed much smaller than those in the control treatment. However, the interior of the cells had characteristic dense structures, as observed with the NaCl treated cells. However, the microscope used does not have a scale bar and therefore measurable size differences are not given.

The observed cellular changes in either NaCl or sorbitol treatments possibly indicates that following each respective stress treatment, the cells lost water into the extracellular medium through the process of osmosis. As the loss of water progressed, cells plasmolysed (Bidwell, 1979), as indicated by the cell's plasma membrane (PM) detaching from the cell wall (CW), subsequently resulting in the cytoplasmic contents condensing and/or shrinking (CC; Figure 7.2B and C). This is indicated by the large gap between the cell wall and plasma membrane that is visible under the light microscope (white block arrows; Figure 7.2B and C). The different levels of cytoplasmic condensation and shrinkage can also be observed (i and ii; Figure 7.2B), thus showing different phases of death of the cells in each respective stress treatment. From these microscopy results it can be confirmed that both the salt and sorbitol treatment of the sorghum cell suspension cultures induce changes in cellular structure. The levels of stress used in this experiment are thus suitable for analysing proteomic changes of either cytosolic or secretory proteins in response to the respective stresses.

7.2.3 pH Changes in the Culture Medium

Changes in the pH of sorghum cell suspension culture medium following stress treatment (200 mM NaCl or 400 mM sorbitol) and without treatment (control) was measured as described in Section 2.5.4.3. The measurements were taken every hour up to 6 hrs. Figure 7.3 illustrates changes in the pH of the culture medium following each treatment. It was observed that for all three treatment groups (control, 200 mM NaCl and 400 mM sorbitol) the pH of the medium gradually decreased following treatment. The control group

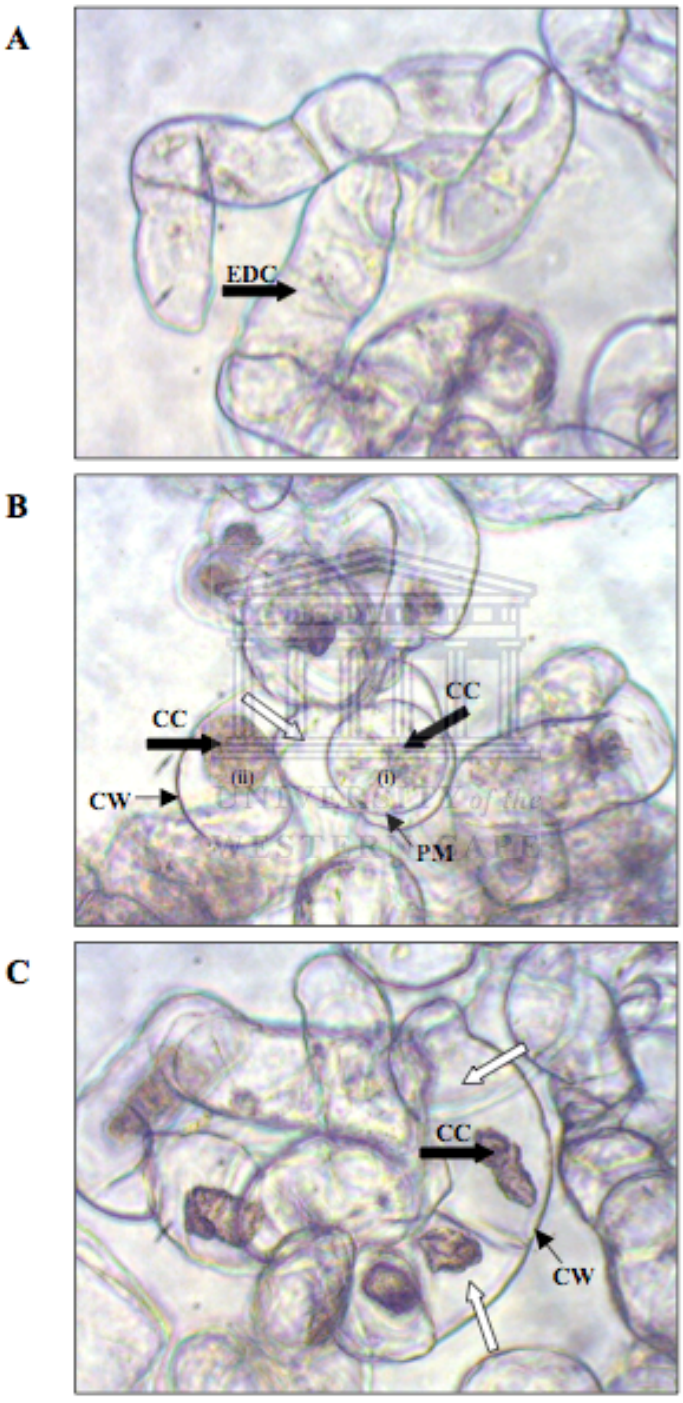

A
EDC
B
CC
CC
(ii)
(i)
CW
PM
C
CC
CW

Figure 7.2: Morphological changes in sorghum cell cultures following 6 hrs of stress treatment.

Ten-day old cell cultures were treated with (A) water (control), (B) 200 mM NaCl (salt stress) and (C) 400 mM sorbitol (hyperosmotic stress). The microscopic pictures were taken using a Nikon Inverted Light Microscope fitted with a Leica Firecam digital camera at a magnification of 10X but without a scale bar. Black block arrows indicate the appearance of the cytoplasmic contents of the cells; EDC, evenly distributed cytoplasmic contents; CC, condensed and shrinking cytoplasm; CW, cell wall; PM, plasma membrane; white block arrows indicate the empty gap between the cell wall and plasma membrane. (i) and (ii) represent different phases of cytoplasmic shrinking.

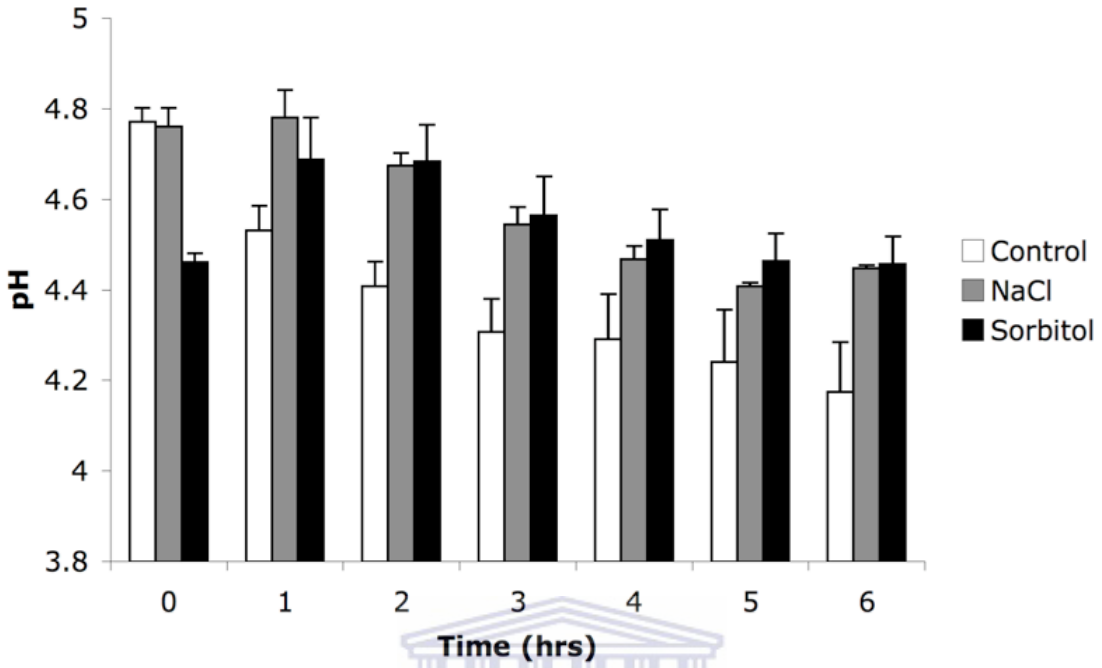


Figure 7.3: pH changes in the sorghum cell suspension culture medium following stress treatments.

The pH measurements of the culture medium were taken every hour up to 6 hrs following each stress treatment; control (water); salt stress (200 mM NaCl) and hyperosmotic stress (400 mM sorbitol). The measurements were taken for three independently established cell culture lines. Vertical bars indicate standard error.

maintained a lower pH than either the NaCl or sorbitol treatment groups for the entire 6 hr period. The culture medium of control and NaCl treated cells had a pH of approximately 4.8 at time 0 hrs and gradually declining to pH 4.17 and 4.44 at 6 hrs respectively. However, at time 0 hrs, the culture medium of sorbitol treated cells had the lowest pH reading of 4.46, which increased to 4.68 at 1 hr then gradually decreasing to 4.45 at 6 hrs.

7.3 Confirmation of Lack of Contamination of the Secretome with Cytoplasmic Proteins

As previously discussed in Chapter 5, Section 5.1, one of the primary objectives of proteomic analysis of the secretome is to ensure that proteins identified in this compartment are *bona fide* residents of the ECM. The secretome of cell suspension cultures may inevitably have varying levels of cytoplasmic protein contaminants that are released as some cells die and leak out their cytosolic constituents. In this study, the presence of cytoplasmic contaminants was evaluated by western blotting analysis of Hsp70, one of the abundant stress proteins (Ndimba *et al.*, 2005) that is located in the cytoplasm (Wang *et al.*, 2004). In addition, Hsp70 has also been used as a cytosolic marker protein in the secretome of rice calli (Cho *et al*., 2009). Therefore, the use of Hsp70 western blotting in this experiment had a dual function; firstly, to check if the secretome was contaminated with this cytoplasmic protein under control conditions; and secondly, to establish if the respective stress treatments caused cytosolic leakages due to membrane damage. Total soluble proteins (TSP) and culture filtrate (CF) protein samples of sorghum cell suspension cultures were prepared as described in Sections 2.7.1 and

2.7.2 respectively. Protein samples were quantified using the Bradford assay and separated on 1D SDS-PAGE gels as described in Sections 2.8 and 2.9 respectively. Western blotting analysis of the Hsp70 protein was carried out on 1D SDS-PAGE profiles of control, salt and sorbitol stressed CF and TSP samples using the human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody as described in Section 2.13. Figure 7.4 illustrates the Hsp70 immuno-detection results of (A) the CF and (B) the TSP samples following stress treatment. Heat shock proteins were detected in the positive control samples (+ve control) on both experimental gels (Lanes 7 and 8; Lanes 13 and 14 for Figure 7.4A and B respectively). This indicates that the western blotting experiment worked in both cases. However, no Hsp70 proteins were detected in either the experimental controls (Lanes 1 and 2), 200 mM NaCl (Lanes 3 and 4) or 400 mM sorbitol (Lanes 5 and 6) treatment samples of the CF (Figure 7.4A). The above results indicate that the culture filtrate medium contains no Hsp70, and based on this result, the CF is most likely free of cytoplasmic proteins.

Of the four biological replicates used for each of the treatments for the TSP samples (Figure 7.4B), Hsp70 was detected in only two of the control samples (Lanes 2 and 3); one of the 100 mM NaCl treated samples (Lane 8); and in all four of the 400 mM sorbitol treated samples (Lanes 9-12). These observations indicate that heat shock protein 70s, are intracellular proteins and none of the stress treatments evoked significant levels of cell damage and subsequent leakages of the cellular contents into the extracellular culture medium. However, since not all TSP protein fractions had detectable Hsp70 (Figure 7.4), the Hsp70 was probably not the best cytosolic marker protein in this study. Therefore, the

lack of detectable contamination in the CF fraction could be due to chance. Furthermore, since cell viability tests using Evans blue (Figure 7.1A) indicated that both salt and sorbitol stress treatments caused cell death by affecting the integrity of plasma membranes, it is possible that much lower molecular weight proteins (smaller than the 77 kDa Hsp70; Figure 7.4) would have leaked out of the cell into the culture medium. Based on this reasoning, future studies on sorghum secretomics should use more lower molecular weight cytosolic marker proteins in western blotting and/or enzyme assays to assess the degree of cytosolic protein contaminants in the CF fractions.

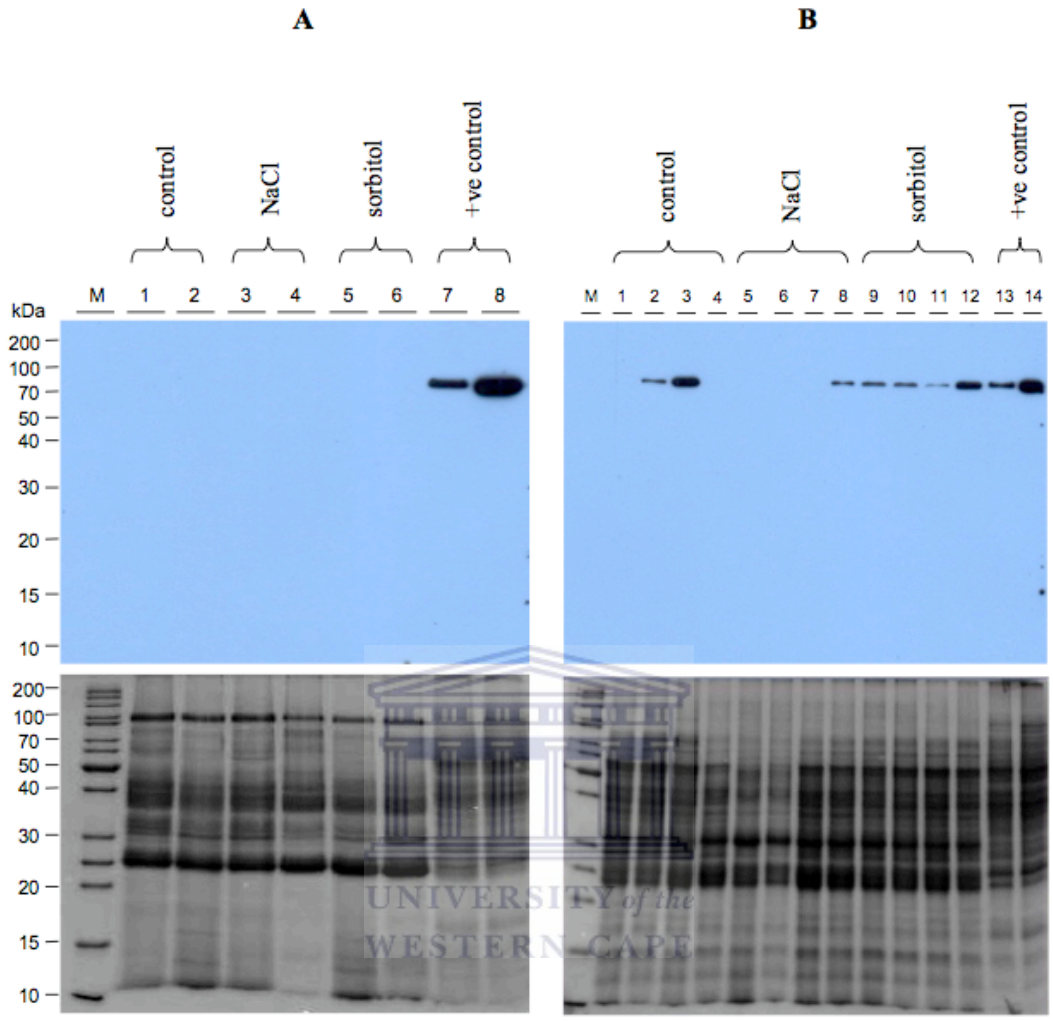


Figure 7.4: Hsp70 Western blotting analysis of sorghum culture filtrate and total soluble protein extracts to verify cytoplasmic protein contaminants.

(A) Culture filtrate and (B) total soluble protein fractions. Ten-day old sorghum cell suspension cultures were treated with, either water (control), 200 mM NaCl (salt stress) or 400 mM sorbitol (hyperosmotic stress). A positive control (+ve control) was included in each experimental gel to verify the immunoblotting procedure. Both the immunoblots and the corresponding CBB stained 1D gels are shown.

7.4 Identification of Salt and Hyperosmotic Stress Responsive Proteins of the Sorghum Secretome

The sorghum secretome (CF) samples treated with either 200 mM NaCl or 400 mM sorbitol or without stress treatment (control) were separated by 2DE as described in Section 2.10. Because of problem with protein solubility, and protein quantification procedures in these particular CF fractions, equal loading on 1D SDS-PAGE was used as a protein quantification measure prior to 2D SDS-PAGE. Figure 7.5 shows representative CBB stained 2D gels of (A) control; (B) 200 mM NaCl; and (C) 400 mM sorbitol treated sorghum secretome samples. Proteome expression changes between the control, salt and sorbitol treatment were visually assessed. One spot (spot CF1; Figure 7.5) was selected for identification by a combination of MALDI-TOF MS and database searching as described in Section 2.14.1. This spot was selected because its expression was increased in two independent stress conditions (salt and sorbitol treatments; Figure 7.5) in comparison with the control. Table 7.1 gives a summary of the identity of this protein, the plant species of origin and corresponding NCBI accession number. Both the theoretical and experimental MW/pI of the protein are also shown in the same table. The protein spot matched the identity of a putative wall-associated kinase from rice (accession AAK52581). Both the theoretical and experimental MW and pI values were in close approximation, thus confirming the close similarities between rice and sorghum WAK proteins.

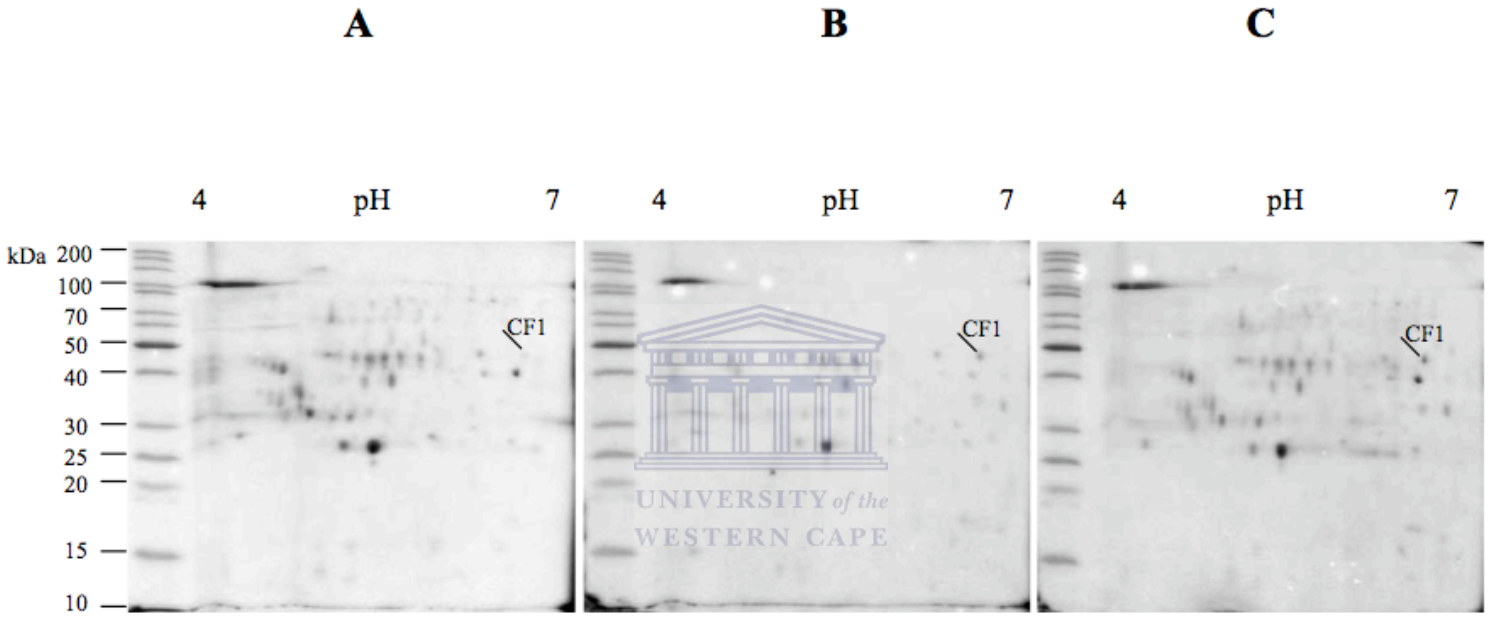


Figure 7.5: Representative CBB stained 2D gels of sorghum culture filtrate protein extracts with or without stress treatment.

Ten- days old cell cultures were treated with (A) water (control), (B) 200 mM NaCl (salt stress) or (C) 400 mM sorbitol (hyperosmotic stress). Equal loading of culture filtrate protein samples were separated by IEF on 7 cm linear IPG strips, pH 4-7 and by 12% SDS-PAGE and stained with CBB. Protein spot CF1, was upregulated following both salt and sorbitol stresses and subsequently selected for identification by MALDI-TOF MS and database searches.

Table 7.1: Identification of a salt and sorbitol stress responsive sorghum culture filtrate protein identified using MALDI-TOF MS and database searching.

Spot[a)]	Best Protein Match	Plant Species	Accession No. [b)]	MOWSE score	Theoretical MW/pI [c)]	Experimental MW/pI [d)]
CF1	Putative wall-associated protein kinase	*Oryza sativa*	AAK52581	73	53/6.01	48/6.2

[a)] Spot number as indicated on the 2D gel images (Figure 7.5).

[b)] Accession number in the National Center for Biotechnology Information (NCBI) database.

[c)] Theoretical MW and pI were taken directly from the MASCOT search results.

[d)] Experimental MW and pI were estimated from the 2D gels shown in Figure 7.5.

7.4.1 Verification of the Subcellular Localisation of the WAK Protein

As previously mentioned in Chapter 4, Section 4.5.3, the prediction of subcellular localisation of proteins helps to clarify protein function and possibly the mechanism of action (Kumar *et al.*, 2002; van Wijk, 2004). The localisation of the identified sorghum WAK protein (spot CF; Figure 7.5; Table 7.1) was predicted using a combination of SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al.*, 2004); TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson *et al.*, 2007), Predotar version 1.03 (http://urgi.versailles.inra.fr/predotar/predotar.html; Small *et al.*, 2004) and literature sources. All these bioinformatics analysis were carried out using the amino acid sequence of the best matching rice putative WAK (NCBI accession AAK52581; Table 7.1). Results of these subcellular localisation predictions are

summarised in Table 7.2. According to the SignalP 3.0 prediction server, the rice WAK protein was predicted not to have a cleavable signal peptide, which targets proteins for the secretory pathway. Based on this prediction, the identified sorghum WAK protein (spot CF1; Figure 7.5) is not a secretory protein (Table 7.2). As a result, other bioinformatics algorithms, TargetP and Predotar were used to predict the subcellular location of this protein. Both algorithms predicted the rice WAK protein and thus the sorghum WAK to having "other" locations other than the secretory, endoplasmic reticulum, chloroplastic or mitochondrial ones (Table 7.2).

The amino acid sequence of the best matching rice WAK protein (accession AAK52581; Table 7.1) was also analysed for the prediction of transmembrane helices using the HMMTOP version 2.0 (http://www.enzim.hu/hmmtop/html/submit.html; Tusnady and Simon, 2001) and TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/; Krogh *et al.*, 2001). Both the HMMTOP and TMHMM servers predicted that the rice WAK was a transmembrane protein with a single transmembrane helix between amino acid positions 80-104 and 84-106 respectively (Table 7.2). This transmembrane nature of WAK proteins is also confirmed in literature (He *et al.*, 1996; Verica and He, 2002). However, the in/out orientation of the protein relative to the membrane differed between the two transmembrane prediction servers. Using the HMMTOP server, the N-terminus of rice WAK was predicted to be outside the cell, while the TMHMM server predicted it to be inside the cell. The observed conflicting prediction in the orientation of this transmembrane protein across the plasma membrane complicates the putative assignment of a function to this protein. However,

Krogh *et al*. (2001) acknowledges that one of the common mistakes of the TMHMM prediction server is to reverse the orientation of proteins, which have only one transmembrane helix. For this reason, orientation results obtained for this rice WAK protein are to be treated with great caution. Based on the transmembrane predictions of the rice WAK (accession AAK52581), the identified sorghum WAK protein (spot CF1; Figure 7.5; Table 7.1) is considered to be a transmembrane protein with a single helix (Table 7.2).

Table 7.2: Bioinformatics analysis of the MALDI-TOF MS identified sorghum culture filtrate salt and sorbitol responsive protein.

Protein name	Spots [a]	Location [b]	Signal peptide [c]	Transmembrane protein [d]
Wall-associated kinase	CF1	Other	None	Yes; single transmembrane helix

The bioinformatics analyses were conducted using the amino acid sequence of the rice WAK (accession AAK52581).

[a] Spot number as indicated on the 2D gel images (Figure 7.5).

[b] Subcellular location of the protein as predicted by TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP/) and Predotar version 1.03 (http://urgi.versailles.inra.fr/predotar/predotar.html).

[c] Presence of a signal peptide predicted using the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/).

[d] Prediction of transmembrane helices using the HMMTOP version 2.0 (http://www.enzim.hu/hmmtop/html/submit.html) and TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

7.4.2 Putative Functional Classification of the Identified Sorghum WAK Protein

If the identified sorghum WAK protein is indeed a transmembrane protein, the fundamental questions that arise are why is it found in the secretome of sorghum cell suspension cultures and what role does it play in this compartment. To fully understand the function(s) of this protein in the secreted protein fraction of sorghum cells suspension cultures, a combination of similarity searches on the Arabidopsis database

(http://www.arabidopsis.org), and TIGR Rice genome Project BLAST server (http://blast.jcvi.org/euk-blast/index.cgi?project=osa1) as well as literature sources were used. Additionally, the domain composition and organisation of the protein was analysed on the SMART database (http://smart.embl-heldelberg.de; Schultz *et al.*, 1998) using the amino acid sequence of the rice WAK protein (accession AAK52581).

Amino acid similarity searches on the Arabidopsis and Rice databases showed that the rice WAK protein (accession AAK52581) was 37% similar to WAK2 (Arabidopsis database accession At1g21270) and 89% similar to OsWAK97 (TIGR rice database accession Os10g02284) respectively. Both the Arabidopsis WAK2 (Anderson *et al.*, 2001; Verica and He, 2002; Kanneganti and Gupta, 2008) and the rice OsWAK97 (Zhang *et al.*, 2005) are known wall-associated receptor-like protein kinases. Using the SMART database for domain composition and organisation, the rice WAK protein (accession AAK52581) was predicted to have a calcium-binding EGF-like domain (amino acid position 29-75), a transmembrane domain (amino acid position 84-106) and a serine/threonine protein kinase domain (amino acid position 142-411). Figure 7.6 illustrates a schematic representation of the domain composition of the best matching rice WAK protein (accession AAK52581). These domain predictions confirm the known structure of WAK receptor-like kinases in literature (He *et al.*, 1999).

To establish whether or not CF1 protein spots (Figure 7.5) had all three domains possessed by its best matching rice WAK protein (Table 7.1; Figure 7.6), the peptide

matching queries of the trypsinised CF1 protein were superimposed on the amino acid sequence of the rice WAK (accession AAK52581). Figure 7.7 illustrates the distribution of the matching resultant peptide fingerprints of the trypsinised spot CF1 on the amino acid sequence of rice WAK accession AAK52581. Out of the 21 mass values that were searched in the MSDB 20060831 (3239079 sequence; 1079594700) database, a total of seven peptides, (indicated in red colour; Figure 7.7A) matched with various sections of the rice WAK. The amino acid positions matched were between 7-386 albeit with missing gaps (Figure 7.7B), resulting in a 19% sequence coverage. Of these seven matching peptides, a total of six were within the serine/threonine protein kinase domain (Figure 7.7B). This result indicated that the sorghum secretome CF1 protein spot (Figure 7.5) indeed has a protein kinase domain. The other two domains, calcium-binding EGF-like domain and the transmembrane domain were not represented by the matching peptides but lie within the region flanked by the seven matching peptides (Figure 7.7B**)**. These additional observations could point out to the fact that the protein spot CF1 (Figure 7.5) identified as being similar to a rice WAK (NCBI accession AAK52581; Table 7.1) could be significantly different at both the calcium-binding EGF-like domain and the transmembrane domain sections.

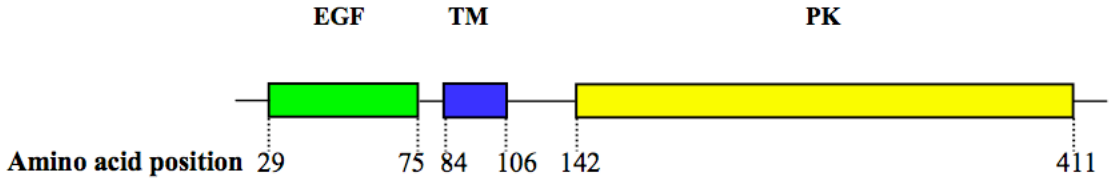


Figure 7.6: Schematic representation of the domain composition of the rice WAK protein.

The domain composition of the rice WAK (accession AAK52581) was predicted using the SMART database (http://smart.embl-heldelberg.de; (Schultz *et al.*, 1998). Three domains were found. **EGF**: a Ca^{+}-binding EGF-like domain, amino acid position 29-75. **TM**: a transmembrane domain, amino acid position 84-106. **PK**: a serine/threonine protein kinase domain, amino acid position 142-411. The diagram is not drawn to scale.

A

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  1 MVSLVKGIAS SQGGMEKSQR SLYAPMYADI NECDPSNKDK YPCYGVCKNI
 51 VGDYECSCHT GYQPSGGGPK KQECNPKFPV AAQLALGVSL GFSFLVVVVL
101 FTLMMLQRRK MNEYFKKNGG SILQNVDNIV IFSKDEMKKI LKNNSEVIGQ
151 GGFGKVYKGR LKDNTLVAVT TSIEVTEAQK EDFTNEVIIQ SRMMHNNIIK
201 LLGCCLEMDV PMLVYEFAAN GSLKDILHSD ASHLVPLTLD LRLDIAIESA
251 EGLRYMHSSI SHTIRHGDVK PANILLTDKF VAKISDFGTS KLLTVDKEFT
301 MVVAGSMGYI DPVFYMTGHL TQKSDVFSFG VVLLELISRR QTIYGKNRSL
351 IIEFQEAYDQ ANSGRLLFDK DIAIEEDVLI LEEIGRLAME CLNEKIDERP
401 DMKEVVARLM MLRRSRKLKQ ENYNISRQQF FEENSIDELP KSFDDNSSSS
451 SAELLSNLAT KNSLTYRSTT GRD
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B

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  1 MVSLVKGIAS SQGGMEKSQR SLYAPMYADI NECDPSNKDK YPCYGVCKNI
 51 VGDYECSCHT GYQPSGGGPK KQECNPKFPV AAQLALGVSL GFSFLVVVVL
101 FTLMMLQRRK MNEYFKKNGG SILQNVDNIV IFSKDEMKKI LKNNSEVIGQ
151 GGFGKVYKGR LKDNTLVAVT TSIEVTEAQK EDFTNEVIIQ SRMMHNNIIK
201 LLGCCLEMDV PMLVYEFAAN GSLKDILHSD ASHLVPLTLD LRLDIAIESA
251 EGLRYMHSSI SHTIRHGDVK PANILLTDKF VAKISDFGTS KLLTVDKEFT
301 MVVAGSMGYI DPVFYMTGHL TQKSDVFSFG VVLLELISRR QTIYGKNRSL
351 IIEFQEAYDQ ANSGRLLFDK DIAIEEDVLI LEEIGRLAME CLNEKIDERP
401 DMKEVVARLM MLRRSRKLKQ ENYNISRQQF FEENSIDELP KSFDDNSSSS
451 SAELLSNLAT KNSLTYRSTT GRD
```


Figure 7.7: Distribution of peptide matches between the sorghum CF1 and the rice WAK proteins.

(A) Peptides shown in red are the resultant peptide fingerprints of the CF1 protein, which match with those on the rice WAK amino acid sequence. (B) Predicted domains of rice WAK are superimposed on the actual amino acid sequence of the protein together with the matching peptides of CF1 (in red). Ca^{+}-binding EGF-like domain (amino acid position 29-75); a transmembrane domain (amino acid position 84-106); and a serine/threonine protein kinase domain (amino acid position 142-411).

7.5 The Known Biological Functions of Wall-Associated Kinases

Wall-associated kinases (WAKs) belong to a unique class of receptor-like kinases (Cosgrove, 2001; Kanneganti and Gupta, 2008) that are tightly bound to the cell wall (He *et al.*, 1996; Li *et al.*, 2009). Structurally, WAKs are transmembrane proteins, which consist of a highly conserved serine/threonine protein kinase domain on the cytoplasmic side and an extracellular domain with high similarity to the epidermal growth factor (EGF) of vertebrates (He *et al.*, 1996; He *et al.*, 1999; Verica and He, 2002). Because of their structural organisation, WAKs are considered to be physical linkers of the cell wall/ECM and the cytoplasm and thus might have signalling functions in response to both abiotic and biotic stresses (Anderson *et al.*, 2001; Kanneganti and Gupta, 2008; Li *et al.*, 2009).

Although the precise function of the extracellular EGF-like domain in plants is still unknown (Anderson *et al.*, 2001; Zhang *et al.*, 2005), they possibly perceives stimuli in the extracellular environment resulting in signals being transmitted to the cells via the cytoplasmic protein kinase domain (Morris and Walker, 2003). This assumption is reinforced by the observation that the abundances of spot CF1 increases following both salt and sorbitol stress treatment of sorghum cell suspension cultures (Figure 7.5). Furthermore, according to results of the domain composition and organisation using the SMART database, the extracellular EGF-like domain has Ca^{+} binding properties (Figure 7.6). As discussed in Chapter 1, Section 1.4, both salt and hyperosmotic stresses have a common element of inducing osmotic stress in plants. Furthermore, osmotic stress is known to induce calcium signalling in plants (Bressan *et al.*, 1998; Zhu, 2002). With this

reasoning, it could be possible that the extracellular Ca^{+} binding EGF-like domain (Figure 7.6) functions as the signalling component in both salt and sorbitol stress responses in sorghum.

The cytoplasmic serine/threonine protein kinase domain predicted in Figure 7.6 subsequently functions in ATP binding and protein phosphorylation at the serine/ threonine residues. Protein phosphorylation in plants is one of the PTM events that occur when plants are exposed to either abiotic or biotic stress (Chitteti and Peng, 2007; Kersten *et al.*, 2009). Reversible phosphorylation regulates cellular processes by changing protein structure, activity, stability, subcellular locations as well as interactions with other biological molecules (Kersten *et al.*, 2009). Wall-associated protein kinases are thus important in facilitating signal transduction processes between the external environment and the cytoplasm where various cellular responses to salt and/or osmotic stress occur.

7.6 Discussion

The sorghum cell suspension culture system was used to investigate proteome changes that occur in the extracellular culture medium in response to either salt (200 mM NaCl) and/or hyperosmotic (400 mM sorbitol) stress relative to the control. Prior to proteomic analysis, parameters such as cell viability, morphological changes in cell structure and pH changes of the culture medium were monitored to determine if the levels of stress used were sufficient to induce any structural or biochemical changes in the cells.

Estimation of cell viability was carried out using both the Evans blue and MTT tests at 0, 6 and 24 hrs following the introduction of the stress. Evans blue measures cell death, the converse of which is regarded as cell viability, while the MTT tests measures the metabolic activity of living cells. Therefore, the use of both methods in estimating cell viability would give more comprehensive information on the viability of sorghum cell cultures under stress. It was observed that relative to the controls, both 200 mM NaCl and 400 mM sorbitol treatments caused increased cell death (Figure 7.1A) as well as a decrease in the metabolic activities of the cells (Figure 7.1B). Although the viability of sorghum cells under stress was much lower than that of the control at 6 hrs, both the salt and sorbitol treatments did not cause total death of the cells. This is confirmed by the increase in cell viability between 6 and 24 hrs (Figure 7.1). Special mention is given to the remarkable increase in cell viability of sorbitol treated cells at 24 hrs following the introduction of the stress. It is possible that during sorbitol stress, cells perceive the stress via cell surface receptors, transmit the signals to the cytoplasm where cell growth and metabolic activities are rapidly increased as a response mechanism to the stress. This is supported in part by reports that when plants are exposed to salt stress in laboratory experiments, there is usually a rapid and temporary reduction in the plants' growth rate. With time, the plants then resume growth albeit at a reduced rate (Munns, 2002; Munns and Tester, 2008).

Microscopic analysis of the cells under both salt and sorbitol stress indicated that both stresses induced morphological changes in cell structure (Figure 7.2). Much of these changes are presumably due to osmotic stress induced by both the salt and sorbitol

treatments. The results are also consistent with the knowledge that plant cells under osmotic stress lose water to the external environment resulting in plasmolysis (Bidwell, 1979; Munns, 2002). As the duration of exposure to the stress increases, cells might die. Plant cell death under stress thus occurs in phases as indicated by the different appearances of the cytosolic constituents of the cells (i and ii; Figure 7.2B). Cytoplasmic condensation and shrinkage observed in this study are some known features of cell death that occur in plant cells during normal physiological growth as well as under abiotic stress conditions (McCabe *et al.*, 1997; McCabe and Leaver, 2000). These features have also been reported in suspension cultures of carrot under low cell density and heat stress (McCabe *et al.*, 1997) and tobacco under heat stress (Burbridge *et al.*, 2007).

Measurement of changes in pH of the culture medium showed that for all three treatment groups (control, NaCl and sorbitol), there was a gradual decline in pH over the 6 hr period (Figure 7.3). The control group maintained a lower pH than the NaCl and the sorbitol treated cells. A decrease in pH (acidification) of the culture medium treatment of *A. thaliana* cell suspension cultures following both 200 mM NaCl and 400 mM sorbitol stress has also been reported by Ndimba *et al*. (2005). However, the results obtained by Ndimba and co-workers indicated a transient acidification of the medium following one hour of stress treatment and a gradual increase of the pH up to 6 hours following treatment. It is not clear why sorghum cell suspension culture system in this current study continuously maintained a gradual decline in pH over the 6-hour period. Nevertheless, this decrease in pH of the culture medium was also observed between day 0 and day 3 when sorghum cell suspension cultures were subcultured into fresh medium for

propagation purposes (Chapter 3; Figure 3.6A). As discussed in Section 3.5, a decline in pH in cell cultures may be due to either differential absorption of nutrients such as phosphates, which have buffering properties and/or an increase in proton transport from the cytoplasm into the extracellular medium (Beyl, 1999; Evans *et al.*, 2003).

In order to verify that the culture medium was not contaminated with cytosolic proteins, western blotting analysis of the CF and TSP fractions was carried out using Hsp70 a known cytoplasmic protein. Because Hsp70 is also a stress responsive protein (Wang *et al.*, 2004; Ndimba *et al.*, 2005), its use in this experiment served dual purposes; to check (i) if plants undergoing normal physiological growth leaked out cytoplasmic constituents into the cell free culture medium; and (ii) if both salt and sorbitol stress treatments caused an increase in the rate of cell death, membrane damage and thus an increase in intracellular leakages into the culture medium. Using this immunoblotting approach, it was shown that none of the stress treatments resulted in detectable amounts of Hsp70 in the CF fraction (Figure 7.4A). However, this protein was present in the some of the cytosolic TSP fractions (Figure 7.4B). Based on these results, the CF fractions are less likely to be contaminated with cytoplasmic proteins. However, in future studies of sorghum secretomics, other lower molecular weight cytosolic proteins are to be used for assessing the level of cytosolic protein contamination of the CF fractions. The verification of cytoplasmic contaminants in the extracellular medium is an important routine step during secretome analysis. Western blotting techniques using RuBisCo (Dani *et al.*, 2005) and tubulin (Zhang *et al.*, 2009) have also been used in other studies of the secretome as a verification procedure for contaminants prior to secretome identification.

An inspection of protein changes in the sorghum cell suspension culture medium following 6 hrs of salt (200 mM NaCl) and hyperosmotic (400 mM sorbitol) stress treatment indicated an induction of only one spot that was robust enough to be seen with a naked eye within our experimental parameters (CF1; Figure 7.5). Using a combination of MALDI-TOF MS and database searching, the trypsinised peptide masses of protein spot CF1 matched the identity of a putative WAK from rice (accession AAK52581; Table 7.1). Both the theoretical and experimental MW/pI were in close approximation possibly showing that sorghum and rice WAK are highly similar as expected. This could be expected taking into account the close phylogenetic relationship between these two grasses (Rajendrakumar *et al.*, 2008).

Using the SMART bioinformatics tool for identification of protein domains, the rice WAK protein was shown to be composed three domains; a calcium-binding EGF-like domain, a transmembrane domain and a serine/threonine protein kinase domain (Figure 7.6). This domain structure and composition of rice wall-associated kinases has also been reported by (Zhang *et al.*, 2005). Although the functions of EGF-like domains in plants are not clearly known, they might be involved in ligand binding (Cosgrove, 2001). The EGF-like domain identified in the rice WAK has properties in calcium-binding, and thus may possibly implicate calcium signalling in sorghum in response to both salt and sorbitol stress. The cytoplasmic protein kinase domain then participates in signal transduction processes between the extracellular and the intracellular environment where stress response mechanisms are effected. Using this model, the identified sorghum WAK may also function as a physical link between culture medium and cell's cytoplasm during

abiotic stress conditions as has been already proposed for Arabidopsis WAKs (Anderson *et al.*, 2001; Kanneganti and Gupta, 2008; Li *et al.*, 2009).

Is not clear however why this putative wall-associated protein kinase (CF1; Figure 7.5), which is known to be tightly linked to the cell wall (He *et al.*, 1996; Zhang *et al.*, 2009) is found in the cell free extracellular medium of sorghum cell suspension cultures. As would be expected, wall-associated protein kinases have been reportedly found in proteomic studies of cell wall preparations of chickpea (*Cicer arietinum* L.) under dehydration stress (Bhushan *et al.*, 2007). Four other different transmembrane proteins have also been identified in a proteomic study of apoplastic fluids of *A. thaliana* rosettes (Boudart *et al.*, 2005). The proteins were identified as 1RX1, and homologs to CLAVATA1, fimbrin protein and a FAD-linked oxidoreductase. However, unlike CF1 identified in this current study (Figure 7.5; Table 7.1), which was shown to flank amino acid positions 7-386 of the 473-amino acid long rice WAK (Figure 7.7), all four transmembrane proteins identified by Boudart *et al*. (2005) were proteolytic fragments of their respective extracellular domains. The latter results are expected because of the high chances of proteolytic activities in the extracellular space.

The identification of spot CF1 (Figure 7.5), a homolog of rice WAK (accession AAK52581; Table 7.1) in the extracellular medium of sorghum cell suspension cultures (Figure 7.5) thus raises questions on the precise function of this protein in that compartment. More so with the observation that bioinformatics predictions of the in/out orientation of this protein using HMMTOP and TMHMM tools (Section 7.4.1) were not

totally convincing because these two algorithms gave antagonistic results. With these observations, it is noteworthy stating that although bioinformatics tools serve as useful prediction tools for a multitude of purposes in proteomics (Rose *et al.*, 2004), the results obtained from using such tools should always be substantiated experimentally (Lee *et al.*, 2004). In this case, immunolocalization experiments or localizations by green fluorescent protein (GFP) fusions (Jamet *et al.*, 2008) could possibly offer more insightful outcomes on the precise localization of the observed CF1 protein observed in this study. Furthermore, once the fully annotated sorghum genome sequence database becomes available to the general public, it is envisaged that more research groups would begin to invest their time and resources on sorghum proteomics and/or genomics and thus resulting in a larger pool of sorghum specific proteins in the public domain. For all we know, the CF1 protein spot (Figure 7.5) could be an extracytosolic homolog of the rice WAK protein, which does not possess a transmembrane domain. Such protein kinases have already been reported in Arabidopsis (Chivasa *et al.*, 2002) and the apoplast of rice leaves (Haslam *et al.*, 2003).

CHAPTER 8

General Discussion and Conclusion

This general discussion gives an overview of the some of the main findings of the study. More detailed discussions on each aspect of the research are at end of each respective Chapter within the main document.

The aim of this research project was to work on both descriptive and differential expressional proteomics of sorghum in response to salt and drought stress. The use of sorghum experimental systems in this study was motivated by several factors including (i) the crop's natural tolerance to drought stress (Rosenow *et al.*, 1983); (ii) the wide genetic diversity of the crop to salt tolerance (Krishnamurthy *et al.*, 2007); (iii) the sorghum genome sequencing initiative (Sorghum Genomic Planning Workshop Participants, 2005), which has just been completed (Paterson *et al.*, 2009); and (iv) the fact that sorghum proteomics is virtually an unknown field worldwide. Therefore, the results generated from the current study would provide insight on how sorghum proteomes change in response to salt and drought stresses. Consequently, this will result in bridging the knowledge gap that currently exists in sorghum proteomics. These results can also be used as reference tools by researchers working on sorghum as well as other cereal proteomics worldwide.

8.1 Experimental Systems Used in the Study

Sorghum cell suspension cultures as well as whole plants were used to study differential protein expression in sorghum in response to salt and/or hyperosmotic stresses. Each one of these systems has specific advantages over the other when used in proteomics studies. For instance, cell suspension cultures provide large amounts of uniform, undifferentiated cells, while whole plant systems provide tissue specific proteomes for studying changes in protein expression that occur at different levels of organisation within the whole plant. In addition, cells in suspension provide a source of easily extractable secreted proteins in the culture medium. These secreted proteins of suspension cells are thus equivalent to those of the apoplasts of whole plants. In whole plants, extraction of secreted proteins from the apoplast mainly relies on the vacuum infiltration method (Dani *et al.*, 2005; Soares *et al.*, 2007; Zhang *et al.*, 2009). However, as reported by Haslam and co-workers, this method has a potential of causing cell damage, which ultimately results in the contamination of apoplastic fractions with cytoplasmic proteins (Haslam *et al.*, 2003). In proteomics studies, high sample purity is of prime importance as it reduces the chances of identifying protein contaminants in the proteome under study. Without high sample purity, protein contaminants would otherwise be erroneously identified in their non-native cellular compartments. Since protein function is related to cellular localisation (Kumar *et al.*, 2002; van Wijk, 2004), the identification of protein contaminants in specific compartments/proteomes ultimately affects the precision of assigning putative functions to these contaminants. For this reason, in the current study, the sorghum cell culture system was used as source of secreted proteins while the whole plant system was used as a source to leaf, sheath and root tissue specific proteomes. Within the whole plant

system, comparative expressional proteomics in response to salt stress between two sorghum varieties AS6 and MN1618, which differed on their relative tolerance to salt stress (Figure 6.2) were also studied.

8.2 Protein Extraction, Separation and Visualisation by 2DE

In this study, a gel-based proteomics approach using 2DE in combination with MS was used to study sorghum proteomes because of several reasons: (i) this system has the potential to separate hundreds of proteins in one gel; (ii) is useful for differential expressional proteomics; (iii) is still relatively cheaper than other gel based techniques such as the fluorescent two-dimensional difference in-gel electrophoresis (2D-DIGE) and non-gel based techniques such as liquid chromatrography-mass spectrometry (LC-MS; Monteoliva and Albar, 2004); and (iv) the full complement of the 2DE system (IEF platform; mini and large format gel systems; PDQuest™ 2D Analysis Software; ExQuest™ spot cutter) and MALDI-TOF MS are all readily available in our proteomics facility.

Protein extraction procedures (reviewed in Section 1.7.1.1) are critical steps in the 2DE-based proteomics workflow as they influence the quality of protein extracts as well as the coverage of the proteome prior to downstream separation (using 2DE), visualisation (by protein staining and image analysis) and identification (by mass spectrometry). In this study, the TCA/acetone method was used for the extraction of sorghum proteomes. The established sorghum cell suspension culture system (Figure 3.2) was used as a source of TSP from the cells as well as CF proteins that were secreted in the culture medium

(Section 3.4). These two proteomes (TSP and CF) showed different expression profiles on both 1D (Figure 3.7) and 2D gels (Figures 3.8 and 3.9). The TSP (Figure 3.8) was more complex and was well separated on large format 18 cm gels over a pH range of 4-7. In contrast, the CF (Figure 3.9) was less complex, being composed of a smaller number of proteins, which were well resolved on mini format 7 cm gels. For the CF samples, both pH ranges of 3-10 and 4-7 were used due to their differential protein separation advantages (Figure 3.9A and B).

For whole plants, leaf, sheath and root tissue proteomes were extracted from both sorghum varieties (AS6 and MN1618). Of these three tissues, the root samples had the least quantities of protein per starting material, thus limiting the amount of protein sample available for further comparative proteome analysis. These observations are consistent with reports by Brechenmacher *et al*. (2009) who reported low total protein content in soyabean root tips. For all three tissues (leaf, sheath and root), protein extracts were of high quality as indicated by 1D gel electrophoresis (Figure 4.1). Furthermore, biological replicate protein extractions for the leaf and sheath tissues also showed high levels of reproducibility for each tissue type in AS6 and MN1618 sorghum varieties (Figure 4.1). This is particularly important when extracts are to be used in comparative analysis as it reduces the detection of non-reproducible protein expression differences (artefacts).

8.3 Protein Identification by Mass Spectrometry

Proteomic tools using 2DE and MS were used in this study because of their potential to allow for the separation, visualisation and identification of proteins in complex biological

systems. In this study, both MALDI-TOF MS and MALDI-TOF-TOF MS, in combination with genomic database searches, were used for the identification of protein spots of interest. Protein identification by these two types of mass spectrometers (reviewed in Section 1.6.5) are based on complementary principles; MALDI-TOF MS generates peptide masses, while MALDI-TOF-TOF MS further fragments the resultant peptides of MALDI-TOF MS to yield fragmented amino acid ion masses. The MS and MS/MS spectra generated by MALDI-TOF MS and MALDI-TOF-TOF MS respectively are subsequently matched with theoretical peptide masses and fragmented amino acid ion masses against the corresponding genomic sequence data of the organism under study or closely related species thereof. As would be envisaged, peptide mass fingerprinting may not always yield positive matches due to reasons such as non availabilities of complete genome sequence for the organism under study, sequence errors on the genomic databases, mutations as well as PTMs (Aebersold and Goodlett, 2001; Westermeier, 2005) amongst others. In such instances, MALDI-TOF-TOF MS, which generates an amino acid sequence on the trypsinised protein spot of interest, provides an alternative method for the identification of proteins from organisms, which have limited genome sequence data. All sorghum protein identifications in this study were carried out before the completion of sorghum genome sequencing project (Paterson *et al.*, 2009). For this reason, database searches relied on limited sorghum sequence data as well as similarity searches against sequence information from other green plants (Section 2.14).

Out of all the 59 protein spots that were picked for MS analysis in this study [40 for the leaf proteome map (Figure 4.4); 15 for the CF proteome map (Figure 5.1); three for the

heat shock proteins (Figure 6.6); and 1 for the WAK (Figure 7.5)], a total of 45 were positively identified as summarised in Tables 4.1, 5.1, 6.1 and 7.1 respectively. The 45 positively identified proteins out of the 59 protein spots picked for MS analysis equated to about 76% positively identified proteins. This high success rate in the identification of sorghum proteins, using partial genome sequence data has also been reported in soyabean (67%; Brechenmacher *et al*., 2009), whose full genome sequence data is not yet available. In the current study, the high success rate in protein identification can therefore be attributed in part to the high number of conserved genes in higher plants as well as to the added advantage of using MS/MS for the *de novo* sequencing of previously uncharacterised proteins.

Of the 14 unidentified proteins in this study [spots 3, 4, 15, 16, 19, 22, 23, 25, 27, 30, 31, 38 (Table 4.1); spot 10 (Table 5.1) and spot S3 (Table 6.1)], a total of 13 (all excluding spot S3) were analysed by both MALDI-TOF and MALDI-TOF-TOF MS. The fact that neither the MS nor MS/MS spectra of these protein spots were positively matched with high confidence to any protein when searched against all entries in the NCBI database (Section 2.14.2), further reinforces the need of an accurately annotated, complete genome sequence data to allow for positive identification of proteins in proteomics. In addition, these spots might represent sorghum specific proteins, which have not yet been characterised. For this reason, the recent announcement of the completion of the sorghum genome-sequencing project (Paterson *et al.*, 2009) is without doubt, a milestone in sorghum proteomics as well as proteomics studies of other cereals.

8.4 The Establishment of Sorghum 2DE Proteome Reference Maps

One of the objectives of this study was to work towards the establishment of sorghum proteome reference maps, which can be used as reference tools by sorghum and/or cereal proteomics research groups worldwide. Using 2DE and protein identification by mass spectrometry, a sorghum cell suspension culture secretome map (Figure 5.1; Table 5.1) and leaf proteome map (Figure 4.4; Table 4.1) were established. Although these maps are not fully saturated, they represent the first large-scale proteomics work on sorghum to date.

As observed in Figures 5.1, 5.4 and Table 5.1, proteins secreted by sorghum cell suspension cultures are mainly peroxidases, germin proteins, oxalate oxidases and alpha-galactosidases. These proteins function in defense mechanisms against pathogenic attack, signal transduction processes as well as cell wall metabolism. Although all these functions are commonly known among secreted proteins of cultured cells or the apoplast of whole plants (reviewed in Sections 5.1 and 7.1), the apparent dominance of oxidoreductases (peroxidases) and defense proteins (germins and oxalate oxidases) in our sorghum secretome is striking. Indeed, it is accepted that cell suspension cultures are non-physiological systems that exert both mechanical and oxidative stresses during continuous agitation in liquid culture medium (Isaacson and Rose, 2006; Jamet *et al.*, 2006). For this reason, the presence of peroxidases and germins in the culture medium might be a reflection of responses towards the stresses inherent to this culture system. Alternatively, this proteome composition might also reflect *bona fide* constituents of sorghum secretome, a virtually unknown field at present. This rationale is supported in

part by (i) results from comparative proteomic study of apoplast proteins of *Arabidopsis thaliana* and two grasses, rice (*Oryza sativa*) and wheat (*Triticum aestivum*) conducted by Haslam and co-workers (Haslam *et al.*, 2003). These co-workers identified germin-like proteins to dominate the proteomes of both rice and wheat. Since Haslam and co-workers conducted their study using the apoplast of leaf material from whole plant systems, the dominance of germins in our study might be a true reflection of the ECM constituents of grasses; (ii) the presence of H_2O_2 producing enzymes (oxalate oxidases and germins with oxalate oxidase activity) as well as those that utilise H_2O_2 (peroxidases) may be a reflection of a naturally balanced biological system in sorghum; and (iii) since germin protein synthesis is upregulated by auxins (Caliskan, 2000; Bernier and Berna, 2001), the high abundance of these proteins in our sorghum cell culture system could possibly be due to the presence of two auxins, 2,4-D and NAA, in the formulated sorghum callus and cell suspension MS media (Section 2.4).

The leaf proteome map was established using the MN1618 sorghum material (Section 4.5). Of the 40 spots that were picked for MS analysis, 28 were positively identified (Table 4.1). The identified protein spots represented six broad functional categories, which will remain putative until the precise functions of these sorghum proteins are experimentally determined. The sorghum leaf proteome was dominated by proteins involved in carbohydrate metabolism (60.7%), and others in proton transport (17.9%), protein synthesis (7.1%), hydrolytic functions (7.1%), nucleotide metabolism (3.6%) and detoxification (3.6%) as illustrated in Figure 4.5. The dominance of carbohydrate metabolism related proteins in our leaf proteome is in line with the primary

photosynthetic function of green leaves. Our current findings are also consistent with those from other leaf proteomics studies of maize (Porubleva *et al.*, 2001), barrel medic (Watson *et al.*, 2003), pea (Schiltz *et al.*, 2004), rice (Nozu *et al.*, 2006) and rapeseed (Albertin *et al.*, 2009). Out of these 28 identified leaf proteins, only one (spot 37) representing chlorophyll a/b-binding proteins type III precursor (Table 4.2) was an integral protein. Our results are also consistent with reports of the under representation of integral proteins in other proteomic studies (Porubleva *et al.*, 2001; Albertin *et al.*, 2009; Brechenmacher *et al.*, 2009). The analysis of membrane proteins in 2DE is limited by the proteins' low solubility in most 2DE extraction and solubilisation buffer (Molloy, 2000; Santoni *et al.*, 2000) and therefore specialised protocols are to be optimised if this class of proteins is to be extensively studied (Friso *et al.*, 2004).

A comparative proteomic analysis between the leaf proteomes of two sorghum varieties AS6 and MN1616 was also conducted (Section 4.6). Differences in protein expression (qualitative, quantitative and Student's *t*-test significant differential expression) between the two sorghum varieties were observed (Table 4.3; Figure 4.7). Of particular interest would be qualitative spots, such as spots X and 31 (Figure 4.7), which represent AS6 and MN1618 variety specific proteins. The identification of such proteins using 2DE techniques confirms the usefulness of proteomic technologies in allowing for the separation and visualisation of protein expression differences between different plant varieties. Such qualitative spot differences between varieties are important marker proteins, which link phenotypic characters to expressed genes (Zivy and de Vienne, 2000; Thiellement *et al.*, 2002).

8.5 Sorghum Stress Proteomics

The main objective of this study was to investigate protein expression changes, which occur in sorghum proteomes in response to salt and hyperosmotic stresses. To achieve this goal, both sorghum cell suspension cultures and whole plants were used as experimental systems. In the cell suspension culture system, changes in the expression pattern of proteins secreted in the culture medium were analysed following 200 mM NaCl (salt stress) and 400 mM sorbitol (hyperosmotic stress) treatments. To assess whether or not these treatments induced the expected stress responses in the cells, cell viability, changes in cell structure as well as pH of the culture medium were monitored following treatment (Section 7.2). Both Evans blue and MTT tests for cell viability indicated both stress treatments induced cell death (Figure 7.1A) as well as reduced metabolic activities (Figure 7.1B) following 6 hrs of treatment respectively. However, these effects were only transient, since cell viability gradually increased at 24 hrs for both stresses (Figure 7.1A and B). For this reason, the 6 hr treatment regime was selected for subsequent experiments. Microscopic analysis of the 6 hr salt and sorbitol treated cells also showed cellular changes associated with osmotic stress. In comparison with the controls, salt and sorbitol treated cells showed condensation and shrinking of cytoplasmic contents (Figure 7.2), an indication of plasmolyis (Bidwell, 1979) and cell death in plant cells under stress (McCabe and Leaver, 2000; Burbridge *et al.*, 2007). The CF protein extracts (control, NaCl and sorbitol treated) were also analysed for the presence of cytoplasmic contaminants using Western blotting techniques with Hsp70 antibodies. The immunoblotting results showed that none of the CF fractions with or without treatments has detectable heat shock protein 70s when compared to the TSP extracts (Figure 7.4).

This result confirmed that the CF protein fraction was less likely to be contaminated with cytoplasmic proteins. Therefore, the culture medium of sorghum cell suspensions provided an experimental system for studying protein expression changes, which occur in the ECM of plant cells in response to abiotic stresses.

Spot CF1 was shown to be induced by both salt and hyperosmotic stresses (Figure 7.5). Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) and database searches showed that the CF1 is similar to a rice WAK (accession AAK52581; Table 7.1). Bioinformatics analysis using the amino acid sequence of the rice WAK indicated that it is a single helix transmembrane protein (Table 7.2) composed of three domains; a calcium-binding EGF-like domain; a transmembrane domain and a serine/threonine protein kinase domain (Figure 7.6). If CF1 is indeed a transmembrane protein like its best matching protein, it is still not clear why CF1 is present in the ECM of sorghum cell suspensions. Amino acid sequence alignment of the matching peptides between the CFI spot and the rice WAK showed that out of the seven matching peptides, six formed part of the serine/threonine protein kinase domain with none representing either the calcium-binding EGF-like domain or the transmembrane domain (Figure 7.7). Based on this result, it is possible that the CF1 protein spot identified in this study might be similar to the rice WAK only at the serine/threonine protein kinase domain and not at the other two. In this case, CF1 protein (Figure 7.5) could represent an ECM protein kinase, with putative signalling functions in response to stress. Although this type of protein kinase has not yet been reported in sorghum proteomics studies because of limited proteome data of this plant, extracytoplasmic protein kinases have been identified

in the apoplast of rice leaves (Haslam *et al.*, 2003) and in Arabidopsis (Chivasa *et al.*, 2002).

The effect of 100 mM NaCl on the protein expression of leaf tissues of 14 day-old sorghum seedlings was investigated. Two sorghum varieties AS6 (salt sensitive) and MN1618 (salt tolerant) were selected for use in this experiment from a collection of 13 different sorghum varieties on the basis of their relative tolerance to 100 mM NaCl (Section 6.2; Figure 6.2). Protein extracts were prepared from the leaf, sheath and root tissues of both sorghum varieties and separated on 1D gels (Figures 6.3 and 6.4). Western blotting analysis of these 1D separated tissue protein extracts using Hsp70 antibodies showed that 100 mM NaCl treatment induced Hsp70 expression in both sorghum varieties (Figure 6.5), a known salt stress response (Wang *et al.*, 2004; Ndimba *et al.*, 2005) The immuno-reactive Hsp70 proteins were resolved by Hsp70 Western blotting analysis of 2DE separated sheath extracts (Figure 6.6). It was observed that the immuno-reactive region was composed of three protein spots (S1-S3) of MW of approximately 77 kDa and pIs of 5.3, 5.4 and 5.5 (Figure 6.6). A combination of MALDI-TOF MS and database searching confirmed the identity of S1 and S2 as a 70 kDa hsc protein 2 and a putative heat shock protein respectively, while S3 was not matched to any protein (Table 6.1). Based on these results, the 100 mM NaCl treatment of sorghum seedlings for 14 days was selected as a treatment regime for comparative analysis of salt stress responsive proteins of the two varieties.

Using the above experimental design, a comparative analysis of differential protein expression of leaf tissues of the varieties was carried out. Analysis was done using the PDQuest^{TM} Advanced 2D Analysis Software version 8.0.1 build 055 (BIO-RAD), with three CBB stained biological replicate gels per treatment group per sorghum varieties. Analysis sets of qualitative, quantitative and Student's *t*-test significant protein expression patterns between the control and salt treatment groups were created. In general, MN1618 was more responsive to salt stress than AS6 (Table 6.2; Figure 6.9; Figure 6.11A). In particular, MN1618 had more qualitative salt responsive spots (Figure 6.9A) of varying expression/abundance levels. With the exception of products of protein degradation, which occurs due to proteolytic activities and/or oxidative stress damage (reviewed in Section 1.8), the identification of upregulated proteins may lead to the identification of expressed genes which function in stress adaptations. Such genes could then be used as target genes for the improvement stress tolerance in agriculturally important crops (summarised in Table 1.1). For this reason, we used the partially annotated leaf proteome map (Figure 4.4; Table 4.1) to landmark some of the salt stress responsive protein in both sorghum varieties. Of the 40 spots that were picked for the mapping exercise, a total of 7 and 30 spots were responsive in AS6 and MN1618 respectively. Of these responsive proteins, a total of 2 and 19 were upregulated in AS6 and MN1618 respectively (Tables 6.3 and 6.4). The biological significance of these observed differences in protein expression between the two sorghum varieties were inferred from literature (Section 6.6). All six functional categories were responsive to salt between the two seed types (Table 6.4).

A few protein expression patterns observed in this comparative proteomic study are note worthy. These include (i) the increased abundances of GST (spot 26) in both sorghum varieties following salt stress. As summarised in Table 1.2 and Section 6.6.4, GSTs are known stress responsive proteins, which scavenge for ROS produced during secondary oxidative stress in plants under salt stress. The increased abundance of this protein in both varieties validates the importance of ROS detoxification mechanisms in plants as well as our experimental design for future stress proteomics studies in sorghum; (ii) the observed contrast in the expression/abundance of the large subunits of RuBisCo (spots 5 and 6; Table 6.4) between AS6 and MN1618 sorghum varieties; being downregulated in AS6 but upregulated in MN1618. Although the cause and/or significance of this difference in protein expression is not clear, it could illustrate differential effects of salt stress on the regulatory mechanisms and/or rates of degradation of this protein in the two sorghum varieties; (iii) the increased expression/abundance of hydroxynitrile lyases (spots 20 and 28; Table 6.4) in both sorghum varieties following salt stress. Although hydroxynitrile lyases are well known for their functions in cyanogenesis, a defense mechanism of plants against herbivores and microbial attack (Section 6.6.3), their roles in salt stress adaptation is poorly documented. For this reason, this study may provide initial findings of the involvement of these enzymes in salt stress responses. However, further studies would need to be conducted to verify their mode of action under conditions of salt stress; and (iv) the observed salt stress induced spots such as spot 3 (Table 6.4; Figure 6.12). Spot 3 is absent in both the control and salt treatment groups of AS6 sorghum variety. In MN1618, this spot is absent in the control treatments but is induced by salt stress (Figure 6.12). However, both MALDI-TOF and MALDI-TOF-TOF MS, and

database searching did not match this protein to any on the NCBI database. This could possibly indicate a previously uncharacterised (novel) variety specific salt stress responsive protein in sorghum. When identified, such proteins could possibly lead to the identification of expressed genes, which could be used as targets for genetic improvement of agricultural crops for salt tolerance.

The increased abundance of protein spots on 2DE proteome maps can be as a result of increased protein expression and/or a decrease in the rate of degradation of the proteins. In plants under stress, protein degradation can either be due to protease dependent or independent mechanisms (Salekdeh and Komatsu, 2007). Although a decrease in protein abundance is usually associated with susceptibility to stress, the identification of down regulated proteins could possibly result in the discovery of control mechanisms, whose reduced expression/abundance positively influences the abundance of other proteins under stress conditions (Bray, 2002). In this study, one of the downregulated proteins, spot 31 was an MN1618 variety specific protein (Figure 4.7 and 6.12). Since this sorghum variety was more salt responsive compared to AS6 (Figure 6.11; Table 6.4), the identification of such spots could possibly results in the discovery of control mechanisms, which positively influence the abundance of other proteins in the proteome. Currently, MS (both MALDI-TOF and MALDI-TOF-TOF MS) analysis of spot 31 did not yield a positive protein identity, thus making it and other unidentified proteins interesting candidates for further characterisation.

8.6 Conclusion

In this research, both descriptive and differential expressional proteomics of sorghum in response to salt and hyperosmotic stresses were studied, making it the first such study on sorghum to date. Using a combination of 2DE and mass spectrometry for the separation, visualisation and identification of proteins, this research worked towards the establishment of two sorghum proteome maps; a secretome map and a leaf proteome map. These two maps can be used as reference tools by proteomics researchers worldwide. Furthermore, stress proteomics conducted in this study provided the first proteomics study of this nature in sorghum. Comparative differential protein expression between the two sorghum varieties with or without salt stress has also indicated that the proteomic tools used in this study are able to separate and allow for the detection of qualitative, variety specific proteins in sorghum. Such qualitative proteins are potential protein markers, which can be used to link specific phenotypes to expressed genes. Expressed genes, which confer desired phenotypic traits can therefore be used in the genetic breeding programmes of agriculturally important crops. Overall, the findings of this study are a step in the process of bridging the gap that currently exists in sorghum proteomics and also provide a foundation for future studies in the same crop.

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APPENDIX I

Publication

African Journal of Biotechnology Vol. 7 (6), pp. 744-749, 18 March, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Establishment of sorghum cell suspension culture system for proteomics studies

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Accepted 22 January, 2008

This study describes the establishment of sorghum cell suspension culture system for use in proteomics studies. Friable sorghum callus was initiated from young shoots under completely dark conditions on MS medium supplemented with 3 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2.5 mg/L 1-naphthaleneacetic acid (NAA). Additionally, sorghum cell suspension cultures have been initiated from the friable callus masses in liquid medium with the same composition as the callus initiation medium. Total soluble proteins (TSP) and culture filtrate (CF) proteins were extracted from the cell culture system and solubilised in urea buffer (9 M urea, 2 M thiourea and 4% CHAPS). Both one-dimensional (1D) and two-dimensional (2D) gel analysis of these two proteomes show that the TSP and CF proteomes have different protein expression profiles. The sorghum TSP proteome, which is highly complex, is best resolved when separated on large format, 18 cm, pH 4 - 7 isoelectric focusing (IEF) immobilised pH gradient (IPG) strips. On the other hand, the sorghum CF proteome (secretome) is less complex with most proteins being resolved on mini format, 7 cm, pH 3 - 10 IPG strips. Furthermore, narrowing down the pH range from 3 - 10 to 4 - 7 for the CF proteome resulted in improved protein spot resolution.

Key words: Sorghum, proteomics, callus, cell suspension cultures, total soluble protein, secretome.

INTRODUCTION

Sorghum, a cereal crop native to Africa, is drought-tolerant, surviving periods of water deficit (Rosenow et al., 1983). The crop is grown in the semi-arid regions of Africa and Asia primarily as a human food source and in the United States as stock-feed. In terms of both area planted and production yields, sorghum is ranked the fifth most important cereal crop in the world (Doggett, 1988) and second after maize in Africa (FAO, 1995). Sorghum survives in hot and dry environments least suitable for other crops, thus making it an important food and energy source for the future. Sorghum is also a potential model plant for studying the complex mechanisms of drought tolerance in cereal crops.

Over the years, mechanisms of plant stress tolerance have been studied largely through measurements of mRNA expression profiles of genes of interest between stressed and unstressed plants. Although these transcriptomic studies provide important information regarding gene expression (Dubey and Grover, 2001) in particular, which genes are turned on and when (Abbott, 1999), there have been reports of the possibility of poor correlation between mRNA and protein levels (Gygi et al., 1999). This poor correlation may be attributed to the different rates of degradation of individual mRNAs and proteins as well as to the post-translational modification of many proteins (Abbott, 1999). In such cases therefore, the functions of genes may be better understood through the direct analysis or measurement of protein levels using proteomics technologies.

The field of proteomics is increasingly gaining momentum in plant sciences with several studies having been made and reported on agriculturally important crops such as maize (Riccardi et al., 1998), rice (Rakwal and Agrawal, 2003; Parker et al., 2006) and *Medicago truncatula* (Watson et al., 2003; Lei et al., 2005). Using

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proteomic approaches, both cell and tissue specific protein expression profiles under various stress conditions may be studied. Although there has been major proteomic advances using several other plant species, much of the knowledge of plant stress response mechanisms has been gained from work using *Arabidopsis* (Chivasa et al., 2002; Ndimba et al., 2005; Oh et al., 2005) mainly because of its small and fully sequenced genome (The Arabidopsis Genome Initiative, 2000). Even though *Arabidopsis* provides for an excellent model system for studying various plant processes under either natural or stress conditions, this plant is a dicot and is agriculturally unimportant (van Wijk, 2001). For this reason, there is need to understand stress tolerance mechanisms from the perspective of agriculturally significant monocots.

Despite the vast economic potential of sorghum in semi-arid areas as well the promising technique of proteomic approaches in understanding proteome expression profiles during abiotic stress conditions, no global sorghum proteomics studies have been reported to date. Several researchers have reported on the physiological distinction between pre- and post flowering drought tolerance (Rosenow et al., 1983), mRNA expression of dehydrin proteins (Cheng et al., 1993; Buchanan et al., 2005), dehydrin protein expression (Wood and Goldsborough, 1997), the increased accumulation of osmolytes such as glycine betaine (Wood et al., 1996) and soluble carbohydrates and organic acid (Newton et al., 1986) among various cultivars of *Sorghum bicolor*. It is our motivation therefore, to begin a comparative analysis of the global proteome expression of sorghum cell suspension cultures under a range of osmotic and salinity stress conditions. These efforts are encouraged by the ongoing sorghum sequencing project (Sorghum Genomics Planning Workshop Participants, 2005).

In this study, we report the establishment of sorghum callus, the initiation and maintenance of sorghum cell suspension cultures, as well as the evaluation of their potential for application in proteomics studies. Callus is an unorganised mass of undifferentiated cells which forms as a plant's protective response mechanism to seal off damaged plant tissue (Evans et al., 2003). In plant tissue culture, callus may be induced from various parts of the plant grown on solid media with appropriate combinations of plant hormonal supplements. Callus is particularly useful for the production of cells in suspension, which are a population of undifferentiated cells grown in liquid culture. In proteomics studies, cell suspension cultures provide an unlimited supply of uniform cells, which are grown in liquid culture. In comparison with whole plant systems, which have a relatively longer growth cycle as well as complex tissue specific proteomes, cells in suspension have a relatively shorter life cycle and remain undifferentiated. The shorter life cycle provides for a continuous supply of experimental units, which are grown under tightly controlled environmental conditions thus increasing reproducibility within and between experiments.

MATERIALS AND METHOD

Plant material

White sorghum seeds were purchased from Agricol, South Africa.

Plant tissue culture

White sorghum seeds were surface sterilised using 70% ethanol followed by 2.5% sodium hypochlorite solution for 20 min and then rinsed three times with sterile distilled water. The seeds were air-dried on filter paper before plating on half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium supplemented with 1% (w/v) sucrose, 5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) and 0.8% (w/v) agar, pH 5.8. Seeds were left to germinate in completely dark conditions at 25°C for about 3 - 4 days. Young shoots were cut into pieces of approximately 5 mm in length and used as explants for callus initiation on full strength MS medium supplemented with 3% (w/v) sucrose, 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 2.5 mg/L 1-naphthaleneacetic acid (NAA) and 0.8% (w/v) agar, pH 5.8 in the dark at 25°C on 10 cm diameter tissue culture dishes (Techno Plastic Products, Switzerland). Callus growth was visually assessed over a 4 week period. Friable callus was maintained in culture by sub-culturing 4-week old callus onto fresh callus initiation media.

Friable callus cultures were used to initiate cell suspension cultures in liquid MS media with the same composition and plant hormone supplements as the callus induction media. About 3 large clumps of 4-week old actively growing friable callus were placed in a 250 ml conical flask containing 50 ml of media. Flasks were incubated on a horizontal incubator-shaker under completely dark conditions at 25°C with agitation at 130 rpm until cultures reached the desired density. Cells were sub-cultured into fresh media by transferring about 40 ml of the cell suspension into a 250 ml flask containing 60 ml of fresh media.

Protein extraction

Sorghum cell suspension culture total soluble proteins

Ten-day old cell suspension cultures were separated from culture media by filtering over 4 layers of Miracloth (Merck, Darmstadt, Germany) and washed once with sterile distilled water. The cells were transferred into sterile tubes, pelleted by centrifugation at 2500 x *g* for 5 min and were then frozen in liquid nitrogen and stored at -20°C until use in protein extraction procedures.

Liquid nitrogen frozen cells (approximately 2 g) were ground using a pestle and mortar, transferred into 15 ml sterile tubes and precipitated with 10 ml of 10% trichloroacetic acid (TCA) in acetone. The cell debris and precipitated protein were collected by centrifugation at 13400 x *g* for 10 min at room temperature. The pellet was washed 3 times with 10 ml of ice-cold 80% acetone by centrifuging at 13 400 x *g* for 10 min per wash. The pellet was then 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 h with vigorous vortexing at room temperature. Soluble protein was collected by vortexing at 15700 x *g* for 10 min. Protein content of total soluble protein was estimated by a modified Bradford assay using BSA as standards. One-dimensional 12% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed to evaluate the quality of protein extracts.

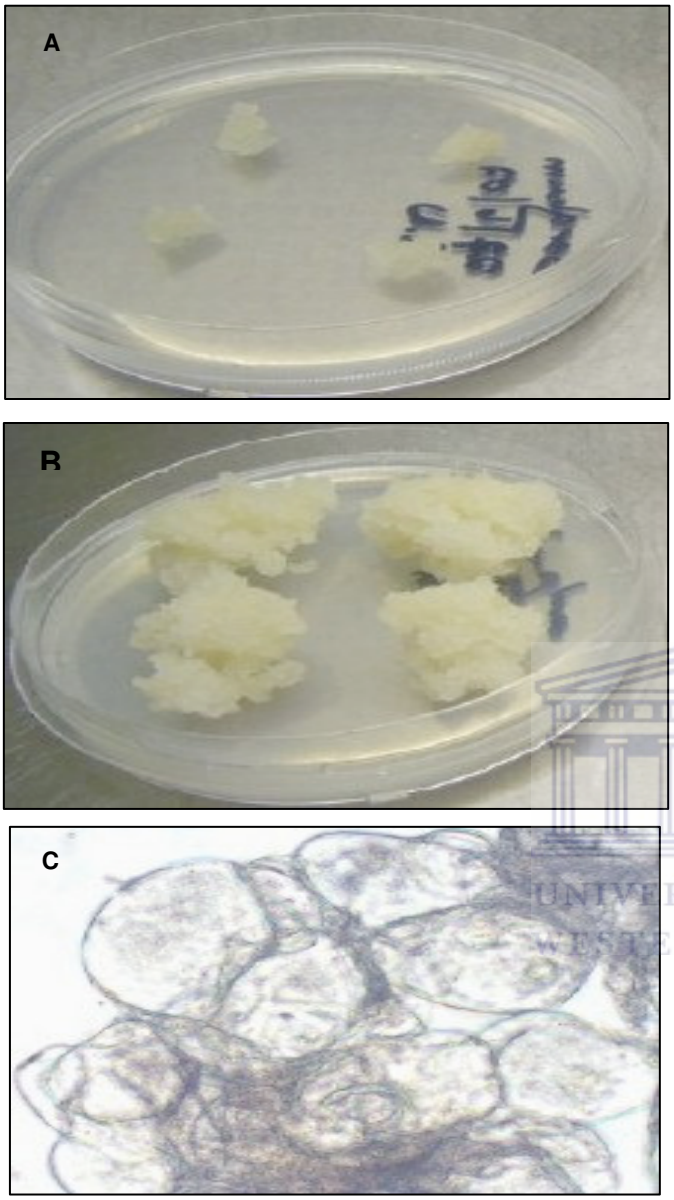

Figure 1. Sorghum callus cultured on tissue culture dishes (∅ 10 cm) and suspension culture cells grown under complete dark conditions on MS media supplemented with 2.4D and NAA. A. Callus masses used for subculturing and maintenance of callus. B. Friable sorghum callus masses after a 4 - 5 week incubation period. C. Microscopic pictures of sorghum suspension culture cells growing in liquid culture taken using a Nikon Inverted Light Microscope fitted with a Leica Firecam digital camera at a magnification of 10X.

Culture filtrate proteins

The culture medium was collected after filtering suspension cell cultures through 4 layers of Miracloth. Cell free culture filtrate was collected by centrifuging the culture medium at 2500 x *g* for 10 min. Culture filtrate proteins were precipitated in 80% acetone for at least 1 h at -20°C and collected in the pellet fraction after centrifugation at 15700 x *g* for 10 min. The pellet was washed 3 times using ice-cold 80% acetone, air dried at room temperature and re-suspended in 2 ml of urea buffer as described above.

2D Electrophoresis of protein

Total soluble proteins

Total soluble protein samples (400 and 800 µg) were mixed with 0.8% (v/v) dithiothreitol (DTT), 0.2% (v/v) ampholytes (BIO-RAD, Hercules, CA), a tiny pinch of bromophenol blue and made up to a final volume of 315 µl using urea buffer. The sample was then used to passively rehydrate linear 18 cm IPG strips, pH 4 - 7 (BIO-RAD) overnight at room temperature. The strips were subjected to isoelectric focusing (IEF) on an Ettan™ IPGphor II™ (GE Healthcare, Amersham, UK) in a step wise programme for a total of 66 000 Vhrs at 20°C. After IEF, the strips were equilibrated twice in an equilibration base buffer (6 M urea, 2% SDS, 50 mM Tris/HCl, pH 8.8 and 20% glycerol), firstly containing 2% (w/v) DTT followed by 2.5% (w/v) iodoacetamide for 15 minutes each with gentle agitation. Equilibrated strips were placed on 12% SDS PAGE gels (18 x 18 cm with 1 mm spacers) and electrophoresed on an Ettan™ DALTtwelve System (GE Healthcare), initially at 5 W/gel for 30 min and then at 17 W/gel (at a constant temperature of 25°C) until the bromophenol blue dye reached the bottom of the gel plates as described by Ndimba et al. (2005). The gels were stained overnight in modified Coomassie Blue stain, destained and imaged using a Molecular Imager PharosFX Plus System (BIO-RAD).

Culture filtrate proteins

CF protein (100 µg) was mixed with 0.8% (v/v) DTT, 0.2% (v/v) ampholytes (BIO-RAD), a tiny pinch of bromophenol blue and made up to a final volume of 125 µl using urea buffer. The sample was then used to passively rehydrate linear 7 cm IPG strips either pH 3 - 10 or pH 4 - 7, (BIO-RAD) overnight at room temperature. The strips were subjected to IEF on an Ettan™ IPGphor II™ (GE Healthcare) in a step wise programme for a total of 12 000 h at 20°C. After IEF, the strips were equilibrated in DTT and iodoacetamide as described above, placed on 12% SDS-PAGE and electrophoresed using the Mini-PROTEAN® 3 Electrophoresis Cell (BIO-RAD). After the second dimension, gels were Coomassie stained, destained and imaged using a Molecular Imager PharosFX Plus System (BIO-RAD).

RESULTS AND DISCUSSION

Plant tissue culture

Sorghum callus was successfully initiated from young shoot explant material cultured on MS media supplemented with 2,4-D and NAA. Visual assessment of callus growth under alternate 16 h light and 8 h dark conditions versus completely 24 h dark conditions showed that sorghum callus grew better under dark conditions. White friable callus masses consisting of soft easily separable cells were maintained in culture by transferring approximately 3-4 mm callus masses (Figure 1A) onto fresh media every 4 - 5 weeks. Suspension culture cells were also established from friable callus masses (Figure 1B) and maintained in liquid culture over several generations.

Microscopic analysis of cultured cells showed cell clusters and aggregates of various sizes (Figure 1C). Individual cells were also observed having a dense cytoplasm with numerous cytoplasmic vesicles possibly indicating high cell metabolic activities. The cells grew

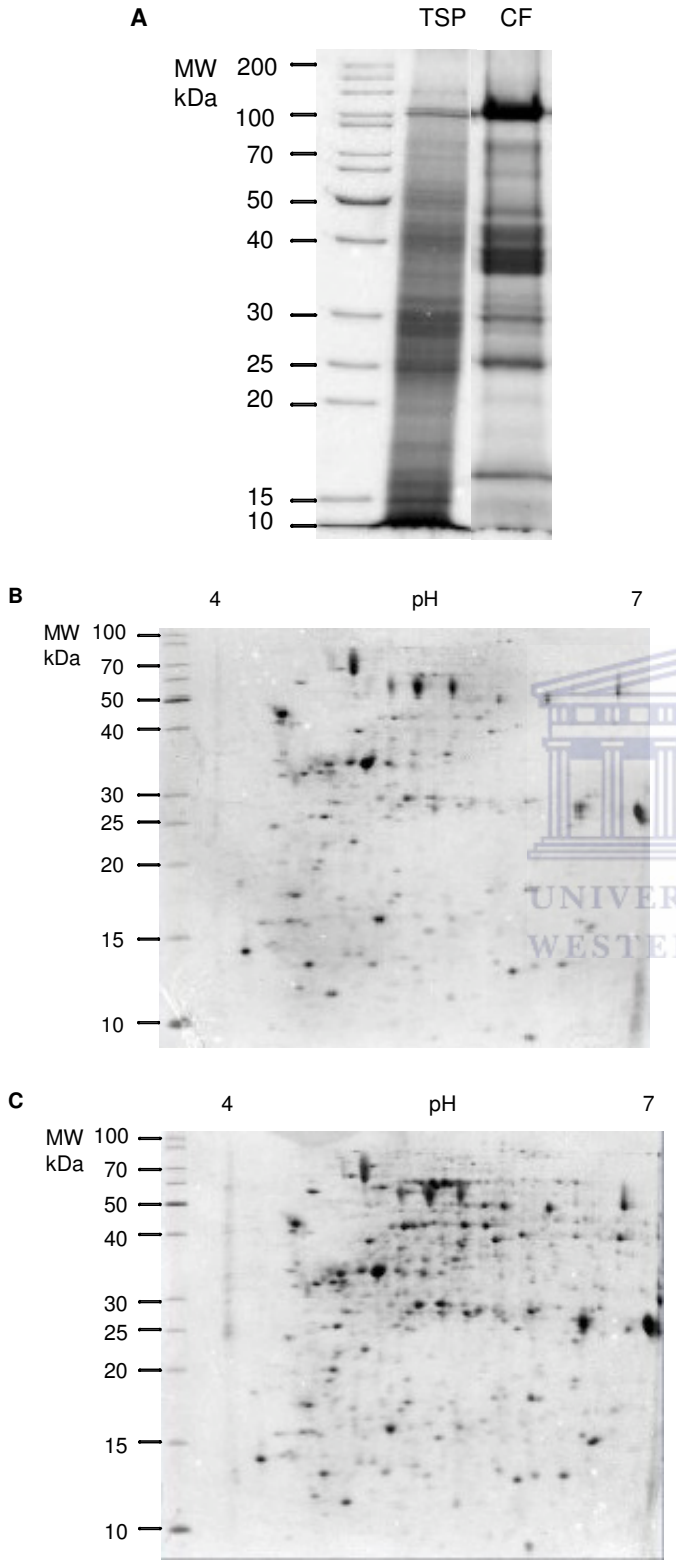

Figure 2. 1D and 2D SDS PAGE protein expression profiles of urea- buffer extracted total soluble proteins of sorghum suspension culture cells stained with Coomassie blue. A. 1D SDS-PAGE profile of sorghum total soluble protein (TSP) and culture filtrate proteins (CF). B. 2D expression profile of sorghum total soluble protein resolved on 18 cm IPG strips, pH 4-7 re-swelled with 400 µg. C. 800 µg of protein.

from a thin density immediately after subculturing, to a thick suspension of cells within 14 days. However, the measurement of cell growth by packed cell volume (PCV) has been rather difficult mainly because of two reasons. Firstly, sorghum cells occur in clusters of various sizes, making sampling even with a wide-bore pipette rather challenging. Secondly, although the cell clusters could be fairly distributed when agitated, they settled quickly resulting in inconsistencies in packed cell volume readings. So far therefore, we have not yet established a more reproducible growth curve of our sorghum cell suspension culture system.

1D and 2D expression profiles of sorghum proteomes

The sorghum cell suspension culture system established here is an invaluable system, which provides for an undifferentiated cell type that can be subjected to various experimental conditions. 1D gel analysis of total soluble protein (TSP) and culture filtrate (CF) showed that the two proteomes display different expression profiles (Figure 2A). The TSP exhibits numerous low abundant proteins while CF is composed of fewer but relatively higher abundant proteins. The isolated proteomes were separately subjected to 2D gel analysis on IPG strips of different lengths and pH ranges depending on the nature, composition and complexity of the protein mixture.

The TSP profile was better resolved on 18 cm strips, pH range of 4-7. Doubling TSP protein load on the IPG strips from 400 µg (Figure 2B) to 800 µg (Figure 2C) resulted in an increased intensity of Coomassie stained protein spots without causing any spot streaking due to protein overloading. Due to the less complex nature of sorghum CF fractions, mini gels (7 cm) were sufficient to resolve the whole CF proteome without any spot overlaps. Although the whole CF fraction could be resolved on the broad range IPG strips, pH 3 - 10 (Figure 3A), narrowing down the pH range to 4 - 7, resulted in an increase in spot resolution (Figure 3B). Protein spots, which were observed to be either closely migrating or co-migrating on IPG strips pH 3 - 10, became relatively well separated on IPG strips pH 4 - 7.

The observed differences in 2D proteome expression profiles of these fractions (TSP and CF) reinforces the fact that different proteomes, even if from the same plant system, are dynamic and heterogeneous, being composed of a complex mixture of proteins with different chemical and functional properties. The suspension culture system here described provides for two sets of proteomes, which although different in complexity play distinct but equally vital roles in maintaining biological processes in plant cells. Additionally, this experimental system demonstrates that we can separate hundreds of cellular soluble proteins and dozens of secreted proteins with high proficiency and efficiency.

Cell suspension culture systems have also been established for a wide range of plant species and applied

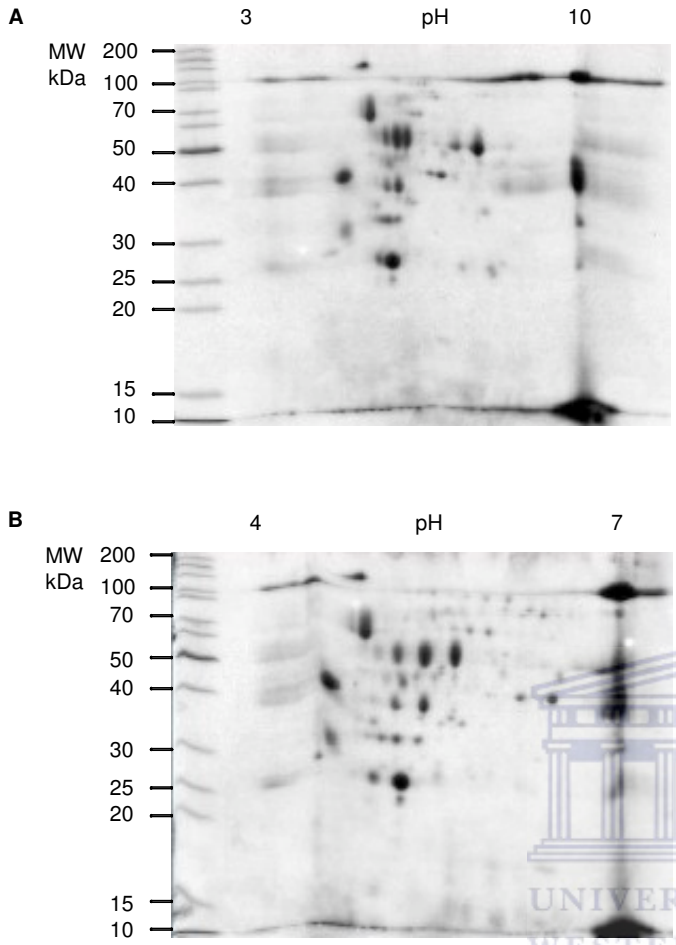

Figure 3. 2D SDS PAGE expression profiles of sorghum cell suspension culture filtrate (CF) stained with Coomassie blue. A. CF proteins resolved on 7 cm IPG strips pH 3 -10. B. pH 4 - 7. Each strip was passively re-swelled with urea- buffer containing 100 µg of protein.

in several proteomic-based studies (Slabas et al., 2004; Lei et al., 2005; Ndimba et al., 2005; Oh et al., 2005). There is no doubt therefore, that the culture system here described would provide the experimental material required for use in global proteomic studies based on both biotic and abiotic stress responses. The fractionation of culture systems into TSP and CF proteomes will help in the biochemical analysis of the separate biological processes that occur in the inter and intracellular matrix of plant cells.

The use of *Sorghum bicolor* suspension culture system, and high technology proteomics, provides a unique experimental model that will help in further understanding plant cell biology. Additionally, we intend to use this experimental system towards further understanding the molecular basis for sorghum's tolerance to drought. Future work will therefore involve further analysis of sorghum cell suspension culture proteomes by mapping and identifying as many expressed proteins as possible via mass spectrometry. This exercise will provide invaluable reference maps of the sorghum soluble cellular proteome and secretome. We also intend to study stress responsive proteins, both in terms of their abundance and post-translational changes.

ACKNOWLEDGEMENTS

We are grateful to the National Research Foundation of South Africa (NRF) for their financial support. Special gratitude goes to the University of the Western Cape's Special Grant for our proteomics equipment. We also thank Dr Mervin Meyer for assisting us with the microscopy work.

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