

**A COMPARATIVE PHYSIOLOGICAL, PROTEOMIC AND
BIOCHEMICAL ANALYSIS OF SORGHUM SEEDLINGS UNDER
SALT STRESS**

Sellwane Jeanette Mloi

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A dissertation submitted in fulfilment of the requirements in respect of the Masters Degree qualification in the Department of Plant Sciences in the Faculty of Natural and Agricultural Sciences at the University of the Free State, QwaQwa Campus.



Supervisor: Dr. Rudo Ngara
Co-supervisor: Dr. Nemera Shargie

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DECLARATION

I, Sellwane Jeanette Moloji, declare that the Masters Degree research dissertation that I herewith submit for the Masters Degree qualification in Botany at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

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DEDICATIONS

I dedicate this work to the most special person in my life my daughter, Lindokuhle Mosa Moloji. You have been a blessing in my life and I will always try my best to make you proud and be the mother you deserve.

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1propanesulfonate
DTT	Dithiothreitol Cleland's reagent
HILIC	Hydrophilic interaction chromatography
iTRAQ	Isobaric tags for relative and absolute quantitation
kDa	kilo Dalton
LC/MS	Liquid chromatography mass spectrometry
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
1D	One-dimensional
2D	Two-dimensional
1D-SDS-PAGE	One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
v/v	volume to volume
w/v	weight to volume

ABSTRACT

Soil salinity negatively affects plant growth and development, causing crop losses, worldwide. Understanding plant response mechanisms towards salt stress is thus an important step in ensuring food security. Sorghum [*Sorghum bicolor* (L.) Moench] is a drought and moderately salt tolerant crop and thus provides a potentially good model system for stress response studies. In this study, a comparative physiological, biochemical and proteomic analysis of two sorghum varieties was conducted under salt stress. SA 1441 is drought tolerant, while ICSB 338 is both drought and salt susceptible. The sorghum seedlings were grown in soil until the V3 growth stage before salt-stress treatment with 0-400 mM NaCl for seven days was applied. Physiological parameters such as relative water content, leaf water loss, stomatal conductance, chlorophyll content, shoot and root length and weight were measured following the salt-stress treatment. The results indicated a statistically significant decrease in the measured parameters with an increase in salt stress in ICSB 338, compared to SA 1441. Furthermore, the leaf relative water content of ICSB 338 was significantly lower than that of SA 1441. Proline and glycine betaine accumulation was also analysed in both sorghum varieties across all NaCl treatments. The results showed that proline played a major role in osmotic adjustment in both sorghum varieties when compared to glycine betaine. However, SA 1441 accumulated more proline levels at higher salt treatment levels than ICSB 338. Based on the physiological and biochemical results, the 100 mM NaCl treatment was selected for proteomic analysis. The isobaric tags for relative and absolute quantitation (iTRAQ) proteomic method was used to identify root and leaf salt-stress responsive proteins in both sorghum varieties. In the root proteome, 522 and 544 proteins were positively identified in SA 1441 and ICSB 338 sorghum varieties, respectively. From these root proteins, 26 (SA 1441) and 31 (ICSB 338) were responsive to 100 mM NaCl-induced salt

stress. Most of the differentially expressed root proteins were involved in disease/defence functions in both sorghum varieties. In the leaf proteome, 829 (SA 1441) and 591 (ICSB 338) proteins were positively identified. From these leaf proteins, 75 (SA 1441) and 24 (ICSB 338) were responsive to salt-stress. Most of the differentially expressed leaf proteins were associated with disease/defence functions in SA 1441, followed by energy and metabolism in both sorghum varieties. Collectively, the physiological and proteomic results suggest that SA 1441, the drought tolerant variety was more protected against the salt stress better than ICSB 338, the drought and salt susceptible variety. Therefore, the results also highlight the differences in salt tolerance between the two sorghum varieties and possibly reinforce the cross-link between drought and salt-stress response mechanisms. The results of this study can be used as reference tools in studies focusing on the differences in salt stress tolerance between varieties.

Keywords: Sorghum, salinity stress, physiological and growth parameters, chlorophyll content, proline, glycine betaine, proteomics, iTRAQ.

CHAPTER 1

LITERATURE REVIEW

1.1 Sorghum, Origin and Importance

Sorghum [*Sorghum bicolor* (L.) Moench; Figure 1.1] is an annual, interbreeding cereal crop, which originated in Africa. It evolved across different environments and exhibits a wide genetic diversity (Kimber *et al.*, 2013). This crop is used for a variety of purposes such as food for human consumption, animal feed, fiber and fuel (Borrell *et al.*, 2014). In terms of production, sorghum is ranked the fifth most important cereal crop after maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (FAOSTAT, 2016).



Figure 1.1: Image of sorghum crop stand, accessed on (July 2016), <http://b4fa.org/sorghum>.

Sorghum is drought tolerant (Rosenow *et al.*, 1983) and more salt tolerant than maize and other crops (Krishnamurthy *et al.*, 2007). At germination, it is considered more salt tolerant than the later stages of growth (Kafi *et al.*, 2013). This crop is able to grow in semi-arid regions, mainly due to its extensive root system, which enables it to draw moisture from deep within the soil (Reddy *et al.*, 2012). Sorghum also reduces transpirational water loss through leaf rolling and a waxy leaf surface. Under drought conditions, sorghum reduces metabolic processes to near dormancy (Pavli *et al.*, 2013). This extensive range of sorghum's adaptability allows it to be easily cultivated in dry conditions.

Furthermore, sorghum's adaptation to marginal lands could help in alleviating crop losses in areas affected by abiotic stresses. It is also viewed as an ideal candidate for crop improvement programmes in agriculture and in the emerging biofuel industry (Dahlberg *et al.*, 2011). Sorghum is also second in the grass family after rice (International Rice Genome Sequencing Project, 2005) and the first C₄ plant to have its genome fully sequenced (Paterson *et al.*, 2009). Fully sequenced genomes are useful resources for the identification of genes in understanding the genetic properties and networks that contribute to the development of plants (Perez-de-Castro *et al.*, 2012), and their adaptations to stressful conditions.

1.2 Abiotic Stresses and their General Effects on Plants

An abiotic stress is any environmental factor which negatively affects the optimal functioning of an organism (Bhatt *et al.*, 2015). Examples include drought, salinity, flooding, heat, cold, ultraviolet radiation and heavy metal toxicity. These environmental stresses negatively affect plant growth and development, and may reduce agricultural productivity by more than 50% in average yields of most crops (Mahajan and Tuteja, 2005; Atkinson and Urwin, 2012). Reductions in crop yield subsequently cause production losses thus threatening the

agricultural industries (Mahajan and Tuteja, 2005). With the climate change and the growing world population, food security then also becomes a global concern (Savvides *et al.*, 2016).

Abiotic stresses occur simultaneously in nature. As such, plants are often exposed to combinations of stress factors at some stage during their life cycle. For example, the effect of heat stress on plants is often followed by water deficiency, while drought stress is accompanied by salinity stress (Slama *et al.*, 2015). These abiotic stresses may result in similar cellular damage in plants (Wang *et al.*, 2003). The most common effects of abiotic stresses on plants are osmotic and oxidative stresses, which ultimately affect plant growth and development (Chinnusamy *et al.*, 2004). For example, both drought and salt stress induce osmotic stress in plants, which disrupts cellular homeostasis and ion distribution. High temperature, salt, and drought stresses all cause oxidative stress, which denatures functional and structural proteins, and damages lipids and DNA (Wang *et al.*, 2003; Gill and Tuteja, 2010). Understanding the mechanisms used by plants to sense and respond to different abiotic stresses can be beneficial in programmes used to breed crops for improved productivity under unfavourable conditions.

Due to their sessile nature, plants have developed different response mechanisms to overcome the challenges imposed on them by abiotic stresses. Upon sensing a stressful environment, plants activate signalling pathways and cellular response mechanisms such as accumulation of compatible solutes, an increase in detoxifying enzymes and production of stress responsive genes and proteins (Wang *et al.*, 2003). These activated molecular networks ultimately result in the re-establishment of cellular homeostasis, and protection and repair of damaged proteins and membranes (Vinocur and Altman, 2005). Figure 1.2 illustrates the complexity of plant responses mechanism towards different abiotic stresses.

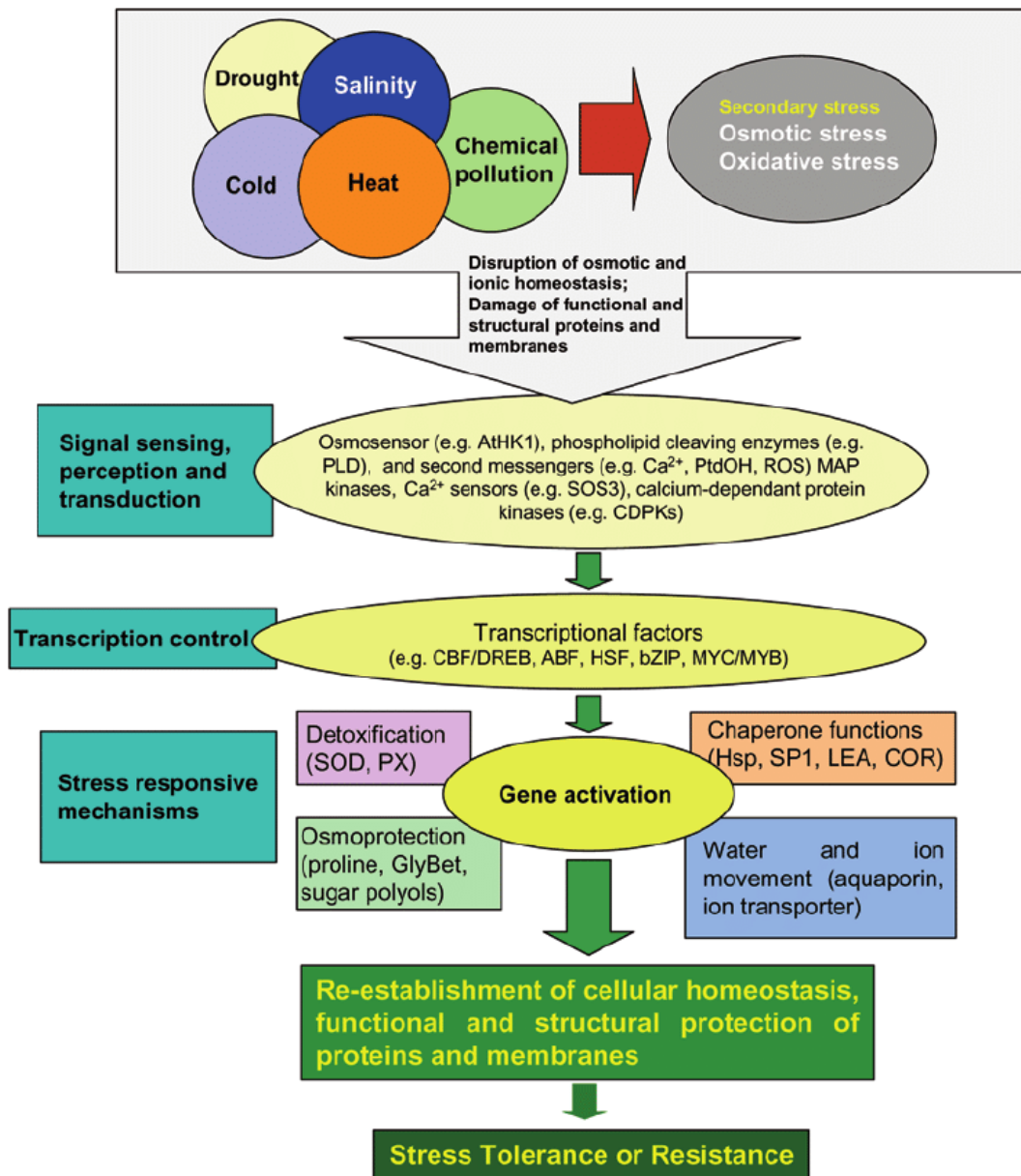


Figure 1.2: Plant response mechanisms toward different abiotic stresses (Wang *et al.*, 2003).

1.3 Salt Stress and its Effects on Plants

Salt stress is defined as the accumulation of high concentrations of soluble salts in a soil solution and is measured in electrical conductivity (ECe in dS/m). Soil is considered to be

saline when the electrical conductivity exceeds 4 dS/m, equivalent to 40 mM NaCl (Munns and Tester, 2008). Soluble salts include sodium (Na^+), chloride (Cl^-), calcium (Ca^{2+}), sulphate (SO_4^{2-}) and bicarbonate (HCO_3^-) (Bhatt *et al.*, 2015). Of these, sodium chloride (NaCl) is regarded as the most soluble and widespread salt affecting the development and growth of plants (Munns and Tester, 2008).

Saline soils can be located at different altitudes ranging from below sea level to mountainous areas (Carillo *et al.*, 2011), and are estimated to cover about 5-10% of the world's arable land (Krishnamurthy *et al.*, 2007). A total of 45 million out of the 230 million hectares of irrigated land worldwide, and 32 million out of 1500 million hectares under dry land agriculture are salt-affected (Parihar *et al.*, 2015). Apart from natural factors such as weathering of rocks, poor irrigation practices largely contribute to the increase in soil salinity (Chen and Jiang, 2010).

Salt stress causes osmotic stress and ion toxicity (Ashraf and Harris, 2004), which result in nutritional deficiencies and oxidative stress in plants (Chinnusamy *et al.*, 2006). Like other environmental factors, salt stress negatively affects normal plant growth and development, causing crop losses worldwide. The effects inflicted by salt stress on plants can be observed in various physiological and metabolic processes depending on the severity and duration of the stress (Gupta and Huang, 2014). At the whole-plant level, the effects of salt stress can be observed during seed germination, vegetative growth and reproductive stages (Parida and Das, 2005). Examples of the major effects of salt stress on plants include the inhibition of germination and photosynthesis, growth reduction, altered water uptake and nutrient imbalance (Hasanuzzaman *et al.*, 2013).

1.3.1 Responses of Plants to the Effects of Salt Stress

1.3.1.1 Osmotic Stress

The presence of high salt concentrations in the soil decreases the ability of a plant to absorb water, subsequently causing cellular dehydration and ultimately reduced plant growth (Munns *et al.*, 2006). Plants respond to the osmotic effect of salt stress through osmotic adjustment (Kosová *et al.*, 2013).

Osmotic adjustment involves the accumulation of organic solutes and inorganic ions (Chen and Jiang, 2010). Organic solutes (also referred to as compatible solutes, osmoprotectants or osmolytes) are low molecular weight and highly soluble compounds, which are non-toxic at relatively high cellular concentrations (Munns, 2005). These organic solutes act as protective mechanisms towards salt stress effects by detoxifying reactive oxygen species (ROS), protecting membrane integrity, and stabilizing proteins and enzymes (Ashraf and Foolad, 2007). Organic solutes also reduce the cellular osmotic potential against an osmotic gradient between root cells and the outside saline solution, which ultimately restore water uptake by the roots (Horie *et al.*, 2012).

Examples of organic solutes include proline, sucrose, polyols, trehalose and quaternary ammonium compounds such as glycine betaine, alinine betaine and proline betaine. Proline and glycine betaine accumulate in many plant species when exposed to a range of abiotic stresses (Ashraf and Foolad, 2007). According to Munns and Tester (2008), in halophytic plants such as *Thellungiella halophila*, the production of proline and glycine betaine is high in the leaves and contributes to the osmotic pressure in the cells. However, the concentration of these compatible solutes is lower in glycophytes such as *Arabidopsis thaliana*, sugarbeet (*Beta vulgaris*) and rice.

Osmotic adjustment also occurs due to the accumulation of inorganic ions such as potassium (K^+) in the cytosol and Na^+ in the vacuole (Horie *et al.*, 2012). In halophytic plants, inorganic ions accumulate to maintain the osmotic gradient for water uptake (Chen and Jiang, 2010). However, the degree of osmotic adjustment in plants may differ among plant species, depending on the growth stage of the plant, duration and severity of the stress (Acosta-Motos *et al.*, 2017).

In a study by Meloni *et al.* (2001), the osmotic adjustment of cotton (*Gossypium hirsutum*) cultivars under 50, 100 and 200 mol m^{-3} NaCl salt-stress treatment was a result of accumulation of Na^+ and Cl^- in both leaves and roots. Proline accumulation in both tissues did not show large increases. In another study on wheat, an increase in osmolyte concentrations of proline and glycine betaine under two levels (5.4 and 10.6 dS m^{-1}) of salt-stress treatment was reported (Sairam *et al.*, 2002). In sorghum, there was an increase in osmolyte concentrations of proline and soluble carbohydrates with an increase in NaCl concentrations of 100 and 200 mM (Heidari, 2009).

1.3.1.2 Ionic Stress

Ionic stress is a long term effect of salinity, and depends on the duration of exposure of plants to the stress as well as the level of salinity (Kosová *et al.*, 2013). High salt concentrations in a plant can reach toxic levels, especially in older leaves (Munns and Tester, 2008). Furthermore, the over-accumulation of Na^+ ion in the cytoplasm may ultimately disrupt cellular metabolic processes such as photosynthesis, protein synthesis and enzyme activity (Horie *et al.*, 2012). In order to avoid the toxic effect of ionic stress, plants can either prevent salt entry or minimize salt concentrations that accumulate in cells.

Salt exclusion occurs in the roots. Consequently, Na⁺ ions are excluded from leaf blades, thus preventing their accumulation to toxic levels in cells. Additionally, plants can also compartmentalize the large amounts of salts in vacuoles of different tissues in order to facilitate the progression of metabolic functions (Parida and Das, 2005; Munns and Tester, 2008; Chaves *et al.*, 2009). Studies have reported reductions in plant growth as a result of the effect of salt stress in crops such as sugar beet (Ghoulam *et al.*, 2002) and canola (*Brassica napus*) (Bandehagh *et al.*, 2011).

1.3.1.3 Nutritional Imbalance

Salt stress results in nutritional imbalances, mainly because of the limitations of nutrients and their distribution within the plant (Grieve *et al.*, 2012). High levels of Na⁺ and Cl⁻ concentrations in the soil cause a reduction in the uptake of other essential nutrients such as Ca²⁺, K⁺ and manganese (Mn²⁺). For example, K⁺ is required for maintaining osmotic balance and cell turgor as well as in protein synthesis and photosynthetic processes (Nawaz *et al.*, 2010). Reasonable amounts of K⁺ and Ca²⁺ are also required to maintain the functioning of cell membranes (Nawaz *et al.*, 2010). Reductions in nutrient uptake as a result of salt stress has been observed in root and leaf tissues of sorghum varieties (Bavei *et al.*, 2011).

1.3.1.4 Oxidative Stress

Under normal growth conditions, ROS are produced in plants during cellular processes occurring in chloroplasts, mitochondria and plasma membranes, but they remain at low concentrations. However, environmental factors such as drought, salinity and high temperature disrupt cellular homeostasis in plants by increasing the production of ROS (Sharma *et al.*, 2012). This overproduction of ROS in plant cells causes oxidative damage to DNA, proteins, lipids and carbohydrates (Gill and Tuteja, 2010). Examples of ROS include

free radicals such as hydrogen peroxide, superoxide, hydroxyl radical, and singlet oxygen (Ashraf, 2009). For example, Nxele *et al.* (2017) reported an increase in the accumulation of hydrogen peroxide which resulted in lipid peroxidation in roots and leaves of sorghum in response to 100 mM NaCl.

To detoxify or eliminate high levels of ROS, plants produce a variety of enzymatic and non-enzymatic antioxidant systems. Enzymatic antioxidants include ascorbate peroxidase, superoxide dismutase, catalase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase, while non-enzymatic antioxidants include glutathione, ascorbate, carotenoids and tocopherols (Hung *et al.*, 2005). Antioxidants may also influence gene expression associated with different biotic and abiotic stress responses in order to maximize their protective functions in plants (Foyer and Noctor, 2005). The activities of antioxidative enzymes were increased in roots and leaves of sorghum with increasing salinity levels up to 150 mM NaCl (Omari and Nhiri, 2015). In another study by Taïbi *et al.* (2016), an increase in the activity of antioxidants was observed with an increase in NaCl concentrations from 50-200 mM in leaves of two bean (*Phaseolus vulgaris*) genotypes.

As discussed above, it is evident that plants employ a variety of biochemical, physiological and metabolic strategies to withstand the prevailing stresses. In order to better understand plant systems in stress response, “omics” approaches such as genomics, transcriptomics, proteomics and metabolomics are employed. These technologies are however complementary, and are aimed at detecting genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) and their complex interactions in biological samples (Horgan and Kenny, 2011). The current study focused mainly on the applications of proteomics and metabolomics approaches in plant stress responses. Figure 1.3

shows the “omics” technologies employed in studying plant stress responses with the ultimate aim of improving plant breeding.

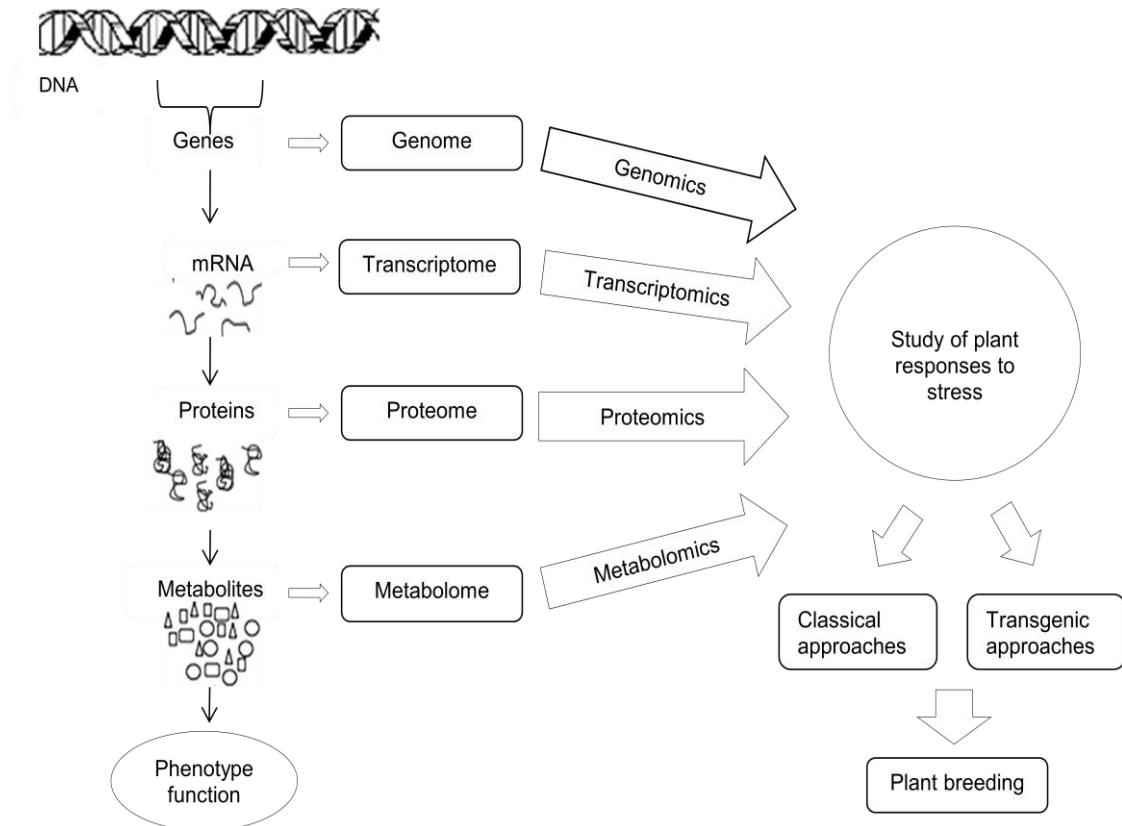


Figure 1.3: “Omics” technologies in plant stress response studies (Adapted from Pérez-Clemente *et al.*, 2013).

1.4 Metabolomics and Proteomics Approaches in Studying Plant Stress Response Mechanisms

1.4.1 Metabolomics

Metabolomics analyses metabolites in a given cell at a specific time (Cramer *et al.*, 2011).

Plants synthesize a variety of metabolites, which form part of the defence mechanism against

salt stress. Examples of such metabolites include polyols mannitol, glycine betaine, sucrose, trehalose and proline (Shulaev *et al.*, 2008). Different techniques such as gas or liquid chromatography mass spectrometry (GC-MS or LC-MS) and nuclear magnetic resonance (NMR) can be used for the separation, detection and identification of metabolites expressed under a range of stress conditions (Obata and Fernie, 2012).

Hossain *et al.* (2017) conducted a metabolite profiling on sugar beet at cellular and subcellular levels in response to 300 mM NaCl treatment for 3 hours and 14 days, respectively. Metabolite profiling using GC-MS resulted in the identification of 83 metabolites in leaves under control and stress conditions. Metabolites of the Calvin cycle decreased under salinity at the whole-leaf level, while proline, inositol and leucine increased. Glycolate and serine levels also increased, indicating that photorespiratory metabolism was stimulated in salt-stressed sugar beet. The accumulation of compatible solutes such as proline, mannitol, and putrescine was mostly observed outside the chloroplasts. On the other hand, putrescine had the highest relative level within the chloroplast and probably assisted in the acclimation of sugar beet to high salinity stress. The results of the study provided information on the contribution of chloroplasts and the extra-chloroplastic space in salinity tolerance in sugar beet (Hossain *et al.*, 2017).

In another study by Pavli *et al.* (2013), the GC-MS technique was used to comparatively analyse metabolite profiling of sorghum leaves and roots treated with 2.5% and 5% polyethylene glycol (PEG) for 7 days. A comparison between stressed and control plants revealed that the metabolite content in both leaves and roots of sorghum plants were substantially altered under drought stress conditions. Increased levels of sugars were reported in both leaf and root tissues of osmotic stressed sorghum plants. The most increase was

observed in the level of D-mannose, D-glucose, isomaltose, fructose and sucrose, but also myo-inositol and L-asparagine in leaves. In roots, D-glucose, fructose, sucrose and D-(+)trehalose as well as D-mannitol showed a significant increase upon stress at both PEG concentrations.

Zhao *et al.* (2014) used GC-MS to analyse metabolite levels in leaves and roots of two rice genotypes with contrasting salt tolerance in a salt-stress time course experiment in response to 100 mM NaCl. A total of 92 metabolites in the leaves and roots of both rice genotypes were identified. Sugars and amino acids significantly increased in the leaves and roots of both genotypes, while organic acids such as citrate acid and malic acid increased in roots and decreased in leaves. The salt-tolerant genotype in relation to the salt-sensitive one exhibited a greater increase in sugars and amino acids, and a greater decrease in organic acids in both tissues. A total of 11 metabolites including amino acids and sugars were specifically increased in the salt-tolerant genotype during the course of salt-stress treatment. Out of the 11 metabolites, lactose, sorbitol and melicitose were identified as being related to salt tolerance (Zhao *et al.*,2014).

1.4.2 Proteomics

Proteomics is defined as the analysis of proteins in a cell, tissue or organism (Graves and Haystead, 2002). Proteomics can be divided into three approaches; namely expression, structural and functional proteomics. Expression proteomics quantifies protein expressions between samples, structural proteomics maps out the proteins structures, while functional proteomics provides information on protein signalling, disease mechanisms and/or protein interactions (Graves and Haystead, 2002).

Expressional proteomic studies allows both a qualitative and quantitative analysis of protein changes (Hayward, 2014). It can thus be used to identify all expressed proteins in a biological sample and/or in comparative analysis between the proteome of a stressed sample and that of the untreated control (Gupta *et al.*, 2013). Such plant proteomic studies have been conducted to monitor the developmental changes or the effect of environmental factors on protein patterns (Cánovas *et al.*, 2004).

Proteomic analysis can be performed through gel-based or non-gel based methods. Gel-based methods include one-dimensional (1D) gel electrophoresis and two-dimensional (2D) gel electrophoresis, while non-gel based methods include isotope-coded affinity tags (ICATs) and isobaric tags for relative and absolute quantitation (iTRAQ) (Hu *et al.*, 2015). Two-dimensional gel electrophoresis in combination with mass spectrometry has been the main approach used for protein separation and identification (Abdallah *et al.*, 2012). However, drawbacks for gel-based methods such as low reproducibility and the inability to characterise complete proteomes, have made non-gel based techniques more valuable (Eldakak *et al.*, 2013). Although relatively more expensive than the gel-based methods, the non-gel based techniques provide a broader and thus high proteome coverage. These techniques are thus now commonly used in proteomic studies (Chen and Harmon, 2006).

1.4.2.1 iTRAQ Analysis and its Application in Plant Stress Response Studies

The iTRAQ technique is used for protein quantitation in plant stress response studies. Protein samples are labelled with 4-plex or 8-plex isobaric tags, and further processed for identification by mass spectrometry (Sur, 2013). This technique quantifies proteins from multiple samples, either those in time-course studies or when comparing normal and stressed samples at a single time point (Yan and Chen, 2004). Recently, the iTRAQ technique was

used to comparatively analyse differentially expressed proteins in soybean (*Glycine max*) leaves and roots in response to 12 hours of 200 mM NaCl-induced salt stress treatment (Ji *et al.*, 2016). The study identified a total of 278 and 440 differential expressed salt-stress responsive proteins in leaves and roots, respectively. From these proteins, a total of 50 proteins were positively identified in both tissues, of which 45 were up-regulated and 5 were down-regulated in the leaves; while in the roots, 46 were up-regulated and 4 were down-regulated. The differentially expressed root proteins identified included signal transduction, metabolism, transport, and stress defence proteins, which played an important role in salt stress response of the soybean plants (Ji *et al.*, 2016).

In another study by Li *et al.* (2015), an iTRAQ analysis was conducted to compare the abundance of proteins in control and salt-stressed roots of cotton in response to 200 mM NaCl after 24 hours. A total of 128 differentially expressed proteins were identified. Of these proteins, the abundance of 76 increased while 52 decreased under the salt stress. The study revealed differentially expressed root proteins with functions related to stress and defence, protein metabolism, carbohydrate and energy metabolism, membrane and transport, cell wall and cytoskeleton metabolism, transcription and signal transduction.

In the above mentioned studies, a large number of stress-responsive proteins were positively identified and quantified by iTRAQ-based methods, thus indicating the power of sensitivity and quantitation of this technique in comparative analysis. Collectively however, physiological, biochemical and proteomic based studies allow for the identification and characterisation of complex response mechanisms in plants under various environmental conditions.

1.5 Aim, Objectives and Significance of the Study

The aim of this study was to conduct a comparative physiological, proteomic and biochemical analysis of two sorghum varieties under salt stress. The specific objectives were to:

- i. Evaluate the growth and physiological parameters of two sorghum varieties exposed to salt stress,
- ii. Evaluate the osmolyte and chlorophyll contents in the two sorghum varieties in response to salt-stress,
- iii. Identify differentially expressed root and leaf salt-stress responsive proteins of the two sorghum varieties using proteomic tools, and
- iv. Perform bioinformatics analysis for putative functional annotation of the positively identified salt-stress responsive proteins.

Abiotic stresses such as drought and salinity reduce plant growth and development, thus posing a serious threat to agricultural productivity, worldwide. In the current study, a combination of physiological, iTRAQ-based proteomic and metabolomics analyses were used in a comparative study between two sorghum varieties with contrasting phenotypes. The findings of the study were targeted at providing more information on salt adaptive mechanisms in sorghum. Furthermore, the identified salt-stress responsive proteins could help in the identification of expressed genes involved in stress tolerance. This knowledge could be potentially applied to plant breeding initiatives aimed at producing salt-tolerant crops, thereby ensuring food security under the expanding environmental stress conditions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material

Eleven sorghum varieties obtained from the Agricultural Research Council (ARC)-Grain Crops Institute (GCI), Potchefstroom, South Africa; Capstone Seeds South Africa, Howick, South Africa; and Agricol, Pretoria, South Africa were used. A list and description of this germplasm is shown in Table 2.1.

Table 2.1: Sorghum germplasm used in the study.

Variety	Traits	Source
ICSV 213	Drought resistant	ARC-GCI
ICSV 210	Drought resistant	ARC-GCI
ICSV 112	Drought resistant	ARC-GCI
SA 1441	Drought tolerant	ARC-GCI
Macia-SA	Drought tolerant	ARC-GCI
ICSB 73	Drought susceptible	ARC-GCI
ICSB 338*	Drought susceptible	ARC-GCI
Sweetfeed	Unknown	Capstone seeds
Cap 1003	Unknown	Capstone seeds
Ns5511	Unknown	Agricol
Macia (Capstone)	Drought tolerant	Capstone seeds

*ICSB 338 is also salt sensitive (Satish *et al.*, 2016).

2.2 Methods

2.2.1 Pot Planting

Sorghum seeds were imbibed in distilled water for 30 minutes at room temperature before sowing on soil. Eleven sorghum varieties were sown on potting soil (Culterra, Muldersdrif, South Africa) in 10 cm diameter plastic pots. Two pots were allocated to each of the sorghum varieties and five seeds per pot were sown per seed variety. The soil was fully saturated with Nitrosol Nutrient Solution [Envirogreen (Pty) Ltd., Braamfontein, South Africa] containing macro and micronutrient content [N (80 g/kg); P (20 g/kg); K (58 g/kg); Ca (6 g/kg); Mg (7 g/kg); S (4 g/kg); Mn (40 mg/kg); Mo (15 mg/kg); Fe (60 mg/kg); Cu (1 mg/kg); Zn (1 mg/kg); Bo (23 mg/kg)] and left to drain for 3 hours. The potted seeds were then incubated at a temperature range of 27/19°C and a 16/8 hour light/dark photoperiod in a growth chamber (Model: GC-539DH, Already Enterprise Inc., Taipei, Taiwan) for 10 days. The pots were well-watered with distilled water every second day.

Germination rates of all sorghum varieties were recorded on day 10 post-sowing. In this experiment, germination was defined as emergence of shoots. Based on the germination results, early seedling vigour and the phenotypic characteristics of the germplasm, two varieties, namely SA 1441-a drought tolerant variety and ICSB 338- a drought and salt susceptible variety were selected for use in salt stress treatment experiments.

2.2.2 Preliminary Salt Stress Treatment Experiment

SA 1441 and ICSB 338 sorghum seeds were sown and grown on soil, with adequate watering until they reached the V3 growth stage as described above. The nutrient solution was applied to the sorghum plants at day 14 after sowing. At the V3 growth stage (plants with three fully expanded leaves with the fourth emerging), the plants were divided into five treatment

groups. The first group consisted of the control, which continued to be watered with distilled water every second day throughout the experiment. The other four groups were each watered every second day with 40 ml of 100, 200, 300 and 400 mM NaCl solutions for 7 days to induce salt stress. After the salt stress treatment period, the plants were harvested and a range of growth and physiological parameters were measured.

2.2.3 Measurement of Growth and Physiological Parameters

2.2.3.1 Growth Measurements

Shoot length was measured in centimeters from the base of the shoot to the tip of the growing point. Root length was measured from the base of the shoot to the tip of the growing root. Fresh shoot and root weight was measured soon after harvesting the plants. The shoot and root tissues were then dried in an oven at 60°C for 48 hours to determine the dry weight. Five biological replicates were used for all measurements.

2.2.3.2 Relative Shoot Water Loss

The relative shoot water loss (RSWL) was measured as previously described by Rahman *et al.* (2000). Briefly, shoots of five biological replicate plants were cut from the roots and their initial weight was measured. The shoots were placed on a bench top at room temperature for a total of 6 hours and weight readings were recorded every hour. After 6 hours, the shoots were dried in an oven at 60°C for 48 hours to determine the dry weight. The water loss was determined according to the formula as described by Rahman *et al.* (2000) and the results were plotted on a line graph.

2.2.3.3 Chlorophyll Content

The leaf chlorophyll content was measured using a CCM-200 plus Chlorophyll Content Meter (Opti-Sciences, ADC BioScientific Ltd., Hoddesdon, UK) and recorded in Chlorophyll Content Index (CCI) units. The measurements were recorded on the third oldest leaf of the control and each of the salt stress treatment groups, at the same time, everyday for 7 days. Ten biological replicate plants were used for the measurements.

2.2.3.4 Stomatal Conductance

The leaf stomatal conductance was measured in $\text{mmol/m}^2\text{s}$ using a leaf porometer (Decagon Devices Inc., Washington, USA). The measurements were taken on the third oldest leaf of the control and each of the salt stress treatment groups, at the same time, everyday for 7 days. Ten biological replicate plants were used for the measurements.

2.2.3.5 Leaf Relative Water Content

The leaf relative water content (RWC) was estimated as previously described (Barrs and Weatherley, 1962). All measurements were taken on the third oldest leaf of five biological replicate plants of each treatment group. Briefly, the third oldest leaf samples were cut off from the plants and the fresh weight (FW) was immediately measured. The leaves were immersed in 50 ml Falcon tubes containing distilled water and incubated at 4°C for 24 hours. After incubation, the leaves were lightly blotted on paper towel and weighed to determine the turgid weight (TW). Thereafter, leaf samples were dried in an oven at 60°C for 48 hours to determine the dry weight (DW). The leaf RWC was estimated according to a formula described by Barrs and Weatherley (1962):

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$$

Where, RWC = relative water content; FW = Fresh weight; DW = Dry weight; and TW = Turgid weight.

2.2.3.6 Calculation of Physiological Indices

Shoot and root length stress tolerance (PHSI, RLSI); shoot and root fresh weight stress tolerance (SFSI, RFSI); shoot and root dry weight stress tolerance (SDSI, RDSI) were calculated according to the formulas previously described by Kausar *et al.* (2012):

$$\text{PHSI} = (\text{Plant height of stressed plants} / \text{Plant height of control plants}) \times 100$$

$$\text{RLSI} = (\text{Root length of stressed plants} / \text{Root length of control plants}) \times 100$$

$$\text{SFSI} = (\text{Shoot fresh weights of stressed plants} / \text{Shoot fresh weights of control plants}) \times 100$$

$$\text{RFSI} = (\text{Root fresh weights of stressed plants} / \text{Root fresh weights of control plants}) \times 100$$

$$\text{SDSI} = (\text{Shoot dry weights of stressed plants} / \text{Shoot dry weights of control plants}) \times 100$$

$$\text{RDSI} = (\text{Root dry weights of stressed plants} / \text{Root dry weights of control plants}) \times 100$$

2.2.4 Determination of Osmolyte Concentrations

Four biological replicates were used for both leaf and root tissue for proline and glycine betaine quantification. Three leaf discs of about 1 cm in diameter were cut off from the third oldest leaf of the control treatment and each of the 100-400 mM NaCl salt stress treatment groups, respectively. Root material from the control and 100-400 mM NaCl salt stress treatment groups was separately bulked into one and washed using distilled water. Approximately 150 mg of root tissue from the control and each of the 100-400 mM NaCl salt stress treatment groups was transferred to 2 ml eppendorf tubes, respectively. A volume of 125 μ l of 0.25N HCl was added to the leaf and root samples before incubating on a heating

block at 60°C for 5 minutes. Thereafter, the leaf and root osmolyte extracts were collected into 1.5 ml eppendorf tubes and stored at -20°C prior to osmolyte quantification at Durham University (UK). However, all data analysis was conducted by myself at the University of the Free State, Qwaqwa Campus.

2.2.4.1 Chromatographic Separation of Root and Leaf Extracts for Proline Content Analysis

The leaf and root proline content was estimated using the Hydrophilic Interaction Chromatography (HILIC) Liquid Chromatography-Mass Spectrometry (LC-MS) as previously described by Prinsen *et al.* (2016). The chromatographic separation of the root and leaf osmolyte samples was performed using an Acquity BEH Amide column (2.1x 100 mm, 1.7 µm particle size) (Waters, Manchester, UK) coupled to the Acquity UPLC system (Waters). A fraction (2 µl) of each diluted (1:10) sample was injected on the column and maintained at 30°C. Optimal chromatographic separation was achieved at a flow rate of 200 µl/minutes using a gradient with solvent A (10 mM ammonium formate, 0.15% formic acid in 85% acetonitrile) and solvent B (10 mM ammonium formate, 0.15% formic acid in water). Initially, solvent A was 100%. After 6 minutes a gradient started for 0.1 minutes (6.0–6.1 minutes) and solvent A decreased to 94.1% and solvent B increased to 5.9%. From 6.1 to 10 minutes, solvent A decreased to 82.4% and solvent B was set at 17.6% and from 10 to 12 minutes, solvent A was set at 70.6% and solvent B was set at 29.4%. The column was equilibrated for 6 minutes in 100% solvent A. Total run time was 18 minutes including column equilibration. The proline was analysed by a Multiple Reaction Monitoring (MRM) using a QTRAP 6500 hybrid triple-quadrupole mass spectrometer system (AB Sciex, Redwood city, USA). Peaks of interest were integrated using Analyst software (Sciex) and quantified with reference to external standards.

2.2.4.2 Chromatographic Separation of Leaf and Root Extracts for Glycine Betaine Content Analysis

The leaf and root glycine betaine content was estimated using the HILIC LC-MS as previously described (Prinsen *et al.*, 2016). The chromatographic separation of the leaf and root osmolyte samples was performed using an Acquity HILIC column (2.1 x 100 mm, 1.7 µm particle size) (Waters) coupled to the Acquity UPLC system (Waters). A fraction (2 µl) of each diluted (1:10) sample was injected on the column, which was maintained at 30°C. Optimal chromatographic separation was achieved at a flow rate of 400µl/minutes using a gradient with solvent A (10 mM ammonium formate, 0.15% formic acid in 85% acetonitrile) and 200 µl/minutes using a gradient with solvent B (10 mM ammonium formate, 0.15% formic acid in water). Initial conditions were 100% solvent A. Solvent A was set on hold for 2 minutes and solvent B was ramped to 100% at 5 minutes. This was then held for 5 minutes before equilibrating at 100% for 5 minutes. The glycine betaine was analysed by a MRM using a QTRAP 6500 hybrid triple-quadrupole mass spectrometer system (AB Sciex).

2.2.5 Salt Stress Treatments Using 100 mM NaCl

On the basis of results obtained from the growth and physiological measurements of plants treated with 100-400 mM NaCl, a salt concentration of 100 NaCl was selected for mainstream salt stress treatment experiments of SA 1441 and ICSB 338 sorghum varieties. The seeds were sown and grown in soil (section 2.2.1) until they reached the V3 growth stage. Thereafter, the plants of each sorghum variety were divided into two groups. The control group was watered with distilled water, while the salt stressed group was supplemented with a 100 mM NaCl solution every second day for 7 days. After 7 days of salt treatment, the plants were harvested for proteomic analysis.

2.2.6 Protein Extraction from Sorghum Leaf and Root Tissue

Sorghum root and leaf total soluble proteins were extracted from the control and 100 mM NaCl salt stressed plants as previously described by Ngara (2009) with minor modifications. Four and five biological replicates were used for the root and leaf tissue, respectively. Four biological replicates were used for the root tissue due to limited amount of root tissue obtained after stress treatment. Approximately 1 g of each tissue type was used for protein extraction. Briefly, the root and leaf tissues were separately ground in liquid nitrogen using a pestle and mortar. The ground material was precipitated with 10% (w/v) trichloroacetic acid (TCA) and centrifuged at $15\ 000 \times g$ for 10 minutes at room temperature. The resultant pellets were washed four times with ice-cold 80% (v/v) acetone and centrifuged at $15\ 000 \times g$ for 10 minutes after each wash. The pellets were air-dried for 30 minutes at room temperature and resuspended in urea buffer [7 M urea, 2 M thiourea and 4% 3-[(3-Cholamidopropyl) dimethylammonio] 1-propanesulfonate (CHAPS)] overnight with vigorous vortexing. The samples were subsequently centrifuged for 10 minutes at $15\ 000 \times g$. The supernatant containing total soluble proteins was collected and stored at -20°C prior to protein quantification and proteomic analysis.

2.2.7 Protein Quantification

The extracted total soluble leaf and root proteins were quantified using the Bradford Assay (Bradford, 1976), with minor modifications as described by Ngara (2009). A 5 mg/ml stock solution of bovine serum albumin (BSA) was used to prepare standard solutions as illustrated in (Appendix 1). The BSA standards and protein samples were prepared in duplicate and absorbance measured in 2ml plastic cuvettes. Root samples contained 10 μl of protein extracts, 10 μl of 0.1 M HCl and 80 μl of distilled water. Due to the low protein content of root samples, a large volume of the extract was used in protein quantification. Leaf samples

contained 5 μ l of protein extracts, 5 μ l of urea buffer, 10 μ l of 0.1 M HCl and 80 μ l of distilled water. A volume of 900 μ l of a diluted (1:4) Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, California, USA) was added to all standards and protein samples, mixed well by pipetting and incubated at room temperature for 5 minutes. Absorbance was measured at 595 nm using a Jenway 7300 spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK). For all absorbance readings, the 0 μ g/ml BSA solution was used as a blank solution. A standard curve was plotted from which the concentrations of all unknown protein samples were determined.

2.2.8 One Dimensional (1D) Polyacrylamide Gel Electrophoresis (PAGE)

The quantified protein samples together with an Unstained Protein Ladder (New England Biolabs Inc, Massachusetts, USA) with known sizes were electrophoresed on a 12% (v/v) 1D sodium dodecyl sulfate (SDS) polyacrylamide gel (Appendix 1), as previously described (Laemmli, 1970). The gels were cast on 1 mm thick plates using the Mini PROTEAN Tetra Cell gel casting system (Bio-Rad) according to the manufacturer's instructions. Protein samples were mixed with 2X sample buffer [100 mM Tris-HCL pH 6.8, 200 mM (w/v) DTT, 4% (w/v) SDS, 20% glycerol and a pinch of bromophenol blue] and the protein extracts at a ratio of 1:1. The samples were pulse vortexed and centrifuged prior to heating on a heat block for 3 minutes at 100°C. All denatured protein samples were loaded into the gel wells.

Gel electrophoresis was carried out using electrode running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS] on a Mini PROTEAN Tetra Cell (Bio-Rad) using a Basic PowerPac (Bio-Rad). The gel was initially run at 100 V for 30 minutes and then at 150V until the bromophenol blue dye reached the bottom of the gel plates. After gel electrophoresis, the gels were stained with Coomassie Brilliant blue (CBB) R-250 staining solution [0.1% (w/v)

CBB R250, 40% (v/v) methanol and 10% (v/v) acetic acid] for overnight and destained [40% (v/v) acetic acid and 10% (v/v) glycerol] until the gel background was clear. The gels were scanned and imaged using the molecular imager Gel Doc™ XR+ with Image Lab™ Software version 5.2.1 (Bio-Rad).

2.2.9 Acetone Precipitation of Protein Samples

The root and leaf protein samples were acetone precipitated and sent to Durham University (UK) for isobaric tags for relative and absolute quantitation (iTRAQ) analysis. However, all data analysis was conducted by myself at the University of the Free State, Qwaqwa Campus. Briefly, the protein extracts were precipitated using 80% (v/v) acetone, overnight at -20°C. The samples were centrifuged at 3 000 rpm for 10 minutes. The supernatants were discarded and the pellets washed 3 times with ice cold 80% (v/v) acetone. The protein precipitates were centrifuged at 15 000 × *g* for 10 minutes and the acetone was discarded. Protein pellets were subsequently stored in 50 µl of 80% (v/v) acetone prior to shipping.

2.2.10. iTRAQ Analysis

2.2.10.1 Sample Preparation for iTRAQ Analysis

iTRAQ analysis was performed as previously described by Smith *et al.* (2015) with minor modifications. The acetone precipitated leaf and root protein samples were centrifuged at 15 000 × *g* for 5 minutes and the supernatant discarded. The pellets were air-dried at room temperature and resuspended in 100 µl urea buffer. The samples were centrifuged for 10 minutes at 15 000 × *g* and the supernatant was collected. Protein samples were subsequently quantified using the Bradford assay (Section 2.2.7) and electrophoresed on a 12% (v/v) SDS polyacrylamide gel (Section 2.2.8) to analyse protein quantity and quality.

2.2.10.2 Sample Labelling

Sample labelling was conducted on four biological replicates of control and salt-stressed root and leaf protein samples. For each sample, 50 µg root and 12.5 µg leaf protein concentrations were used. The protein samples were acetone precipitated overnight at -20°C and centrifuged for 10 minutes at 15 000 × g. The samples were air-dried and resolubilised using an iTRAQ Reagent-Multiplex Buffer Kit (AB Sciex) according to the manufacturer's instructions. For the leaf samples, 2.5 µl of the denaturant was added to each pellet and incubated for 1 hour at 60°C. Thereafter, 47.5 µl of dissolution buffer was added and the samples vortexed for 20 minutes and centrifuged for 10 minutes at 15 000 × g. The supernatant was collected and mixed with 1 µl of reducing agent. The samples were incubated for 1 hour at 60°C and alkylated with 0.5 µl of cysteine blocking agent, vortexed and incubated at room temperature for 10 minutes.

The root samples were prepared for labelling as described above, but with four times the volumes of all reagents and solutions in the iTRAQ Reagent-Multiplex Buffer Kit. All leaf and root protein samples were separately digested with trypsin (Promega, Madison, USA), overnight at 37°C. The digested protein samples were vacuum-dried, resuspended in MilliQ water before adjusting the pH to 7.5 using the dissolution buffer. All the samples were labelled with an 8-plex iTRAQ reagent kit (AB Sciex) according to the manufacturer's instructions. The four control replicates were labelled using the tags with molecular weights 113, 114, 115, 116 and the salt-stressed replicates were labelled 117, 118, 119, and 121, respectively. Root and leaf samples were separately pooled to make one composite sample, which was vacuum dried and resuspended in 3 ml of buffer A (10 mM K₂HPO₄/25% acetonitrile, pH 2.8).

2.2.10.3. iTRAQ Sample Clean-up

Samples were cleaned-up using HILIC SPE cartridges (Poly-LC Inc), containing 300 mg of 12 μm polyhydroxyethyl-A, to remove unincorporated label and buffer salts. The cartridges were equilibrated by sequential addition of 4×3 ml releasing solution (5% acetonitrile, 30 mM ammonium formate pH 3.0) followed by 4×3 ml binding solution (85% acetonitrile, 30 mM ammonium formate pH 3.0). The dried iTRAQ-labelled peptide residue was dissolved in 75 μl of 3% acetonitrile, 0.1% formic acid followed by 150 μl of 0.3 M ammonium formate, pH 3. The pH of the mixture was checked and adjusted to 3.0 using trifluoroacetic acid if necessary. After clarifying by centrifugation ($10\,000 \times g$, 10 minutes), the samples were mixed with 1275 μL acetonitrile. The resulting 1.5 ml sample was added to the SPE cartridge and the flow-through retained and passed through a second time. The column was then washed twice with 2 ml binding solution. Finally, the peptides were eluted with 2×1 ml releasing solution. The eluate was freeze-dried and re-suspended in 3% acetonitrile, 0.1% formic acid for liquid chromatography-mass spectrometry (LC-MS).

2.2.10.4 LC-MS Analysis

LC-MS/MS was performed using a Triple TOF 6600 mass spectrometer (AB Sciex) linked to an Eksigent 425 LC system via a Sciex Nanospray III source. Peptides originating from 5 μg protein were used for each LC-MS run and chromatographic separations of peptides used a trap and elute method. Samples were loaded and washed on a Triart C18 guard column 1/32", 5 μm , 5×0.5 mm (YMC) acting as a trap, and online separation of peptides performed over 87 minutes on a Triart C18 1/32", 3 μm , 150×0.3 mm column (YMC) at a flow rate of 5 μl /minute. Buffer A was 0.1% formic acid in water and buffer B 0.1% formic acid in acetonitrile. Sequential linear gradients of 3 to 5% B over 2 minutes, 5 to 30% B over 66 minutes, 30 to 35 % B over 5 minutes and 35 to 80% B over 2 minutes were followed by a 3

minute column wash in 80% B. Return to 3% B was over 1 minute before column re-equilibration for 8 minutes. Data-dependent top-30 MS-MS acquisition, with collision energy adjusted for iTRAQ-labelled peptides, was started immediately upon gradient initiation and was for 85 minutes. Throughout this period, precursor-ion scans (400 to 1600 m/z) of 250 ms enabled selection of up to 30 multiply-charged ions (>500 cps) for CID fragmentation and MS/MS spectrum acquisition (m/z 100-1500) for 50 ms. The cycle time was 1.8 second and a rolling precursor exclusion of 15 seconds was applied to limit multiple fragmentation of the same peptide. Analyst TF 1.7.1 instrument control and data processing software (AB Sciex) was used to acquire spectrometer data.

2.2.10.5 Mass Spectra Data Analysis

Mass spectra data were analysed as previously described by Smith *et al.* (2015) with minor modifications. Protein identification and relative quantification was performed by processing the raw data-files against the Uniprot database (downloaded in October 2013 for the root samples and May 2018 for the leaf samples) sequences of *Sorghum bicolor* only using ProteinPilot™ 5.0.1 version 4895 software, incorporating the Paragon™ Algorithm 5.01.04874 (AB Sciex). An iTRAQ 8-plex (peptide-labelled) Paragon method, for tryptic peptides with iodoacetamide cys-modification and data acquired on a TripleTOF 6600 spectrometer, was used. Label bias-correction was activated in this, the ‘Thorough ID’ and ‘Run False Discovery Rate Analysis’ options were selected, and the detected protein threshold was set at 0.05 (10%) [Unused ProtScore (conf)]. Peptide and protein tables were exported from ProteinPilot for subsequent manual data-handling and filtering. All proteins identified on the basis of a single peptide were removed from the dataset. The abundance of each protein in all samples was calculated as a ratio to the 113-tagged sample. Averages of the ratios for each protein across the four replicates in control and in salt stressed samples

were calculated. The fold-change in protein expression was denoted by the ratio of control average to salt-stressed treatment average. For down-regulated proteins, the salt treatment average was the numerator and the control average the denominator, with a negative sign denoting down-regulation. A probability value for the comparison of control average to salt average was computed using the Student's t-test at 95% confidence level.

2.2.10.6 Bioinformatic Analysis

The identified proteins were functionally annotated using UniProt database (<http://www.uniprot.org>) and Gene Ontology (GO) analysis. Proteins were categorized according to their biological process, biological function, and cellular localization. The conserved domains and family names of the identified proteins were determined using the Interpro database (<http://www.ebi.ac.uk/interpro/>).

2.2.11 Statistical Analysis

For statistical analysis, a Mann-Whitney test was used to compare the means for physiological and biochemical results at a 5% level of significance, using the GraphPad Prism 5.00 software.

CHAPTER 3

PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF SORGHUM PLANTS UNDER SALT STRESS

3.1 Introduction

Salt stress is one of the primary limiting factors of crop productivity worldwide. It is caused by high accumulation of soluble salts such as Na⁺ and Cl⁻ in the soil (Parida and Das, 2005). In plants, salt stress causes a range of primary and secondary effects, such as osmotic, ionic and oxidative stresses (Chinnusamy *et al.*, 2006), which affect their normal growth and development. For example, as soluble salts accumulate in the soil, outside the roots, water uptake by roots is disturbed due to osmotic stress (Munns and Tester, 2008). As the salt stress prevails, ion toxicity may follow due to high concentrations of salts accumulating in the plant (Munns and Tester, 2008). Other effects of salt stress on plants include the inhibition of seed germination and photosynthesis, stunted growth and nutritional imbalance (Hasanuzzaman *et al.*, 2013).

Plants have developed a range of morphological, physiological and molecular mechanisms to cope with salt stress (Parida and Das, 2005). However, the degree with which plants survive the stress differs between species and genotypes (Kosova *et al.*, 2013), as well as the intensity of stress and duration of exposure to the stress. For example, halophytic plants such as *Thellungiella halophila* are capable of completing their life cycles at salt concentrations higher than 200 mM, as they are able to withstand long term exposure to high levels of salt stress (Munns and Tester, 2008; Kosova *et al.*, 2013). However, the growth of glycophytes such as *Arabidopsis thaliana* is severely inhibited at 100-200 mM NaCl (Munns and Tester, 2008). Although sorghum has been characterised as being moderately salt tolerant

(Krishnamurthy *et al.*, 2007), this level of tolerance varies between sorghum genotypes and varieties (Satish *et al.*, 2016).

Plants acclimatize to salinity by employing mechanisms such as osmotic tolerance, Na⁺ exclusion from leaf blades and tissue tolerance (Munns and Tester, 2008). Osmotic tolerance involves the ability of a plant to tolerate the osmotic stress effect of salinity and also maintaining leaf expansion and stomatal conductance. Na⁺ exclusion by roots minimizes the amount of Na⁺ that accumulate in the cytosol, ensuring that Na⁺ does not accumulate to toxic levels in the leaf blades. On the other hand, tissue tolerance involves Na⁺ and Cl⁻ compartmentalization at cellular and intercellular levels in order to avoid their toxic effects within the cytoplasm (Munns and Tester, 2008; Carillo *et al.*, 2011). Plants also accumulate compatible osmolytes such as proline, glycine betaine, sugars and polyols (Parida and Das, 2005; Chen and Jiang, 2010) in response to salt stress. The accumulation of these compounds results in a decrease in the cell osmotic potential, maintains water absorption and cell turgor pressure, which all contribute towards sustaining important physiological processes (Serraj and Sinclair, 2002).

The main objective of this chapter was to conduct a comparative physiological and biochemical analysis of two sorghum varieties; a drought tolerant (SA 1441) and a drought and salt susceptible (ICSB 338) under salt stress.

3.2 Results

3.2.1 Seed Germination Experiment

Eleven sorghum varieties (Table 2.1) were germinated in a well-watered potting soil mix in a growth chamber for 10 days. Germination rates of the different sorghum varieties are presented in Figure 3.1. Sorghum varieties ICSB 338, Ns5511, Cap1003 and SA 1441 had the highest germination rate of 100%, while ICSV 213 and Macia (Capstone) had the lowest at 40%. Sorghum varieties SA 1441 and ICSB 338 were subsequently selected for salt treatment experiments based on high germination rates (Figure 3.1) as well as the contrasting phenotypic responses to drought stress (Table 2.1).

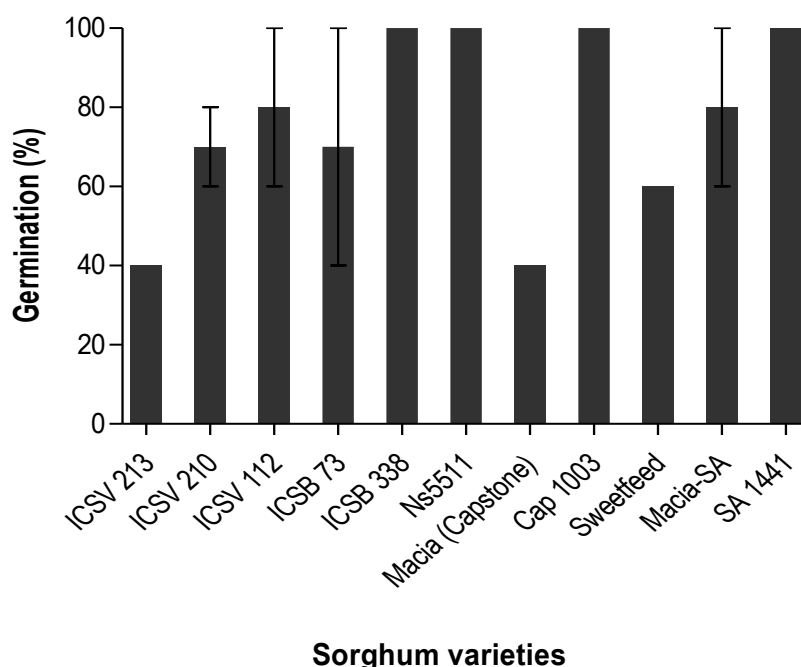


Figure 3.1: Germination rates of different sorghum varieties. The sorghum seeds were sown and grown in potting mix soil and germination counts were taken 10 days after sowing. Data represented as mean \pm SE ($n = 5$).

3.2.2 Preliminary Salt Stress Treatment Experiment

Different concentrations of NaCl ranging from 100, 200, 300 and 400 mM were used for the preliminary salt stress treatment experiments on the two sorghum varieties, SA 1441 and ICSB 338 (Figure 3.2 A-B). The objective of this experiment was to identify the most appropriate concentration to use in the mainstream experiment. The plants were exposed to salt stress every second day for 7 days, while the control plants were watered with distilled water. Figure 3.2 shows the sorghum plants after 7 days of salt stress exposure. Generally, plant growth decreased with an increase in NaCl concentration in both sorghum varieties. However, the decrease was more prominent in ICSB 338 at 300 and 400 mM NaCl concentrations. At these concentrations, the leaves of ICSB 338 started to wilt and became chlorotic with some of the older leaves falling off the plants.

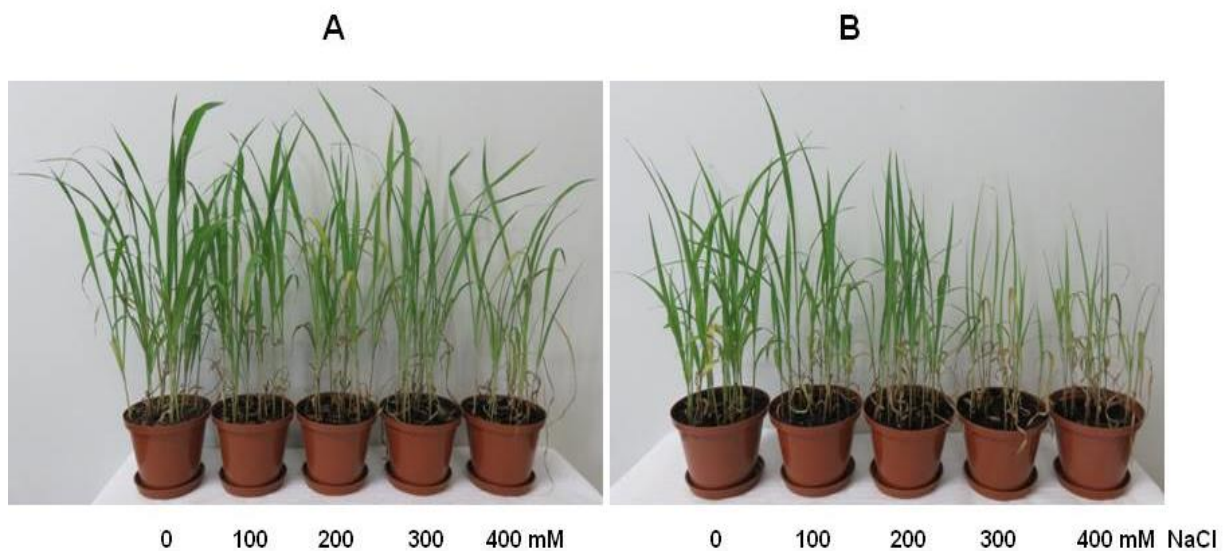


Figure 3.2: The effect of salt stress on the growth of sorghum plants. (A) shows SA 1441, a drought tolerant variety and (B) shows ICSB 338, a drought and salt susceptible variety. The plants were grown until the V3 stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM. The control group with 0 mM NaCl was watered with distilled water. The pictures were taken on day 7 of the experiment.

3.2.3 Leaf Relative Water Content (RWC)

Salt stress treatment of the plants using 100-400 mM NaCl for 7 days differentially influenced the leaf RWC of the two sorghum varieties. As illustrated in Figure 3.3, SA 1441 plants maintained a relatively constant leaf RWC across the salt treatment levels applied. However, ICSB 338 showed a statistically significant decrease in leaf RWC at 300 and 400 mM NaCl concentrations when compared to the control.

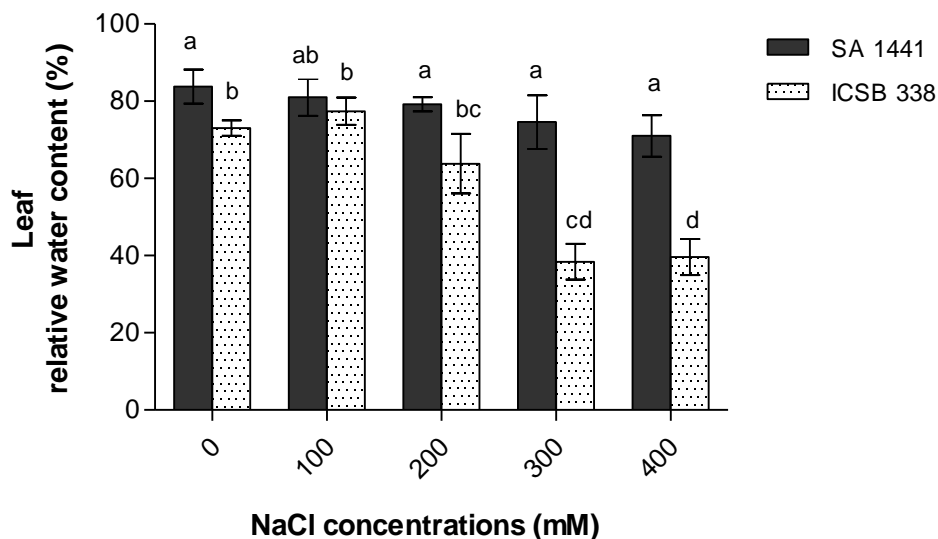


Figure 3.3: Leaf relative water content of sorghum varieties at different salt concentrations. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. The leaf relative water content was estimated on the third oldest leaf, 7 days after stress treatment. Data represented as mean \pm SE ($n = 5$). Bars with different letters are significantly different at $p \leq 0.05$ according to a Mann-Whitney test.

3.2.4 Relative Shoot Water Loss

Figure 3.4 shows a gradual decrease in shoot water loss in both sorghum varieties with an increase in NaCl concentrations over a period of six hours. The shoots of ICSB 338 (the drought and salt susceptible variety) lost more water at a faster rate than SA 1441 (the drought tolerant variety). Generally, SA 1441 shoots were better able to control shoot water loss, 6 hours after being excised from the whole plant.

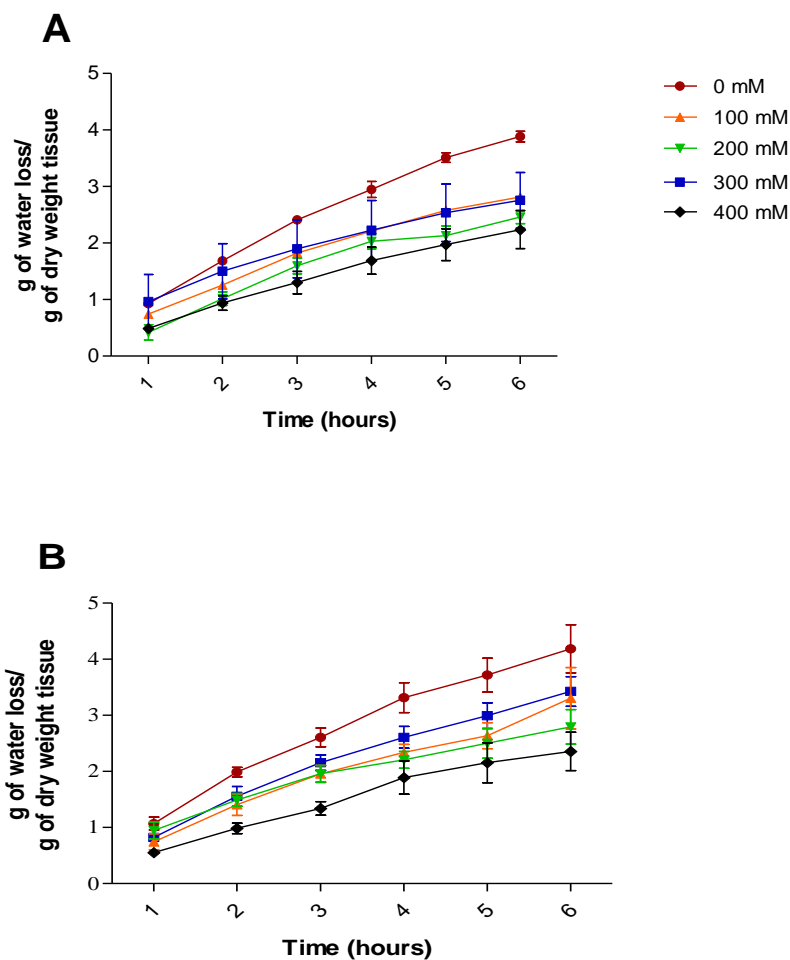


Figure 3.4: The effect of salt stress on shoot water loss of sorghum varieties at different salt concentrations. (A) shows SA 1441, a drought tolerant variety and (B) shows ICSB 338, a drought and salt susceptible variety. The plants were grown until the V3 stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. Shoot water loss measurements were taken on day 7 of stress treatment. Data represented as mean \pm SE ($n = 5$).

3.2.5 Effect of Salt Stress on Growth Parameters

The effect of salt stress on shoot/root weight was assessed in the two sorghum varieties. The shoot fresh and dry weight results are shown in Figures 3.5 A & B, respectively. Generally, both the fresh and dry shoot weight of ICSB 338 gradually decreased with an increase in NaCl concentrations. For example, when compared to the control, the dry weight of salt-stressed ICSB 338 shoots was statistically significantly lower from 100 mM NaCl up to the highest concentrations of 400 mM. The change in shoot fresh weight in ICSB 338 was observed from 200-400 mM NaCl. However, for SA 1441 there was no statistical significant difference in the shoot fresh weight from the control across all salt concentrations. The shoot dry weight measurements were statistically significantly different from the control only at 400 mM NaCl.

The root fresh and dry weight results of the two sorghum varieties are shown in Figures 3.5 C & D, respectively. The root fresh and dry weights of SA 1441 plants following salt exposure were not statistically significantly different from the control across all the salt concentrations. However, for ICSB 338 a significant decrease in root fresh and dry weight was observed at 300 and 400 mM NaCl when compared to the control.

The shoot and root length measurements of the two sorghum varieties following salt stress are shown in Figures 3.6 A & B, respectively. The shoot length of SA 1441 and ICSB 338 was statistically different from their respective controls at higher concentrations of 300 mM and 400 mM NaCl. The change in root length in ICSB 338 was observed from 300-400 mM NaCl. However, for SA 1441 there was no statistical significant difference in root length from the control across all salt concentrations. Collectively, the shoot/root weight and length results illustrate the different effects of salt stress on the growth of the two varieties.

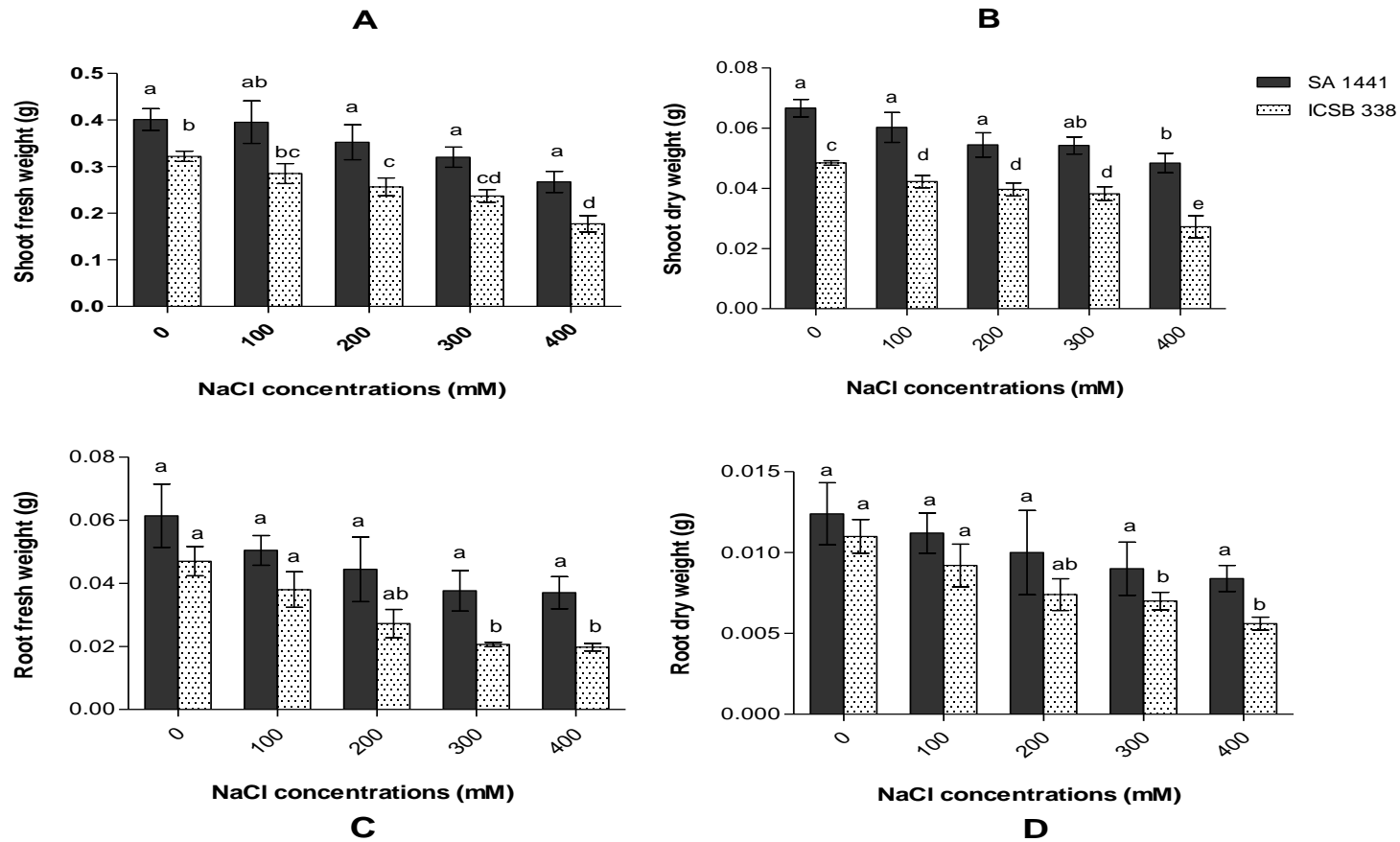


Figure 3.5: Shoot and root fresh and dry weight of sorghum varieties at different salt concentrations. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. Measurements were taken on day 7 of stress treatment. A & B shows fresh and dry shoot weight, while C & D shows fresh and dry root weight. Data represented as mean \pm SE ($n = 5$). Bars with the same letter are not significantly different at $p \leq 0.05$ according to a Mann-Whitney test.

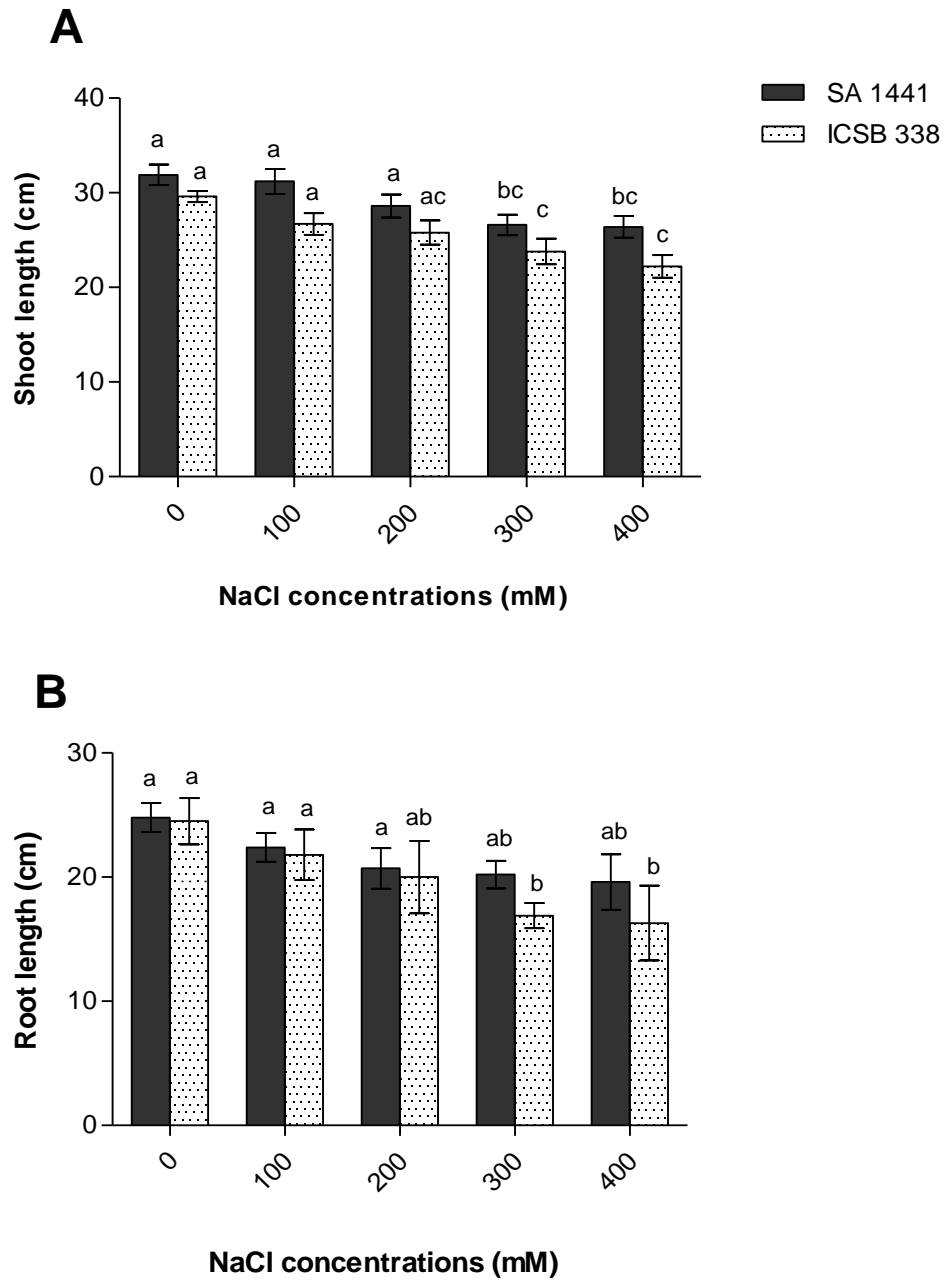


Figure 3.6: Shoot and root length of sorghum varieties at different salt concentrations. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. Measurements were taken on day 7 of stress treatment. (A) shows the shoot length and (B) shows the root length of both varieties. Data represented as mean \pm SE ($n = 5$). Bars with different letters are significantly different at $p \leq 0.05$ according to a Mann-Whitney test.

3.2.6 Physiological Indices

Using the fresh/dry weight and shoot/root length readings presented above in Figures 3.5 & 3.6 respectively, a range of physiological indices were calculated for the two sorghum varieties under salt stress. These physiological indices of the plants indicated that the growth of the two sorghum varieties was affected by the application of different NaCl concentrations. Generally, the physiological indices gradually decreased with increasing levels of NaCl. Overall, the growth of ICSB 338 was more affected with an increase in NaCl compared to SA 1441 (Table 3.1).

Table 3.1: Sorghum plant physiological indices under salt stress.

Sorghum varieties	NaCl concentrations	PHSI	RLSI	SFSI	RFSI	SDSI	RDSI
SA 1441	100	97	90	98	82	90	90
ICSB 338		90	88	88	80	87	83
SA 1441	200	89	83	87	72	81	80
ICSB 338		87	81	79	57	81	67
SA 1441	300	82	81	79	61	81	72
ICSB 338		80	68	73	43	78	63
SA 1441	400	83	79	66	60	72	67
ICSB 338		75	66	55	42	56	50

Plant height stress tolerance index (PHSI), root length stress tolerance index (RLSI), shoot fresh stress tolerance index (SFSI), root fresh stress tolerance index (RFSI), shoot dry stress tolerance index (SDSI) and root dry stress tolerance index (RDSI).

3.2.7 Stomatal Conductance

Stomatal conductance was measured on the third oldest leaf using a portable leaf porometer, at the same time each day for 7 days. Stomatal conductance gradually decreased with an increase in NaCl concentration in both sorghum varieties as shown in Figure 3.7. However, the effect of salt stress on stomatal conductance was more prominent in ICSB 338 (Figure 3.7 B) compared to SA 1441 (Figure 3.7 A). For example, SA 1441 plants treated with 100-400 mM NaCl has stomatal conductance readings ranging between 20.39 – 17.59 mmol/m²s at day 7 of salt-stress treatment. However, within the same treatment range, ICSB 338 plants had 14.64 - 12.98 mmol/m²s at day 7 of salt-stress treatment. Overall, SA 1441 was able to maintain stomatal opening during periods of salt stress.

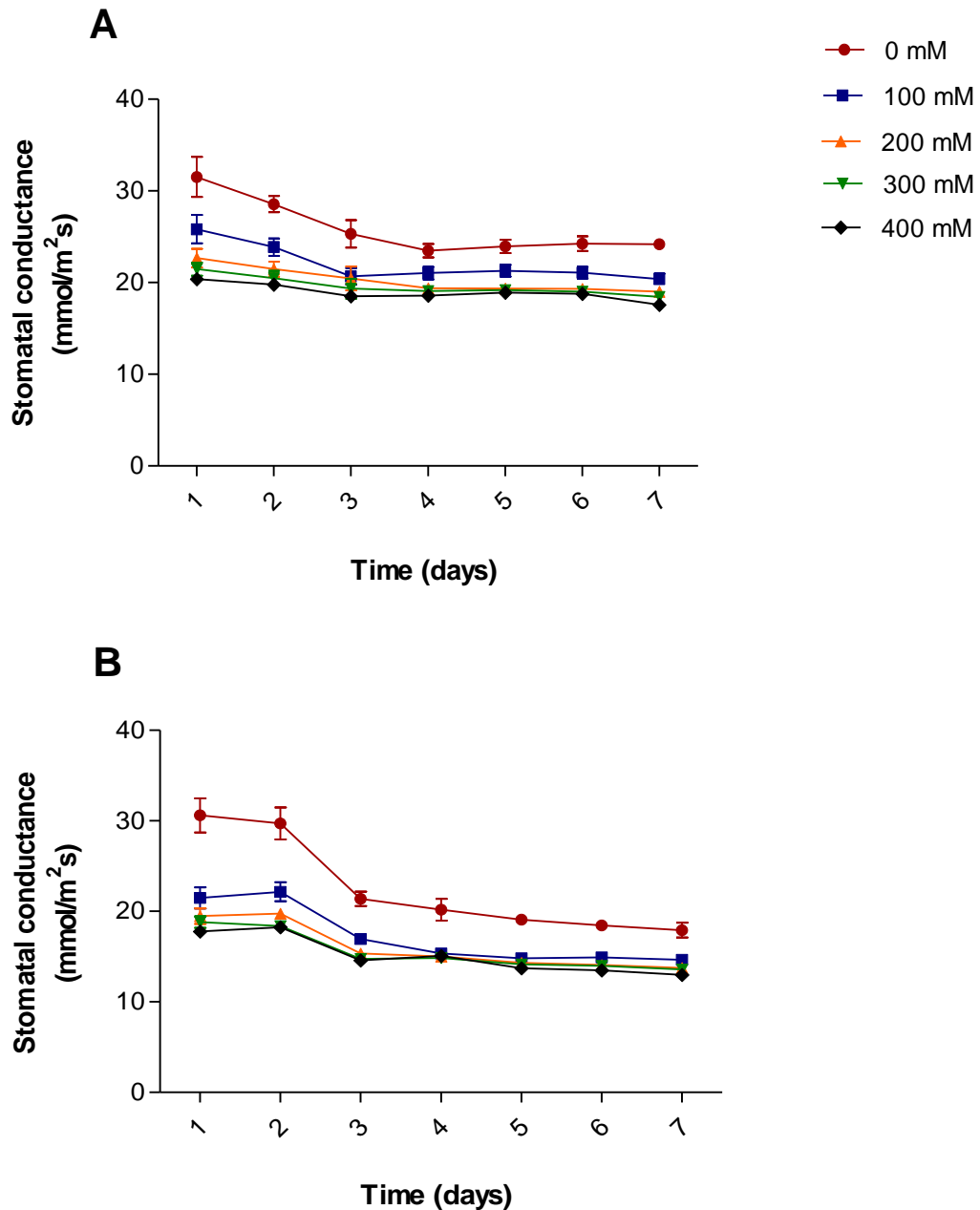


Figure 3.7: The effects of salt stress on stomatal conductance of two sorghum varieties. Changes in stomatal conductance for (A) SA 1441, a drought tolerant variety and (B) ICSB 338, a drought and salt susceptible variety were measured in response to salt stress. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. Measurements of stomatal conductance were taken every day during stress treatment. Data represented as mean \pm SE ($n = 10$).

3.2.8 Chlorophyll Content

The chlorophyll content was measured using a Chlorophyll Content Meter on the third oldest leaf, at the same time each day for 7 days. The results represented as chlorophyll content index (CCI) are shown in Figure 3.8. The chlorophyll content gradually decreased with an increase in NaCl concentration over the treatment period in both sorghum varieties. However, the chlorophyll content of salt stressed ICSB 338 plants (Figure 3.8 B) decreased more than that of SA 1441 (Figure 3.8 A). These results also correlate well with the levels of chlorosis that was observed in ICSB 338 plants at 300 and 400 mM NaCl stress treatments (Figure 3.2).

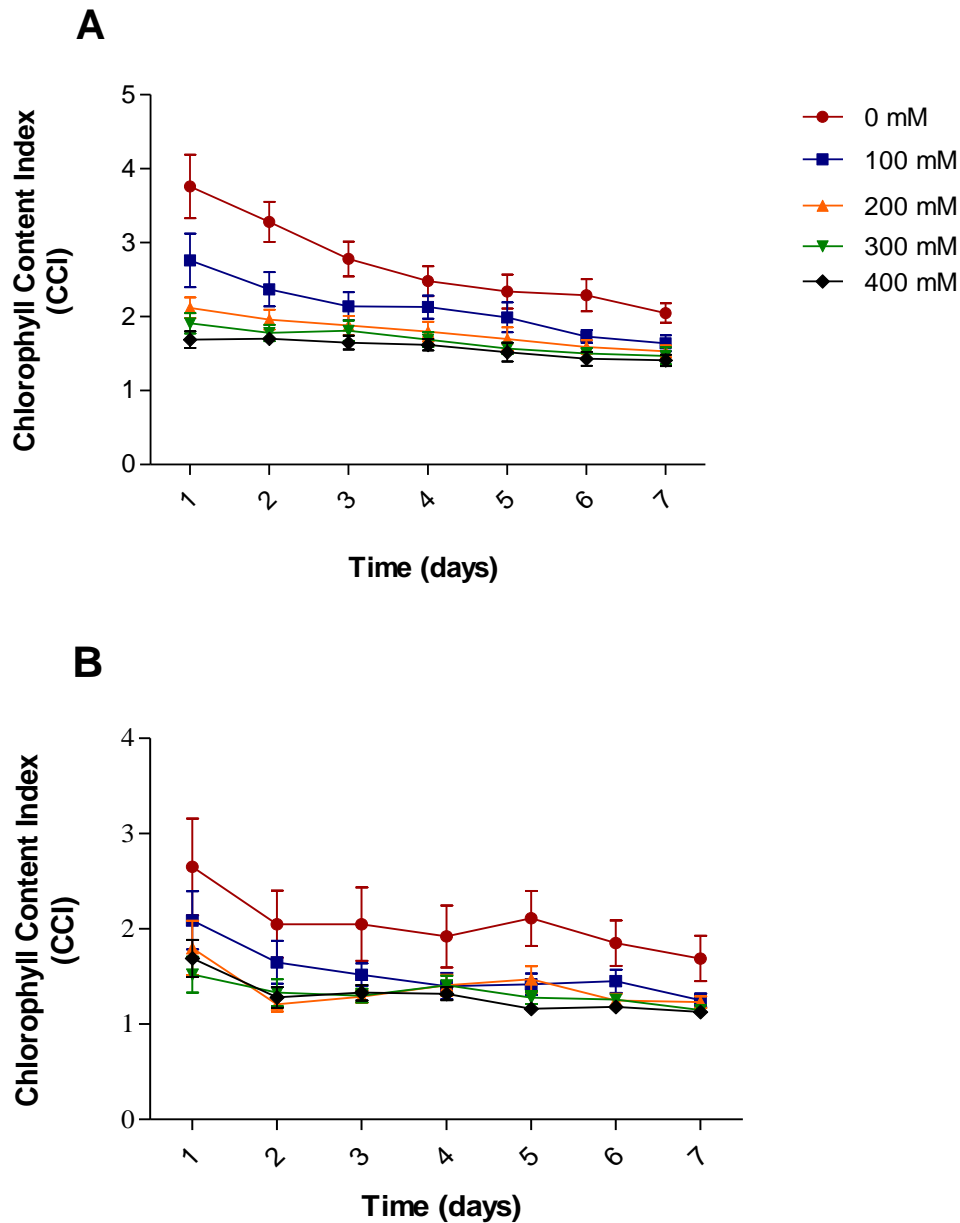


Figure 3.8: The effects of salt stress on chlorophyll content of two sorghum varieties. Changes in chlorophyll content for (A) SA 1441, a drought tolerant variety and (B) ICSB 338, a drought and salt susceptible variety were measured in response to salt stress. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. Measurements of chlorophyll content were taken every day during stress treatment. Data represented as mean \pm SE ($n = 10$).

3.2.9 Leaf and Root Proline Content

The effect of salt stress on the proline content of the two sorghum varieties was assessed using the Hydrophilic Interaction Chromatography (HILIC) Liquid Chromatography-Mass Spectrometry (LC-MS). The leaf and root proline content results are shown in Figures 3.9 A & B, respectively. For SA 1441, there was no change in proline content in response to NaCl at 100-300 mM. However, there was a statistically significant increase in proline content at 400 mM NaCl. For ICSB 338, no change in proline content was observed at 100 mM and 400 mM NaCl relative to the control. No proline accumulated at 200 and 300 mM NaCl concentrations.

For SA 1441, no change in proline accumulation was observed at 100 mM NaCl, whereas a significant increase was observed at salt concentrations ranging from 200-400 mM. For ICSB 338, no proline was detected in roots of control plants and those treated with 100 mM NaCl. However, proline was detected in the roots of SA 1441 plants treated with 200, 300 and 400 mM NaCl.

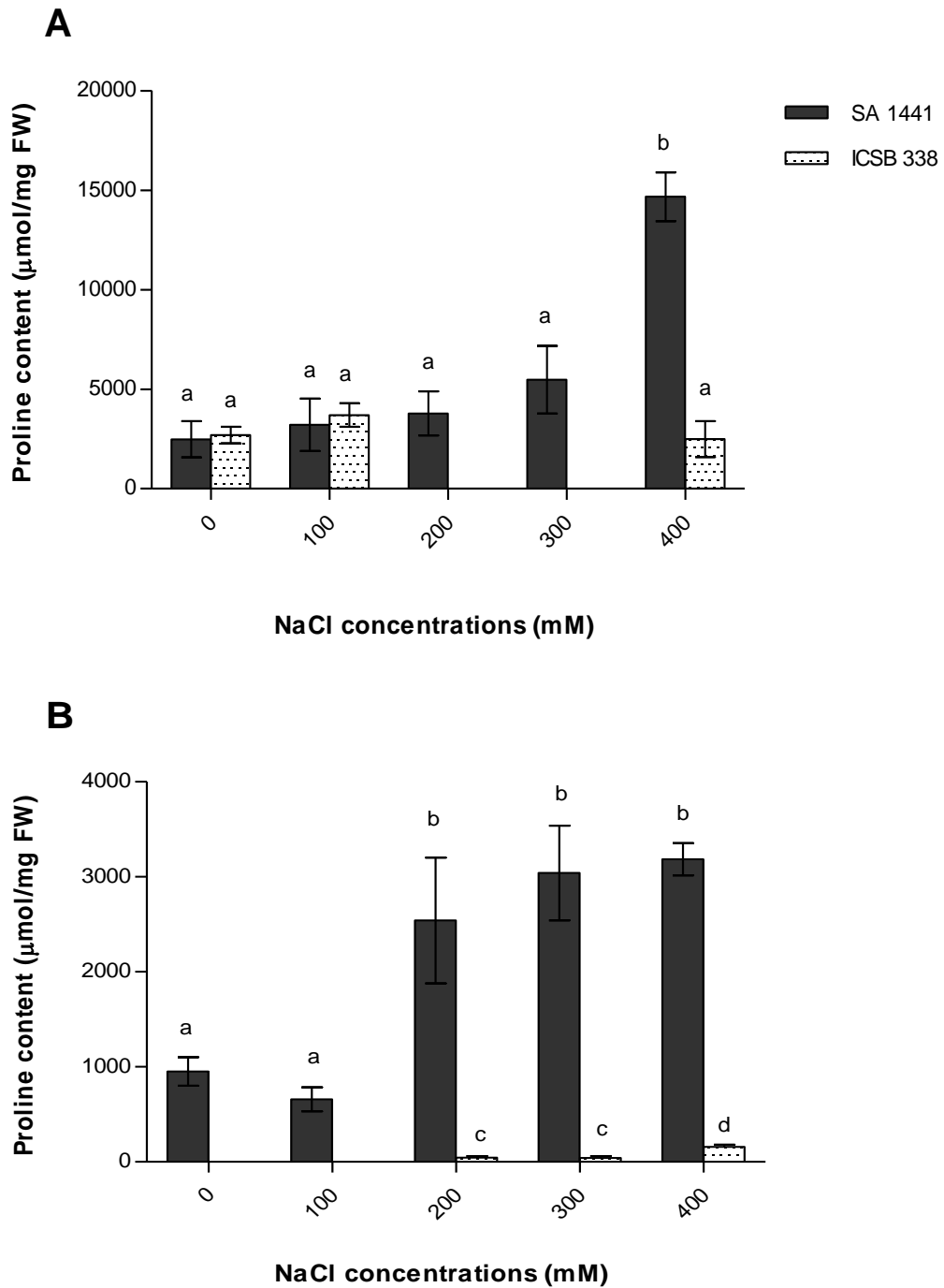


Figure 3.9: The effects of salt-stress on leaf and root proline content of two sorghum varieties. (A) shows the leaf and (B) the root proline content of both varieties. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. The proline content was estimated 7 days after stress treatment using the HILIC LC-MS. Data represented as mean \pm SE ($n = 4$). Bars with different letters are significantly different at $p \leq 0.05$ according to a Mann-Whitney test.

3.2.10 Leaf and Root Glycine Betaine Content

The effects of salt stress on glycine betaine content of the two sorghum varieties was assessed using the HILIC LC-MS. Figures 3.10 shows the leaf and root glycine betaine content results of the two sorghum varieties. For SA 1441, there was no statistical significant difference in leaf glycine betaine across all the salt concentrations relative to the control. For ICSB 338, no glycine betaine accumulation was detected in leaves of control plants and those treated with 100, 300 and 400mM NaCl. Glycine betaine content was only detected in the leaves treated with 200 mM NaCl.

For SA 1441, no glycine betaine accumulation was detected in roots of control plants and those treated with 100 mM NaCl. Glycine betaine content was only detected in the roots of plants treated with 200, 300 and 400 mM NaCl. However, for ICSB, there was no detection of glycine betaine in roots of control plants and those treated with 100,200 and 300 mM NaCl. The accumulation of glycine betaine was only observed in the roots of plants treated with 400 mM NaCl.

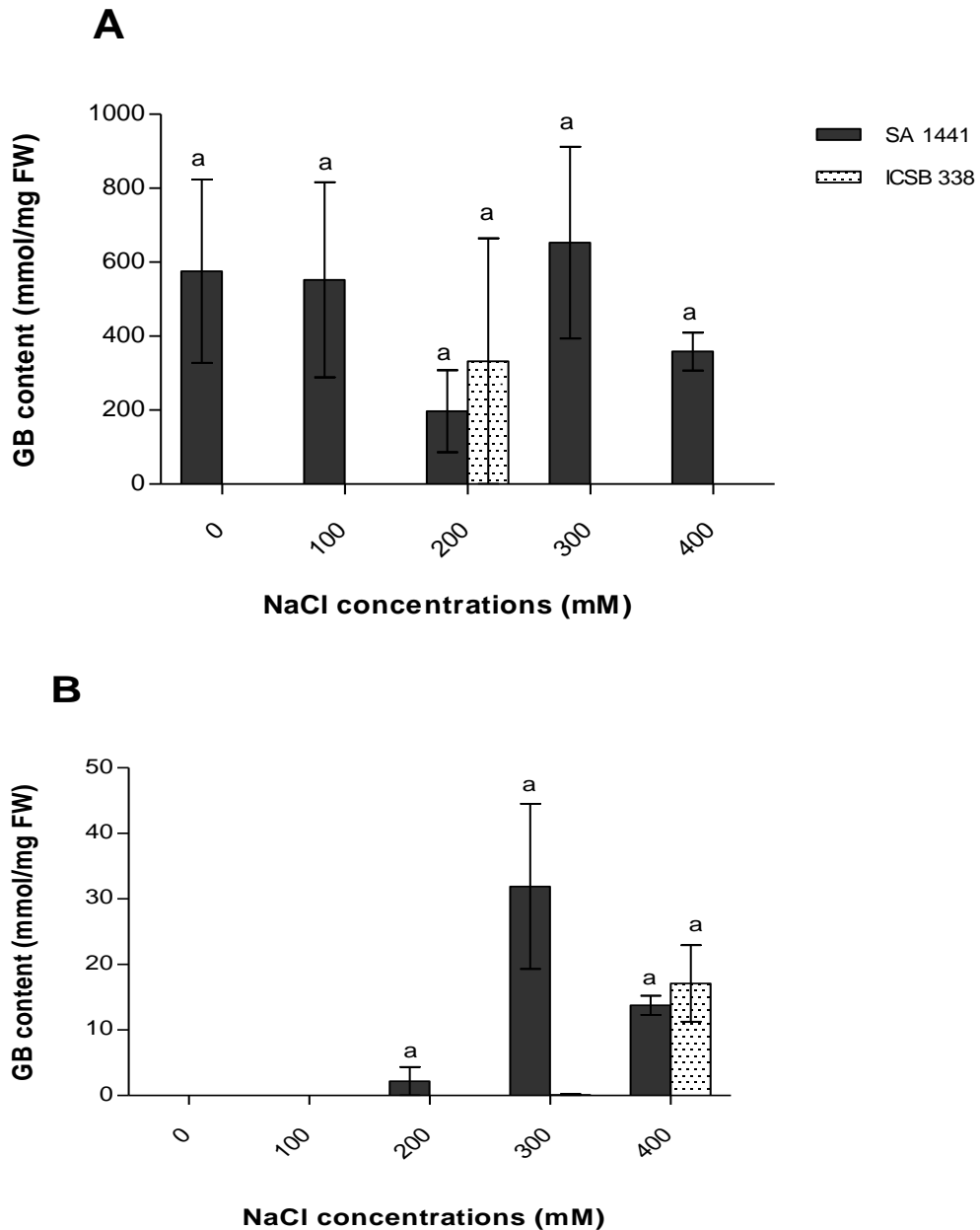


Figure 3.10: The effects of salt-stress on leaf and root glycine betaine content of two sorghum varieties. (A) shows the leaf and (B) the root glycine betaine content of both varieties. SA 1441 and ICSB 338 plants were grown until the V3 growth stage and salt stress was induced by watering the plants every second day with different concentrations of NaCl ranging from 100-400 mM for 7 days. The control group with 0 mM NaCl was watered with distilled water. The glycine betaine content was estimated 7 days after stress treatment using the HILIC LC-MS. Data represented as mean \pm SE ($n = 4$). Bars with different letters are significantly different at $p \leq 0.05$ according to a Mann-Whitney test.

3.3 Discussion

In this study, a comparative physiological, growth and biochemical analysis of two sorghum varieties under salt stress was conducted. The two varieties used, SA 1441 and ICSB 338 have contrasting tolerance to drought (Table 2.1). Furthermore, ICSB 338 is also characterised as being salt sensitive (Satish *et al.*, 2016). Salinity stress was induced on the two sorghum varieties using different NaCl concentrations ranging from 100-400 mM over a period of 7 days. Due to limited plant growth facilities in our research laboratory, the selection of the two sorghum varieties for the main stream experiments was based on high germination rates (Figure 3.1) and contrasting phenotypes to drought stress (Table 2.1) rather than preliminary salt stress screening experiments. It is also well-known that drought and salt stresses exert similar primary and secondary effects on plants such as osmotic and oxidative stress. Furthermore, plants may respond to both drought and salt stresses using common response mechanisms (Wang *et al.*, 2003), making the selection of the two varieties valuable in the current study.

Salt stress causes osmotic stress on plants, resulting in negative effects on the physiological growth parameters (Munns and Tester, 2008). However, the overall effects may differ with species, genotypes and varieties, as well as the intensity and duration of the stress. A range of physiological, growth and biochemical parameters were measured between two sorghum varieties under control and salt stress conditions in order to improve our understanding of stress responsive mechanisms in sorghum.

Relative water content measures the current water status in plants and is considered a useful indicator of water stress (Anjum *et al.*, 2011). In this study, we observed that the leaf RWC of ICSB 338 (the drought and salt susceptible variety) decreased with an increase in salt stress

(Figure 3.3), while that of SA 1441 remained relatively constant. A decrease in the leaf RWC in response to salt stress has also been observed in wheat (*Triticum aestivum*) (Guo *et al.*, 2012) and soybean (*Glycine max*) (Wu *et al.*, 2014) genotypes. The observed decrease in leaf RWC in ICSB 338 indicated that salinity resulted in dehydration at cellular levels (Figure 3.3), possibly due to the osmotic effect of salt stress on plants. Furthermore, the leaf RWC difference between the two varieties is consistent with their known phenotypic responses to drought (Table 2.1). RWC was also reduced in a comparative study on two sorghum varieties with contrasting drought tolerance (Jedrowski *et al.*, 2014; Ogbaga *et al.*, 2014).

The photosynthetic process is often affected by the exposure of plants to salt stress (Chaves *et al.*, 2009), due to a decrease in leaf surface area, chlorophyll content, stomatal conductance and also reduced photosystem II activities (Shrivastava and Kumar, 2015). In this study, the observed decrease in the leaf stomatal conductance in both sorghum varieties with an increase in NaCl concentrations (Figure 3.7) could be attributed to the plant's responses to the osmotic stress effects of salinity stress. As the plant senses a decrease in water supply due to high salt content in the soil, they close their stomata as a way of reducing transpirational water loss (Ashraf and Harris, 2013). Although this stomatal closure restricts water loss, it also limits the uptake of carbon dioxide and ultimately the rate of photosynthesis (Parida and Das, 2005; Maiti and Satya, 2014). Therefore, biomass production and yield are significantly reduced (Almeida *et al.*, 2017).

Coupled with the changes in stomatal conductance, there was an observed decrease in chlorophyll content with an increase in salt stress intensity (Figure 3.8). Similar trends have also been reported in sorghum (Sui *et al.*, 2015). A decrease in chlorophyll content may be due to the impaired pigment biosynthesis and/or its increased degradation under salt stress

(Ashraf and Harris, 2013), leading to leaf chlorosis. Leaf chlorosis is one of the most commonly observed effects of salinity stress in plants (Swami *et al.*, 2011) and was observed in ICSB 338 at high concentrations of NaCl (Figure 3.2 B). Furthermore, the chlorophyll content of plants can be considered as a physiological parameter that shows good correlation with salinity tolerance (Saleh, 2012). Using this parameter, SA 1441 may be considered more salt tolerant than ICSB 338. Overall, as the chlorophyll content gradually decreased in both sorghum varieties (Figure 3.8), the rate of photosynthesis was possibly negatively affected, thereby reducing plant growth (Figures 3.5 and 3.6).

A decrease in stomatal conductance, transpiration and photosynthetic rates leads to an overall decline in plant growth (Hanin *et al.*, 2016). In this study, all growth parameters (shoot and root length and biomass) decreased with an increase in salt stress as shown in Figures 3.5 & 3.6. Although roots may be the first organs to be exposed to salt stress, shoots are more salt sensitive than roots. A reduction in leaf area development relative to root growth would decrease water use, thus allowing plants to conserve soil moisture and prevent a rapid increase in the salt concentration in the soil (Munns and Tester, 2008).

The shoot biomass of ICSB 338 was more affected by salt stress when compared to SA 1441 (Figure 3.5 A & B). The observed decrease in shoot biomass may indicate that salinity reduced growth by suppressing leaf initiation and expansion, internode growth, and by accelerating leaf abscission (Farooq *et al.*, 2015). Leaf abscission of older leaves in the sorghum plants treated with high concentrations of NaCl was observed in both sorghum varieties. However, this was more prominent in ICSB 338 (Figure 3.2). The root biomass was also reduced with an increase in NaCl concentrations, but the decrease was more prominent in ICSB 338 at 300 and 400 mM NaCl concentrations (Figure 3.5 C & D). Generally, the

reduction in plant growth under salt stress may be due to the osmotic effect, which reduced the availability of water but increased accumulation of ions in tissues (Turan *et al.*, 2009), or even increased salinity-induced oxidative stress (Taïbi *et al.*, 2016) in plants.

Salt tolerance has been estimated using parameters such as germination, shoot and root development under salt stress (Bafeel, 2014). In this study, physiological indices (Table 3.1) were calculated in order to evaluate the differences in salt tolerance between the two sorghum varieties. Overall, the physiological growth of ICSB 338 was largely affected by salt stress when compared to SA 1441. As such, the drought-tolerant SA 1441 sorghum variety tolerated salt stress better than the drought and salt-sensitive ICSB 338 sorghum variety across all measured parameters. The results showed the interconnection between drought and salinity stress response mechanisms in plants (Wang *et al.*, 2003). The observed results also justify the classification of ICSB 338 as a salt sensitive variety (Satish *et al.*, 2016).

Plants also need to synthesize compatible organic solutes such as proline and glycine betaine in order to withstand the stress imposed on them by salt stress (Chen and Jiang, 2010). Proline accumulation is a common protection mechanism in salt stressed crops. However, the rate of accumulation varies between tolerant and sensitive genotypes (Misra and Gupta, 2005). This is consistent with the results obtained in this study, as the proline content increased in SA 1441 as compared to ICSB 338 (Figure 3.9). Meloni *et al.* (2001), also observed a slight increase in the proline content in both leaf and root tissues of cotton (*Gossypium hirsutum*). The increase in the proline content in SA 1441 under different NaCl treatments may suggest that plants attempted to stabilize their protection mechanism towards salt-stress. An increase in proline accumulation in plants has been proposed to play an important role in salt tolerance (Mansour and Ali, 2017).

However, glycine betaine content was undetectable at most salt concentrations in both leaf and root tissues of ICSB 338 in response to salt-stress treatment (Figure 3.10). According to Ashraf and Foolad (2007), not all plants are able to accumulate glycine betaine or proline in sufficient amounts to reduce the effects caused by different abiotic stresses. A low accumulation of glycine betaine was observed in root tissues of sorghum genotypes (Yang *et al.*, 2003). Overall, the osmolyte results showed that proline played the main role in osmotic adjustment in both sorghum varieties when compared to glycine betaine. Furthermore, SA 1441 (drought tolerant variety) can readily switch on the osmolyte biosynthetic process compared to ICSB 338 (drought and salt susceptible variety). Therefore, SA 1441 exhibits a greater stress protective mechanism.

After conducting the physiological, growth and biochemical analyses of sorghum varieties following salt stress, 100 mM NaCl stress treatment was selected for subsequent use in proteomic analysis of the root (Chapter 4) and leaf (Chapter 5) tissues. This relatively low salt concentration was selected to identify initial common/different salt stress signalling events in the two sorghum varieties, before cellular damage occurs.

CHAPTER 4

A COMPARATIVE PROTEOMIC IDENTIFICATION OF SALT-STRESS RESPONSIVE ROOT PROTEINS OF TWO SORGHUM VARIETIES

4.1 Introduction

Salt stress affects various aspects of plant cell structure, growth, development, and metabolism. It also induces signalling events that result in changes in gene expression, protein relative abundance and activity (Kosova *et al.*, 2013). Therefore, quantitative protein expression analysis is important in determining the effects of a stress factor on plants and their subsequent response mechanisms (Sobhanian *et al.*, 2011). Proteomic studies can thus contribute towards the identification of cellular functions that are affected by the stress factor, and also possible relationships between protein abundances and/or modification and plant stress tolerance (Hu *et al.*, 2015).

Most proteomic studies on stress responses are based on the comparison of proteome composition between stressed and the untreated control, and also in differentially-tolerant genotypes (Gupta *et al.*, 2013). Such studies have led to the identification of stress-related proteins, and thus expressed genes that could be used in the genetic improvement of plants against the stresses (Ahmad *et al.*, 2016). Quantitative proteomic approaches such as isotope-coded affinity tags (ICATs) and isobaric tags for relative and absolute quantitation (iTRAQ) have been used in both descriptive and comparative analysis of plant development during stress adaptation (Hu *et al.*, 2015). These liquid chromatography mass spectrometry (LC-MS) based approaches quantify proteins or peptides at a global level (Ghosh and Xu, 2014).

According to Zhang *et al.* (2012), iTRAQ analysis enhances the discovery of low abundant proteins and novel regulatory mechanisms in salt- stress signalling and metabolic pathways.

Roots are the primary site for sensing salinity and respond by passing signals to the shoot, for appropriate changes in plant function (Zhao *et al.*, 2013). Comparative studies on root, salt stress-induced proteomic changes have been investigated in crops such as barley (*Hordeum vulgare*) (Witzel *et al.*, 2009), soybean (*Glycine max*) (Alam *et al.*, 2011) and maize (*Zea mays*) (Luo *et al.*, 2018). In the maize study, iTRAQ analysis was used in a comparative analysis of two genotypes; Jing724 (salt tolerant) and D9H (salt sensitive) in response to 100 mM NaCl treatment for 7 days. A total of 513 differentially expressed salt-stress responsive root proteins were identified, with 83 and 386 being specific to Jing724 and D9H, respectively. The up-regulated root proteins in Jing724, were associated with the pentose phosphate pathway, glutathione metabolism, and nitrogen metabolism, while in D9H, they were associated in fatty acid degradation (Luo *et al.*, 2018).

In the current study, an iTRAQ analysis was conducted to assess salt-stress responsive root proteins in two sorghum varieties. The main objective of this chapter was to conduct a comparative root proteomic analysis of a drought tolerant (SA 1441) and a drought and salt susceptible (ICSB 338) sorghum variety exposed to 100 mM NaCl-induced salt stress for 7 days.

4.2 Results

4.2.1 One Dimensional Protein Profiles of Sorghum Root Extracts

SA 1441 and ICSB 338 sorghum plants were grown as described in section 2.2.1. Thereafter, salt-stressed plants were watered with 100 mM NaCl every second days for 7 days. The control plants were watered with distilled water for the duration of the experiment. All plants were harvested after 7 days of salt treatment. Total soluble proteins were extracted from the control and salt-stressed root tissue of both sorghum varieties (Section 2.2.6) and quantified (Section 2.2.7). One dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to evaluate the quality and quantity of the root protein extracts prior to iTRAQ analysis.

Figure 4.1 shows Coomassie Brilliant blue (CBB) stained 1D SDS-PAGE gels of the root protein extracts. Four biological replicates of each of the control and 100 mM NaCl stressed root samples were electrophoresed and each lane was loaded with 7 µg of protein extract. Lane M shows the molecular weight marker. The biological replicates generally showed similar banding patterns in both sorghum varieties and all extracts were of good quality with no visible signs of protein degradation (Figure 4.1).

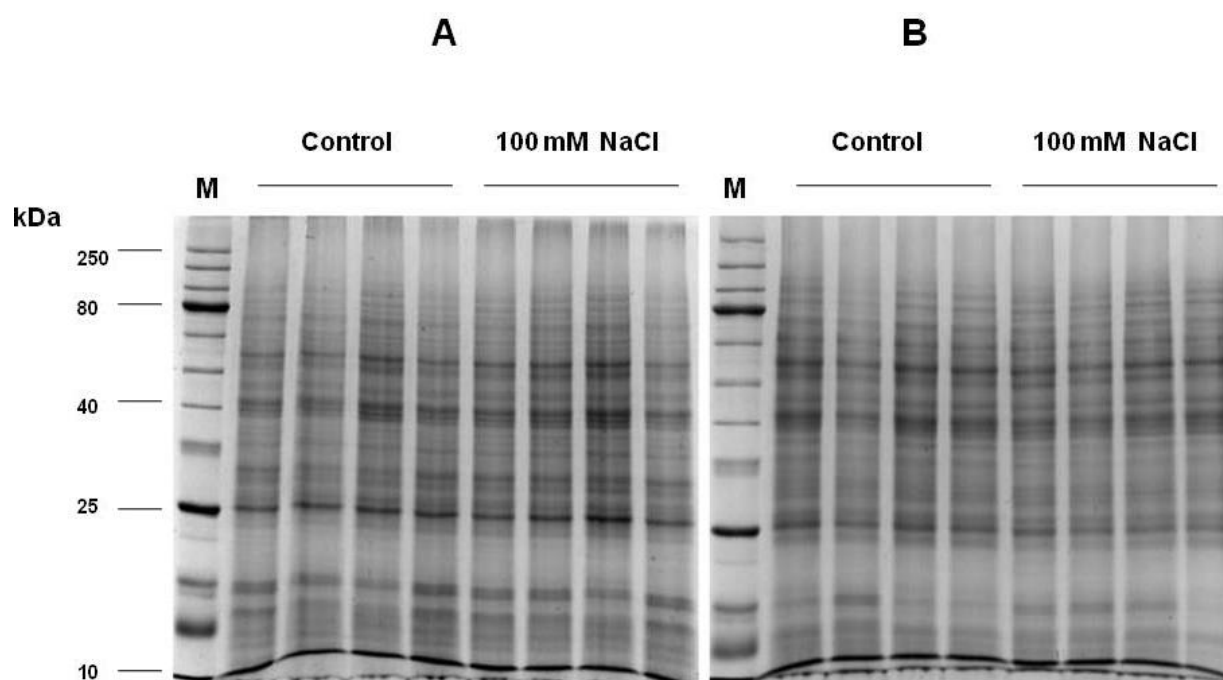


Figure 4.1: Comparative 1D SDS-PAGE analysis of sorghum root total soluble proteins. (A) shows SA 1441 and (B) shows ICSB 338 root samples loaded on a 12% (v/v) SDS-PAGE gel. About 7 μg of protein extracts was loaded in each lane. Four biological replicates of both the control and 100 mM NaCl salt-stressed samples are shown. The gels were stained with CBB stain. Lane M represents the protein molecular weight marker measured in kDa.

4.2.2 iTRAQ Analysis of the Sorghum Root Proteome in Response to Salt Stress

The iTRAQ-based proteomic technique was used to positively identify sorghum root proteins in response to 100 mM NaCl salt stress treatment. Four biological replicate samples of 50 μg total protein content, from each of the control and 100 mM NaCl salt-stressed groups were used for protein identification. The samples were iTRAQ labelled, digested with trypsin, separated by liquid chromatography and subsequently identified by tandem mass

spectrometry as described in Section 2.2.10. A total of 522 and 544 root proteins were positively identified for SA 1441 and ICSB 338, respectively. Out of these positively identified root proteins, a total of 379 (73%) SA 1441 and 405 (74%) ICSB 388 were uncharacterised.

For SA 1441, 26 (Table 4.1) of the 522 positively identified proteins were responsive to 100 mM NaCl-induced salt stress with a significant difference ($p \leq 0.05$). For ICSB 338, 31 (Table 4.2) of the 544 positively identified proteins were responsive to 100 mM NaCl-induced salt stress with a significant difference ($p \leq 0.05$). There were similar proteins in each of the two varieties, uncharacterised proteins (protein no. 85 & 289) in SA 1441 and (protein no. 74 & 293) in ICSB 338.

Of the 26 differentially expressed salt-stress responsive root proteins for SA 1441, 12 (46%) proteins were up-regulated, while 14 (54%) proteins were down-regulated in the same treatment. The fold changes for the up-regulated proteins ranged from 1.06 (protein no. 189) to 1.21 (protein no. 504), whereas the fold changes for the down regulated proteins ranged from -1.24 (protein no. 102) to -1.06 (protein no. 130 & 368). For ICSB 338, of the 31 differentially expressed root proteins, 22 (71%) proteins were up-regulated and 9 (29%) proteins were down-regulated in the same salt treatment. The fold changes for the up-regulated proteins ranged from 1.06 (protein no. 8 & 54) to 1.47 (protein no. 594), whereas the fold changes for the down regulated proteins ranged from -1.21 (protein no. 307) to -1.06 (protein no. 52).

Table 4.1: List of SA 1441 sorghum salt-stress responsive root proteins identified using the iTRAQ and database searches.

Prot No. ^a	Accession No. ^b	Protein name	Scor ^c	% Cov ^d	Seq Pep ^e	Ratio ^f	SD ^g	p-value ^h	GO analysis ⁱ			Conserved domains and family names ^j
									P	F	C	
Disease/defence												
16	C5YB22	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBI_3006G277500	23.21	44.47	22	1.12	0.03	2.47E-02	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; peroxidase family
25	Q4VQB2	Pathogenesis-related protein 10b OS= <i>Sorghum bicolor</i> GN=PR10	21.63	76.88	27	1.17	0.07	1.40E-02	Plant defense	Pathogenesis-related protein	None predicted	BetVI domain and family
85	C5WWQ2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G342600	12.07	13.86	8	1.07	0.04	4.40E-02	Cell redox homeostasis	Thioredoxin-disulfide reductase activity	Cytoplasm	Thioredoxin domain; thioredoxin-like superfamily
86	Q4VQB4	Pathogenesis-related protein 10c OS= <i>Sorghum bicolor</i> GN=PR10	19.93	72.32	19	1.19	0.06	1.18E-02	Plant defense	Pathogenesis related protein	None predicted	BetVI domain and family
126	C5Z0E3	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBI_3009G186500	10.04	24.23	6	-1.16	0.04	3.54E-02	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; peroxidase family
212	A0A1B6PH65	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3007G112100	7.7	15.36	5	-1.09	0.02	9.75E-03	None predicted	None predicted	None predicted	Domain not predicted; short-chain dehydrogenase/ reductase family
289	C5WMM1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G401100	18.03	66.86	17	1.19	0.03	1.33E-03	Plant defense	Pathogenesis related protein	None predicted	BetVI domain and family
342	C5X780	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G007200	4.39	12.08	3	-1.19	0.03	8.89E-03	Electron transfer chain	Electron transfer activity	Plasma membrane	Phytocyanin domain; cupredoxin superfamily
390	C5WPD8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G418200	4.01	21.38	3	1.15	0.07	2.67E-02	None predicted	None predicted	None predicted	Gnk2-homologous domain and superfamily
509	A0A1B6QJR7	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBI_3001G189000	2.91	5.98	2	-1.10	0.03	2.53E-02	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; peroxidase family
Metabolism												
113	A0A194YKZ5	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G227400	11.05	12.09	5	-1.07	0.04	2.65E-02	None predicted	None predicted	None predicted	Domain not predicted; Glycosyl hydrolase family 31
130	A0A194YT53	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G345800	10.04	18.14	5	-1.06	0.02	4.65E-02	Metabolic process	Transferase activity	None predicted	Thiolase domain and family

163	C5Y8Y2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3006G235600	8.82	6.78	4	-1.13	0.03	3.59E-03	Carbohydrate metabolic process	Hydrolase activity	None predicted	Glycoside hydrolase family3C-terminal domain and superfamily
255	A0A1B6Q513	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G241500	6.12	10.21	3	-1.07	0.01	1.08E-02	Isocitrate metabolic process	Isocitrate dehydrogenase (NADP+) activity	None predicted	NADP-dependent isocitrate dehydrogenase domain; isocitrate dehydrogenase family
299	C5Y9F6	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3006G244400	5.64	11.72	3	-1.12	0.04	1.38E-02	DNA catabolic process	Endonuclease activity	None predicted	Domain not predicted; S1/P1 nuclease family
368	A0A1B6QPV9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G465400	4.13	7.43	2	-1.06	0.03	3.25E-02	None predicted	None predicted	None predicted	Dihydroliipoamide succinyltransferase domain; family not predicted
Protein destination & storage												
209	C5YWH3	Carboxypeptidase OS= <i>Sorghum bicolor</i> GN=SORBI_3009G247100	7.72	13.16	4	1.08	0.03	1.51E-02	Proteolysis	Serine-type carboxypeptidase activity	None predicted	Domain not predicted; peptidase S10 family
269	C5YXY3	Peptidyl-prolyl cis-trans isomerase OS= <i>Sorghum bicolor</i> GN=SORBI_3009G001500	6.08	18.47	3	1.08	0.03	1.55E-02	NAD(PH) dehydrogenase complex assembly	Peptidyl-prolyl cis-trans isomerase activity	Chloroplast	Cyclophilin-type PPIase domain and family
294	C5WW86	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G196700	5.76	8.62	3	-1.10	0.04	2.89E-02	Proteolysis	Serine-type peptidase activity	None predicted	Domain not predicted; peptidase S28 family
403	C5Y171	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G142800	4	10.24	2	-1.16	0.03	2.87E-02	Proteolysis	Cysteine-type endopeptidase activity	Extracellular region	Peptidase C1A domain and family
Signal transduction												
189	C5YHK9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3007G186800	15.09	32.40	7	1.06	0.01	9.01E-04	None predicted	Protein-specific binding	None predicted	14-3-3 domain and family
230	C5XYN4	Annexin OS= <i>Sorghum bicolor</i> GN=SORBI_3004G232300	6.77	13.38	3	1.17	0.07	2.28E-02	None predicted	Calcium ion binding	None predicted	Domain not predicted; annexin family
504	C5X978	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G307600	3.24	26.32	7	1.21	0.08	1.71E-02	None predicted	None predicted	None predicted	C2 domain and superfamily
Protein synthesis												
26	A0A194YJY9	Elongation factor 1- alpha OS= <i>Sorghum bicolor</i> GN=SORBI_3010G182100	21.57	32.65	17	1.14	0.08	3.02E-02	Translation elongation	Translation elongation factor activity	Cytoplasm	Transcription factor_GTP- binding domain; classic translation factor GTPase family
421	C5X163	40S ribosomal protein S27 OS= <i>Sorghum</i>	4	34.88	6	-1.12	0.04	2.29E-02	Ribosomal small subunit	Structural constituent of	Cytosol	Domain not predicted; eukaryotic ribosomal

Cell structure		<i>bicolor</i> GN=SORBL_3001G091 801							assembly	ribosome		protein eS27 family
102	A0A1B6QID3	Tubulin alpha chain OS= <i>Sorghum bicolor</i> GN=SORBL_3001G107200	11.74	18.17	6	-1.24	0.05	1.10E-02	Microtubule- based process	GTPase activity	Cytoskeleton	Tubulin domain and family

^a Protein number assigned in ProteinPilot software.

^b Protein accession numbers obtained from the UniProt database (<http://www.uniprot.org>).

^c Protein score generated by ProteinPilot software relating to the confidence of protein identification.

^d Percentage coverage is determined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

^e Sequenced peptide refers to the number of peptides that were sequenced and gave rise to protein identity.

^f Ratio represents the average fold change. A negative value indicates down-regulation.

^g Standard deviation obtained from comparing root protein control and treatment values.

^h Probability-value of the quantified difference between the treatment and control root proteins.

ⁱ Gene ontology analysis as predicted from the UniProt database (<http://www.ebi.ac.uk/QuickGO/>). P denotes Biological Process, F denotes Functional Process, and C denotes Cellular Component.

^j Conserved domains family and superfamily names as predicted by Interpro database (<http://www.ebi.ac.uk/interpro/>).

Table 4.2: List of ICSB 338 sorghum salt-stress responsive root proteins identified using the iTRAQ and database searches.

Prot No. ^a	Accession No. ^b	Protein name	Scor ^c	% Cov ^d	Seq Pep ^e	Ratio ^f	SD ^g	p-value ^h	GO analysis ⁱ			Conserved domains and family names ^j
									P	F	C	
Disease/defence												
8	A0A1B6PQR2	Protein disulfide-isomerase OS= <i>Sorghum bicolor</i> GN=SORBI_3005G074400	38.41	44.08	20	1.06	0.02	1.19E-02	Cell redox homeostasis	Protein disulfide isomerase activity	Endoplasmic reticulum	Thioredoxin domain; protein disulfide isomerase family
52	C5Z475	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBI_3010G162000	16.69	42.46	13	-1.06	0.01	2.65E-03	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; peroxidase family
74	C5WWQ2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G342600	14.62	16.10	8	1.08	0.01	2.12E-02	Cell redox homeostasis	Thioredoxin-disulfide reductase activity	Cytoplasm	Thioredoxin domain; thioredoxin-like superfamily
77	A0A1B6QNQ4	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G400700	22.28	81.12	22	1.11	0.04	4.71E-02	Plant defense	Pathogenesis related protein	None predicted	BetVI domain and family
126	C5XY5	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBI_3004G105100	12.43	37.85	16	-1.12	0.03	1.02E-02	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; peroxidase family
187	C5YYX3	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3009G017800	8	27.57	5	-1.15	0.05	3.35E-02	Glutathione metabolic process	Glutathione transferase activity	Cytoplasm	Glutathione S-transferase, C-terminal domain, and superfamily
255	C5WMM0	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G400900	19.52	80.62	22	1.14	0.07	3.93E-02	Plant defense	Pathogenesis related protein	None predicted	BetVI domain and family
293	C5WMM1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G401100	18.61	75.62	18	1.11	0.04	1.71E-02	Plant defense	Pathogenesis related protein	None predicted	BetVI domain and family
307	A0A1B6QM42	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G310300	11.56	21.43	6	-1.21	0.07	2.52E-02	None predicted	Protein binding	None predicted	Glutathione S-transferase, C-terminal domain and superfamily
322	C5WZ08	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G514200	4.95	9.73	3	-1.15	0.06	2.47E-02	Glutathione metabolic process	Glutathione transferase activity	Cytoplasm	Glutathione S-transferase C-terminal domain and superfamily
350	C5Y1X4	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3005G103300	6.97	10.29	3	1.39	0.23	4.16E-02	Oxidation-reduction process	Oxidoreductase activity	None predicted	Alcohol dehydrogenase domain; zinc-containing alcohol dehydrogenase family
550	C5Z8E7	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G110800	2.48	14.38	2	-1.09	0.04	4.95E-02	Electron transport chain	Electron transfer activity	Plasma membrane	Phycocyanin domain; cupredoxin superfamily

Energy

3	C5XX52	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Sorghum bicolor</i> GN=SORBI_3004G205100	42.6	73.00	41	1.42	0.23	3.19E-02	Glucose metabolic process	Oxidoreductase activity	None predicted	Glyceraldehyde-3-phosphate dehydrogenase domain and family
6	C5XFH6	Fructose-bisphosphate aldolase OS= <i>Sorghum bicolor</i> GN=SORBI_3003G393900	39.41	67.32	31	1.16	0.07	1.58E-02	Glycolytic process	Fructose-bisphosphate aldolase activity	None predicted	Domain not predicted; class I fructose-bisphosphate aldolase family
9	A0A194YGY2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G027000	37.04	61.43	39	1.15	0.07	4.11E-02	Glycolytic process	Phosphopyruvate hydratase activity	Cytosol	Enolase, C-terminal TIM barrel domain; enolase family
11	A0A1B6Q6S1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G336000	33.41	38.99	18	1.10	0.02	2.63E-02	Glucose catabolic process	Phosphoglycerate mutase binding	Cytoplasm	Metalloenzyme domain; phosphoglycerate mutase,2,3-bisphosphoglycerate-independent family
30	A0A194YMM6	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Sorghum bicolor</i> GN=SORBI_3010G262500	37.91	65.28	34	1.18	0.08	3.88E-02	Glucose metabolic process	Oxidoreductase activity	None predicted	Glyceraldehyde-3-phosphate dehydrogenase domain and family
48	C5XQ07	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G072300	17.4	62.05	13	1.17	0.06	7.06E-03	Glycolytic process	Triose-phosphate isomerase activity	Cytosol	Domain not predicted; triosephosphate isomerase family
80	C5XRQ7	Fructose-bisphosphate aldolase OS= <i>Sorghum bicolor</i> GN=SORBI_3004G146000	32.84	53.35	30	1.10	0.01	1.19E-03	Glycolytic process	Fructose-bisphosphate aldolase activity	None predicted	Domain not predicted; class I fructose-bisphosphate aldolase family

Metabolism

92	A0A1B6QL52	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G260800	12.99	16.12	6	1.11	0.05	1.97E-02	Biosynthetic process	Pyridoxal phosphate binding	None predicted	Aminotransferase, class I/ class II domain; pyridoxal phosphate-dependent transferase superfamily
103	C5WNH9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G405500	12.01	13.44	8	1.11	0.02	5.84E-03	None predicted	Thiamine pyrophosphate binding	Intracellular region	Thiamine pyrophosphate-dependent enzyme domain and family
284	C5YAK8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3006G269200	5.7	15.21	3	1.08	0.03	2.36E-02	Asparagine biosynthetic process	Protein homodimerization activity	None predicted	Domain of unknown function DUF3700; nucleophile aminohydrolases, N-terminal superfamily
594	C5XVW3	Uncharacterized protein OS= <i>Sorghum</i>	3.77	4.90	2	1.47	0.23	4.33E-02	Ubiquinone-6 biosynthetic	NADH dehydrogenase	None predicted	NAD-dependent epimerase/dehydratase

		<i>bicolor</i> GN=SORBI_3004G3 44800							process	activity		domain; NAD(P)- binding domain superfamily
Protein destination & storage												
54	C5WZF2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G228200	16.42	16.98	10	1.06	0.02	4.38E-03	Protein folding and refolding	ATP binding	Cytoplasm	Domain not predicted; chaperonin (HSP60) family
289	C6JS77	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=Sb0111s002010	5.6	18.13	3	-1.18	0.04	8.71E-03	Intracellular protein transport	GTP and nucleotide binding	Endoplasmic reticulum	Small GTP-binding protein domain; SAR1 family
602	C5XN14	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G186700	2.11	3.77	2	1.18	0.02	3.54E-02	Ubiquitin- dependent protein catabolic process	Thiol-dependent ubiquitinyl hydrolase activity	Nucleus	Ubiquitin domain; peptidase C19 family
Signal transduction												
363	C5XQD5	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G214900	4.28	5.07	2	1.35	0.05	3.33E-03	Protein phosphorylation	Protein kinase activity	Integral component of membrane	Protein kinase domain; protein kinase-like domain superfamily
Protein synthesis												
274	C5XJI8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G003000	5.93	26.71	3	-1.07	0.04	4.77E-02	None predicted	None predicted	None predicted	Domain not predicted; nucleic acid binding OB-fold superfamily
Cell growth/division												
329	C5XTU6	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G030000	4.92	13.75	3	-1.19	0.03	2.81E-02	Pollen development	Cobalt, copper and zinc ion binding	Chloroplast	Domain not predicted; male gametophyte defective 1 family
Cell structure												
633	C5WSQ3	Tubulin alpha chain OS= <i>Sorghum bicolor</i> GN=SORBI_3001G453700	6.11	13.16	5	1.11	0.01	4.89E-03	Microtubule- based process	GTPase activity	Cytoskeleton	Tubulin domain and family
Unclassified												
82	A0A194YLC1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G248150	13.61	16.89	9	1.09	0.03	3.12E-02	None predicted	None predicted	None predicted	None predicted

^a Protein number assigned in ProteinPilot software.

^b Protein accession numbers obtained from the UniProt database (<http://www.uniprot.org>).

^c Protein score generated by ProteinPilot software relating to the confidence of protein identification.

^d Percentage coverage is determined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

^aSequenced peptide refers to the number of peptides that were sequenced and gave rise to protein identity.

^fRatio represents the average fold change. A negative value indicates down-regulation.

^gStandard deviation obtained from comparing root protein control and treatment values.

^hProbability-value of the quantified difference between the treatment and control root proteins.

ⁱGene ontology analysis as predicted from the UniProt database (<http://www.ebi.ac.uk/QuickGO/>). P denotes Biological Process, F denotes Functional Process, and C denotes Cellular Component.

^jConserved domains, family and superfamily names as predicted by Interpro database (<http://www.ebi.ac.uk/interpro/>).

4.2.3 Gene Ontology Analysis

All the differentially expressed sorghum salt stress responsive root proteins were submitted for Gene Ontology (GO) annotation on the Uniprot database for the determination of their biological process, molecular function and cellular component. Due to the fact that most proteins were uncharacterised, the Interpro database (<http://www.ebi.ac.uk/interpro/>) was used to identify conserved domains and family names of the salt-stress responsive proteins. Protein domains are regions of a protein's structure that have distinct functions (Buljan and Bateman, 2009). Protein families are a group of related proteins sharing a common ancestry and similar sequence with common domain architecture (Wu *et al.*, 2004). Therefore, protein domain and family name annotation assisted in understanding the putative functions of the identified proteins. The GO annotation results for SA 1441 and ICSB 338 sorghum varieties are illustrated in Tables 4.1 and 4.2, respectively. Furthermore, comparative diagrammatic representations of the three GO terms, namely biological process, molecular function and cellular component of the two sorghum varieties are shown in Figures 4.2, 4.3 and 4.4, respectively.

Figure 4.2 below shows the biological processes of the differentially expressed salt-stress responsive root proteins. The majority of the identified proteins in both SA 1441 and ICSB 338 were involved in responses to oxidative stress, followed by those with an unpredicted biological process. Interestingly, only ICSB 338 had 23% stress responsive proteins involved in the glycolytic pathway.

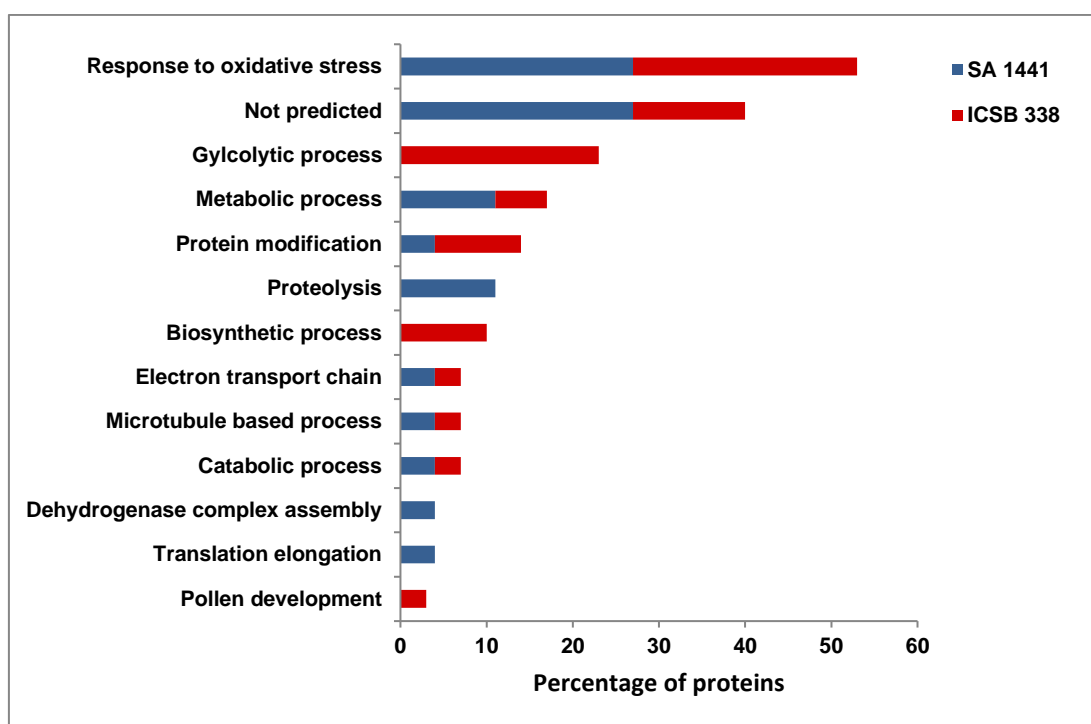


Figure 4.2: Biological process predictions of the differentially expressed sorghum root salt responsive proteins.

The majority (20%) of the differentially expressed salt-stress responsive root proteins of SA 1441 had unpredicted molecular functions (Figure 4.3). Apart from this category, the highly represented molecular functions were pathogenesis-related proteins, proteins involved in protein binding, transferase and peroxidase activity in both sorghum varieties.

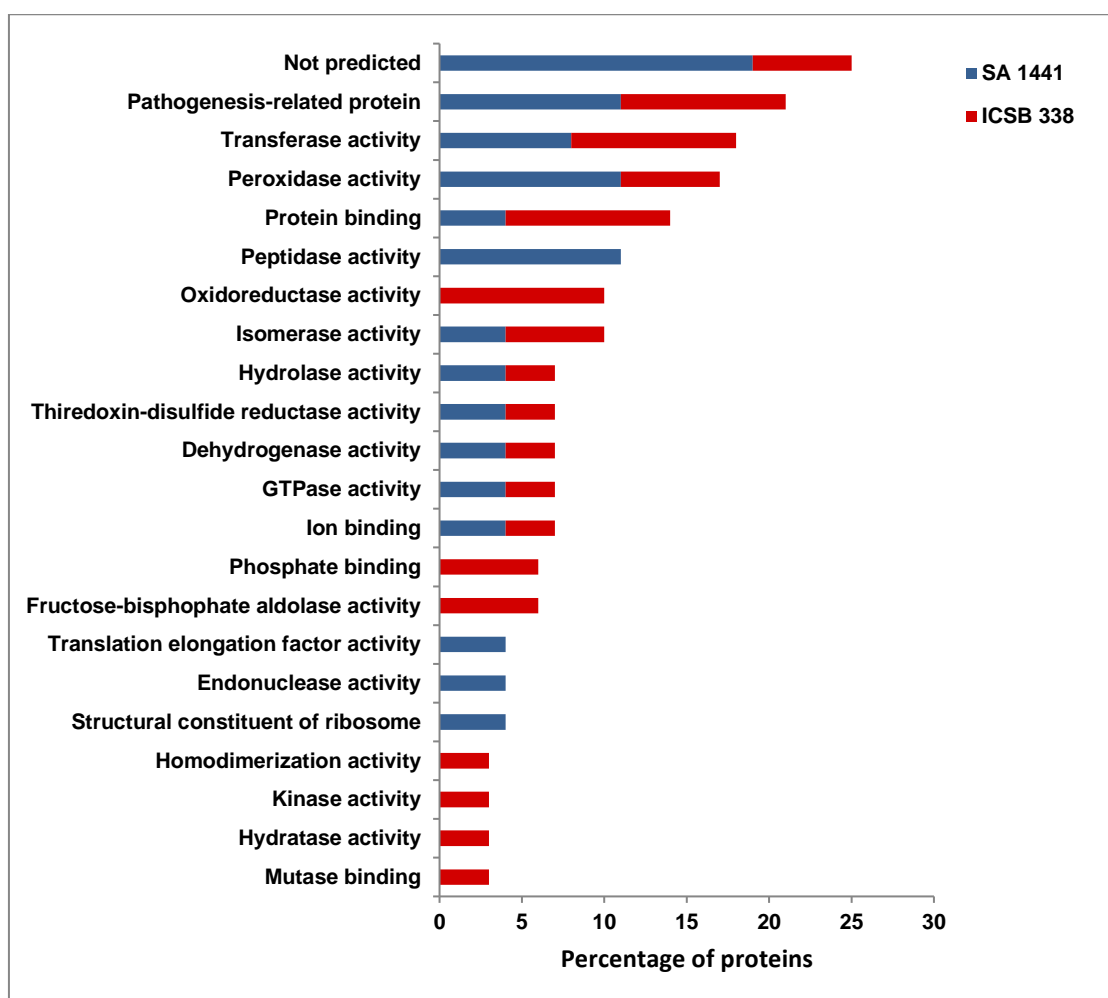


Figure 4.3: Molecular function predictions of the differentially expressed sorghum root salt responsive proteins.

Figure 4.4 below shows the different locations of the differentially expressed sorghum salt-stress responsive root proteins. Most of the differentially expressed proteins in SA 1441 (62%) and ICSB 338 (45%) did not have any predicted cellular components. Apart from these, ICSB 338 had the majority of its proteins localised in the cytoplasm/cytosol/intracellular region with SA 1441 proteins mainly in the extracellular region.

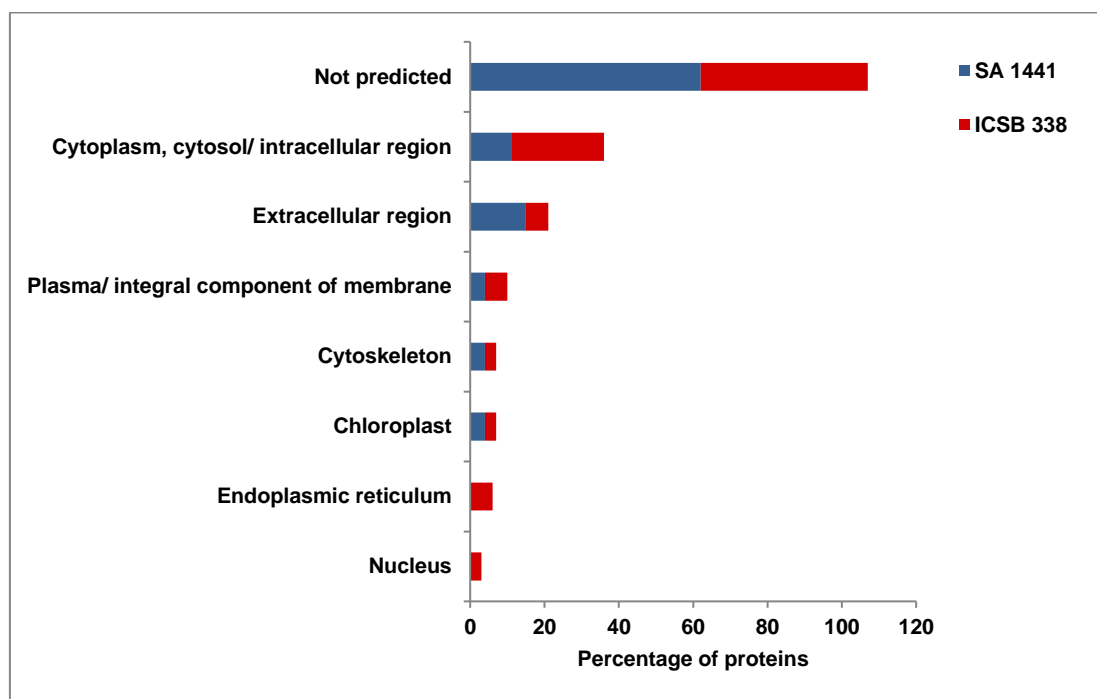


Figure 4.4: Cellular component predictions of the differentially expressed sorghum root salt responsive proteins.

4.2.4 Functional Categories of the Differentially Expressed Sorghum Root Proteins

The differentially expressed sorghum root proteins of both SA 1441 (Table 4.1) and ICSB 338 (Table 4.2) were grouped into functional categories according to Bevan *et al.* (1998) and various literature sources. For the uncharacterised proteins, the information obtained on the conserved domain and protein family names assisted with the overall functional grouping of these proteins. Figure 4.5 A shows the differentially expressed proteins of SA 1441, which were classified into 6 functional categories; namely disease/defence (38%); metabolism (23%); protein destination and storage (15%); signal transduction (12%); protein synthesis (8%) and cell structure (4%).

Nine functional categories were identified in ICSB 338 salt stress responsive root proteins, namely; disease/defence (39%); energy (23%); metabolism (13%); protein destination and storage (10%); signal transduction (3%); protein synthesis (3%); cell structure (3%) cell growth/division; (3%) and unclassified (3%) as shown in Figure 4.5 B. Interestingly, the most represented functional group in both sorghum varieties was disease/defence, followed by energy, which was only present in ICSB 338. Although the metabolism functional group was present in both sorghum varieties, it was more dominant in SA 1441. A brief description of each of the functional categories is given below.

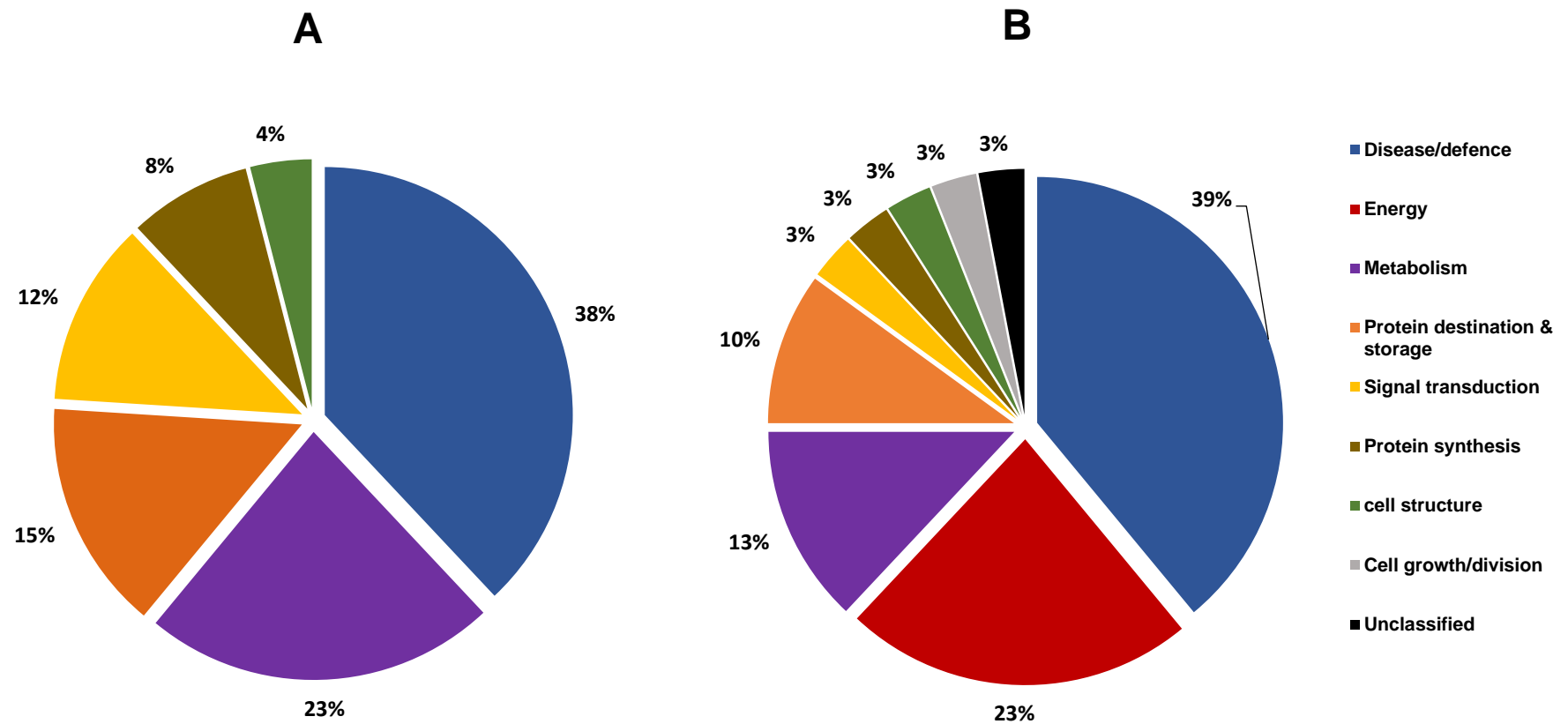


Figure 4.5: Functional categories of differentially expressed sorghum root salt-stress responsive proteins. (A) shows results of SA 1441, while (B) shows that of ICSB 338. Proteins were grouped into functional categories using data available on the UniProt database (www.uniprot.org) and according to Bevan *et al.* (1998) and literature sources.

4.2.4.1 Disease/defence

Of the 26 differentially expressed SA 1441 salt stress responsive root proteins, a total of 9 (38%) proteins were involved in disease and defence (Table 4.1; Figure 4.5 A). This functional category consists of peroxidases (protein no.16, 126 & 509), pathogenesis-related proteins (protein no. 28 & 86) and uncharacterised proteins (protein no. 85, 212, 289, 342 & 390) involved in plant defence. Most of the proteins in this category were up-regulated (60%), while (40%) were down-regulated in response to the salt stress as illustrated in Table 4.1.

For ICSB 338, a total of 12 (39%) differentially expressed salt stress responsive root proteins were involved in disease and defence (Table 4.2; Figure 4.5 B). This functional group consists of peroxidases (protein no. 52 & 126), protein disulfide-isomerase protein (protein no. 8) and uncharacterised proteins (protein no. 74, 77, 187, 225, 293, 307, 322, 350 & 550). The uncharacterised proteins are involved in cell redox homeostasis, plant defence, glutathione metabolic and oxidation-reduction processes. Fifty percent of these proteins were each up- and down-regulated in response to the salt stress (Table 4.2). The up-regulation of proteins involved in reactive oxygen species (ROS) scavenging was also observed in the root proteome of barley genotypes in response to 100 and 150 mM NaCl-induced salt stress (Witzel *et al.*, 2009). A down-regulation of defence proteins was also observed in the root proteome of tomato genotypes in response to 100 mM NaCl-induced salt stress (Manaa *et al.*, 2011).

4.2.4.2 Metabolism

A total of 6 (23%) root proteins were involved in metabolism (Table 4.1; Figure 4.5 A) in SA 1441, and all were uncharacterised (protein no. 113, 130, 163, 255, 299 & 368). According to

the GO annotation analysis (Table 4.1), some of these uncharacterised proteins (protein no.130, 163, 255 & 299) had biological processes involved in carbohydrate and isocitrate metabolic, and DNA catabolic processes, while (protein no.133) belongs to the glycosyl hydrolase family. Furthermore, all the SA 1441 metabolic-related proteins were down-regulated in response to the salt stress (Table 4.1). Metabolism related proteins were also down-regulated in soybean in response to 40 mM NaCl-induced salt stress (Sobhanian *et al.*, 2010).

For ICSB 338, a total of 4 (13%) salt stress responsive root proteins were involved in metabolism (Table 4.2; Figure 4.5 B). Similarly, for ICSB 338, all the proteins involved in metabolism were uncharacterized (protein no. 92, 103, 284 & 594). According to the GO annotation analysis (Table 4.2), these uncharacterised proteins had biological processes involved in biosynthetic, asparagine biosynthetic and ubiquinone-6 biosynthetic processes and there were all up-regulated in response to the salt stress (Table 4.2).

4.2.4.3 Signal Transduction

About 3 (12%) of the 26 differentially expressed salt stress responsive root proteins for SA 1441 were involved in signal transduction processes (Table 4.1; Figure 4.5 A). This functional group consist of an annexin protein (protein no. 230), and uncharacterised proteins (protein no. 189 & 504) with 14-3-3 and C2 domains, respectively. The annexin protein and the uncharacterised proteins were up-regulated in response to salt stress (Table 4.1). The up-regulation of the 14-3-3 protein was also observed in soybean roots in response to 150 mM NaCl-induced salt stress (Ma *et al.*, 2014). For ICSB 338, only one uncharacterised protein (3%) was involved in signal transduction processes (Table 4.2; Figure 4.5 B). This protein

(protein no.363) is involved in protein phosphorylation and was up-regulated in response to the salt stress (Table 4.2).

4.2.4.4 Protein Destination and Storage

A total of 4 (15%) proteins of the 26 differentially expressed SA 1441 salt stress responsive root proteins were categorised to function in protein destination and storage (Table 4.1; Figure 4.5 A). The proteins in this functional group include carboxypeptidase protein (protein no. 209), peptidyl-prolyl cis-trans isomerase protein (protein no. 269) and uncharacterised proteins (protein no. 294 and 403). Carboxypeptidase and peptidyl-prolyl cis-trans isomerase proteins were up-regulated in response to the salt stress while the uncharacterised proteins involved in proteolysis were down-regulated (Table 4.1).

For ICSB 338,3 (10%) proteins were predicted to function in protein destination and storage (Table 4.2; Figure 4.5 B). All the proteins in this functional group were uncharacterised (protein no. 54, 289 & 602). According to the GO annotation analysis (Table 4.2), these uncharacterised proteins had biological processes involved in protein folding and refolding, intracellular protein transport and ubiquitin-dependent protein catabolic process. Furthermore these uncharacterised proteins were up-regulated in response to the salt stress (Table 4.2).

4.2.4.5 Energy

This functional group was only found in ICSB 338. A total of 7 (23%) differentially expressed salt stress responsive root proteins were involved in energy related processes (Table 4.2; Figure 4.5 B). These proteins included glyceraldehydes-3-phosphate dehydrogenase (protein no. 3 & 30); fructose-bisphosphate aldolase (protein no. 6 & 80) and uncharacterised proteins (protein no. 9, 11, & 48) which were involved in glycolytic and

glucose catabolic processes. All the proteins in this group were up-regulated in response to the salt stress (Table 4.2). Similar increases in glycolytic related proteins was observed in rice roots under salt stress (Nam *et al.*, 2012).

4.2.4.6 Protein Synthesis

Of the 26 differentially expressed SA 1441 salt stress responsive root proteins, a total of 9 (8%) proteins were involved in protein synthesis (Table 4.1; Figure 4.5 A). These proteins included elongation factor 1-alpha (protein no. 26) and a 40s ribosomal protein S27 (protein no. 421) which were up- and down-regulated in response to the salt stress, respectively (Table 4.1). Ribosomal proteins also decreased in Arabidopsis under salt stress (Jiang *et al.*, 2007). For ICSB 338, only one uncharacterised protein (protein. no 274) was involved in protein synthesis. This uncharacterised protein was down-regulated and predicted to have functions in protein synthesis due to its classification into the nucleic acid binding OB-fold superfamily.

4.2.4.7 Cell structure

This group consists of a similar protein found in both sorghum varieties, tubulin alpha chain protein (protein no. 102) in SA 1441 and (protein no.633) in ICSB 338. The tubulin alpha chain protein was down-regulated in SA 1441 (Table 4.1). Similar results were reported in soybean in response to 50 mM NaCl-induced salt stress (Yin *et al.*, 2018). However in ICSB 338, the tubulin protein was up-regulated in response to the salt stress (Table 4.2). The up-regulation of this protein was also observed in barely roots in response 100 mM NaCl-induced salt stress (Mostek *et al.*, 2015).

4.2.4.8 Other Functional Categories

This group consists of functional categories that were only classified in one of the sorghum variety ICSB 338 (Figure 4.5 B). These categories include a protein involved in cell growth/division and an unclassified protein. The protein categorised in cell growth/division was an uncharacterised protein (protein no.329) involved in pollen development and was down-regulated in response to salt stress. The unclassified protein (protein no. 82) did not have any predicted functions, protein family or conserved domains and it was up-regulated in response to salt stress treatment (Table 4.2).

4.3 Discussion

Plant responses to salt stress involve a range of physiological, biochemical and molecular mechanisms (Parida and Das, 2005), which ultimately contribute to the plant's overall degree of tolerance/susceptibility to the stress imposed. Salt stress alters gene and protein expression resulting in changes in cellular functions and metabolite levels (Kosova *et al.*, 2013). Gel based and non-gel based proteomic tools quantify changes in protein expression in response to stress, which may result in either a decrease or increase in protein abundance. However, the degree of change may depend on various factors such as the plant species, genotype, developmental stage, intensity of the stress and duration of exposure (Pérez-Torres *et al.*, 2008).

In this study, a comparative root proteomic analysis was conducted between two sorghum varieties in response to 100 mM NaCl-induced salt stress. The two varieties used, SA 1441 and ICSB 338 have contrasting tolerance to drought (Table 2.1). SA 1441 is drought tolerant, while ICSB 338 is both drought and salt sensitive (Satish *et al.*, 2016). An iTRAQ method was used to investigate the differential protein expression in 100 mM NaCl salt stressed

roots. Furthermore, a mild salt stress treatment using 100 mM NaCl for 7 days was also used to target any differences in early signalling processes and stress perception between the two sorghum varieties. Roots are essential in plant growth as they absorb water and nutrients to maintain the normal functioning of the plant (Nouri and Komatsu, 2014). Under salt stress, roots sense salinity and are able to respond by passing the signal to the shoot for appropriate changes in shoot function (Zhao *et al.*, 2013).

In this study, a total of 522 and 544 root proteins were identified in SA 1441 and ICSB 338 sorghum varieties, respectively. Of these positively identified root proteins, a total of 379 (SA 1441) and 405 (ICSB 338) were uncharacterised. Although the sorghum genome is fully sequenced (Paterson *et al.*, 2009), a large number of sorghum genes have not been functionally annotated. A total of 26, (SA 1441) (Table 4.1; Figure 4.5 A) and 31, (ICSB 338) (Table 4.2; Figure 4.5 B) root proteins were differentially expressed in response to the salt stress treatment. These proteins were subsequently grouped into different functional categories according to Bevan *et al.* (1998) and other literature sources. Interestingly, the most represented differentially expressed root proteins in response to 100 mM NaCl in both sorghum varieties were associated with disease/defence (Figure 4.5). These proteins include peroxidases, pathogenesis related-proteins and cell redox proteins (Table 4.1 & 4.2).

In plants, salt stress causes a high accumulation of reactive oxygen species (ROS), which results in oxidative stress. Such high levels of ROS disrupts the normal metabolic processes in root cells (Zhao *et al.*, 2013). In turn, plants have developed a range of enzymatic and non-enzymatic antioxidant systems to eliminate or reduce high levels of ROS (Foyer and Noctor, 2005). Examples of enzymatic antioxidants include ascorbate peroxidase, superoxide dismutase, catalase, monodehydroascorbate reductase, dehydroascorbate reductase and

glutathione reductase. The non-enzymatic antioxidants include glutathione, ascorbate, carotenoids and tocopherols (Hung *et al.*, 2005). Oxidative stress also activates several defence and pathogenesis-related proteins in plants (Kosová *et al.*, 2013). In this study, most of the proteins involved in disease/defence were up-regulated in both sorghum varieties. This observation possibly illustrates the importance of proteins in reducing the toxic effects of ROS during salt stress. However, SA 1441, the drought tolerant variety had more up-regulated proteins compared to ICSB 338. Examples of the up-regulated disease/defence proteins include pathogenesis-related proteins, peroxidase and uncharacterised proteins involved in plant defence and cell redox homeostasis.

In ICSB 338 the up-regulated proteins included protein disulfide isomerase and uncharacterised proteins involved in plant defence, cell redox homeostasis, oxidation-reduction and glutathione metabolic processes. However, some peroxidases were down-regulated in ICSB 338 in response to the salt stress (Table 4.2). Overall, it is interesting to note that even at low salt stress treatment levels of 100 mM NaCl, sorghum plants were able to recruit a range of antioxidant systems to protect the cells against oxidative damage, irrespective of the variety. The up-regulation of proteins involved in disease/defence such as pathogenesis-related and peroxidase proteins has also been observed in wheat roots exposed to a range of 0.5%, 1.5% and 2.5% NaCl-induced salt stress for 2 days (Guo *et al.*, 2012).

Plants exposed to abiotic stresses, including salinity respond by sensing and transferring signals through signal transduction networks which, lead to molecular and metabolic changes to maintain normal functioning (Komatsu and Hossain, 2013). In this study, the signal transduction category had an uncharacterised protein, belonging to the 14-3-3 family in SA 1441, the drought tolerant variety. This protein was up-regulated in response to the salt stress.

The 14-3-3 proteins interact with a signal-regulated kinase pathway in order to regulate cell growth and survival (Mhaweche, 2005). According to Zhang *et al.* (2012), 14-3-3 proteins are involved in response to a variety of stresses including salinity stress and regulate target proteins with functions including signalling, transcription activation and defence. A study on soybean roots also revealed the up-regulation of 14-3-3 proteins under 150 mM NaCl-induced salt stress for 1, 12, 72, and 144 hours (Ma *et al.*, 2014).

Another protein, which was up-regulated in SA 1441 in response to the salt stress was an annexin. Plant annexins are calcium and membrane binding proteins possibly involved in signalling pathways. The expression, abundance, and cellular position of annexins can respond to osmotic stress, salinity, drought and abscisic acid (ABA) treatment (Mortimer *et al.*, 2008). The annexin protein was also up-regulated in barely root proteome under 100 mM NaCl-induced salt stress for 6 days (Mostek *et al.*, 2015).

The drought and salt susceptible variety, ICSB 338 had one uncharacterised protein which relates to signal transduction. This protein was involved in protein phosphorylation and was up-regulated in response to the salt stress. During salt stress in plants, protein phosphorylation is a major type of post-translation modification (Chitteti and Peng, 2007). It regulates many cellular processes in eukaryotes and plays a major role in signal transduction pathways (Hunter, 1995).

Metabolism represents the basic processes that maintain cellular function and consists of both primary and secondary metabolic processes. In response to stress, plants alter their metabolism by producing osmolytes that stabilize proteins and their cellular structures (Krasensky and Jonak, 2015). In this study, all SA 1441 responsive proteins involved in

metabolism were uncharacterised and down-regulated in response to the salt stress treatment. These proteins are involved in carbohydrate metabolic and DNA catabolic processes. On the other hand, all the salt stress responsive root proteins of ICSB 338 were also uncharacterised but involved in biosynthetic, asparagine biosynthetic and ubiquinone-6 biosynthetic processes. However, these proteins were all up-regulated in response to the salt stress treatment. It is however unclear why different metabolic processes were differentially affected in the two sorghum varieties.

Protein synthesis plays a major role in abiotic stress adaptation. An elongation factor protein was identified in SA 1441 and it was up-regulated in response to salt stress. This protein was also up-regulated in rice roots exposed to 150 mM NaCl-induced salt stress for either 3 or 7 hours (Nam *et al.*, 2012). A ribosomal protein was also identified in SA 1441 but it was down-regulated in response to the salt stress treatment. Only one protein related to protein synthesis was identified in ICSB 338. However, this protein (protein. no 274) was down-regulated in response to the salt stress treatment. The activation of proteins involved in protein synthesis in SA 1441 possibly shows their importance in plant salt tolerance as compared to ICSB 338.

The balance between protein synthesis and proteolysis results in growth and development in most organisms (Palma *et al.*, 2002). In plants, protein degradation occurs by a mechanism referred to as proteolysis caused by hydrolytic enzymes called proteases. Plants ensure the quality of intracellular proteins by eliminating misfolded or damaged polypeptides (Goldberg, 2003). In SA 1441, carboxypeptidase and peptidyl-propyl cis-trans isomerase were up-regulated in response to the salt stress treatment. Peptidyl-propyl cis-trans isomerase proteins maintain normal protein folding and repair stress-damaged proteins (Zhang *et al.*,

2012). In ICSB 338, two uncharacterized proteins involved in protein folding and refolding and ubiquitin-dependent protein catabolic process were up-regulated in response to the salt stress treatment. Under salt stress conditions, proper protein folding and processing is important in the functioning of roots (Zhao *et al.*, 2013). Ubiquitin-mediated degradation of proteins regulates cellular processes such as signal transduction and transcription (Zhang *et al.*, 2012).

Plant cells also generate energy in the form of ATP to fuel all metabolic functions and growth and development processes. Under salt stress, ATP synthesis is enhanced for energy-consuming adaptive mechanisms such as ion homeostasis, ROS defence and osmotic adjustment (Che-Othman *et al.*, 2017). Energy related proteins accumulated only in ICSB 338 (Table 4.2; Figure 4.5 B). All the glycolytic enzymes such as fructose bisphosphate aldolase and glyceraldehyde 3-phosphate dehydrogenase were up-regulated in ICSB 338 in response to the salt stress treatment. These proteins were also up-regulated in rice roots exposed to 150 mM NaCl-induced salt stress for either 3 or 7 hours (Nam *et al.*, 2012).

Under salt stress, the cytoskeleton allows cell size adjustment for the maintenance of cell turgor (Zhang *et al.*, 2012). The cell structure category in both sorghum varieties had a similar protein, the tubulin alpha-chain protein. Microtubules play a key role in division and growth of plant cells (Ban *et al.*, 2013). The tubulin alpha chain protein was down-regulated in SA 1441 and up-regulated in ICSB 338 in response to the salt stress treatment. The tubulin alpha chain protein was also up-regulated in barely roots in response 100 mM NaCl-induced salt stress for 6 days (Mostek *et al.*, 2015). On the other hand Yin *et al.* (2018) observed its down regulation in soybean under 50 mM NaCl-induced salt stress for 4 days.

After observing that the sorghum root samples exposed to 100 mM NaCl for 7 days showed minor changes in response to the salt stress, a leaf proteomic analysis (Chapter 5) was performed to further analyse any differences in salt tolerance between the two sorghum varieties.

CHAPTER 5

A COMPARATIVE PROTEOMIC IDENTIFICATION OF SALT-STRESS RESPONSIVE LEAF PROTEINS OF TWO SORGHUM VARIETIES

5.1 Introduction

Leaves play an important role in the growth and survival of a plant, as they are primarily involved in photosynthesis. Leaves also assist in the transportation of essential elements and water from the roots to the aerial parts of the plant (Komatsu and Hossain, 2013). Under salt stress, leaf growth is often reduced more than that of roots, mainly due to the osmotic effect on plants (Munns, 2002). Furthermore, salts accumulate to toxic levels in leaves causing ion toxicity, which results in the death of older leaves. If the rate at which older leaves die is greater than the rate at which new leaves develop, the photosynthetic capacity of a plant will be negatively affected (Munns and Tester, 2008).

Several studies have used proteomic tools to investigate the mechanism underlying salt tolerance in plants. Leaf proteomic studies on wheat (*Triticum aestivum*) (Donnelly *et al.*, 2005), soybean (*Glycine max*) (Xu *et al.*, 2006) and sorghum (Swami *et al.*, 2011; Ngara *et al.*, 2012) have revealed that the majority of the identified proteins are associated with energy production and metabolism. These observations are consistent with the primary photosynthetic functions of leaves.

Comparative studies on leaf salt stress-induced proteomic changes have been conducted in crops such as cowpea (*Vigna unguiculata*) (de Abreu *et al.*, 2014), tomato (*Solanum lycopersium*) (Manaa *et al.*, 2013) and cotton (*Gossypium hirsutum*) (Gong *et al.*, 2017). In

the cotton study, iTRAQ analysis was conducted between two cotton genotypes; Earlistaple 7 (salt tolerant) and Nan Dan Ba Di Hua (salt sensitive) in response to 200 mM NaCl treatment for 4 and 24 hours. A total of 58 differentially expressed salt-stress responsive leaf proteins were identified, 29 of the 58 proteins were not genotype specific in response to the salt treatment. The other 29 salt-responsive leaf proteins were genotype specific, and 62.1 and 27.6% of these proteins were related to chloroplast and defence responses, respectively (Gong *et al.*, 2017).

In this study, an iTRAQ analysis was used to assess sorghum salt-stress responsive leaf proteins in two sorghum varieties. The main objective of this chapter was to conduct a comparative proteomic analysis of two sorghum varieties; SA 1441 and ICSB 338 exposed to 100 mM NaCl-induced salt stress for 7 days.

5.2 Results

5.2.1 One Dimensional Protein Profiles of Sorghum Leaf Extracts

SA 1441 and ICSB 338 sorghum plants were grown as described in section 2.2.1. Thereafter, the salt stressed group of plants were watered with 100 mM NaCl every two days for 7 days. The control plants were watered with distilled water for the duration of the experiment. All plants were harvested after 7 days of salt treatment. Total soluble proteins were extracted from the control and salt-stressed leaf tissue of both sorghum varieties (Section 2.2.6) and quantified (Section 2.2.7). One dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to evaluate the quality and quantity of the leaf protein extracts prior to iTRAQ analysis.

Figure 5.1 shows Coomassie Brilliant Blue (CBB) stained 1D SDS-PAGE gels of the leaf protein extracts. Five biological replicates of each of the control and 100 mM NaCl stressed leaf samples were electrophoresed and each lane was loaded with 10 μ g of protein extract. Lane M shows the molecular weight markers. The biological replicates showed similar banding patterns in both sorghum varieties and the quality of the leaf protein extracts was good with no visible signs of protein degradation (Figure 5.1).

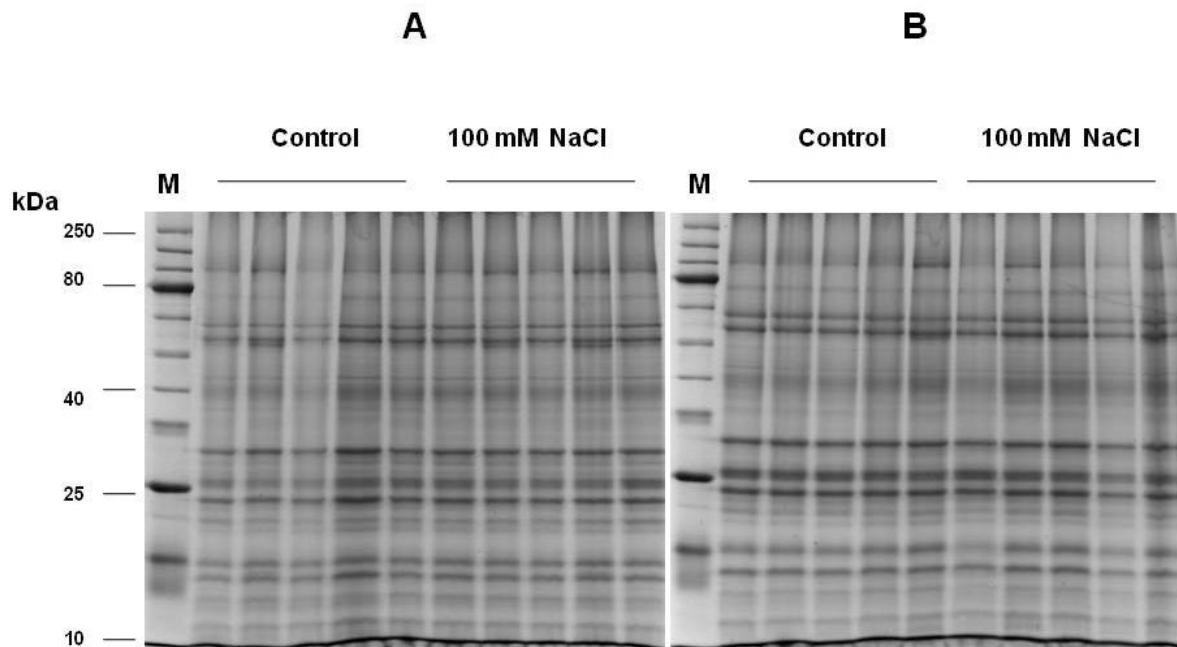


Figure 5.1: Comparative 1D SDS-PAGE analysis of sorghum leaf total soluble proteins. (A) shows SA 1441 and (B) shows ICSB 338 leaf samples loaded on a 12% (v/v) SDS-PAGE gel. About 10 μ g of protein extracts was loaded in each lane. Five biological replicates of both the control and 100 mM NaCl salt-stressed samples are shown. The gels were stained with CBB stain. Lane M represents the protein molecular weight marker measured in kDa.

5.2.2 iTRAQ Analysis of the Sorghum Leaf Proteome in Response to Salt Stress

The iTRAQ-based proteomic technique was used to positively identify sorghum leaf proteins in response to 100 mM NaCl salt stress treatment. Four biological replicate samples of 12.5 µg total protein content, from each of the control and 100 mM NaCl salt-stressed groups were used. The samples were iTRAQ labelled, digested with trypsin, separated by liquid chromatography and subsequently identified by tandem mass spectrometry as described in Section 2.2.10. A total of 829 and 591 leaf proteins from SA 1441 and ICSB 338 respectively were positively identified in this study. Of these positively identified leaf proteins, a total of 559 (67%) for SA 1441 and 408 (69%) for ICSB 338 were uncharacterised.

For SA1441, 75 (Table 5.1) of the 829 positively identified proteins were responsive to 100 mM NaCl-induced salt stress with a significant difference ($p \leq 0.05$). For ICSB 338, 24 (Table 5.2) of the 591 positively identified proteins were responsive to 100 mM NaCl-induced salt stress with a significant difference ($p \leq 0.05$). There was a similar protein in each of the two varieties, an uncharacterised protein (protein no. 75) in SA 1441 and (protein no. 108) in ICSB 338.

Of the 75 differentially expressed salt-stress responsive leaf proteins for SA 1441, 37 (49%) were up-regulated, while 38 (51%) were down-regulated in the same stress treatment. The fold changes for the up-regulated proteins ranged from 1.11 (protein no. 59) to 1.62 (protein no. 989), whereas the fold changes for the down-regulated proteins ranged from -2.80 (protein no. 745) to -1.12 (protein no. 87). For ICSB 338, of the 24 differentially expressed leaf proteins, 14 (58%) were up-regulated and 10 (42%) were down-regulated in the same salt treatment. The fold changes for the up-regulated proteins ranged from 1.08 (protein no. 7) to

1.60 (protein no. 509), whereas the fold changes for the down-regulated proteins ranged from -1.60 (protein no. 356) to -1.19 (protein no. 54).

Table 5.1: List of SA 1441 sorghum salt-stress responsive leaf proteins identified using the iTRAQ and database searches.

Prot No. ^a	Accession No. ^b	Protein name	Scor ^c	% Cov ^d	Sep Pep ^e	Ratio ^f	SD ^g	p-value ^h	GO analysis ⁱ			Conserved domains and family names ^j
									P	F	C	
Disease/defence												
4	A0A1B6QIR3	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3001G129000	62.68	48.39	43	-1.19	0.04	6.66E-03	Protein folding	ATP binding	None predicted	Domain not predicted; heat shock protein 70 family
23	A0A1B6QPW6	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3001G472000	39.6	50	25	1.24	0.13	2.69E-02	Oxidation-reduction process	Oxidoreductase activity	None predicted	Amine oxidase domain; flavin amine oxidase family
45	A0A1B6PMT8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3006G204000	32.06	53.58	18	-1.20	0.01	6.36E-05	Response to oxidative stress	Peroxidase activity	None predicted	Secretory peroxidase domain; plant peroxidase family
62	A0A1B6PQR2	Protein disulfide-isomerase OS= <i>Sorghum bicolor</i> GN=SORBL_3005G074400	27.36	30.01	12	-1.13	0.02	2.34E-02	Endosperm development	Protein disulfide isomerase activity	Endoplasmic reticulum lumen	Thioredoxin domain; protein disulphide isomerase family
117	C5Z469	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBL_3010G161600	19.06	47.13	20	-1.16	0.04	4.23E-02	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
166	C5YN49	Superoxide dismutase [Cu-Zn] OS= <i>Sorghum bicolor</i> GN=SORBL_3007G166600	15.24	50.49	13	-1.17	0.08	1.76E-02	Superoxide metabolic process	Superoxide dismutase activity	Extracellular region	Superoxide dismutase, copper/zinc binding domain; superoxide dismutase (CI/ZN) family
176	C5YT85	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3008G063100	14.54	38.08	10	-1.35	0.04	5.50E-03	Cell redox homeostasis	Oxidoreductase activity	Chloroplast	Thioredoxin domain and family
187	C5WUH5	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3001G317400	14.23	28.15	8	1.24	0.03	2.18E-03	Glutathione metabolic process	Glutathione transferase activity	Cytoplasm	Glutathione S-transferase, C-terminal-like domain; glutathione S-transferase, C-terminal domain superfamily
219	C5XG44	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3003G254300	12.05	64.81	7	-1.30	0.04	1.84E-03	Cell redox homeostasis	Oxidoreductase activity	Cell	Thioredoxin domain; peroxiredoxin-5-like family
253	A0A1Z5RKL6	Catalase OS= <i>Sorghum bicolor</i> GN=SORBL_3004G011566	10.93	15.37	6	1.55	0.23	9.67E-03	Hydrogen peroxide catabolic process	Catalase activity	None predicted	Catalase core domain; catalase, mono-functional, haem-containing family
336	C5ZIZ3	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3010G270300	8.83	21.26	4	1.26	0.02	8.93E-05	None predicted	None predicted	None predicted	Rhodanese-like domain; rhodanese-like domain superfamily
357	A0A1B6P6N7	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3009G043000	8.37	17.19	4	-1.20	0.08	1.44E-02	Oxidation-reduction process	Protein disulfide oxidoreductase activity	Cell	Thioredoxin domain and family
360	C5WRH6	Uncharacterized protein	7.54	12.94	4	1.23	0.13	2.87E-02	Oxidation-	Peptide-methione	Mitochondri	Peptide methione

		OS= <i>Sorghum bicolor</i> GN=SORBL_3001G295300							reduction process	(S)-S-oxide reductase activity	on	sulphoxide reductase MsrA domain and superfamily
414	Q6JAG4	Glutathione peroxidase OS= <i>Sorghum bicolor</i> GN=SB20O07.21	6.68	17.26	3	-1.31	0.12	9.27E-03	Response to oxidative stress	Glutathione peroxidase activity	None predicted	Domain not predicted; glutathione peroxidase family
444	C5Z0N8	Peroxidase OS= <i>Sorghum bicolor</i> GN=SORBL_3009G055100	6.06	10.08	3	1.27	0.16	2.59E-02	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
465	C5WNS2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3001G122300	5.96	14.55	3	-1.36	0.08	5.06E-03	None predicted	None predicted	None predicted	Plastid lipid-associated protein/fibrillin conserved domain; family not predicted
468	C5Y8T1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3006G088000	5.92	7.56	3	1.21	0.11	2.22E-02	Chloroplast organization	None predicted	Chloroplast	Heat shock chaperonin-binding domain; family not predicted
603	C5Z3A0	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3010G020200	4	14.29	2	-1.27	0.10	3.33E-02	None predicted	None predicted	Extracellular region	Pathogenesis-related protein 1-like, SCP domain; cysteine-rich secretory protein, allergen V5/Tpx-1-related family
636	C5YZF7	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3009G029600	3.93	3.19	2	1.20	0.08	1.18E-02	Glutathione biosynthetic process	Glutamate-cysteine ligase activity	Chloroplast	Domain not predicted; glutamate-cysteine ligase, plant-type family
718	C5XD56	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3002G361100	2.98	6.14	2	1.31	0.09	3.35E-03	Glutathione metabolic process	Glutathione transferase activity	Cytoplasm	Glutathione S-transferase, C-terminal domain superfamily
989	C5YLK6	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3007G140700	1.85	5.62	2	1.62	0.34	1.69E-02	Oxidation-reduction process	Oxidoreductase activity	None predicted	Aldehyde dehydrogenase domain and family
Energy												
19	C5Z0B5	Phosphoglycerate kinase OS= <i>Sorghum bicolor</i> GN=SORBL_3009G183700	44.77	54.04	46	-1.17	0.05	4.64E-03	Glycolytic process	Phosphoglycerate kinase activity	None predicted	Domain not predicted; phosphoglycerate kinase family
21	A0A194YL80	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3010G241000	39.86	57.53	82	-1.30	0.02	4.05E-06	Photosystem II assembly	Oxygen evolving activity	Photosystem II oxygen evolving complex	Domain not predicted; Photosystem II Psbo, manganese-stabilising family
55	C5X9F7	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3002G030200	29.02	62.84	60	-1.22	0.05	3.40E-04	Photosynthesis	Calcium ion binding	Photosystem II	Domain not predicted; PsbP family
72	A0A1B6QQR5	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Sorghum bicolor</i> GN=SORBL_3001G519800	38.71	44.82	43	-1.21	0.04	2.26E-03	Glucose metabolic process	NADP binding	Chloroplast	Glyceraldehyde3-phosphate dehydrogenase domain and family
79	C5YTW1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3008G080400	23.53	50.32	23	1.40	0.13	2.22E-03	Beta-glucosidase activity	Glycosyl compound metabolic process	None predicted	Domain not predicted; glycoside hydrolase family 1

122	A0A1Z5RFN9	Chlorophyll a-b binding protein, chloroplastic OS= <i>Sorghum bicolor</i> GN=SORBL_3006G264201	19.15	44.31	19	-1.17	0.07	1.28E-02	Photosynthesis	Chlorophyll binding	Chloroplast	Domain not predicted; chlorophyll A-B binding protein family
153	A0A1W0W3H4	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3002G119500	16.52	57.75	64	-1.29	0.12	3.28E-02	Photosynthesis	None predicted	Photosystem I	Domain not predicted; photosystem I Psae, reaction centre subunit IV family
154	A0A1B6QHQ8	Glucose-6-phosphate isomerase OS= <i>Sorghum bicolor</i> GN=SORBL_3001G071800	16.47	17.28	9	1.13	0.07	1.65E-02	Glycolytic process	Glucose-6-phosphate isomerase activity	None predicted	Phosphoglucose isomerase, SIS domain 1; phosphoglucose isomerase family
158	C5Y5Q8	Fructose-bisphosphate aldolase OS= <i>Sorghum bicolor</i> GN=SORBL_3005G056400	37.62	60.05	31	-1.13	0.07	2.52E-02	Glycolytic process	Fructose-bisphosphate aldolase activity	None predicted	Domain not predicted; fructose-bisphosphate aldolase, class-I family
183	A0A1Z5R6D4	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3008G131700	13.93	29.43	8	-1.21	0.05	1.07E-02	Photosynthesis	Calcium ion binding	Chloroplast	Domain not predicted; PsbP family
191	C5XX52	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Sorghum bicolor</i> GN=SORBL_3004G205100	32.01	45.01	20	1.28	0.18	4.16E-02	Glucose metabolic process	NADP binding	None predicted	Glyceraldehyde 3-phosphate dehydrogenase, domain and family
246	A1E9S3	Photosystem I P700 chlorophyll a apoprotein A2 OS= <i>Sorghum bicolor</i> GN=psaB	11.08	13.35	6	1.29	0.18	3.35E-02	Photosynthesis	Chlorophyll binding	Chloroplast	Domain not predicted; photosystem I Psae/PsaB family
388	A0A1B6PCC8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3008G063500	7.08	9.43	7	-1.28	0.06	2.34E-02	Photosynthesis	None predicted	Chloroplast	Domain not predicted; photosystem I reaction centre subunit N, chloroplastic family
392	C5YRS9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3008G037800	7.07	14.65	4	-1.22	0.08	3.47E-02	None predicted	None predicted	None predicted	ACT domain; family not predicted
408	C5X320	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3002G233500	6.07	30.97	4	-1.25	0.07	2.57E-02	Electron transport chain	Electron transfer activity	Plasma membrane	Phycocyanin domain and family
435	A0A1B6QP29	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3001G415400	6.04	29.31	9	-1.16	0.05	3.64E-02	Photosynthesis	Calcium ion binding	Chloroplast	Domain not predicted; PsbP family
626	C5WWE9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3001G202400	4.28	14.08	2	-1.23	0.12	2.74E-02	None predicted	Heme binding	Endomembrane system	Cytochrome b5-like heme/steroid binding domain; cytochrome b5 family
730	C5XSC0	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3004G012900	2.92	7.01	2	1.14	0.05	1.59E-02	Photosystem II assembly	None predicted	Chloroplast	Domain of unknown function DUF1995; family not predicted

Metabolism

18	C5XSW9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3004G311100	40.47	74.65	35	-1.24	0.04	4.65E-03	Phosphate-containing compound metabolic process	Inorganic diphosphatase activity	Cytoplasm	Domain not predicted; inorganic diphosphatase family
33	C5XPV6	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3003G359100	35.42	42.04	31	-1.15	0.03	1.67E-02	Fructose 1,6-bisphosphate metabolic process	Fructose 1,6-bisphosphate 1-phosphate activity	Cytoplasm	Fructose-1-6-bisphosphate class I, N-terminal domain and family
51	A0A1B6PEC9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3008G178800	30.7	24.08	15	1.12	0.05	8.63E-03	Methionine biosynthetic process	Zinc ion binding	None predicted	Cobalamin-independent methionine synthase MetE, N-terminal domain ; cobalamin-independent methionine synthase family
63	C5YMV5	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3007G166300	27.17	36.36	20	-1.16	0.08	1.71E-02	Carbohydrate metabolic process	Malate dehydrogenase activity	Chloroplast	Lactate/malate dehydrogenase, N-terminal domain; malate dehydrogenase, type 2 family
107	C5XHR8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3003G421700	20.03	43.07	29	-1.30	0.05	1.12E-03	Carbohydrate metabolic process	Hydrolase activity	Plasma membrane	Domain not predicted; glycoside hydrolase family 17
190	C5YG66	Aminomethyltransferase OS= <i>Sorghum bicolor</i> GN=SORBL_3006G220800	13.71	22.06	7	1.14	0.07	3.18E-02	Glycine catabolic process	Aminomethyl-transferase activity	Mitochondrion	Aminomethyl-transferase, folate-binding domain; aminomethyl-transferase-like family
216	A0A1B6QG06	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3002G402700	12.75	5.86	8	1.16	0.06	8.58E-03	Glutamate biosynthetic process	Glutamate synthase activity	None predicted	Glutamate synthase domain; glutamate synthase alpha subunit, C-terminal domain superfamily
321	A0A1B6QH30	Uncharacterized protein (Fragment) OS= <i>Sorghum bicolor</i> GN=SORBL_3001G029600	8.52	32.67	5	-1.33	0.08	4.12E-03	None predicted	None predicted	None predicted	Domain not predicted; thylakoid soluble phosphoprotein TSP9 family
330	B5B9V8	Glutamine synthetase OS= <i>Sorghum bicolor</i> GN=gs	16.37	27.17	8	1.16	0.11	4.00E-02	Glutamine biosynthetic process	Glutamate-ammmonia ligase activity	None predicted	Glutamine synthetase , catalytic domain; family not predicted
347	A0A1B6P873	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3009G119200	7.79	20.06	4	-1.46	0.10	4.28E-02	Carbohydrate metabolic process	Hydrolase activity	None predicted	Domain not predicted; glycoside hydrolase family 17
504	C5XB38	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3002G055600	5.34	14.01	4	1.27	0.17	3.82E-02	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; family not predicted
517	A0A1B6PCA2	Uncharacterized protein	4.89	9.61	3	1.31	0.10	1.24E-02	None predicted	Hydrolase activity	None	GDSL lipase domain;

527	A0A194YPE9	OS= <i>Sorghum bicolor</i> GN=SORBL_3008G077800 Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3004G080100	4.63	7.17	2	1.55	0.20	1.50E-02	Tyrosine catabolic process	Catalytic activity	Cytosol	predicted	SGNH hydrolase superfamily Fumarylacetoacetase, N-terminal domain; fumarylacetoacetase family
647	C5XRZ8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3004G296800	3.72	8.8	2	1.33	0.13	5.54E-03	Metabolic process	None predicted	None predicted		3-isopropylmalate dehydratase, swivel domain and superfamily
745	A0A1Z5RLH0	Glycosyltransferase OS= <i>Sorghum bicolor</i> GN=SORBL_3004G071200	2.83	8.57	2	-2.80	0.34	2.18E-02	Metabolic process	Transferase activity	None predicted		UDP-glycosyltransferase family, conserved site domain; UDP-glycosyltransferase family
840	C5YW13	Ketol-acid reductoisomerase OS= <i>Sorghum bicolor</i> GN=SORBL_3009G239900	7.76	8.51	4	1.57	0.32	4.43E-02	Isoleucine biosynthetic process	Ketol-acid reductoisomerase activity	None predicted		Ketol-acid reductoisomerase, N-terminal domain; ketol-acid reductoisomerase family
Protein destination & storage													
31	A0A1Z5RAB0	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3007G116950	36.44	55.16	30	-1.20	0.06	2.87E-02	Protein peptidyl-prolyl isomerization	Peptidyl-prolyl cis isomerase activity	Chloroplast		Cyclophilin-type peptidyl-prolyl cis-trans isomerase domain; cyclophilin-like domain superfamily
59	C5YW53	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3009G098100	37.23	39.86	21	1.11	0.03	1.31E-02	Chaperone-mediated protein folding	Protein binding involved in protein folding	Cytoplasm		Domain not predicted; chaperonin Cpn60/TCP-1 family
171	C5YXY3	Peptidyl-prolyl cis-trans isomerase OS= <i>Sorghum bicolor</i> GN=SORBL_3009G001500	15.09	33.33	12	-1.17	0.04	5.70E-03	Protein peptidyl-prolyl isomerization	Peptidyl-prolyl cis – trans isomerise activity	Chloroplast		Cyclophilin-type peptidyl-prolyl cis-trans isomerase domain and family
229	C5YV24	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3009G223300	11.69	28.44	6	1.22	0.13	3.89E-02	None predicted	Protein binding	None predicted		WD40-repeat-containing domain; WD40-repeat-containing domain superfamily
304	A0A1B6PRP0	Adenosylhomocysteinase OS= <i>Sorghum bicolor</i> GN=SORBL_3005G112800	9.11	8.45	4	1.22	0.10	1.70E-02	S-adenosyl-homocysteine catabolic process	Adenosyl-homocysteinase activity	None predicted		S-adenosyl-L-homocysteine hydrolase, NAD binding domain; adenosyl-homocysteinase family
564	C5YBN2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3006G007100	4.16	10.67	5	1.40	0.11	4.98E-02	Proteolysis	Cysteine-type endopeptidase activity	Extracellular space		Peptidase C1A, papain C-terminal domain; peptidase C1A family

717	C5WZS8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G371400	7.22	26.57	4	-2.54	0.17	1.29E-02	None predicted	None predicted	Nascent polypeptide-associated complex	Nascent polypeptide-associated complex NAC domain; nascent polypeptide-associated complex subunit alpha family
Signal transduction												
87	A0A1B6Q0N0	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G005800	21.86	32.00	10	-1.12	0.03	9.81E-03	Cellular response to stimulus	None predicted	Plasma membrane	Domain not predicted; DREPP family
200	A0A1B6QEM9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G324500	13.24	19.09	7	1.41	0.20	1.76E-02	Protein phosphorylation	Phosphotransferase activity	Chloroplast	Domain not predicted; bifunctional kinase-pyrophosphorylase family
225	A0A1B6PBB3	Nucleoside diphosphate kinase OS= <i>Sorghum bicolor</i> GN=SORBI_3009G259200	11.94	20.58	6	1.15	0.07	2.54E-02	Nucleoside diphosphate phosphorylation	Nucleoside diphosphate kinase activity	None predicted	Nucleoside diphosphate kinase-like domain; nucleoside diphosphate kinase family
296	A0A194YR04	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G220000	9.37	23.39	7	1.27	0.16	2.46E-02	Purine ribonucleoside salvage	Adenosine kinase activity	None predicted	Carbohydrate kinase PfkB domain; adenosine kinase family
348	C5YPK6	Nucleoside diphosphate kinase OS= <i>Sorghum bicolor</i> GN=SORBI_3008G123600	7.79	15.77	5	1.16	0.07	1.45E-02	Nucleoside diphosphate phosphorylation	Nucleoside diphosphate kinase activity	Intracellular region	Nucleoside diphosphate kinase-like domain; nucleoside diphosphate kinase family
475	C5YJQ8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3007G226100	11.92	21.24	6	1.23	0.06	1.20E-02	None predicted	Protein domain specific binding	None predicted	14-3-3 domain and family
Transcription												
179	A0A1B6QIY5	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G141300	14.44	64.12	9	1.21	0.09	4.92E-03	None predicted	RNA binding	None predicted	RNA recognition motif domain; RNA-binding domain superfamily
419	C5X6U9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G278700	6.6	10.84	3	1.18	0.07	4.85E-02	RNA phosphodiester bond hydrolysis endonuclease	RNA binding	None predicted	Domain not predicted; ribonuclease T2-like family
Cell structure												
310	A0A1B6P5M1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3009G005900	29.76	50.39	20	1.19	0.12	4.40E-02	None predicted	ATP binding	None predicted	Domain not predicted; actin family
Protein synthesis												
459	A0A1B6QFD0	Eukaryotic translation initiation factor 5A OS= <i>Sorghum bicolor</i> GN=SORBI_3002G363100	6.29	23.13	4	1.19	0.02	1.87E-02	Positive regulation translation elongation	Translation elongation factory activity	None predicted	Translation elongation factor, IF5A C-terminal domain; translation elongation factor, IF5A-like family

Unclear classification

174	C5XQ28	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBL_3003G074700	14.62	57.00	10	-1.18	0.05	2.11E-02	None predicted	None predicted	Chloroplast	None predicted
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Unclassified

90	C5XF29	Uncharacterized protein OS=Sorghum bicolor GN=SORBL_3003G106500	21.47	76.1	17	-1.31	0.06	4.71E-03	None predicted	None predicted	None predicted	None predicted
126	C5WWF4	Uncharacterized protein OS=Sorghum bicolor GN=SORBL_3001G203000	18.51	52.04	18	-1.43	0.12	3.48E-02	None predicted	None predicted	None predicted	None predicted

^a Protein number assigned in ProteinPilot software.

^b Protein accession numbers obtained from the UniProt database (<http://www.uniprot.org>).

^c Protein score generated by ProteinPilot software relating to the confidence of protein identification

^d Percentage coverage is determined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

^e Sequenced peptide refers to the number of peptides that were sequenced and gave rise to protein identity.

^f Ratio represents the average fold change. A negative value indicates down-regulation.

^g Standard deviation obtained from comparing leaf protein control and treatment values.

^h Probability-value of the quantified difference between the treatment and control leaf proteins.

ⁱ Gene ontology analysis as predicted from the UniProt database (<http://www.ebi.ac.uk/QuickGO/>). P denotes Biological Process, F denotes Functional Process, and C denotes Cellular Component.

^j Conserved domains, family and superfamily names as predicted by Interpro database (<http://www.ebi.ac.uk/interpro/>).

Table 5.2: List of ICSB 338 sorghum salt-stress responsive leaf proteins identified using the iTRAQ and database searches.

Prot No. ^a	Accession No. ^b	Protein name	Scor ^c	% Cov ^d	Seq Pep ^e	Ratio ^f	SD ^g	p-value ^h	GO analysis ⁱ			Conserved domains and family names ^j
									P	F	C	
Energy												
4	A1E9T1	ATP synthase subunit beta, chloroplastic OS= <i>Sorghum bicolor</i> GN=atpB	57.53	78.71	110	1.13	0.07	1.91E-02	ATP biosynthetic process	ATP binding	Chloroplast	ATPase, F1 complex, alpha/beta subunit, N-terminal domain; ATP synthase, F1 complex, beta subunit family
6	C5YSP7	Fructose-bisphosphate aldolase OS= <i>Sorghum bicolor</i> GN=SORBI_3008G053200	48.72	74.55	77	1.10	0.07	4.43E-02	Glycolytic process	Fructose-bisphosphate aldolase activity	None predicted	Domain not predicted; fructose-bisphosphate aldolase, class-I family
54	A0A1B6Q6S1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3003G336000	21.93	25.04	13	-1.19	0.03	4.96E-02	Glucose catabolic process	Phosphoglycerate mutase activity	Cytoplasm	Metalloenzyme domain; phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent family
141	A0A1Z5RB19	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3007G212400	12.01	40.23	9	1.39	0.10	3.94E-04	Photosynthesis	Calcium ion binding	Photosystem II	Domain not predicted; PsbP family
144	C5XYR1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G235200	11.57	44.53	19	1.37	0.26	4.70E-02	ATP synthesis coupled proton transport	Proton-transporting ATP synthase activity, rotational mechanism	Chloroplast	Domain not predicted; ATPase, OSCP/delta subunit family
553	C5WZ31	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G070500	2.27	9.17	3	-1.38	0.05	1.05E-02	Photosynthesis	None predicted	Photosystem I	Domain not predicted; photosystem I Psaf, reaction centre subunit III
Metabolism												
108	C5YTW1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3008G080400	14.58	32.26	10	-1.25	0.08	4.97E-02	Carbohydrate metabolic process	Beta-glucosidase activity	None predicted	Domain not predicted; glycoside family 1
176	C5X8J1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G301700	10.14	13.92	6	-1.57	0.14	8.28E-03	None predicted	None predicted	None predicted	Amidase signature domain and superfamily
219	C5Y7R1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3005G084300	8.47	18.18	7	-1.24	0.03	1.07E-02	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; glycoside hydrolase 18 family
331	A0A1B6PNS7	Glutamine synthetase OS= <i>Sorghum bicolor</i> GN=SORBI_3006G249400	9.84	20.09	8	-1.26	0.11	2.65E-02	Glutamine biosynthetic process	Glutamate-ammonia ligase activity	Chloroplast	Glutamine synthetase, catalytic domain; glutamine synthetase family
509	A0A1Z5R3J0	ATP-dependent 6-phosphofructokinase OS= <i>Sorghum</i>	3.61	4.68	4	1.60	0.31	2.15E-02	Fructose 6-phosphate metabolic	6-phosphofructokinase activity	Cytoplasm	ATP-dependent 6-phosphofructokinase domain and family

		<i>bicolor</i> GN=PFK							process				
Protein destination & storage													
7	A0A194YKU6	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G223200	48.52	46.23	33	1.08	0.05	1.96E-02	Proteolysis	ATP binding	Membrane	AAA+ ATPase domain; peptidase, FtsH family	
81	C5XFP1	Cysteine synthase OS= <i>Sorghum bicolor</i> GN=SORBI_3003G110100	19.65	36.32	13	1.24	0.08	4.60E-03	Cysteine biosynthetic process	Cysteine synthase activity	Cytoplasm	Pyridoxal-phosphate dependent enzyme domain; cysteine synthase family	
501	A0A194YIG2	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G108900	2.97	8.33	2	1.58	0.31	1.14E-02	Nucleotide-excision repair	Damaged DNA binding	Nucleus	Ubiquitin domain; UV excision repair protein Rad23 family	
Disease/defence													
49	A0A194YIH8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G113000	25.09	26.98	21	-1.21	0.07	2.15E-02	Oxidation reduction process	Oxidoreductase activity	None predicted	Aldehyde dehydrogenase domain and family	
337	C5XUC9	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G326500	5.3	13.16	3	-1.33	0.09	4.11E-02	None predicted	None predicted	None predicted	Plastid lipid-associated protein/fibrillin conserved domain; family not predicted	
356	C5WPD8	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G418200	4.92	18.12	5	-1.60	0.10	8.44E-03	None predicted	None predicted	None predicted	Gnk2-homologous domain and superfamily	
Signal transduction													
162	C5X972	Nucleoside diphosphate kinase OS= <i>Sorghum bicolor</i> GN=SORBI_3002G306900	10.36	36.00	6	1.14	0.06	4.44E-02	Nucleoside diphosphate phosphorylation	Nucleoside diphosphate kinase activity	Intracellular region	Nucleoside diphosphate kinase-like domain; nucleoside diphosphate kinase family	
Protein synthesis													
164	C5XE07	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G373800	10.36	24.67	10	1.15	0.06	4.91E-02	Translation elongation	Translation elongation factor activity	Eukaryotic translation elongation factor 1 complex	Translation elongation factor EF1B, beta/delta subunit, guanine nucleotide exchange domain; EF-1-beta family	
415	A0A1Z5S5F3	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3001G130301	4	9.79	3	1.32	0.05	1.69E-05	Translation	Structural constituent of ribosome	Ribosome	Domain not predicted; ribosomal protein S21e	
Unclear classification													
329	C5Z8V5	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3010G249600	5.97	24.35	5	-1.22	0.04	3.96E-03	None predicted	None predicted	Chloroplast	Domain not predicted; macrophage migration inhibitory factor family	
358	C5X2G7	Uncharacterized protein OS= <i>Sorghum</i>	4.56	9.50	2	1.43	0.10	1.36E-03	None predicted	None predicted	None predicted	GRAM domain; GEM-like protein family	

		<i>bicolor</i> GN=SORBI_3002G378800										
491	A0A194YSZ4	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3004G345900	3.04	12.9	2	1.45	0.14	3.33E-02	None predicted	None predicted	None predicted	CBS domain; family not predicted
Unclassified												
554	A0A1B6QAN1	Uncharacterized protein OS= <i>Sorghum bicolor</i> GN=SORBI_3002G118000	2.19	11.96	2	1.37	0.18	3.26E-02	None predicted	None predicted	None predicted	None predicted

^a Protein number assigned in ProteinPilot software.

^b Protein accession numbers obtained from the UniProt database (<http://www.uniprot.org>).

^c Protein score generated by ProteinPilot software relating to the confidence of protein identification

^d Percentage coverage is determined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

^e Sequenced peptide refers to the number of peptides that were sequenced and gave rise to protein identity.

^f Ratio represents the average fold change. A negative value indicates down-regulation.

^g Standard deviation obtained from comparing leaf protein control and treatment values.

^h Probability-value of the quantified difference between the treatment and control leaf proteins.

ⁱ Gene ontology analysis as predicted from the UniProt database (<http://www.ebi.ac.uk/QuickGO/>). P denotes Biological Process, F denotes Functional Process, and C denotes Cellular Component.

^j Conserved domains, family and superfamily names as predicted by Interpro database (<http://www.ebi.ac.uk/interpro/>).

5.2.3 Gene Ontology Analysis

All the differentially expressed sorghum responsive leaf proteins were submitted for Gene Ontology (GO) annotation on the Uniprot database for the determination of their biological process, molecular function and cellular component. As observed with the root salt stress responsive proteins, most leaf proteins were uncharacterised. Therefore, protein domains and family annotations assisted in their functional classifications. The GO annotation results for SA 1441 and ICSB 338 sorghum varieties are illustrated in Tables 5.1 and 5.2, respectively. Furthermore, comparative diagrammatic representations of the three GO terms, namely biological process, molecular function and cellular component of the two sorghum varieties are shown in Figures 5.1, 5.2 and 5.3, respectively.

Figure 5.2 shows the biological processes of the differentially expressed salt stress responsive leaf responsive proteins. The majority of leaf proteins in ICSB 338 (25%) and SA 1441 (19%) did not have predicted biological processes. The other highly represented categories included response to oxidative stress, metabolic and biosynthetic processes.

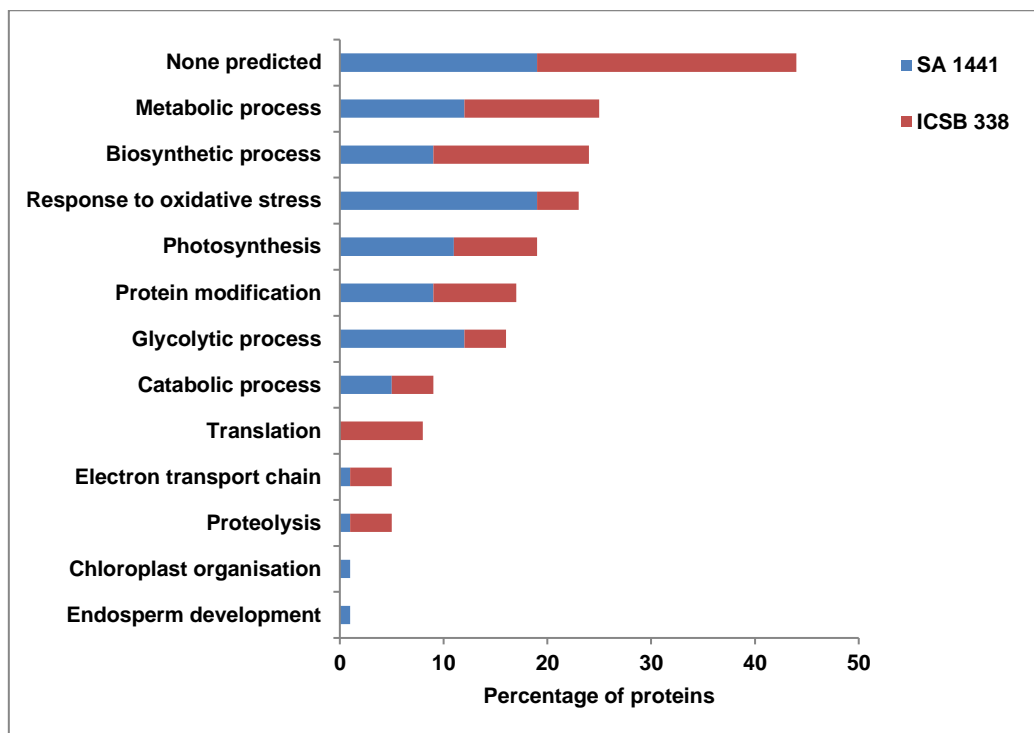


Figure 5.2: Biological process predictions of the differentially expressed sorghum leaf salt responsive proteins.

Figure 5.3 shows the molecular functions of the differentially expressed leaf proteins. Most of the proteins in ICSB 338 (35%) and SA 1441 (19%) did not have any predicted functions. However, protein binding, oxidoreductase, transferase and peroxidase activities were highly represented in both sorghum varieties.

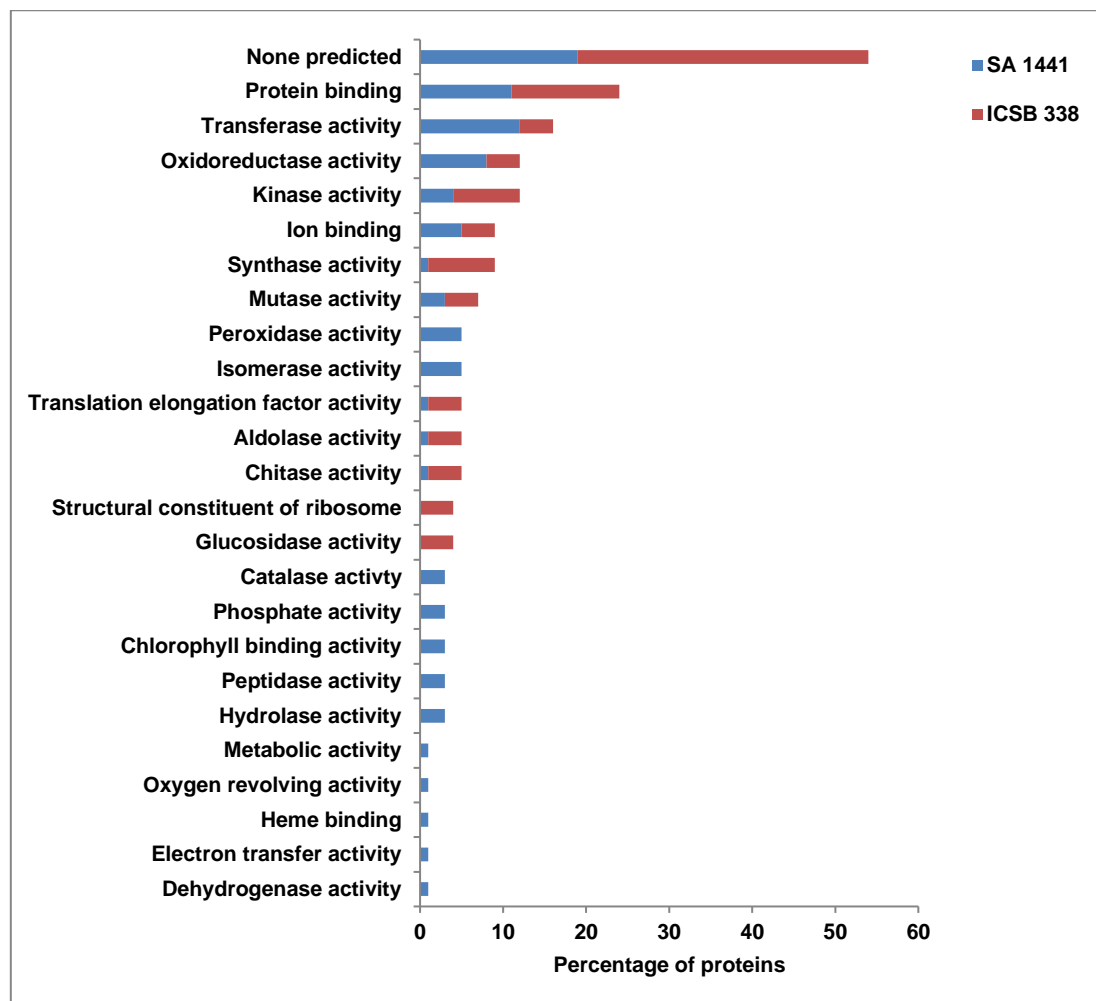


Figure 5.3: Molecular function predictions of the differentially expressed sorghum leaf salt responsive proteins.

The putative locations of each of the sorghum leaf salt stress responsive proteins are represented in Figure 5.4. Most of the sorghum leaf proteins identified in SA 1441 (45%) and ICSB 338 (38%) had no predicted cellular location. Apart from this category, both sorghum varieties had the majority of proteins localised in the chloroplast and cytoplasm.

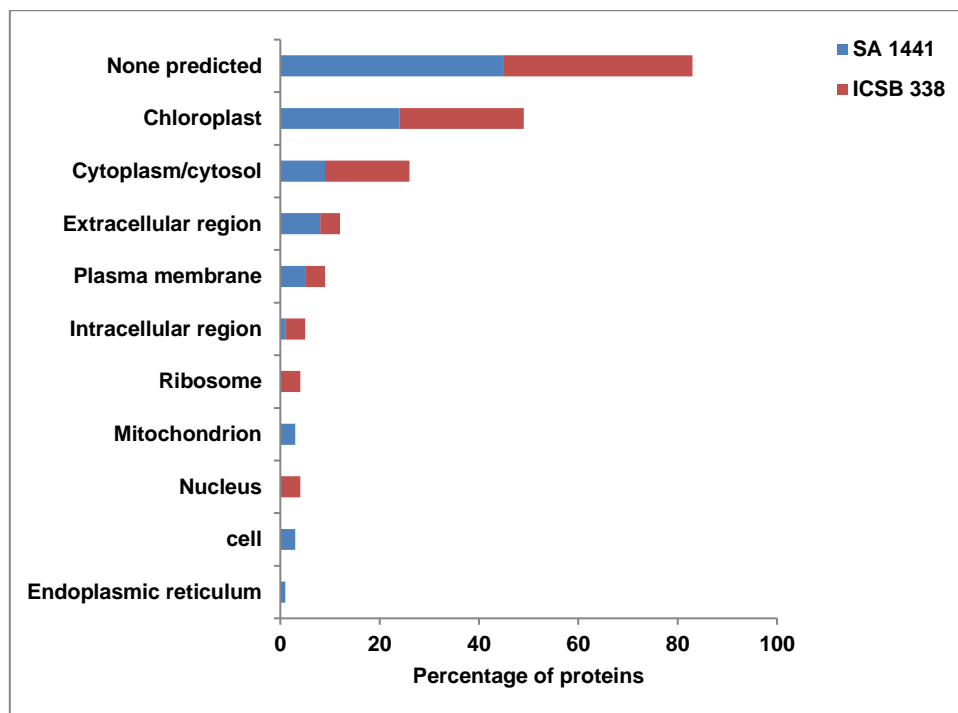


Figure 5.4: Cellular component predictions of the differentially expressed sorghum leaf salt responsive proteins.

5.2.4 Functional Categories of the Differentially Expressed Sorghum Leaf Proteins

The differentially expressed sorghum leaf salt stress responsive proteins of both SA 1441 (Table 5.1) and ICSB 338 (Table 5.2) were grouped into functional categories according to Bevan *et al.* (1998) and various literature sources. For the uncharacterised proteins, information obtained on the conserved domains and protein family names assisted with the overall functional grouping of these proteins. Figure 5.5 A shows the 10 functional categories of SA 1441 proteins namely; disease/defence (28%); energy (24%); metabolism (21%); protein destination and storage (9%); signal transduction (8%); transcription (3%); protein synthesis (1%); cell structure (1%); unclear classification (1%) and unclassified (3%).

Eight functional categories were identified in ICSB 338 salt stress responsive leaf proteins namely; energy (25%), metabolism (21%), disease/defence (13%), protein destination and storage (13%), unclear classification (13%), protein synthesis (8%), signal transduction (4%) and unclassified (4%) as shown in Figure 5.5 B. Most differentially expressed leaf proteins were associated with disease/defence in SA 1441, followed by energy and metabolism in both sorghum varieties. A brief description of each of the functional categories is given below.

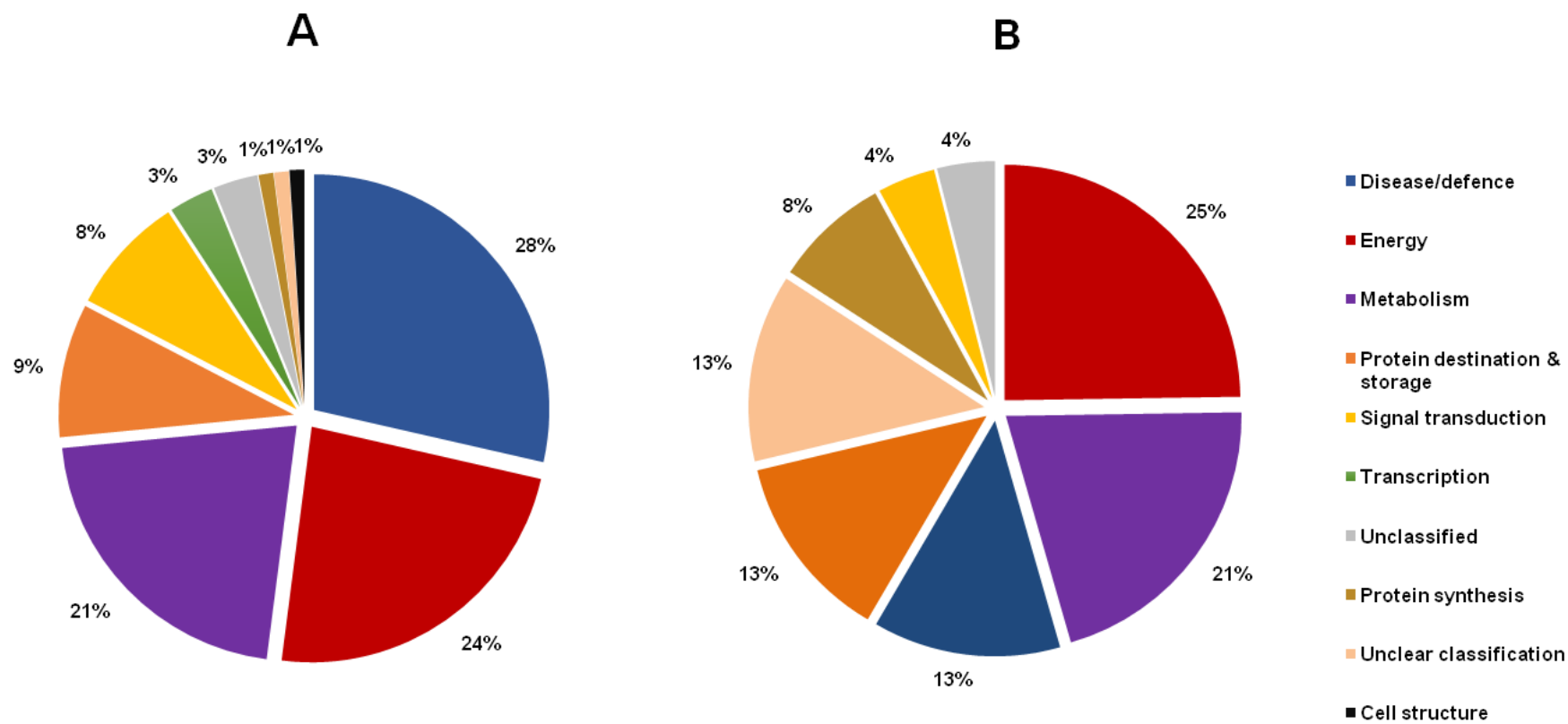


Figure 5.5: Functional categories of differentially expressed sorghum leaf salt stress responsive proteins. (A) shows results of SA 1441, while (B) shows that of ICSB 338. Proteins were grouped into functional categories using data available on the UniProt database (www.uniprot.org) and according to Bevan *et al.* (1998) and literature sources.

5.2.4.1 Disease/defence

Of the 75 differentially expressed SA 1441 salt stress responsive leaf proteins, 21 (28%) were involved in disease/defence (Table 5.1; Figure 5.5 A). This functional category consists of protein disulfide-isomerase (protein no. 62), peroxidases (protein no. 117 & 444), superoxide dismutase [Cu-Zn] (protein no. 166), catalase (protein no. 253), glutathione peroxidase (protein no. 414) and uncharacterised proteins (protein no. 4, 23, 45, 176, 178, 219, 336, 357, 360, 468, 603, 636, 718 & 989). The uncharacterised proteins are involved in responses to oxidative stress. There was an equal number of up- and down-regulated proteins in this functional category as illustrated in Table 4.1. An increase in up-regulation of proteins involved in disease or defence was also observed in rye (*Secale cereale*) leaf proteome in response to 200 mM NaCl (Lee *et al.*, 2013).

In ICSB 338, only three proteins (13%) were involved in disease/defence (Table 5.1; Figure 5.5 B) and all were uncharacterised (protein no. 49, 337 & 356). According to the GO annotation analysis (Table 5.2), one of the proteins had a biological process involved in oxidation-reduction and the other two proteins belong to the plastid lipid-associated protein/fibrillin and Gnk2-homologous domains. All of these proteins were down-regulated in response to salt stress as illustrated in Table 5.2. The lipid-associated protein/fibrillin protein was down regulated in a sorghum leaf proteome in response to 100 mM NaCl (Ngara *et al.*, 2012).

5.2.4.2 Energy

A total of 18 (24%) proteins of the 75 differentially expressed SA 1441 salt stress responsive leaf proteins were involved in energy (Table 5.1; Figure 5.5 A). The proteins in this functional category include phosphoglycerate kinase (protein no. 19), glyceraldehyde-3-

phosphate dehydrogenase (protein no. 72 & 191), chlorophyll a-b binding protein chloroplastic (protein no. 122), glucose-6-phosphate isomerase (protein no. 154), fructose-bisphosphate aldolase (protein no. 158), photosystem I P700 chlorophyll a apoprotein A2 (protein no. 246) and uncharacterised proteins (protein no. 21, 55, 79, 153, 183, 388, 392, 408, 435, 626 & 730). The uncharacterised proteins are involved in photosynthesis, electron transport and beta-glucosidase processes. Most proteins in this functional category were down-regulated (68%), while only 32% were up-regulated in response to the salt stress as illustrated in Table 5.1. Some of the proteins in this functional group were also identified in canola (*Brassica napus*) leaves in response to 175 and 350 mM NaCl-induced salt treatment (Bandehagh *et al.*, 2011).

For ICSB 338, a total of 6 (25%) differentially salt stress responsive leaf proteins were involved in energy (Table 5.2; Figure 5.5 B). These proteins include ATP synthase subunit beta, chloroplastic (protein no. 4), fructose-bisphosphate, aldolase (protein no. 6) and uncharacterised proteins (protein no. 54, 141, 144 & 553). According to the GO annotation analysis (Table 5.1), the uncharacterised proteins had biological processes involved in glucose catabolic process, photosynthesis and ATP synthesis coupled proton transport. Most of the proteins in this group were up-regulated (67%), while 33% were down-regulated in response to the salt stress treatment (Table 5.2). Increased and decreased levels of some of these energy related proteins have also been observed in the leaf proteome of rice (*Oryza sativa*) genotypes under 120 mM NaCl-induced salt treatment (Ghaffari *et al.*, 2014).

5.2.4.3 Metabolism

About 16 (21%) of the 75 differentially expressed salt stress responsive leaf proteins of SA 1441 were involved in metabolism (Table 5.1; Figure 5.5 A). Proteins identified in this

category include aminomethyltransferase (protein no. 190), glutamine synthetase (protein no. 330), glycosyltransferase (protein no. 754), ketol-acid reductoisomerase (protein no. 840) and uncharacterised proteins (protein no. 18, 33, 51, 63, 107, 216, 321, 347, 504, 517, 527 & 647). According to the GO annotation analysis (Table 5.1), these uncharacterised proteins are involved in phosphate-containing compound, fructose 1,6-bisphosphate metabolic, carbohydrate metabolic, glutamate biosynthetic and tyrosine catabolic processes. Most of the proteins in this category were up-regulated (56%), while 44% were down-regulated in response to salt stress as illustrated in Table 5.1. The up-regulation of metabolic related proteins has also been observed in the leaf proteome of cotton genotypes in response to 200 mM NaCl-induced salt stress (Gong *et al.*, 2017).

In ICSB 338, a total of 5 (21%) differentially expressed salt stress responsive leaf proteins were involved in metabolism (Table 5.2; Figure 5.5 B). This functional category consists of glutamine synthetase (protein no. 331), ATP-dependent 6-phosphofructokinase (protein no. 509) and uncharacterised proteins (protein no. 108, 176 & 219) involved in the carbohydrate metabolic process. The majority of proteins in this group were down-regulated (80%), while 20% were up-regulated in response to the salt stress (Table 5.2).

5.2.4.4 Protein destination and storage

Of the 75 differentially expressed SA 1441 salt stress responsive leaf proteins, a total of 7 (9%) proteins were involved in protein destination and storage (Table 5.1; Figure 5.5 A). This functional category consists of peptidyl-prolyl cis-trans isomerase (protein no. 171), adenosylhomocysteinase (protein no. 304) and uncharacterised proteins (protein no. 31, 59, 229, 564 & 717). The uncharacterised proteins had biological processes involved in chaperone mediated protein folding, peptidyl-prolyl cis-trans isomerase and proteolysis. The

majority of proteins in this category were up-regulated, while 29% were down-regulated in response to the salt stress (Table 5.1).

For ICSB338, only 3 (8%) proteins were predicted to function in protein destination and storage (Table 5.1; Figure 5.5 B). This functional group consists of cysteine synthase (protein no. 81) and uncharacterised proteins (protein no. 7 & 501) involved in proteolysis and nucleoside excision repair. Furthermore, all the proteins in this group were up-regulated in response to the salt stress (Table 5.2). The cysteine synthase protein was also up-regulated in rice leaves in response to 250 mM NaCl-induced salt stress (Liu *et al.*, 2014).

5.2.4.5 Signal Transduction

A total of 6 (8%) proteins of the 75 differentially expressed salt stress responsive leaf proteins of SA 1441 were predicted to function in signal transduction (Table 5.1; Figure 5.5 A). The proteins in this functional group include nucleoside diphosphate kinase (protein no. 225 & 348), and uncharacterised proteins (protein no. 87, 220, 296 & 475). According to the GO annotation analysis (Table 5.1), the uncharacterised proteins had biological processes involved in cellular response to stimulus, protein phosphorylation, purine ribonucleoside salvage and protein binding. The majority of the proteins in this functional group were up-regulated, with only one uncharacterised protein that was down-regulated in response to the salt stress treatment (Table 5.1). Nucleoside diphosphate kinases were also up-regulated in barley (*Hordeum vulgare*) in response to 300 mM NaCl-induced salt stress (Fatehi *et al.*, 2012). This same protein was the only one identified in this functional group in ICSB 338 (protein no. 162), and it was up-regulated in response to the salt stress (Table 5.2).

5.2.4.6 Other Functional Categories

This group consists of functional categories that were only classified in either one of the two sorghum varieties. The transcription functional group in SA 1441 had two uncharacterised proteins (protein no. 179 & 419) involved in RNA binding and both were up-regulated in response to the salt stress (Table 5.1). The protein synthesis functional group in SA 1441 consisted of an eukaryotic translation initiation factor 5A protein (protein no. 459), while ICSB 338 had one uncharacterised protein (protein no. 164) involved in translation. These two proteins were up-regulated in response to the salt stress treatment (Table 5.1 & 5.2). Increased level of eukaryotic translation initiation factor 5A protein was also observed in the leaf proteome of rice genotypes under salt stress (Bandehagh *et al.*, 2011). The cell structure functional group in SA 1441 had an uncharacterised protein (protein no. 310), with a putative actin domain and was up-regulated in response to salt stress.

Both sorghum varieties had a group of proteins with unclear classification. For, SA 1441 the proteins were uncharacterised (protein no. 174 & 465), and were down-regulated in response to the salt stress (Table 5.1). For, ICSB 338 the proteins with unclear classification were uncharacterised proteins (protein no. 329, 356, 358 & 491) and they were up- and down regulated in response to salt stress (Table 5.2). The two sorghum varieties also had unclassified proteins, for SA 1441 (protein no. 90 & 126) which were down-regulated, while ICSB 338 (protein no 554) was up regulated in response to the salt stress (Table 5.1 & 5.2).

5.3 Discussion

In this study, a total of 829 and 591 leaf proteins were identified in SA 1441 and ICSB 338 sorghum varieties, respectively. As observed in the root proteome in Chapter 4, a large proportion of these proteins were uncharacterised. For example, SA 1441 and ICSB 338 each

had 67% and 69% of their positively identified leaf proteins being uncharacterised. Of these positively identified leaf proteins, 75, SA 1441 (Table 5.1; Figure 5.5 A) and 24, ICSB 338 (Table 5.2; Figure 5.5 B) proteins were differentially expressed in response to the 100 mM NaCl-induced salt stress. These proteins were grouped into functional categories and the most represented differentially expressed leaf proteins were associated with disease/defence in SA 1441, followed by energy and metabolism in both sorghum varieties.

Under salinity stress, plants experience oxidative stress as a result of the accumulation of reactive oxygen species (ROS) to toxic levels (Sharma *et al.*, 2012). Plants respond to oxidative stress by activating a range of antioxidant mechanisms in order to detoxify or eliminate the ROS (Foyer and Noctor, 2005). In this study SA 1441, the drought tolerant variety responded to the salt stress by activating a large number of proteins involved in redox reactions. These include peroxidases, superoxide dismutase [Cu–Zn], catalase, glutathione peroxidase and uncharacterised proteins involved in responses to oxidative stress. The enhanced up-regulation of these proteins in SA 1441, more than in ICSB 338 possibly indicates an established antioxidant system in the drought tolerant variety. As both drought and salt stresses are known to induce oxidative stress (Wang *et al.*, 2003), the drought tolerance response of SA 1441 may also be linked to an efficient antioxidant system. The up-regulation of such redox-related proteins has also been observed in rye leaves in response to 200 mM NaCl-induced salt-stress for 4 days (Lee *et al.*, 2013). In ICSB 338 the proteins involved in disease/defence were down-regulated in response to the salt stress, possibly indicating that the defence response was suppressed in this drought and salt susceptible sorghum variety.

Plants require energy, water and mineral nutrients for normal growth and development and also in response to abiotic stress factors (Cramer *et al.*, 2011). In this study, the identified energy-related salt stress responsive proteins were involved in photosynthesis and glycolysis. This is consistent with the photosynthetic function of plant leaves. SA 1441, the drought tolerant variety had a large number of characterised energy-related proteins when compared to ICSB 338 the drought and salt susceptible variety. In SA1441, these proteins included phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, glucose-6-phosphate isomerase, chlorophyll a-b binding protein chloroplastic and photosystem I P700 chlorophyll apoprotein A2. These proteins were either up- or down-regulated in response to the salt stress. The chlorophyll a/b binding protein was also up-regulated in the leaf proteome of tomato exposed to 200 mM NaCl-induced salt stress for 14 days (Manaa *et al.*, 2013). Chlorophyll a/b binding proteins are light harvesting proteins involved in photosynthesis (Liu *et al.*, 2013). The photosystem I P700 chlorophyll apoprotein A2 was identified in the leaf proteome of cotton salt-tolerant genotype in response to 200 mM NaCl-induced salt stress for 4 hours (Gong *et al.*, 2017).

In ICSB 338, energy related proteins included fructose-bisphosphate aldolase and ATP synthase subunit beta, chloroplastic which were up-regulated in response to the salt stress. These proteins were also up-regulated in rice leaf proteome in response to 250 mM NaCl-induced salt stress for 30 minutes (Liu *et al.*, 2014). An increase in the expression of photosynthesis-related proteins in SA 1441 could possibly explain the increased plant growth observed under salt stress (Figures 3.5 & 3.6). In contrary, the salt stress negatively affected the rate of photosynthesis in ICSB 338 which resulted in reduced plant growth (Figures 3.5 & 3.6).

Salinity stress alters gene expression and subsequently protein metabolism, protein biosynthesis as well as protein degradation (Kosová *et al.*, 2013). In this study, the metabolism functional group was highly represented in both sorghum varieties. In SA 1441, aminomethyltransferase, glutamine synthetase, glycosyltransferase and ketol-acid reductoisomerase were up-regulated in response to the salt stress. Ketol-acid reductoisomerase is an enzyme which acts as both an isomerase and a reductase, and it is involved in the biosynthesis of branched-chain amino acids (Tyagi *et al.*, 2005). A study on tomato leaves reported an up-regulation of ketol-acid reductoisomerase and aminomethyltransferase in response to 200 mM NaCl-induced salt stress for 25 days (Zhou *et al.*, 2011). Glutamine synthetase protein was also up-regulated in the leaf proteome of soybean genotypes in response to 150 mM NaCl-induced salt stress for 1, 2, 72 and 144 hours (Ma *et al.*, 2012). According to Bernard and Habash (2009), glutamine synthetase is a key enzyme for nitrogen metabolism, and plays a major role in the fixation of ammonium to form the amino acid glutamine. The glutamine protein was down-regulated in response to the salt stress in ICSB 338 the drought/salt susceptible variety.

In plants, normal protein folding is maintained by the ability cells to eliminate misfolded or damaged polypeptides (Goldberg, 2003). Proteins involved in protein destination and storage in SA 1441, included adenosylhomocysteinase and peptidyl-propyl cis-trans isomerase which were up-regulated in response to the salt stress treatment. Peptidyl-propyl cis-trans isomerase proteins are known to function in maintaining normal protein folding and repairing stress-damaged proteins (Zhang *et al.*, 2012). Peptidyl-propyl cis-trans isomerase protein was also identified in canola leaves in response to 175 and 350 mM NaCl-induced salt stress for 15 days (Bandehagh *et al.*, 2011). Adenosylhomocysteinase protein was also up-regulated in the leaf proteome of cotton salt-tolerant genotype in response to 200 mM NaCl-induced

salt stress for 24 hours (Gong *et al.*, 2017). In ICSB 338, cysteine synthase protein was up-regulated in response to the salt stress. Cysteine synthase is an enzyme involved in the biosynthesis of cysteine, which plays an essential role in the structure, stability and catalytic function of proteins (Yasuko and Kaoru, 2004). The cysteine synthase protein was also identified in the leaf proteome of rice in response to 250 mM NaCl-induced salt stress for 30 minutes (Liu *et al.*, 2014).

Salt signalling pathways are important in salt stress tolerance in plants. In this study, the signal transduction functional category had a similar protein nucleoside diphosphate kinase. This protein was up-regulated in response to the salt stress in both sorghum varieties. Nucleoside diphosphate kinase is an enzyme involved in maintaining stable GTP levels through nucleotide homeostasis in protein and DNA synthesis and GTP-mediated signal transduction pathways (Pan *et al.*, 2000). This protein was also up-regulated in barley salt-tolerant genotype in response to 300 mM NaCl-induced salt stress for 3 weeks (Fatehi *et al.*, 2012).

Overall, SA 1441, the drought tolerant variety was better adapted to the stress and had more salt stress responsive leaf proteins as compared to ICSB 338, the drought and salt susceptible variety.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Salt stress disrupts the normal functioning of a plant, which leads to reduced plant growth and development. Together with other abiotic stresses, salt stress thus has a negative impact on agricultural productivity worldwide. With the increasing population growth and extent of marginal lands worldwide, it is important to develop agricultural crops that can withstand the effects of high soil salinity. One of the first steps towards achieving this goal is to understand the basic mechanisms of salt stress responses in crops. An ideal model crop system to be used is sorghum, due to its natural tolerance to drought and moderate tolerance to salt stress. Sorghum is a very diverse crop exhibiting great phenotypic variability among the cultivated races.

In this study, a comparative physiological, biochemical and proteomic analysis was conducted with the aim of understanding sorghum response mechanisms to salt stress. The two sorghum varieties used have contrasting tolerance to drought stress. SA 1441 is drought tolerant, while ICSB 338 is susceptible to both drought and salt stresses. The results revealed that the two sorghum varieties undergo different changes at both physiological and biochemical levels when exposed to NaCl-induced salt stress. The level of salt tolerance was remarkably different between the two varieties at both physiological and biochemical levels.

The difference in stress response between the two varieties was further observed at the proteome level. Using an iTRAQ based proteomic method, a comparative quantitative analysis of the root and leaf salt stress responsive proteins was conducted in the two sorghum varieties. A low salt stress level of 100 mM NaCl was used to identify any initial differences in response elements between the two varieties. For both the root and leaf proteomes, over

70% of the identified proteins were uncharacterised and their functions are yet to be verified experimentally. This illustrates the need to carry out more protein functional characterisation studies in sorghum.

Of the 522 and 544 positively identified root proteins for SA 1441 and ICSB 338 sorghum varieties, only 26 and 31 were statistically differentially expressed in response to 100 mM NaCl, respectively. The functional classification of the identified salt stress responsive proteins illustrated that the most highly represented group was disease/defence. This possibly shows the ability of sorghum to survive under salt stress. This was observed in both sorghum varieties. Overall, the root proteome results showed minor fold changes ranging from -1.24 to 1.47 amongst the salt stress responsive proteins between the two sorghum varieties. Therefore, a comparative leaf proteomic analysis was conducted to further investigate any differences in salt stress response of the two sorghum varieties.

In the leaf proteome, a total of 829 and 591 leaf proteins of SA 1441 and ICSB 338 sorghum varieties were positively identified. However, it is unclear why there was such a huge difference in the total number of identified proteins between the two varieties. Of the 829 and 591 positively identified SA 1441 and ICSB 338 leaf proteins respectively, 75 (SA 1441) and 24 (ICSB 338) were responsive to the 100 mM NaCl-induced salt stress. The most differentially expressed leaf proteins were associated with disease/defence in SA 1441, followed by energy and metabolism in both sorghum varieties. These results indicate that SA 1441, the drought tolerant variety quickly activates protein expression of important defence-related elements in order to counter the effects of salt stress. It is this activation of protective mechanisms that enables the plant to restore cellular homeostasis and growth during stress adaptation.

Overall and based on the combined comparative physiological, biochemical and proteomic analyses of this study, SA 1441, the drought tolerant variety was better adapted to the salt stress imposed compared to ICSB 338, the drought and salt susceptible variety. These results are important given the known cross-linking of drought and salt stress responses in plants. For example, SA 1441 could be utilising its drought tolerance mechanisms to counter the negative osmotic and oxidative stress effects of the imposed salt stress.

Future studies on sorghum salt stress responses are however recommended in order to improve our understanding on how this crop adapts to the stress. For example validation studies using gene expression analysis of a few target gene identities from this study could be conducted. Such studies could utilise other sorghum varieties exposed to similar levels of salt stress as in this study or even at different, higher salt concentrations and several time points. There is also a need to conduct further metabolite profiling studies in the sorghum varieties and also gene expression of key regulatory enzymes involved in osmolyte biosynthesis.

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APPENDICES

Appendix 1: Protein Quantification and 1D SDS-PAGE Preparation

Appendix 1-Table 1: Preparation of BSA standard solutions for protein quantification.

Concentration (μg)	BSA 5 mg/ml stock solution(μl)	Urea buffer (μl)	0.1 M HCl (μl)	Distilled H ₂ O (μl)
0*	0	10	10	80
5	1	9	10	80
10	2	8	10	80
20	4	6	10	80
40	8	2	10	80
50	10	0	10	80

*blank solution

Appendix 1-Table 2: Preparation of resolving and stacking gels for gel electrophoresis.

	Resolving gel 12% (v/v)	Stacking gel 5% (v/v)
Distilled water	4.3ml	3.6ml
40% Acryl-bisacrylamide mix	3ml	0.625ml
0.5M Tris-HCL (pH 6.8)	-	0.63ml
1.5M Tris-HCL (pH 8.8)	2.5ml	-
10% SDS	0.1ml	0.05ml
10% APS	0.1ml	0.05ml
TEMED	0.006ml	0.005ml
Total volume	10 ml	5 ml