# COMPARATIVE ANALYSIS OF SUGAR-BIOSYNTHESIS PROTEINS OF SORGHUM STEMS AND THE INVESTIGATION OF THEIR ROLE IN HYPEROSMOTIC STRESS TOLERANCE

**Anathi Perseverence Njokweni**

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



Supervisor: Prof. Bongani Ndimba

February 2015

**COMPARATIVE ANALYSIS OF SUGAR-BIOSYNTHESIS PROTEINS OF SORGHUM STEMS AND THE INVESTIGATION OF THEIR ROLE IN HYPEROSMOTIC STRESS TOLERANCE**

Anathi Perseverence Njokweni

**KEYWORDS**

Sorghum

Abiotic stress

Osmotic adjustment

Metabolomics

Sugar metabolism

Sucrose-biosynthesis gene expression

Transcriptomics

Proteomics

Drought tolerance

Bio-energy production

## ABSTRACT


### COMPARATIVE ANALYSIS OF SUGAR-BIOSYNTHESIS PROTEINS OF SORGHUM STEMS AND THE INVESTIGATION OF THEIR ROLE IN HYPEROSMOTIC STRESS TOLERANCE

A.P. Njokweni

PhD Thesis, Department of Biotechnology, University of the Western Cape.

*Sorghum bicolor* (L.) *Moench* is an important cereal crop currently explored as a potential bio-energy crop due to its stress tolerance and ability to ferment soluble sugars. Physiological studies on sorghum varieties have demonstrated that part of drought tolerance is attributed to sugar accumulation in the sorghum stems. Despite the agronomic advantages of sorghum as a bio-energy crop, more research efforts towards the molecular elucidation of sorghum traits that confer drought tolerance are necessary. Particular focus on traits, which could potentially contribute to an efficient bio-energy production under environmental constraints, would be an added advantage. This study examined the role of sugar biosynthesis proteins in conferring tolerance to drought-induced hyperosmotic stress, and ultimately osmotic adjustment in sorghum varieties. *Sorghum bicolor* (L.) *Moench* varieties (ICSB338, ICSB73, ICSV213 and S35) with different levels of drought tolerance, were grown under watered conditions until early anthesis after which, a 10-day water deficit period was introduced.

Control (well-watered) and stressed plants were harvested and used to evaluate plant growth, water retention, chlorophyll content, metabolic profiles, and enzymatic activity of sucrose biosynthesis enzymes. GC-MS based metabolic profiling approach was used to determine the changes in the stem metabolome with stress induction. HPLC analysis was carried out to determine the total soluble sugar content in the stems in all four varieties. Q-PCR was used to determine the levels of mRNA expression of sucrose biosynthesis genes in control and stressed plants. Furthermore, two-dimensional gel electrophoresis (2DE) coupled with mass spectrometry was used to identify stress responsive proteins in sorghum plants. A significant reduction in soil water content was observed. As a consequence leaf water content, total chlorophyll content and plant biomass was also reduced. Metabolite analysis revealed a higher abundance of soluble sugars compared to the free amino acid pool, which suggested that osmotic adjustment under stressed conditions was more attributed to sugar content. From a detailed soluble sugar analysis, an increase in total soluble sugars (brix) and hexose concentrations (glucose and fructose) was observed followed by a decrease in sucrose and starch levels in all four sorghum varieties. Gene (mRNA) expression analysis showed an up regulation of sucrose-biosynthesis genes as a consequence of drought stress. However, a direct correlation between the soluble sugar levels and mRNA expression was only evident in ICSV213, indicating that only this variety was responsive to hyperosmotic stress.

Contrary to the observations made in ICSV213, there was no direct correlation between soluble sugars and the mRNA expression for ICSB338. Therefore, ICSV213 was regarded as the most responsive variety to drought-induced hyperosmotic stress induction and ICSB338 the least responsive variety. A comparative proteome analysis between ICSV213 and ICSB338 confirmed the presence of proteins associated with other metabolic pathways that modulate sugar biosynthesis. Fourteen well resolved CBB stained protein spots were positively identified using mass spectrometry. These spots were classified in different functional categories including carbohydrate metabolism (57.2%), amino acid metabolism (14.3%), disease/defense (14.3%), protein synthesis (7.1%), and signal transduction (7.1%). Contrasting expression patterns were observed between the two varieties with two stress-related proteins being up-regulated in variety ICSV213 and down-regulated in ICSB338. The results of this study suggest that the differential regulation of sucrose biosynthesis proteins for ICSB338, ICSB73 and S35 could be related to the physiological requirements of plant cells during environmental stress conditions rather than osmotic adjustment. The synergism observed between the transcriptome and proteome of ICSV213 under water limited conditions indicated a response to drought-induced hyperosmotic stress. These findings highlight the potential role of this variety in genetic engineering studies for increased sugar content for bio-energy production under adverse conditions.

## DECLARATION

I declare that "*Comparative analysis of sugar-biosynthesis proteins of sorghum stems and the investigation of their role in hyperosmotic stress tolerance*" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

**Anathi Perseverence Njokweni** **February 2015**

Signed .....

## ACKNOWLEDGEMENTS


- I would like to express my sincere gratitude to Prof Bongani Ndimba for allowing me the opportunity to pursue this degree, and for his guidance throughout the course of this study. I am also grateful for the financial support received from the Agricultural Research Council and the Department of Science and Technology.

- To Dr Sandile Ndimande, thank you for being a beacon of hope when I was at my lowest point and was ready to throw in the towel. To Dr Omodele Ibraheem, thank you for your guidance, support and friendship throughout this journey. To Dr Ashwil Klein and Dr Hannibal Musarurwa, thank you for your invaluable input during the most challenging period of compiling this document. To Dr Abram Madiehe, thank you for your mentorship.

- To the Proteomics Group, thank you for the good and challenging times we have endured together during this learning phase of my life.

- To my parents, siblings and extended family, thank you for your encouragement and constant belief in my abilities. My family, Akona and Yakhani Nakani, you've been my true inspiration and motivation in keeping the faith and finishing this race. Thank you.

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AMG | amyloglucosidase |
| ANOVA | anaysis of variance |
| BSA | bovine serum albumin |
| Ca $(NO_3)_2$ | calcium nitrate |
| CBB | Coomassie Brilliant Blue |
| CCC | companion cell complex |
| cDNA | complementary DNA |
| $CO_2$ | carbon dioxide |
| CW-INV | cell wall invertase |
| DTT | Dithiothreitol |
| DW | dry weight |
| EDTA | ethylenediaminetetraacetic acid |
| Fe | iron |
| Fru-6-P | fructose-6-phosphate |
| FW | fresh weight |
| Glu-6-P | glucose-6-phosphate |
| G-6-PDH | glucose-6-phosphate dehydrogenase |
| GC-MS | gas chromatography mass spectrometry |
| HPLC | high performance liquid chromatography |
| Hsp70 | Heat shock protein 70 |
| Hsc70 | Heat shock conjugate 70 |
| IEF | isoelectric focusing |
| INV | invertase |
| IPG | Immobilised pH gradient |
| kDa | kilo Dalton |
| $KH_2PO_4$ | potassium phosphate monobasic |
| KOH | potassium hydroxide |
| $KNO_3$ | potassium nitrate |

| | |
|---|---|
| LC-MS | liquid chromatography mass spectrometry |
| LRWC | leaf relative water content |
| LSD | least significant differences |
| MALDI-TOF | Matrix assisted laser desorption /ionization-time of flight |
| MASCOT | Matrix Science |
| ME | mesophyll cells |
| MFS | major facilitator superfamily |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulphate |
| MOWSE | Molecular weight search |
| MS | mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| MSTFA | N-Methyl-N-(Trimethylsilyl) trifluoroacetamide |
| NAD | nicotinamide adenine dinucleotide |
| NADP | nicotinamide adenine dinucleotide phosphate |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase Chain Reaction |
| PEPC | phosphoenolpyruvate carboxylase |
| PGM | phosphoglucomutase |
| PPA | phloem parenchyma cells |
| PVDF | Polyvinylidene difluoride |
| PVPP | polyvinylpolypyrrolidone |
| QTL | qualitative trait loci |
| RNA | ribonucleic acid |
| SDS | Sodium dodecyl sulphate |
| SE | sieve element |
| SPP | sucrose phosphatase |
| SPS | sucrose phosphate synthase |
| Suc-6-P | sucrose-6-phosphate |
| Susy | sucrose synthase |

| | |
|---|---|
| SUT | sucrose transporter |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline containing Tween 20 |
| TFA | trifluoroacetic acid |
| TW | turgid weight |
| UDP | uridine-5’-diphosphate |
| V | Volts |
| Vhrs | Volt hours |
| $ZnSO_4$ | zinc sulphate |
| 1D | One-dimensional |
| 2D | Two-dimensional |
| 2DE | Two-dimensional electrophoresis |

## Table of Contents

# CHAPTER 1

## Literature Review

### 1.1 Introduction

There is great pressure for the production of agricultural crops with higher yield and stability. This is in order to keep pace with the demands of an increasing human population; the need for renewable energy sources; and the uncertain climatic changes (Slewinski, 2012; Khan and Hanjra, 2009). Due to their sessile nature, plants require an efficient response to environmental changes. They have thus acquired different response mechanisms via physiological, biochemical and molecular processes that act in coordination to execute stress responses (Wang *et al.,* 2004; Gill *et al.,* 2001). Plant adaptation to abiotic stresses is associated with metabolic adjustments that lead to the accumulation of organic solutes including carbohydrates and amino acids. These solutes play important roles as osmoprotectants in cell biochemical functions and maintenance of cell structural components (Valliyodan and Nguyen, 2006).



The regulation of carbohydrate metabolism by abiotic stresses has been reported in a variety of plants (Rosa *et al.,* 2009; Mohammadkhani and Heidari, 2008; Gupta and Kaur, 2005). However, research on gene transcriptional and translational regulations of sucrose biosynthesis genes under stressed conditions is still limited (Murray *et al.,* 2008; Tarpley and Vietor, 2007; Roitsch 1999).

Amongst the carbohydrate metabolites, sucrose is considered to be the main source of carbon and energy in the plant sink tissues (Qazi *et al.,* 2012), and a valuable substrate for bio-energy production (Abramson *et al.,* 2010; Waclawovsky *et al.,* 2010). Therefore, further studies on sucrose biosynthesis genes are pivotal for increasing bio-energy production. Such studies would provide a better understanding of the dynamics of whole-plant carbohydrate partitioning under adverse conditions, not only for the development of stress tolerant cultivars, but also for increased plant productivity (Ibraheem *et al.,* 2011; Fischer and Edmeads, 2010).

Sorghum has come under the spotlight as a potential bio-energy crop due to its cultivation under temperate and tropical conditions (Amuda and Balasabramani, 2011; Massaci *et al.,* 1996). It also has multi-purpose uses as food for humans, animal feedstock and as a fermentable crop (Audilakshmi *et al.,* 2010; Rooney *et al.,* 2007). Sorghum, like other plants contains a number of sucrose metabolism genes (Qazi *et al.,* 2012). However, their roles and regulation under adverse environmental conditions still remain undetermined. The complete sequencing of the sorghum genome together with the use of new “omics” approaches, such as transcriptomics, proteomics and metabolomics, has created a platform for further research into networks of interactions between genes, proteins and metabolites involved in stress response mechanisms. Moreover, this will facilitate the development of stress tolerant transgenic sorghum plants (Hayward, 2014; Urano *et al.,* 2010; Paterson *et al.,* 2009).

This review thus focuses on the role of sucrose as an osmoprotectant. It also highlights the significance of “omics” approaches in investigating the potential role of the sucrose metabolic pathways to optimize carbohydrate partitioning for increased productivity under adverse environmental conditions.

### 1.2 Carbohydrate metabolism of $C_4$ plants

Agricultural important crops such as sorghum, maize, sugarcane, etc., are involved in the $C_4$ photosynthesis pathway. This attribute allows these crops to produce and accumulate high nutritional value photoassimilates, such as sucrose and starch (Shakoor *et al.,* 2014; Lawrence and Walbot, 2007). The $C_4$ cycle has evolved to prevent plant photorespiration by maintaining high $CO_2$ concentrations, thus enhancing carbohydrate production (Sage, 2004). The key feature of this pathway is its compartmentation between two photosynthetic cell types: the mesophyll and bundle sheath cells. Carbon dioxide is initially fixed in the mesophyll cell by phosphoenolpyruvate carboxylase (PEPC) forming a $C_4$-oxaloacetic acid, which is converted to malic or aspartic acid and transported to bundle sheath cells (Lunn, 2007). In the bundle sheath malic/aspartic acid is decarboxylated into pyruvate by the chloroplastic NADP-malic enzyme, mitochondrial NAD-malic enzyme or cytosolic PEP carboxylase enzyme. This is followed by the release of $CO_2$, which is refixed by ribulose bisphosphate carboxylase-oxygenase (Rubisco; Sage, 2004; Edwards *et al.,* 2004). The pyruvate is returned to the mesophyll cell where it is converted back to PEP or into $CO_2$.

The PEP then enters the Calvin cycle and is reduced to carbohydrates such as starch, cellulose or sugars depending on the needs of the plant (Smith and Stitt, 2007). The translocation of osmotically active sugars and sugar alcohols, from photosynthetic source tissues to non-photosynthetic sink tissues forms the major component of carbohydrate metabolism (Dinant and Lemoine, 2010; Chaves *et al.,* 2003). These metabolites aid the plant in making physiological adjustments, or developmental responses based on nutrient availability and environmental constraints.

### 1.3 Mechanisms of sucrose transport in $C_4$ plants

#### 1.3.1 Phloem loading strategies

Sucrose is the predominant photosynthetic byproduct and also serves as the major sugar translocated in higher plants (Wind *et al.,* 2010; Rolland *et al.,* 2006; Roitsch, 1999). From the leaf tissues where sucrose is synthesized, it diffuses towards the plant vascular system where it is then transported into the phloem tissue through the sieve-element-companion cell complex (SE-CCC; Truernit, 2001). The high solute content in the phloem sap, due to active sucrose loading, results in a high osmotic pressure in the SE-CCC, compared to the leaf mesophyll cells. This process is known as phloem loading. This helps in the maintenance of a concentration gradient and consequently sink strength (Rolland *et al.,* 2006). From the phloem tissues, sucrose is unloaded into the apoplasmic space where it can be taken up into the sink cells and stored in the vacuoles of storage cells (Lemoine, 2000).

Alternatively, sucrose is cleaved by invertase to hexoses, which are then transported by specific carriers and used for sink growth of plant meristems and developing organs (Amin *et al.,* 2010, Dinant and Lemoine 2010).

There are two principal pathways of sucrose transportation into the phloem cells: (i) active transport (apoplastic loading) and (ii) passive transport (symplastic loading). In apoplastic loading, sucrose is released from the mesophyll cells and is actively taken up by sucrose transporters into the phloem tissues. Symplastic loading entails the passing of sucrose from cell to cell via plasmodesmata from source tissues to the SE-CCC, which makes up the phloem (Figure 1.1; Dinant and Lemoine, 2010; Turgeon and Wolf, 2009; Lalonde *et al.,* 2003). The extent to which plants use either of these two pathways depends on the abundance of the plasmodesmatal connections between the source and phloem tissues, and also on the location, activity and specificity of sucrose transporters (Slewinski and Braun, 2010; Lemoine, 2000). In many plant species, sugars are translocated via the apoplastic route (Figure 1.1), which requires the activity of specific sugar transporters.

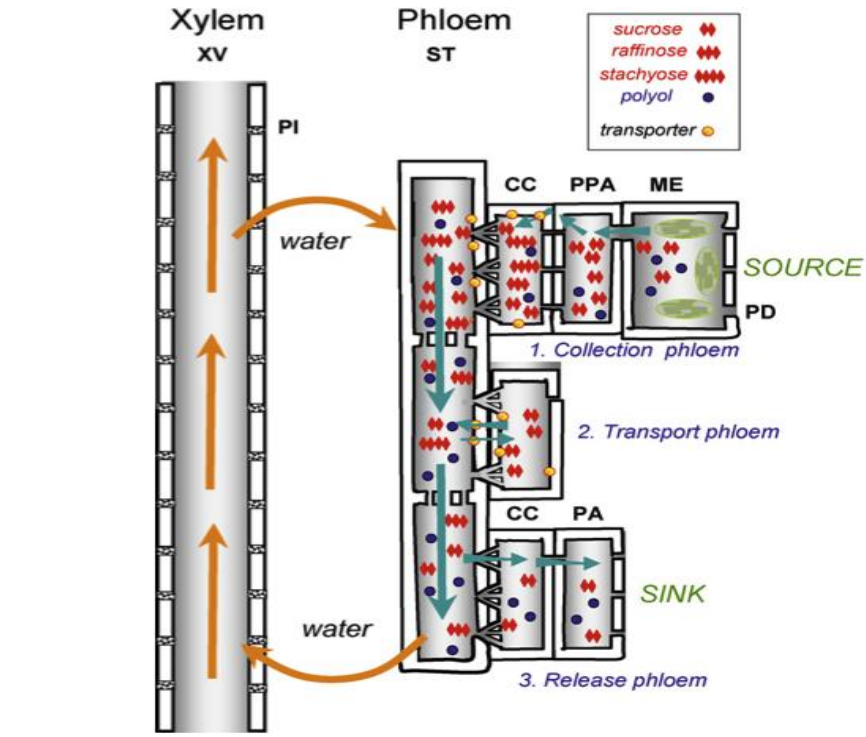


**Figure 1.1:** The path of sugar transport from source to sink tissues in an apoplasmic loader. From mesophyll cells (ME), sucrose and other sugars are transported through plasmodesmatal connections to the phloem parenchyma cells (PPA) and subsequently taken up at the SE-CCC complex by a sugar transporter (yellow circles) energized by a proton pump ATPase. PI: half-bordered pit, CC: companion cells, ST: sieve tube. This picture was adapted from Dinant and Lemoine (2010)

### 1.3.2 Roles and regulation of sucrose transporters

Due to metabolic compartmentation, plant systems require a host of plasma membrane transporters to move metabolites around the cells. These transporters facilitate plant cells in fulfilling part of their nutritive requirements, control long distance transport and assist in responses to environmental signals (Ibraheem *et al.,* 2008; Rae *et al.,* 2005; Li *et al.,* 2003). Among the major facilitator superfamily (MFS), sucrose transporters play a major role in carbohydrate partitioning.

These transporters control the long distance transport of sucrose generated in source leaves to sink organs where it is either utilized or stored (Lalonde *et al.,* 2004). Many sucrose transporters have been shown to function as proton-symporters using a transmembrane proton gradient to drive sucrose transport (Resimer *et al.,* 1992).

An important step towards understanding the importance of sucrose transporters in plants was made when the first plant sucrose transporter SoSUT1, from spinach (*Spinacea oleracea*), was functionally identified using a yeast complementation strategy. This transporter has become instrumental for the development of our current understanding of transport mechanisms (Reismeier, 1993; Reismeier *et al.,* 1992). To date, a number of sucrose transporters have been characterized (Kuhn and Grof, 2010). Though they all transport sucrose, they have a number of distinct functions, which are attributed to differences found in their cellular location, kinetic properties, substrate specificity and expression patterns (Kuhn and Grof, 2010). For example, they are involved in carbohydrate uptake from the apoplast, play a role in sugar sensing, and may be essential for the uptake of other substances (Turgeon and Wolf, 2009; Gibson, 2005). Sucrose transporters have been categorized into four separable classes based on their cellular localization. Members of the class 1 dicotyledonous family are expressed in the plasma membrane of the SE or CC or in both cell types (Barker *et al.,* 2000; Kuhn *et al.,* 1997).

These sucrose transporters have a high affinity for sucrose as a substrate and are mainly responsible for phloem loading. Additional functions have been suggested in sink tissues in relation to sucrose import into developing grains (Ibraheem *et al.,* 2011; Scofield *et al.,* 2007). The class 2 dicotyledonous transporters demonstrate a low affinity for sucrose and are localized in the SE plasma membranes in tomato, plantain and Arabidopsis (Meyer *et al.,* 2004; Barth *et al.,* 2003, Barker *et al.,* 2000). These transporters have been reported in a number of vegetative sinks and developing seeds and are thus responsible for sucrose import (Truernit and Sauer, 1995). The class 3 monocotyledonous members are localized to the plasma membranes of SE in wheat and in both SE and CC in rice, where they are potential carriers involved in phloem unloading in the apoplast (Scofield *et al.,* 2007; Aoki *et al.,* 2004). Class 4 members have been identified in the chloroplast fractions (AtSUT3), vacuoles (AtSUT4; HvSUT2) and plasma membrane (Endler *et al*., 2006; Rolland *et al.,* 2003) and play a key role in sucrose import into the vacuoles.



The diversified roles of sucrose transporters suggest that sucrose transport is tightly regulated. Tight regulation of sucrose allocation is required to modulate carbon allocation for plant growth and development and in response to changing environmental conditions. This has been demonstrated at transcriptional and post-transcriptional levels (Gupta and Kaur, 2005; Lemoine, 2000). Maize *sut1* mutant plants demonstrated reduced sucrose transport, which resulted in carbohydrate accumulation in the leaf and early leaf senescence (Slewinski *et al.,* 2009).

Biomass partitioning, delayed flowering and stunted tassel development were also observed. Antisense suppressed rice *sut1* mutant plants demonstrated impaired seed germination and growth, and reduced grain filling due to reduced sucrose remobilization from the starch endosperm in developing rice seeds (Furbank *et al.,* 2001; Ishimaru *et al.,* 2001). Okadaic acid, a protein phosphatase inhibitor, also demonstrated a negative regulation in both activity and transcript of BvSUT (a phloem sucrose transporter) in sugar beet (Kuhn and Grof, 2010). Evidence of post-translational regulation of sucrose transporters was demonstrated in *Arabidopsis thaliana* by phosphorylation of its plasma membrane AtSUC1 and AtSUC5 (Sauer, 2007), and SUT2 and SUT4 family in Solanaceous species (He *et al.,* 2008; Barker *et al.,* 2000; Weise *et al.,* 2000).



**1.4 Sucrose signaling, physiological roles and regulations of sucrose biosynthesis enzymes**

In addition to its prominent role as a transport molecule, sucrose has also been documented as a signaling molecule that regulates both source and sink metabolism, and ultimately plant developmental and physiological states (Koch, 2004; Sheen *et al.,* 1999). Sucrose-specific regulation of plant development has been demonstrated on plant species, including spruce, where sucrose applied to somatic embryos resulted in regular embryonic cell division (Iraqi *et al.,* 2005).

High sucrose levels were shown to repress radicle elongation of carrot embryos (Yang *et al.,* 2004), whereas externally applied sucrose induced the emergence of lateral roots on the aerial tissues of Arabidopsis (Macgregor *et al.,* 2008). Sucrose has also been postulated to affect the timing with which some plants flower, the onset of senescence, as well as the organ number and shape (Gibson 2005). This disaccharide also regulates several sugar-controlled gene and promoter elements. For example, the patatin-1 promoter in potato tubers is induced specifically by sucrose, whereas the maize *Shrunken* gene and the Arabidopsis plastocyanin gene activity is specifically repressed by sucrose levels (Wind *et al.,* 2010). This suggests that different signal types might be perceived by the same sugar receptor (Chaves *et al.,* 2003). Sucrose metabolism is thus a very dynamic process. During its transport, activities of various enzymes are regulated (Figure 1.2) to ensure that the flow of sucrose is unidirectional (from phloem to sink tissues) and the osmolarity of the cell is maintained (Qazi *et al.,* 2012). These activities regulate the entry of sucrose into distinct biochemical pathways such as respiration or the biosynthesis of cell wall polysaccharides and storage reserves (Sturm and Tang, 1999). This is achieved through differential expression at transcriptional and translational levels (Koch, 2004).

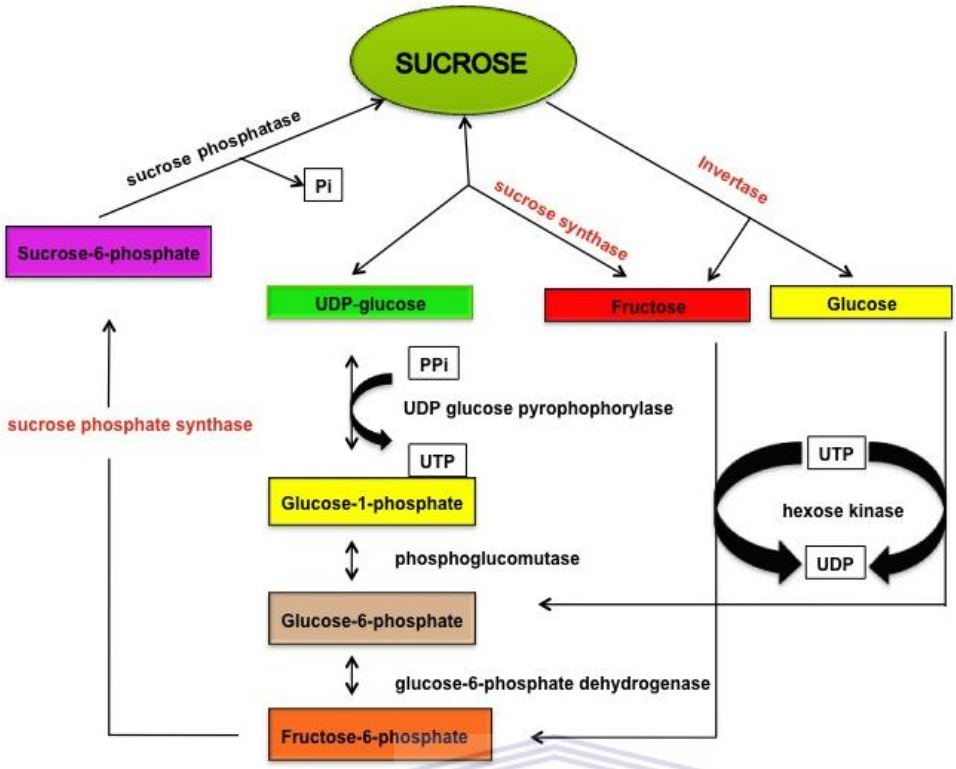


**Figure 1.2:** Schematic diagram of the major reactions in sucrose metabolism in sinks of higher plants. This picture was modified and adapted from Noorkaraju et al. (2010). Refer to the text for the role and function of the various enzymes involved in sucrose metabolism.



Changes in enzyme activities affect sucrose levels and cellular metabolism, thereby influencing plant growth and development (Wind *et al.,* 2010). There are three key enzymes involved in sucrose metabolizing pathways, which have been implicated in carbon partitioning in sink tissues. They include: sucrose phosphate synthase (SPS), sucrose synthase (Susy) and invertases (INV) (Winter and Huber, 2000). These enzymes have been studied in a variety of crops with most plant species containing different isoforms with highly homologous amino acid sequences and similar biochemical properties. By contrast however, these isoforms have distinct organ-specific and developmental expression patterns (Koch, 2004).

### 1.4.1 Invertases

Invertases (EC 3.2.1.26) are members of the glycosyl transferase family responsible for the hydrolysis of sucrose to hexoses (fructose and glucose) in sink tissues (Koch, 2004). Three types of invertase have been identified in higher plants, namely soluble acid, soluble neutral and cell wall bound enzymes. These invertases are distinguished based on their solubility, pH optima, isoelectric point and subcellular localization (Misic *et al.,* 2012).

Soluble forms, neutral or alkaline invertase with pH optima of 7.0 to 8.0 are found in the cytoplasm of mature tissues where they regulate the hexose and sucrose levels (Bosch *et al.,* 2004). Soluble acid invertases are localized in the vacuoles and have pH optima of 5 to 6. They play a role in sucrose metabolism and their activity is high in rapidly growing tissues (Liu *et al.,* 2006). Cell wall invertases have pH optima between 4 and 5. Invertases also play a role in sucrose partitioning between source and sink organs and are involved in plant response to wounding and infection (Huang *et al.,* 2007). These genes are spatially and temporally expressed during plant development and are therefore involved in regulating processes of synthesis, transport and utilization of sucrose, thereby influencing plant growth (Sturm and Tang 1999). Soluble acid invertases have been reported to be the primary determinants of the rate and extent of sucrose levels and storage in tomato (Klann *et al,* 1996) and cucumber (Burger and Schaffer, 2007). In Arabidopsis the down-regulation of invertase activity affected plant growth resulting in shorter primary roots (Barratt *et al.,* 2009; Qi *et al.,* 2007).

Transgenic plants of tomato, carrot, potato and muskmelon with repressed invertase activity had altered sugar composition in their fruits and tubers (Yu *et al.,* 2008; Hajirezaei *et al.,* 2003; Tang *et al.,* 1999; Klann *et al.,* 1996). Maize plants lacking cell wall invertase (ZmCW-INV2) showed reduced sucrose transport to the seeds, resulting in reduced seed weight (Kang *et al*., 2009). In carrots, the down-regulation of cell wall or vacuolar invertases resulted in plants with small roots and increased leaf number (Tang *et al.,* 1999). Yu *et al*. (2008) reported increased sucrose accumulation in muskmelon plants with down-regulated soluble acid invertase. Furthermore, the transgenic fruits showed accelerated ripening. These studies demonstrate that invertases control hexose (glucose) availability and therefore determine where and when hexoses are produced and perceived by the hexose signaling systems (Koch, 2004).



### 1.4.2 Sucrose synthase

Sucrose synthase (Susy) (EC 2.4.1.13) is another sucrose hydrolyzing enzyme, which is pivotal in maintaining a balance between sugar signals and metabolic pathways. It is a member of the glycosyl transferase enzyme family and catalyzes the reversible reaction of sucrose to fructose and UDP-glucose in the presence of uridine 5'-diphosphae (UDP; Dejardin *et al.,* 1999). The same enzyme, in source tissues, is known to play a role in sucrose synthesis using uridine diphosphate (UDP)-glucose and fructose as substrates (Ciereszko and Kleczkowski, 2002).

Sucrose synthase is located in the cytosol or in various subcellular compartments including plasma, mitochondria and vacuolar membranes (Cho *et al.,* 2011). Susy plays a major role in sucrose metabolism in a number of different growth processes within a variety of sink tissues (Zrenner *et al.,* 1995). It is also proposed to supply UDP-glucose for cell-wall synthesis (Winter and Huber, 2000). Further roles of Susy have been demonstrated in other metabolic processes including phloem loading/unloading and nitrogen fixation in legume nodules (Gordon *et al.,* 1999).

Susy encodes multiple genes with distinct and partially overlapping expression patterns. Two Susy isoforms (SS I and SSII) have been reported in Japanese pear with isoform SS I playing a role in the degradation of translocated sucrose in young pears. Isoform SS II was reported to play a role in sucrose synthesis in mature pears (Tanase and Yamaki, 2000). In maize three genes encoding Sus, Sh1 and Sus3 have been reported, with Sh1 having a dominant role in cell-wall synthesis whereas Sus 1 is involved in starch synthesis (Carlson *et al.,* 2002). Similarly in pea and rice, three Susy genes have been identified and characterized with distinct roles been suggested for each gene (Barratt *et al.,* 2001; Huang *et al.,* 1996). Susy is highly regulated in plants, playing a major role in the sucrose import and compartmentation in a variety of sink tissues. Susy was reported to regulate the import of sucrose in the early stage of tomato fruit development (Demnitz-King *et al.,* 1997). The over-expression of Susy resulted in elevated starch levels in the tubers of potato plants, increasing total tuber weight (Baroja-Fernandez *et al.,* 2009).

Similarly, an increase in Susy activity during early stages of tomato fruit development correlated with fruit growth and increased sink strength suggesting the regulatory role of Susy in controlling sugar export (Chengappa *et al.,* 1999). Conversely, antisense repression of Susy in tomato resulted in decreased sink strength and reduced dry matter accumulation in fruits (Noorkaraju *et al.,* 2010). The down regulation of Susy in cotton inhibited fiber biosynthesis and consequently seed development (Ruan *et al.,* 2003). Susy activity therefore plays an important role in maintaining sink metabolism.

### 1.4.3 Sucrose phosphate synthase

Sucrose phosphate synthase (EC 2.4.1.14) is a rate-limiting enzyme in sucrose synthesis, which plays a dominant role in sucrose metabolism in both photosynthetic and non-photosynthetic tissues (Huber, 1996). It catalyzes the conversion of Fruc-6-P and UDP-glucose to Suc-6-P, which is subsequently hydrolyzed to sucrose by sucrose phosphatase (SPP; EC 3.1.3.24). SPP is an enzyme that renders the SPS reaction irreversible (Lunn and MacRae, 2003). The critical role of SPS in carbon partitioning and sucrose accumulation has been demonstrated through genetic manipulations. An over-expression of the maize SPS gene in rice and tobacco was correlated with the increase in sucrose/starch ratio in leaf blades (Okamura *et al.,* 2011; Baxter *et al.,* 2003). Antisense repression of SPS activity in potato however resulted in decreased sucrose synthesis and increased starch synthesis (Ndimande, 2007).

Several studies have associated SPS activity with plant productivity. A transgenic potato with over-expressed maize SPS was shown to improve the photosynthetic rate, inhibit leaf senesces and increase yield (Ishimaru, 2008). In rice, the QTL for plant height coincides with the locus of OsSPS1 gene (Yonekura *et al.,* 2003).

Plants have multiple SPS isoforms, which differ in individual species with development, tissue type and environmental signals (Reimholz *et al.,* 1997). SPS genes have been clustered into four classes A, B, C and D, based on their amino acid sequences (Lutfiyya *et al.,* 2007; Castleden *et al.,* 2004). All higher plants contain at least one representative from each SPS family with a member of each family being expressed. One isoform may be predominant at a particular developmental stage or growth condition (McGregor, 2002). The functional significance of the differential expression of these isoforms is not clear and requires more precise approaches to unravel the roles of individual isoforms to sucrose synthesis.



**1.5 Osmoprotective functions of sucrose metabolism in plants**

Abiotic stresses negatively impact plant cellular processes such as growth, photosynthesis, carbon partitioning, carbohydrate and lipid metabolism, osmotic homeostasis, protein synthesis, and gene expression (Krasensky and Jonak, 2012; Anjum *et al.,* 2011; Huber and Winter, 2000). As a result, plants have developed strategies to cope with stresses. These survival strategies normally involve a mixture of stress avoidance and tolerance mechanisms.

During drought avoidance, plants maintain high tissue water potential by improving water uptake through developing a deep-root system, thus reducing water loss (Amuda and Balasabramani, 2011; Chaves *et al.,* 2009). Drought-tolerance involves metabolic adjustments, mediated by alteration in gene expression, to help improve the plant functionality (Anjum *et al.,* 2011; Valliyodan and Nguyen, 2006; Chaves *et al.,* 2003).

Physiological studies have demonstrated the accumulation of metabolites including sugars, sugar alcohols and amino acids in different plant species, which act as osmotic balancing agents (Rontein *et al.,* 2002; Gill, 2001; Rathinasabapathi, 2000). The changes in these metabolites at cellular level are thought to be associated with maintaining cell turgor and to stabilize cell proteins and structures during abiotic stresses (Mohamadkhani and Heidari, 2008; Seki *et al.,* 2007; Langridge *et al.,* 2006).



When different abiotic stresses affect plant functionality, alterations in photosynthesis and carbon partitioning are common features that take place at the whole plant level. Carbohydrate accumulation in response to adverse environmental conditions has been reported in the temperate grasses and cereals (Slewinski, 2012). Sucrose accumulation is a common metabolic response when plants are exposed to drought stress. It has been identified as a major osmoprotectant molecule involved in regulating osmotic potential in plants, thus offering resistance against drought stress (Cramer *et al.,* 2011; Misic *et al.,* 2002; Massaci *et al.,* 1996).

Sucrose flux under stress conditions involves the modulation of many enzyme activities in the carbohydrate metabolic pathway including sucrose-biosynthesis genes, resulting in whole-plant adjustment (Winter and Huber, 2012; Koch, 2004). Sucrose phosphate synthase (SPS), a highly regulated enzyme with a key role in source-sink relationships has been reported as the main target for the biochemical effect of drought stress in grapevines (Maroco *et al.,* 2002). In stressed maize leaves acid invertase activities were increased, which coincided with the accumulation of glucose and fructose (Trouverie *et al.,* 2003). Similarly glucose, fructose, and sucrose accumulated in both leaf blades and petiole of lupins which were subjected to stress (Pinheiro *et al.,* 2001). Two sucrose synthase encoding genes (Sus1 and SuS2) from *Arabidopsis thaliana* were found to be differentially regulated in leaves exposed to cold and drought stress (Dejardin *et al.,* 1999).



Recent studies for increasing tolerance to abiotic stresses, through metabolic engineering of compatible solutes, have demonstrated the potential of increases in soluble sugars and/or other osmolytes to increase plant tolerance to abiotic stresses such as drought (Capell and Christou, 2004; Wang *et al.,* 2003; Rontein *et al.,* 2002).

## 1.6 ‘‘Omics’’ approaches in studying plant drought stress response mechanisms

Plant productivity is greatly affected by abiotic stresses. As a result; many efforts have been invested in better understanding global plant systems in response to stress conditions to underpin gains in crop productivity under unfavorable conditions (Langridge and Fleury, 2010; Umezwa *et al.,* 2006; Rathinasabapathi, 2000).

The rapid advances in ‘‘omics’’ technologies provide sustainable screening and analysis platforms to improve the efficiency of selection strategies for crop adaptation to various abiotic stresses (Bogyo and Rudd, 2013; Diouf, 2011). The integration of transcriptomics, proteomics and metabolomics has increased our understanding of the complex regulatory networks associated with stress adaptation and tolerance (Hayward, 2014; Urano *et al.,* 2010; Chaves *et al.,* 2009; Hirai *et al.,* 2005). It has also led to the identification of candidate genes and pathways that can be used for the genetic improvement of plants against stresses (Cushman and Bohnert, 2000).



Transcriptome analysis technologies have advanced to a point, which enables the discovery of candidate genes on the basis of expression profiles in various tissues, developmental stages, and environmental conditions such as drought, cold, heat and salinity (Zhuang *et al.,* 2014). The availability of transcriptome technologies including high-density microarrays and next generation sequencing has created a platform to carry out whole genome transcriptome analyses in a high-throughput manner and have thus greatly

expanded comparative analysis within stress physiology (Gregory *et al.,* 2008; Zhang *et al.,* 2006). Transcriptomics facilitates the identification of genes responsible for stress responses and the prediction of their putative functions through the association of co-expressed and differentially expressed genes (Hayward, 2014). Whole genome transcriptomics has enabled researchers to identify cascades of target genes under transcriptional control also capturing changes in the expression of rare transcripts (Wang *et al.,* 2010). These next generation technologies are thus paving way for novel insights into plant abiotic stress responses.

Proteomics studies have also significantly contributed in unraveling potential links between protein abundance and plant responses to abiotic stress (Kosova *et al.,* 2011). Proteomics tools including 2DE and MS are useful for the identification of stress responsive proteins in plants (Thiellement *et al.,* 2002). They provide qualitative and/or quantitative differences in gene expression (Carpentier *et al.,* 2008). Furthermore, proteomics plays a role in the identification of post-translational modifications, which are involved in stress adaptation and thus greatly affecting protein structure, activity and stability (Ytterberg and Jensen, 2010). A short falling with this approach however is the limitation in the global proteome coverage due to the high dynamic range of protein expression in biological systems (Rabilloud, 2002; Park, 2004). As a result, a low copy number proteins such as most signal transduction and regulatory proteins become masked by the more highly expressed proteins and have thus a higher chance of not being detected (Xi *et al.,* 2006).

This problem has however been overcome through the use of more sensitive staining protocols and the incorporation of non-gel based approaches, which provide broader and more distinct proteome coverage (Plomion *et al.,* 2006).

Metabolomics is another viable tool in genomic assisted selection for crop improvement (Hochberg *et al.,* 2013). It provides a basis for the sustainable analysis of both primary and secondary metabolites that respond to environmental challenges and can thus be used as a marker for stress physiology (Dixon *et al.,* 2006). Different metabolomics techniques can be employed to detect changes that occur when plants are in stressed conditions and can be evaluated through metabolite profiling (Chandna *et al.,* 2013). Gas or liquid chromatography-mass spectrometry (GC-MS or LC-MS) and nuclear magnetic resonance (NMR) are the predominantly used approaches in plant literature (Fritz, 2004; Unger, 2004). The limitation with these approaches however includes insufficient global metabolome coverage. Many factors including the sensitivity of the technique employed, and the metabolite libraries available for the system used contribute to these shortfalls (Hayward, 2014). Although this limits the identification of the broad functions of metabolite regulatory networks during abiotic stress responses (Urano *et al.,* 2010), metabolomics is the outcome of both transcript and protein expression, thus directly contributing to plant phenotypes. This creates a platform for the better understanding of fundamental roles of single metabolites and broadly makes omics accessible for many more species, particularly those that are agriculturally important (Shulaev *et al.,* 2008; Capell and Christou, 2004).

## 1.7 Sorghum as a model system for engineering stress tolerant bio-energy crops

Over the years, genomic research has been focused on model crops, particularly Arabidospsis, to provide detailed knowledge on plant response mechanisms to abiotic stresses (Denby and Gehring, 2005; Vinocur and Altman, 2005; Zhu, 2000). Although the use of this plant has provided an excellent model system for the study of various plant processes, it has been slow to find its way to practical crop plant improvement. This has been attributed to Arabidopsis being agriculturally unimportant (van Wyk, 2001), and not adapted to various abiotic stresses such as salt and drought (Vinocur and Altman, 2005; Zhu, 2000). Furthermore, Arabidopsis is fundamentally different in structure, development and osmotic adjustment from agriculturally important monocotyledons (Tester and Bacic, 2005). Thus, the transfer of knowledge gained using this plant to agriculturally important crops does not seem feasible. Amongst the worldwide challenges of food security and climate change, there is an increase in economic and scientific interest in developing bio-fuel crops to mitigate the current energy crisis (Karp and Richter, 2011; Hanjra and Qureshi, 2010). Although rice (an agriculturally important crop) has provided insights into many plant processes, and is considered a model species for studying fuel production (Abbas and Ansumali, 2010; Elmore *et al.,* 2008), its use as a model system is limited. This is attributed to the $C_3$ photosynthesis pathway of this crop, which makes it prone to abiotic stresses (Munns and Tester, 2008).

Other agriculturally important crops including maize and sugarcane are potential candidates for bio-energy initiatives (Quintero *et al.,* 2008). The restriction with these crops however is in production, which requires high water input (Reddy *et al*, 2008). The quarantines imposed on maize production due to biotic stress outbreaks, and the polyploidy nature of sugarcane have also made these crops less suitable as model systems for increased stem sugar content and stress tolerance (Calvino and Messing, 2011; Lawrence and Walbot, 2007).

Sorghum genome sequencing (Peterson *et al.,* 2009), in combination with the availability of "omics" resources and bioinformatic tools, is leading to the discovery of new metabolic pathways in sorghum. This is providing new insights into the compartmentation in sorghum metabolism and is making it possible to identify genes that govern agronomically important traits (Tardieu *et al.,* 2010; Umezwa *et al.,* 2006). Sorghum has been proposed as the key model system for gene discovery relating to biomass yield and quality in the bio-energy grasses under adverse conditions (Calvino and Messing, 2011). This is attributed to its high energy return obtained from its high dry weight biomass yield, high stem sugar content, and abiotic stress tolerance due to its high water and nutrient use efficiencies, and resistance to diseases (Mutava *et al.,* 2011; Shakoor *et al.,* 2004). Therefore, the development and characterization of a diverse sorghum germplasm collection with considerable variation for energy yield, abiotic stress tolerance and pathogen resistance will result in sorghum hybrids dedicated to bio-energy production (Rooney *et al.,* 2007).

The benefits of sorghum as a model plant will further extend to other grass species such as sugarcane and Miscanthus that are being targeted as potential resources for bio-ethanol production. The aim of re-engineering $C_3$ plant species to adapt to $C_4$ photosynthesis, in order to boost yields will also benefit immensely from the sorghum data (Doubnerova and Ryslava, 2011).

### 1.8 Research aim and objectives

Physiological studies on sorghum varieties have demonstrated sugar accumulation in response to drought stress, therefore suggesting that sugars are involved in an unknown mechanism of abiotic stress tolerance. This study investigates the possible cross talks between the sugar biosynthesis and stress-related pathways, focusing on the following objectives:



I. To investigate the effect of drought stress on plant growth and development
II. To investigate changes in the stem metabolome of sorghum varieties in response to drought stress
III. To investigate the effect of drought-induced hyperosmotic stress on the activity and expression levels of sucrose-biosynthesis genes
IV. To investigate changes in the stem proteome of sorghum varieties in response to drought stress

# CHAPTER 2

## The effect of drought stress on physiological and biochemical parameters in *Sorghum bicolor* (L.) *Moench* varieties


### Abstract

Water deficit triggers various responses in plants that are dependent on the stress intensity. Sorghum has acquired immunity from drought stress through various adaptation mechanisms. To date however, research on the physiological and biochemical mechanisms contributing to drought tolerance in sorghum is still limited. In this study physiological and biochemical parameters of four sorghum varieties ICSB338; ICSB73; ICSV213 and S35 were analyzed in order to understand their response to water deficit. At early anthesis, plants were subjected to drought stress by withholding water for a period of 10 days. Stress induction was demonstrated by the continuous decrease in soil water content during experimentation. This was accompanied by an increase in the root length across all four sorghum varieties. It was observed that the relative water content of stressed sorghum leaves was significantly reduced when compared to the well-watered plants, with variety ICSV213 displaying the highest reduction of 46%. The chlorophyll contents under stressed conditions were also reduced; an indication that the photosynthesis machinery was compromised. Drought stress influenced chlorophyll a, b and total chlorophyll levels in all four varieties, with the most pronounced reductions observed in ICSV213. The biomass of the basal stems measured was reduced by water deficit whereas; the shoot length was not affected.

Metabolic analysis showed an increase in proline and fructose whereas, the abundance of leucine and sucrose were reduced under stressed conditions. The results of this study indicate that water deprivation provoked plant responses by the mechanism of osmotic adjustment in the sorghum varieties. These results also highlight the major role of soluble sugars in osmotic adjustment.

## 2.1 Introduction

Drought is generally accepted as the most widespread abiotic stress that adversely affects plant growth and development (Khalili *et al.,* 2008). Effects of drought on the productivity of economic crops are more severe in arid and semi-arid regions around the world where there is limited rainfall, high temperature, poor water quality, and poor soil management practices (Niu *et al.,* 2012). The faster-than-predicted change in climate (Inter governmental Panel on Climate Change, 2007) and the different scenarios of climate change suggest an increase in aridity for the semi-arid areas around the globe. Together with the exponential growth in global population, this will lead to the overexploitation of water resources for food production and less arable land. There are concerted efforts to produce renewable energy from plant sources as an alternative to the limited availability of fossil fuels (Rooney *et al.,* 2007). Thus in order to meet these food and non-food requirements, crop production in marginal areas has become a priority.

As a result, the development of improved levels of drought tolerance has become a priority for many crop-breeding programs focusing on food and bio-energy production (Hanjra and Qureshi, 2010). The ability of most organisms to survive and recover from water restricted conditions is a function of acquired tolerance mechanisms that can improve the overall stress tolerance (Kaplan *et al.,* 2004). Plants that are frequently subjected to periods of drought stress exhibit physiological and metabolic constraints and have thus adopted response mechanisms to overcome such effects (Nasim *et al.,* 2011).

Plant resistance to drought stress has been mainly attributed to high stomatal sensitivity, which consequently affects photosynthesis. Photosynthesis is an essential process in crop growth and development (Rong-hua *et al.,* 2006). Together with cell growth it is among the primary processes to be affected by drought stress. A decrease in $CO_2$ assimilation resulting from closed stomata is generally observed in response to drought stress (Chaves *et al.,* 2009). The aforesaid coupled with the combined effect of reduced water supply required to support cell expansion results in decreased biomass, chlorosis, slowed growth and ultimately plant death (Mahajan and Tuteja, 2005).

*Sorghum bicolor* (L.) *Moench* is a moderately drought tolerant cereal crop, and responds to drought stress through various biochemical metabolic adjustments (Gill *et al*., 2001). These processes are regarded as the primary mechanisms for plants to survive in circumstances of water deficiency, and are mostly dependent on osmotic adjustment that promotes solute

accumulation in plant cells (de Oliveira Neto *et al.,* 2009). Sugars and polyols are considered particularly important metabolites involved in osmotic adjustment. These metabolites are small and biochemically compatible compounds that increase in concentration to increase osmolarity, thus contributing to the maintenance of the water potential gradient between the plant and soil (Mahajan and Tuteja, 2005). Amino acids also function as osmotically active metabolites in response to drought stress although an increase in their contents may not be a global response to drought stress (ElSayed *et al.,* 2014; Barchet *et al.,* 2013). This study explores the effect of drought stress on the physiological and biochemical parameters of four sorghum varieties.



## 2.2 Materials and Methods

### 2.2.1 Plant material

Four sorghum varieties; (ICSB338; ICSB73.ICSV213 and S35.) were obtained from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) India. These varieties were selected because they offer considerable genetic variation in drought tolerance based on the morphological assessments made at ICRISAT.

### 2.2.2 Experimental design

The experiment was laid out in a randomized complete block design (RCBD) with five biological replications. At the early stages of anthesis (80 days after sowing), plants from all four varieties were randomly selected and divided into two groups; well watered (irrigation in all stages of plant growth) and water deprived (drought stressed).

### 2.2.3 Plant growth conditions and drought treatment

The four sorghum varieties (ICSB338; ICSB73; ICSV213 and S35) were sown on the 28$^{th}$ of November 2012 at the Agricultural Research Council, Stellenbosch, Western Cape, (South Africa). The glasshouse conditions under which these varieties were grown included, natural daylight, a temperature at 41˚C (day) and 21˚C (night), and a constant relative humidity of 60%. Pots with a diameter of 25 cm and height of 25 cm each containing a sterilized equal proportion mix (1:1) of loam soil and organic matter were used for sowing the sorghum seeds. Macro and micronutrients were also applied to the soil and these were obtained from a prepared inorganic nutrient solution [Ca $(NO_3)_2$ (164.1 g/l); $KNO_3$ (101.1 g/l); $MgSO_4$ (120.4 g/l); $KH_2PO_4$ (136.1 g/l) and Fe cheloate (16.6 g/l)]. The date of anthesis was determined when 50% of the spikes had extruded anthers.

In this experiment, instead of investigating all parameters continually throughout the stress period, one time point was selected to represent the well watered and water limited conditions. This was at day 10, obtained from preliminary work, where the relative soil water content under water deficit was discovered to be at the lowest compared to the well watered conditions. Water loss was estimated based on the soil measurements taken every second day during the stress induction period. Measurements were taken using a Digital Moisture Meter-MT960 (MAJOR TECH (PTY) LTD, South Africa).

### 2.2.4 Growth analysis and measurement of leaf relative water content



Analysis of plant growth was performed on watered and stressed sorghum plants at the early anthesis stage (80 days after planting). The measurement of leaf relative water status, basal stem biomass, shoot and root length was carried out from means of four plants per sorghum variety per growth condition. A Stabila Measuring Tape (Upat S.A (Pty) Ltd, South Africa) was used to determine shoot and root length. To measure root length, the intact root system was rapidly separated from the bulk of the soil by gentle shaking. A weighing balance (RADWAG Wagi Elektroniczne, Poland (EU)) was used to measure the basal stem fresh weight of each of the different plants. The leaf relative water content (LRWC) was measured using four 20 mm leaf disks excised from the second youngest leaf of each plant. The fresh weight (FW) for each disk was immediately measured. The disks were transferred to a Petri dish and saturated in deionized water at 4°C in the dark for 24 hours,

after which the disks were blotted in between filter papers and weighed in order to determine the turgid weight (TW). The dried weight (DW) was determined by oven drying the turgid disks with forced air circulation at 80°C for 24 hours and then weighing the dried disks. The leaf relative water content (LRWC) was determined according to the formula as contained in Barrs and Weatherly, (1962).

LRWC (%) = [(FW – DW) / (TW – DW)] X 100

### 2.2.5 Determination of chlorophyll content:

Leaf material was ground in liquid nitrogen using a mortar and pestle. Chlorophyll was extracted from 100 mg leaf material using 80% ice cold acetone. Homogenates were centrifuged at 13,000 rpm for 10 minutes. The absorbance of each extract was recorded at (663 nm; 645 nm) using UV-visible spectrophotometer (POLARstar OMEGA, BMG LABTECH GmbH, Germany). Chlorophyll content (chlorophyll a, chlorophyll b and total chlorophyll) was using a method described by Arnon (1949).



### 2.2.6 Metabolite sample preparation

Metabolite analysis by GC-MS was carried out by a modified method described by Glassop *et al.,* (2007). Three biological replicates of basal stem tissues were ground in liquid nitrogen using a mortar and pestle. Approximately 10 mg of tissue was weighed into micro-centrifuge tubes

containing 500 µl methanol (metabolite extracting agent), and 20 µl internal standard (ribitol [10 mg/ml]). Metabolic extracts were incubated at 70°C for 15 minutes. After incubation, 500 µl water and 400 µl chloroform was added to each extract and mixed by vortexing. The polar and non-polar phases were separated by centrifugation, at 12,000 rpm for 10 minutes. The phases were separated into clean tubes and 600 µl of chloroform added to the polar phase, vortexed, centrifuged and separated just as before. Aliquots of the polar phases were vacuum dried. The samples were stored at room temperature in a desiccator until further analysis.

The dried residues were resuspended in 50 µl of methoxyamine hydrochloride (20 mg/ml in pyridine) then incubated at 37°C for 120 minutes at 13,000 rpm in a Thermomixer comfort (Eppendorf South Pacific Pty Ltd, New South Wales, Australia). The samples were treated with N-Methyl-N-(Trimethylsilyl)–triflouroacetamide (MSTFA), and incubated at 37°C for 30 minutes. Samples were left for 2 hours before by centrifugation at 13,000 rpm for 2 minutes.

### 2.2.7 GC-MS analysis

Separation of the analytes was performed on an Agilent 6890 N Gas Chromatograph coupled with an Agilent 5975B Mass Spectrometer detector and a CTC CombiPAL Analytics Autosampler. This GC-MS system was equipped with a polar ZB-5MS GUARDIAN (30 m, 0.25 mm ID, 0.25 µm film thickness) capillary column (Phenomenex). One microliter of samples was

injected with splitless injection with a flow rate of 1 ml/min. The injection temperature was set at 280°C. The oven temperature was as follows: 5 minutes at 70°C, followed by a 1°C/min oven ramp to 76°C and a second ramp to 300 °C at 8 °C/min. The temperature was then equilibrated to 70°C before injection of the next sample. The mass spectra were obtained at two scans per sec with the scanning range of 40-650 *m/z*. ChemStation software was used to control the system for data acquisition. Authentic standards were unavailable so compounds were identified by using the NIST mass spectral library (www.nist.gov). The relative abundances of the metabolites were quantified by dividing the peak area of the compound by the peak area of the internal standard (ribitol).



### 2.2.8 Statistical analysis

Results are reported as the mean ± standard error (SE) of four independent determinants. For statistical analysis, a two-way analysis of variance (ANOVA) test was used to compare the effect of drought stress on leaf relative water content, growth parameters and chlorophyll content for all data. Means were compared according to the Student-T lsd (least significant differences) at 5% level of significance, using the statistical software SAS version 9.3 (SAS, 2012) statistical software.

## 2.3 Results

### 2.3.1 Effect of drought stress on growth parameters

When watering was completely withdrawn for 10 days, the soil water content fell progressively and at day 10 the soil was completely dry as indicated in Figure 2.1. There were however no significant differences in the rate of soil water decline amongst the sorghum varieties.

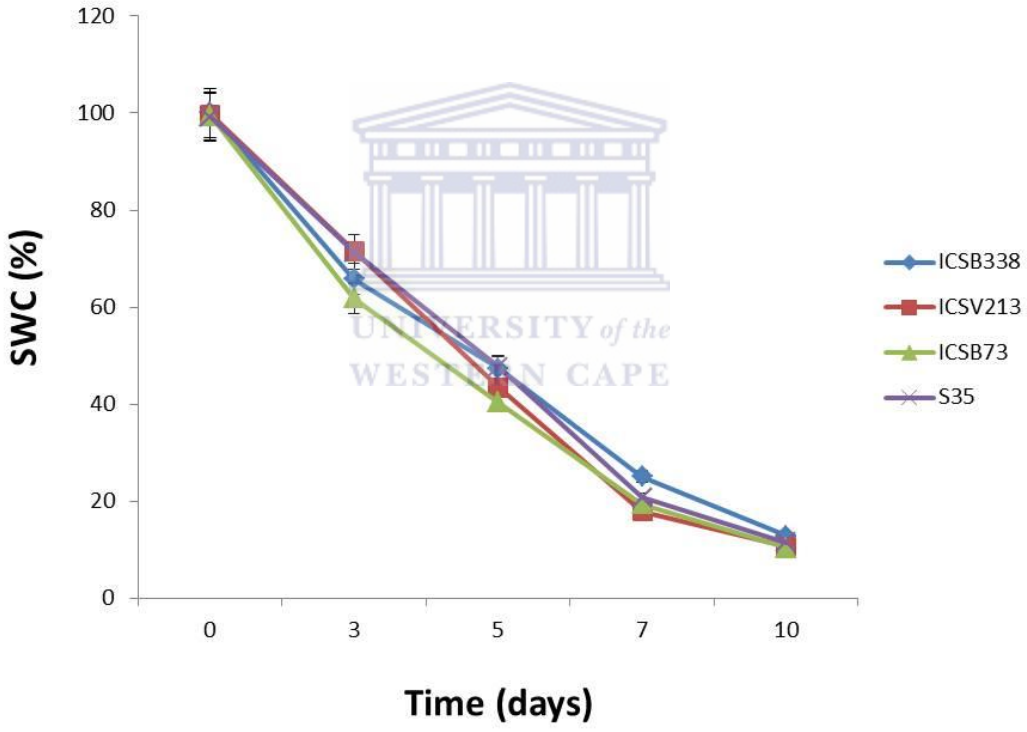


**Figure 2.1:** The change in soil water content of ICSB338, ICSB73, ICSV213 and S35 over 10 day water-deficit period. The data represents the mean ± SE of 4 replicates

Shoot length was not affected by stress induction as the watered plants and stressed plants across the varieties indicated the same height with intra varietal differences attributing to the obtained data (Figure 2.2).

There were distinct differences between varieties ICSB338 and ICSB73 versus ICSV213 and S35. Root length was increased with stress induction in all four varieties (Figure 2.3). Although there were no significant differences in the watered and stressed plants, there were distinct differences in varieties ICSB338 and ICSB73 versus ICSV213 and S35.

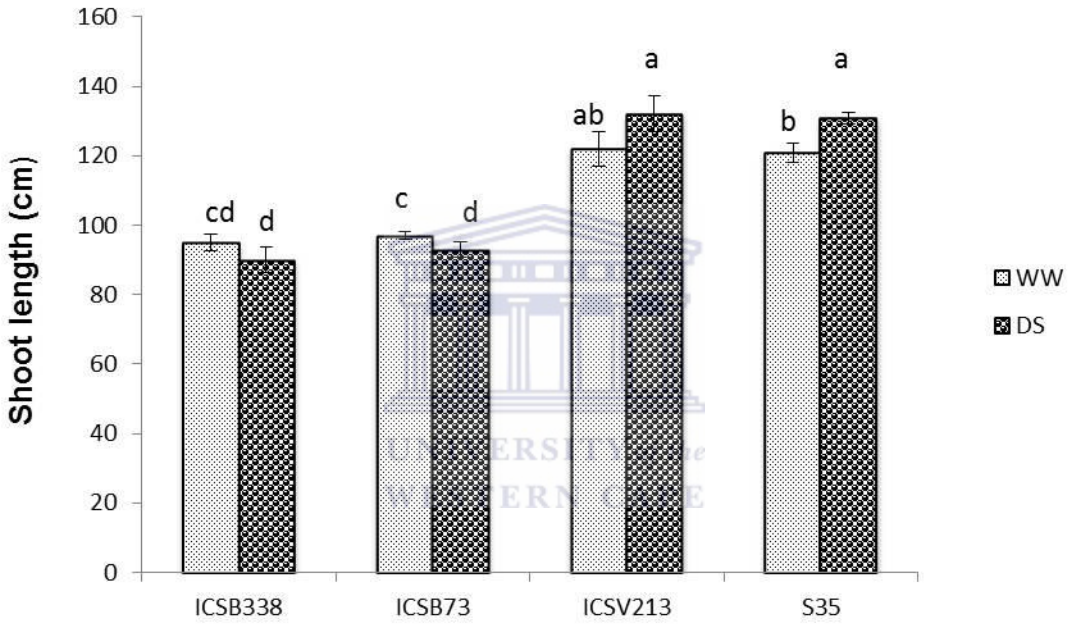


**Figure 2.2:** Shoot length of well watered (WW) and drought stressed (DS) sorghum varieties after 10 days of stress induction. The data represents the mean ± SE of 4 replicates

The basal stem biomasses were reduced with stress induction across varieties ICSB338, ICSB73, ICSV213 and S35 (Figure 2.4). In general the biomass of S35 stems were higher under both growth conditions when compared to varieties ICSB338, ICSB73 and S35 ,which had more or less the same weight.

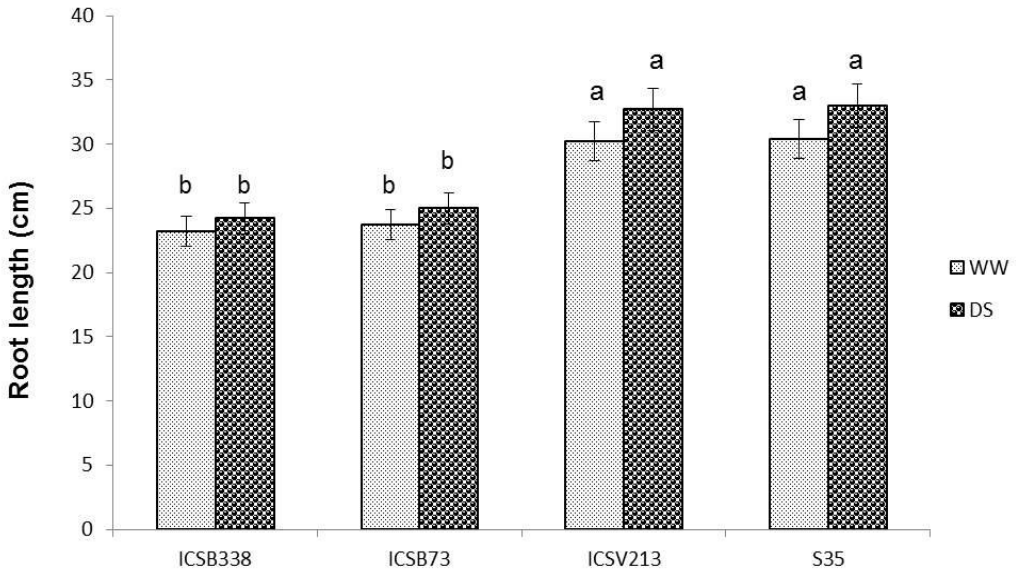


**Figure 2.3:** Root length of well watered (WW) and drought stressed (DS) sorghum varieties after 10 days of stress induction. The data represents the mean ± SE of 4 replicates



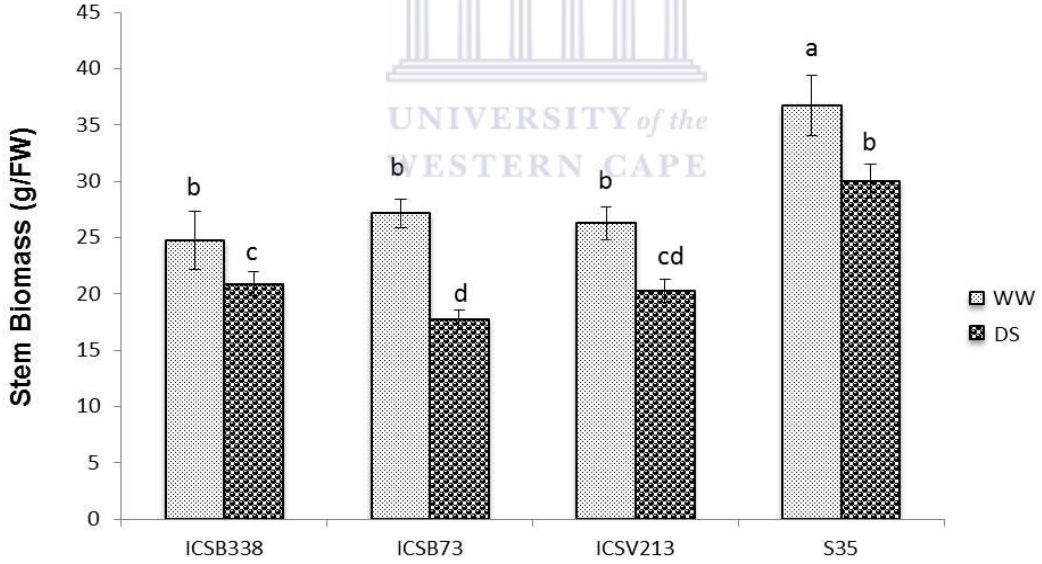


**Figure 2.4:** Stem biomass of well watered (WW) and drought stressed (DS) sorghum varieties after 10 days of stress. The data represents the mean ± SE of 4 replicates

### 2.3.2 Leaf relative water content (LRWC)

Water deficit for a period of 10 days influenced LRWC in all four sorghum varieties. The leaves of stressed plants showed a significant decrease in LRWC compared to the watered plants for all varieties. ICSV213 showed the highest reduction in LWRC with approximately 46%, whereas S35 had the lowest reduction with approximately 18% after 10 days of water deprivation. The LWRC for both ICSB338 and ICSB73 were reduced by approximately 27% compared to the watered plants (Figure 2.5).

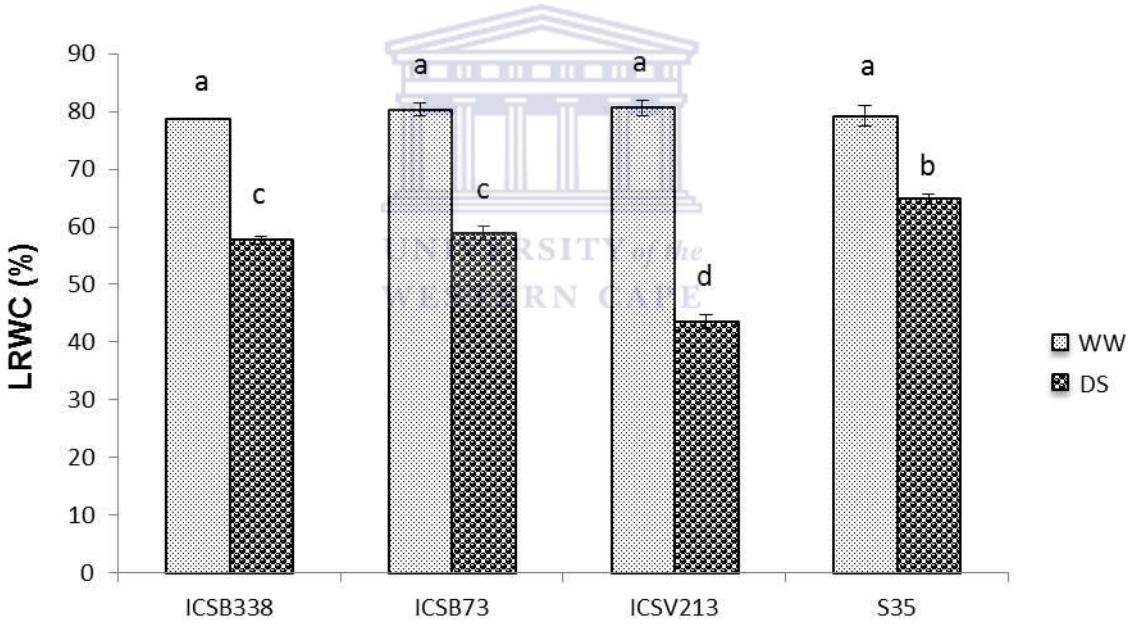


**Figure 2.5:** Percent of leaf relative water content (LRWC) of sorghum varieties ICSB338, ICSB73, ICSV213 and S35 after 10 days of drought stress induction. The data in bars represents the mean ± SE of 4 replicates

### 2.3.3 Chlorophyll content

Drought stress significantly affected the chlorophyll a, b and total chlorophyll levels in all four varieties. Chlorophyll a as shown in Figure 2.6 (A) presented reductions in ICSB338; ICSB73; ICSV213 and S35 at 12%, 21 %, 65 % and 23%, respectively. Chlorophyll b (Figure 2.6 B) showed a similar behavior, indicating that these chlorophyll pigments decrease in response drought stress. The decrease in total chlorophyll for ICSB338, ICSB73, ICSV213 and S35 were at 16%; 20%; 62% and 27%, respectively. Variety ICSV213 indicated the most significant reductions as shown in Figure 2.6 (C).

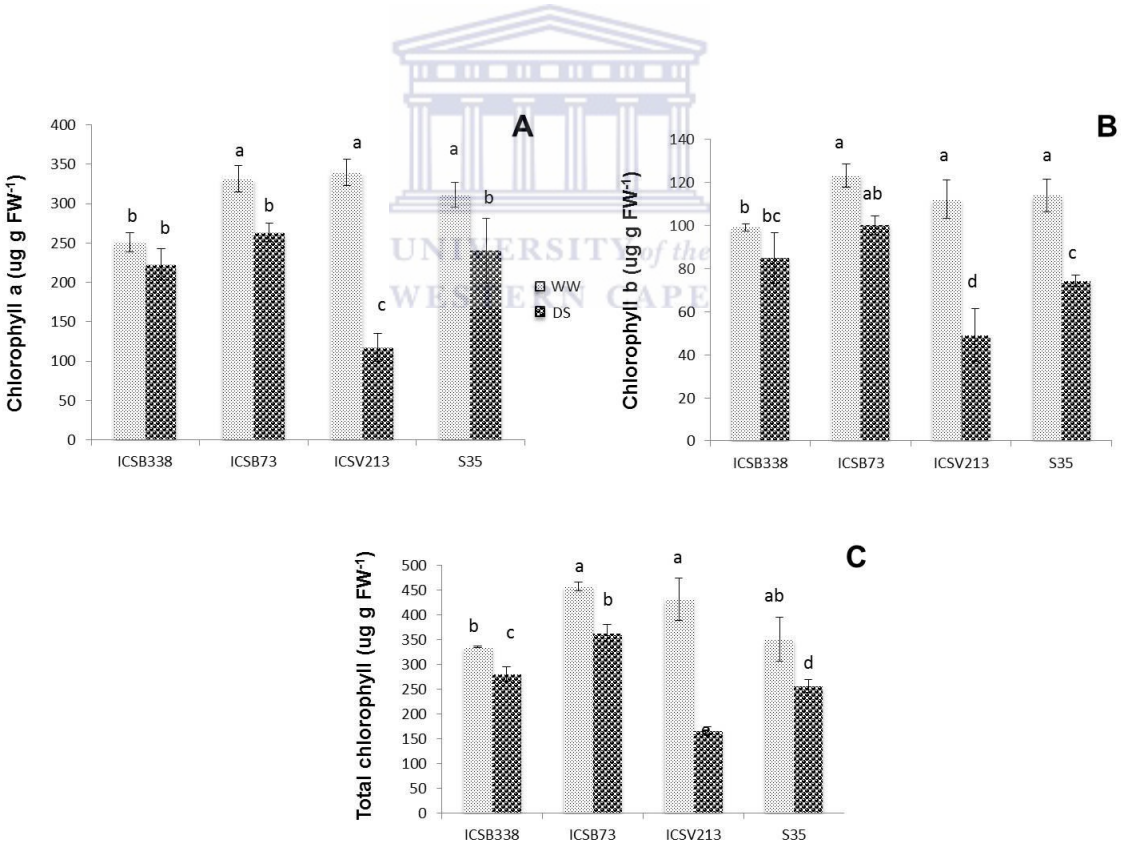


**Figure 2.6:** Analysis of chlorophyll pigments of Sorghum bicolor (L.) Moench varieties ICSB338, ICSV213, ICSB73 and S35 subjected to 10 days of water deficit. (A) is Chlorophyll a; (B) is chlorophyll b; (C) total chlorophyll. The bars represent the mean ± SE of 4 replicates

### 2.3.4 Metabolite profiling

A total of 21 polar metabolites from sorghum stem tissues were analyzed by GC-MS. Eighteen of these selected metabolites could be identified using a comparison of the retention time indices and mass spectra with existing standard library data. Differences in relative abundance of selected metabolites were found among the different varieties. For example, 5/7 amino acids, 1/2 organic acids and 4/9 sugars analyzed showed differences in abundance between watered and stressed plants (Table 2.1)

Metabolites whose amounts increased under drought stress in one or more variety were also evaluated. In particular, significant increases in abundance of proline and fructose were observed in stems of stressed varieties. Sucrose and leucine displayed an opposite behavior under stressed conditions and were found to be reduced in the stressed varieties. Insignificant changes were reported for the rest of the analyzed metabolites between the watered and stressed plants.

**Table 2.1:** Relative abundance (%) of selected compounds of sorghum stems in well watered and drought stressed plants

| Metabolite | 338W | 338DS | 213W | 213DS | B73W | B73DS | S35W | S35DS | Mass Ion | RT | Group |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Alanine** | 9.02 ± 0.05 | 7.94 ± 0.03 | 2.55 ± 0.00 | 17.5 ± 0.04 | 3.7 ± 0.00 | 7.27 ± 0.00 | 1.93 ± 0.01 | 2.79 ± 0.01 | **116** | **9.65** | **aa** |
| **Valine** | 0.34 ± 0.00 | 4.50 ± 0.01↑ | - | - | 12.6 ± 0.00 | 1.78 ± 0.04↓ | 1.25 ± 0.00 | 2.54 ± 0.00 | **144** | **12.29** | **aa** |
| **Serine** | 1.46 ± 0.02 | 3.26 ± 0.01 | 0.26 ± 0.01 | 0.15 ±0.00 | 1.81 ± 0.00 | 8.60 ± 0.02↑ | 1.45 ± 0.00 | 1.93 ± 0.00 | **204** | **15.06** | **aa** |
| **Proline** | 0.16 ± 0.00 | 25.5 ± 0.00↑ | 0.14 ± 0.02 | 2.11 ± 0.03 | 1.01 ± 0.02 | 41.5 ± 0.06↑ | 0.70 ± 0.00 | 12.7 ± 0.01↑ | **142** | **13.86** | **aa** |
| **Glutamine** | 0.25 ± 0.00 | 0.35 ± 0.00 | - | - | 0.16 ± 0.00 | 0.72 ± 0.00 | 0.23 ± 0.01 | 0.52 ± 0.00 | **347** | **21.42** | **aa** |
| **Leucine** | 8.70 ± 0.03 | 6.70 ± 0.02↓ | 4.80 ± 0.02 | 2.40 ± 0.01 | 49.4 ± 0.13 | 19.1 ± 0.03↓ | 25.0 ±0.00 | 5.20 ± 0.00↓ | **73** | **13.40** | **aa** |
| **Glycine** | 0.61 ± 0.00 | - | 0.24 ± 0.00 | 0.32 | 0.20 | - | 0.15 ± 0.00 | 0.41 ± 0.00 | **174** | **10.59** | **aa** |
| **Aspartic acid** | 1.45 ± 0.00 | 2.80 ± 0.01 | 0.34 ± 0.00 | 0.16 ± 0.00 | 1.57 ± 0.00 | 5.46 ± 0.02 | 1.88 ± 0.02 | 1.03 ± 0.00 | **232** | **17.75** | **TCA,oa** |
| **Malic acid** | 7.28 ± 0.03 | 8.10 ± 0.03 | 5.22 ± 0.05 | 6.22 ± 0.04 | 7.28 ± 0.00 | 16.2 ± 0.05↑ | 3.78 ± 0.01 | 12.9 ± 0.01↑ | **147** | **17.22** | **TCA,aa** |
| **Trehalose** | - | 4.12 ± 0.01 | 0.89 ± 0.01 | 0.19 ± 0.00 | 0.82 ± 0.00 | 0.54 ± 0.00 | 0.41 ± 0.00 | 0.54 ± 0.00 | **361** | **32.07** | **sugar** |
| **Maltose** | - | - | 0.61 ± 0.00 | 0.68 ± 0.00 | - | 0.36 ± 0.00 | - | 1.44 ± 0.00 | **361** | **31.98** | **sugar** |
| **Mannitol** | 3.12 ± 0.03 | 0.49 ± 0.00 | 1.72 ± 0.02 | 0.28 ± 0.01↓ | 0.18 ± 0.00 | 0.52 | 0.17 ± 0.05 | 0.94 ± 0.00 | **319** | **23.41** | **s.alc** |
| **Mannose** | 0.31 ± 0.00 | 2.43 ± 0.01 | 4.02 ± 0.03 | 2.05 ± 0.03 | 0.27 ± 0.00 | 4.31 ± 0.02 | 1.12 ± 0.01 | 6.65 ± 0.01 | **319** | **22.86** | **sugar** |
| **Galactose** | 2.16 ± 0.01 | 2.08 ± 0.01 | 7.05 ± 0.05 | 1.84 ± 0.01 | 1.13 ± 0.01 | 1.92 ± 0.01 | 0.24 ± .00 | 2.26 ± 0.00 | **451** | **38.32** | **sugar** |
| **Raffinose** | - | - | 0.21 | - | - | 0.27 ± 0.00 | - | 0.18 ± 0.00 | **319** | **22.91** | **sugar** |
| **Glucose** | 2.60 ± 0.01 | 2.56 ± 0.01 | 8.63 ± 0.06 | 2.21 ± 0.01 | 1.35 ± 0.00 | 2.30 ± 0.01 | 2.17 ± 0.00 | 2.68 ± 0.00 | **319** | **22.94** | **sugar** |
| **Fructose** | 1.87 ± 0.01 | 12.2 ± 0.00↑ | 7.49 ± 0.08 | 15.6 ± 0.05↑ | 1.18 ± 0.00 | 17.7 ± 0.05↑ | 6.70 ± 0.00 | 25.5 ± 0.02↑ | **307** | **23.14** | **sugar** |
| **Sucrose** | 61.8 ± 1.45 | 51.9 ± 1.34↓ | 64.5. ± 2.99 | 33.8 ± 1.00↓ | 94.1 ± 3.20 | 74.4 ± 0.05↓ | 39.2± 0.00 | 39.6 ± 0.19 | **361** | **31.12** | **sugar** |

**RT**- retention time; **aa** - amino acid; **oa** - oxalic acid; **TCA** - tricarboxylic acid cycle; **s.alc** - sugar alcohol

↑ Increase in relative abundance ↓ decrease in relative abundance

## 2.4 Discussion

Abiotic stresses, such as drought, pose a serious threat to crop productivity, more importantly to the sustainability of high crop yields (Slewinski, 2012; Anjum *et al.,* 2011; Mahajan and Tuteja, 2005). Drought stress triggers various responses in plants at different cellular levels. Sorghum is a cereal crop that has acquired immunity from drought stress through various adaptive mechanisms (Gill *et al.,* 2001). To breed for economic crops with improved drought tolerance, a better understanding of the traits associated with drought tolerant plants is crucial so these traits can be transferred into new varieties. In this study, physiological and biochemical assessments of the performance of four sorghum varieties under water limited conditions was carried out in order to improve understanding of stress tolerance mechanisms in sorghum.



The effectiveness of the induced drought stress was demonstrated by the decrease in soil water content and leaf relative water content (Figure 2.1 and Figure 2.5). Relative water content is considered an important indicator of water stress in leaves, which is directly related to soil water content (Hamad and Ali, 2014). The decrease in LRWC indicated that there was a resistance to the water-flow at soil-root interface, or a decrease in hydraulic conductivity of soil at low soil moisture (Kudoyarova *et al.,* 2011; Kholova *et al.,* 2010). The different levels at which LRWC declined amongst the varieties indicated that sensitivity to stress varied at anthesis. This could be attributed to the variations in transpiration rates amongst the varieties, which was determined by the conditions under which the plants were grown.

A decrease in LRWC in response to drought stress has been demonstrated in wide variety of plants, as reported by Nayyar and Gupta (2006). Plants subjected to drought stress generally exhibit reductions in LRWC and water potential, and these have been reported to cause changes in plant growth and metabolism (Anjum *et al.,* 2011; Neto *et al.,* 2009).

Photosynthesis is among the primary metabolic processes affected by water deficit (Flexas *et al.,* 2002; Chaves, 1991). The first response by plants to drought stress is through stomatal closure to prevent transpiration from occurring (Ghobadi *et al.,* 2013; Flexas *et al.,* 2004, Cornic, 2000). Stomatal closure limits the absorption of carbon dioxide thus leading to a decline in photosynthesis (Reddy *et al.,* 2004, Wingler *et al.,* 1999). Photosynthetic activity under stressed conditions was evaluated by determining chlorophyll content. A decline in concentration of the chlorophyll pigments was observed in all four varieties under stress conditions, with the highest reduction displayed in variety ICSV213 (Figure 2.6). The decline in chlorophyll content in stressed plants serves as an indicator that photosynthesis activity was negatively affected by stress. The changes in chlorophyll content, and consequently photosynthesis, are related to changes in carbon partitioning between the stem and roots (DaCosta and Huang, 2006; Massacci *et al.,* 1996; McCutchan and Shackel, 1992). A decrease in chlorophyll content under drought stress has been previously reported in pea, wheat (Huseynova 2012; Alexieva *et al.,* 2001); sorghum (Neto *et al.,* 2009) and apple (Sircelj *et al.,* 2007).

Loss in tissue water content due to stress induction reduces turgor pressure thereby inhibiting cell division and expansion (Namich, 2007; Delfine *et al.,* 2002). Therefore, the reduction in basal stem biomass (Figure 2.4) in all the sorghum varieties was indicative that the turgor and water potential decreased to a level which restricted cell enlargement. The positive correlation between the chlorophyll content and biomass production is an indication that photosynthesis is one of the major driving forces for biomass production in the sorghum varieties. Plants grown under water deficit conditions exhibited an increase in total root length when compared with plants under regularly supplied water. Root elongation is a mechanism sorghum varieties used to extract soil water in order to increase water potential, thus maintaining root turgor at a constant level under drought stress. Root elongation was however not significant to develop a steep water potential gradient to maintain water uptake, as evident from the compromised photosynthesis activity. The effect of stress on plant height amongst the varieties was inconclusive. Varieties ICSB338 and ICSB73 displayed a decrease in height under stress whereas ICSV213 and S35 had higher plant heights under stress. We therefore concluded that the differences observed within the varieties were not a result of the effect of stress but rather intra-plant variation. These results indicated the major impact of the developmental stage on plant response to drought stress.

In addition to alterations in the photosynthetic machinery and growth parameters, drought stress often induces osmotic adjustment, which is considered an important mechanism to allow the maintenance of water uptake under stress (Rosa *et al.,* 2009; Mahajan and Tuteja, 2005; Chaves and Oliveira, 2004). Osmotic adjustment is the active accumulation of solutes in response to imposed water deficit, which have a function in sustaining tissue metabolic activities such as the plant growth and development (Chaves *et al.,* 2009; Blokhina *et al.,* 2003). Of these metabolites, the accumulation of free amino acids, sugars and sugar alcohols is of great significance in osmotic adjustment and osmoprotection from abiotic stresses (Bohnert *et al.,* 2006).



Metabolic analysis was carried out to investigate the role of metabolites in stress response in the sorghum varieties. The metabolites selected for analysis have previously been documented in abiotic stress-related studies (Lu *et al.,* 2013; Gavaghan *et al.,* 2011; Gibon *et al.,* 2006, Taji *et al.,* 2002; Munns, 2002). Following 10-day water deficit period, only a small number of metabolites indicated significant changes in response to stress. An increase in abundance of amino acid content was observed for proline in all four varieties whereas the relative abundance of leucine was reduced with stress induction. The contents of all other amino acids were very low and did not contribute significantly to the amino acid pool. The accumulation of proline is frequently reported in many plants in response to abiotic stresses (Manivannan *et al.,* 2008; Heuer, 1994). The accumulation of this amino acid has been considered a general indicator of drought tolerance (Ahmed *et al.,* 2009; Liu *et al.,* 2003).

Similar results on proline accumulation were observed for pea and wheat grown under water deficit conditions (Alexieva *et al.,* 2001). These observations have also been reported in maize, demonstrating the major role of this amino acid in minimizing damage caused by dehydration (Mohammad and Heidra, 2008). Liu *et al.* (2003) reported that drought susceptible turfgrass varieties accumulated proline in response to drought stress. Gao *et al.,* (2009) reported proline accumulation in drought tolerant pinus species in response to drought stress. Thus, whether proline accumulation plays a role in osmoregulation or osmoprotection remains elusive. In contrast, stress induction had a negative effect on leucine content. Although reported in other experimental studies (Barchet *et al.,* 2013; Widodo *et al.,* 2009), the functional significance of the decline in leucine under drought is however not clearly understood.



Soluble sugars play complex essential roles in plant metabolism. They are products of hydrolytic processes, and act as substrates in signaling systems (Mohammadkhani and Heidari, 2008). Soluble sugars therefore may also function as osmoprotectants, stabilizing cellular membranes and maintaining cell turgor. The accumulation of sugars in response to drought stress has also been well documented (ElSayed *et al.,* 2013; Amudha and Balasubramani, 2011; Rosa *et al.,* 2009; Umezawa *et al.,* 2006; Massacci *et al.,* 1996). In this study, the soluble sugars displayed a variable response to drought stress amongst the four varieties (Table 2.1). Amongst the sugars analyzed, fructose and sucrose abundance was significantly affected by stress with an increase observed for fructose in all four varieties and a

decrease in sucrose in three of the four varietiesSoluble sugars had higher abundance as compared to amino acids. This indicated that the increase observed in the amino acids could therefore not be part of an adaptive response to stress but rather a consequence of reduced water availability (Munns, 2002), and an indicator of general stress and cell damage (Widodo *et al.,* 2009). These results also indicate the importance of these sugars in osmotic adjustment in sorghum.

In sorghum, drought tolerance has been estimated using a variety of parameters, including yield stability, leaf water potential, leaf rolling, root growth, osmotic adjustment, stomatal conductance, ABA accumulation, seedling establishment and growth and proline accumulation to list a few (Anjum *et al.,* 2011). The results of this study were indicative that drought stress brought out some adaptive effects in order to reduce damage experienced by sorghum varieties, with some parameters being more responsive than the others. The differential responses to drought stress amongst the sorghum varieties implied that the mechanisms conveying tolerance differed between these varieties. However these differences were negligible in comparison to the general response of each variety to stress leading us to infer the presence of a basic common response. From an ecological point of view we suggest that ICSV213 was the most responsive variety to drought stress.

The accumulation of osmolytes by this variety under conditions where photosynthesis was decreased is an indication that reengineering of the leaf structure may have occurred in this variety. This would be a mechanism whereby ICSV213 was altering its leaf tissues in order to maximize its survival under drought (Ogbaga *et al.,* 2014).

The physiological analysis carried out in this study was however not sufficient to conclude from an agricultural point of view, which variety would be preferred for crop breeding strategies. Other traits including grain yield stability, stomatal conductance, ABA accumulation and the stay-green ability needed to be taken into consideration in order to better understand the physiological basis of changes in drought resistance. This will allow for the selection or creation of new varieties of crops in order to obtain better productivity under water-limited conditions (Ghobadi *et al.,* 2013). Nevertheless, the highlighted role of soluble sugars in osmotic adjustment indicates that, the selection for high tissue sugar content, rather than the ability to respond dynamically to drought, may be a more successful approach in terms of crop breeding strategies for bio-energy production. Therefore, further studies on the underlying mechanisms regulating sugar metabolism under stress conditions should be considered.

# CHAPTER 3

## Sucrose biosynthesis genes mediate the physiological responses of sorghum varieties to hyperosmotic stress


### Abstract

Sucrose plays a key role in maintaining the osmotic equilibrium and in protecting cellular function in plants under adverse environmental conditions. This study investigated the regulation of sucrose genes after subjecting sweet sorghum varieties to a 10-day water deficit period. A detailed analysis on soluble sugars was carried out using HPLC, to determine the influence of water deficit on soluble sugar concentrations, and subsequently the expression of the sucrose biosynthesis genes. Sugar levels were elevated by drought stress induction in varieties ICSB338, ICSB73, ICSV213 and S35, with ICSV213 demonstrating the highest brix content. Sucrose and starch levels were decreased whereas hexose concentrations (glucose and fructose) were increased by stress in all four varieties. Variety ICSV213 demonstrated the highest glucose and fructose levels under stress. Activity assays of sucrose phosphate synthase, sucrose synthase, soluble acid and neutral invertase enzymes demonstrated increased activities in all varieties under stressed conditions. Overall the activity levels of invertase were high compared to sucrose phosphate synthase (SPS) and sucrose synthase (Susy) in all varieties. Gene expression analysis demonstrated changes in the sucrose biosynthesis genes with stress induction. SPS was up regulated with variety ICSV213 displaying the highest expression levels.

The response of the invertases to stress was evident only in ICSV213, whereas sucrose synthase displayed significant regulation only in ICSB73. Sucrose transporters also demonstrated a response to stress in all four varieties with significant up regulation observed in ICSV213 and S35. The correlation of sugar levels with gene expression in ICSV213 suggested the possible role of sucrose biosynthesis genes in the accumulation and mobilization of sorghum stem reserves during drought stress.

## 3.1 Introduction

Plant metabolic adjustments form the basis of survival under adverse environmental conditions that affect plant cellular processes. Plants accumulate metabolites that act as osmotic balance agents to maintain cell turgor and to stabilize cell proteins and structures during periods drought stress (Seki *et al.,* 2007). Alteration in photosynthesis and carbon partitioning is the most common feature that takes place at a whole plant level under stress conditions and forms the basis of crop productivity (Slewinski, 2012). Sucrose accumulation is a common metabolic response when plants are subjected to drought stress. As a result, sucrose is considered a major osmoprotectant involved in regulating osmotic potential in plants offering resistance against drought stress (Djilianov *et al.,* 2005). Sucrose metabolism is subject to the expression and regulation of enzymes that ensure the unidirectional flow of sucrose from the phloem to the sink tissues where it serves as a source of energy or storage reserves (Sturm and Tang, 1999).

Three key enzymes regulate sucrose metabolism and have been implicated in carbon partitioning thereby influencing plant growth and development (Qazi *et al.,* 2012). Sucrose phosphate synthase (EC 2.4.1.14) catalyzes the synthesis of sucrose in photosynthetic cells using fructose-6-P (Fru-6-P) and glucose and is also active in the futile cycle of simultaneous breakdown and synthesis of sucrose in various tissues (Park *et al.,* 2008). Invertase (EC 3.2.1.26) catalyzes the conversion of sucrose to glucose and fructose and is involved in phloem unloading and the control of cell differentiation and development (Huang, 2003). Sucrose synthase (EC 2.4.1.13) reversibly converts sucrose into UDP-glucose and fructose in the presence of uridine 5'-diphosphate (UDP) and its activity is suggested to be associated with sink strength (Winter and Huber, 2000). Sucrose metabolism is also regulated by a host of membrane transporters that facilitate the movement of sucrose between source and sink tissues (Ibraheem *et al.,* 2011; Ibraheem *et al.,* 2008; Lalonde *et al.,* 2004; Li *et al.,* 2003). Koch (2004) has reported on the modulation of these sucrose biosynthesis genes under stress conditions resulting in sucrose flux, therefore suggesting their possible roles as stress-responsive proteins.

Due to its drought tolerance and multi-purpose traits, sorghum is gaining prominence as a substitute for sugarcane to produce sucrose for food and ethanol production (Rao *et al.,* 2008). There is much interest in developing varieties that will sustain next-generation bio-fuel in arid and semi-arid regions (Slewinski, 2012).

There are different classes of sorghum with grain and sweet being the most commonly grown. These two classes differ in carbohydrate partitioning with sucrose content being predominant in the grain of grain sorghum, and in the stem of sweet sorghum respectively (Rooney *et al.,* 2007). Gaining an understanding in the underlying processes that lead to the differential accumulation in the different sorghum varieties is important for the genetic engineering of super sorghum crops with high nutritional and energy value under adverse environmental conditions.

As mentioned previously, sucrose metabolism is regulated by the differential expression of various enzymes that influence plant physiology and productivity. Therefore, a comparative study of gene expression of these enzymes and regulatory networks involved in sucrose metabolism may be useful in the identification of molecular markers that are involved in molecular adaptation or tolerance mechanisms during drought conditions. These may be exploited in breeding programs to improve the growth performance or increase the yields of sorghum varieties under drought stress. The main objective of this chapter was to determine the effect of drought stress on the expression levels of sucrose-biosynthesis genes, with the aim of elucidating differences in expression in the different varieties under different growth conditions.

## 3.2 Materials and Methods

### 3.2.1 Plant material

*Sorghum bicolor* (L.) *Moench* varieties ICSB338; ICSB73; ICSV213 and S35 were provided by the International Crop Institute for the Semi-arid Tropics (ICRISAT), India. Plants were grown as previously described in Section 2.2.1. At anthesis, the basal stems (5cm from the soil) were separated from the rest of the plant and chosen for soluble sugar, protein and RNA analyses.

### 3.2.2 Extraction and determination of total soluble sugars

The external layer of green parenchyma of the basal stem was rapidly removed to avoid interference caused by pigments during sugar extractions. The stem (500 mg) was finely crushed using a pre-cooled pestle and mortar. The homogenized tissue was put in a 10 ml syringe and the sap collected by pressing the tissue with a plunger into a pre-cooled 1.5 ml microcentrifuge tube. The sap was then centrifuged in a pre-cooled Eppendorf centrifuge 5414 (Eppendorf, GERMANY) at 13000 rpm, at 4°C for 5 min. The supernatant was collected and either used for soluble sugar determination immediately or stored at -20 °C. The brix (total soluble solids expressed as a percentage) was assayed from the sap using a common laboratory refractometer (OTAGO$^{TM}$, Germany). Soluble sugars (sucrose, glucose and fructose) were extracted from stem in 80% acetone (v/v).

After incubating the solution in a water bath at 80°C for 1 hour, the insoluble fraction was removed by centrifugation at 13000 rpm for 10min at room temperature. The extraction process was repeated three times. Supernatants were pooled and vacuum dried in the Savant speedvac (Thermo Scientific, USA) and re-suspended in 1 ml distilled water and used immediately. Soluble sugars (glucose, fructose and sucrose) were quantified using HPLC system (Dionex $^{TM}$ Ultimate 3000) on a Rezex RHM monosaccharide column with operating conditions of 25 °C, and 10 mM $H_2S0_4$ as mobile phase at a flow rate of 0.6 ml/min. Each sample was injected into the column by an Ultimate 3000 auto-sampler and the sugar components were detected using a refractive index detector (Shodex RI 101). Concentrations of sucrose, glucose and fructose were calculated from peak heights using standards (Sigma-Aldrich, Germany). Analyses were performed in triplicate using three separate extractions for each treatment and all results were expressed in g/l.



### 3.2.3 Determination of starch content

To estimate starch content in plant tissue samples, starch is hydrolyzed to its monomeric form, glucose, which is subsequently assayed. Starch was extracted using a modified extraction procedure of Beutler (1984). Starch was determined from remaining pellets after extraction of soluble sugars. The pellets were vacuum-dried and 60mg was boiled at 95 °C for 60 min in 0.2 M KOH in order to gelatinize the starch.

The extracts were then cooled rapidly to room temperature and the pH adjusted to 4.8 by the addition of 50 mM sodium acetate buffer (pH 4.8). The gelatinized starch in the supernatant was hydrolyzed with 4 units of amyloglucosidase (AMG) from *Aspergillus amyloglucosidase* (Sigma) in 50 mM sodium acetate buffer (pH 4.8) at 55 °C overnight. The reaction was terminated by boiling the sample at 100 °C for 15 minutes. Samples were then centrifuged at 12000 rpm for 10 min room temperature, vacuum dried and resuspended in distilled water. The glucose released was detected using the HPLC as described in Section 3.2.2.

### 3.2.4 Activity assays

#### 3.2.4.1 Protein extraction for sucrose-enzyme activity assays



Crude protein extracts were prepared from stem tissues of sorghum varieties and ground in liquid nitrogen using chilled pestle and mortar. The extraction buffer used consisted of 100 mM Hepes-KOH (pH 7.5), 2 mM EDTA, 5 mM DTT, 4 mM $MgCl_2$, 10% glycerol and 2% PVPP. Extracts were centrifuged at 13200 rpm at 4°C for 4 minutes. Supernatants were transferred to new micro-centrifuge tubes and centrifuged as described earlier. For desalting, the crude protein extracts and the supernatants were transferred to Sephadex G-50 spin columns pre-equilibrated in extraction buffer and centrifuged for 2 min at 4000 rpm at 4 °C. The supernatant containing soluble protein was collected and used immediately in protein quantification and activity assays or stored at -80°C.

### 3.2.4.2 Protein quantification for sucrose-enzyme activity assays

The concentration of all protein extracts was determined using a modified Bradford Assay (Bradford, 1976), as previously described by Ndimba *et al.*, (2003). Bovine serum albumin (BSA) standards were prepared in triplicates from a 1 mg/ml BSA stock solution in a bottom-flat micro-titer plate as indicated in Table 3.1. Protein extracts were prepared in triplicates by mixing 10 µl of unknown protein sample with a 190 µl of diluted Bradford reagent (BIO-RAD). The same volume of the diluted Bradford reagent was added to 10 µl of the standards to make up a total volume of 200 µl. The flat-bottom micro-titer plate containing standards and protein extracts was incubated for 5 min at room temperature. Absorbance was measured at 595 nm in a POLARstar OMEGA micro-titer plate reader (BMG LABTECH GmbH, Germany) using Bradford reagent as a blank. BSA standards were used to derive a standard curve from which concentrations of all the unknown protein extract samples were extrapolated.



**Table 3.1:** Preparation of BSA protein standard for protein quantification

| **Standard Concentration [µg/µl]** | **BSA 1mg/ml stock solution (µl)** | **Distilled water (µl)** |
|---|---|---|
| 0 | 0 | 100 |
| 2.5 | 12.5 | 87.5 |
| 5 | 25 | 75 |
| 7.5 | 37.5 | 63.5 |
| 10 | 50 | 50 |
| 12.5 | 62.5 | 37.5 |

### 3.2.4.3 Invertase activity assay

Neutral invertase and acid invertase activities were determined according to Rossouw *et al.,* (2010). Acid invertase was assayed by incubating desalted crude protein samples at 35 °C for 30 min in assay buffer consisting of 50 mM citrate phosphate (pH 5.5) and 125 mM sucrose. The reaction was stopped by incubating the samples at 90 °C for 2 min followed by subsequent freezing in liquid nitrogen after cooling. Samples were used immediately or stored at -80°C until required. Neutral invertase activity was assayed by incubating desalted crude protein extract in assay buffer consisting of 50 mM Hepes-KOH (pH 7.5) and 125 mM sucrose. The reaction was stopped by the addition of 2 M Tris-HCl (pH 8.0) and 22 mM $ZnSO_4$ solution followed by the freezing of samples in liquid nitrogen and storage at -80°C until required.



The amount of reducing sugars in the samples was measured using a NAD coupled reaction according to Huber and Akazawa (1986). The reaction mixture consisted of 50 mM Hepes-KOH (pH 7.5), 2 mM $MgCl_2$, 15 mM KCl, 0.4 mM NAD, 1mM ATP and 4 U Hexokinase/Glucose 6-phosphate dehydrogenase (HK/G6-PDH). The reaction was initiated by the addition of the reducing sugars resulting from the neutral and acid invertase activities. NADH production was monitored at 340 nm.

### 3.2.4.4 Sucrose synthase activity assay (synthesis direction)

Sucrose synthase was assayed using a coupled reaction according to Schafer *et al.,* (2004). Desalted crude protein samples were incubated in assay buffer consisting of 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 20 mM UDP-glucose, 0.2 mM NADH, 1 mM phosphoenolpyruvate and 0.45 U/ml pyruvate kinase/lactate dehydrogenase (PK/LDH). Reactions were started by the addition of 10 mM fructose. The decrease in the absorbace of NADH was monitored at 340 nm. Activity was calculated in terms of pmols NADH oxidized per mg protein per minute.



### 3.2.4.5 Sucrose synthase activity assay (cleavage direction)

The catalytic activity of sucrose synthase in the breakdown direction was assayed using a NAD-coupled reaction according to Schafer *et al.,* (2004). Desalted crude protein samples were incubated with assay buffer consisting of 100 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 400 mM sucrose, 2 mM $NAD^+$, 1.5 mM sodium pyrophosphate, 4 U/ml phosphoglucomutase (PGM) and 4U/ml glucose-6-phosphate dehydrogenase (G-6-PDH). Reactions were started by the addition of 1.5 mM uridine diphosphate (UDP). NADH production was monitored at 340 nm. Sucrose synthase activity was calculated in terms of pmols $NAD^+$ produced per mg protein per minute.

### 3.2.4.6 Sucrose phosphate synthase (SPS) activity assay

SPS activity was assayed according to Baxter *et al.,* (2003) under maximal ($V_{max}$) and limiting ($V_{lim}$) reaction conditions. Desalted crude protein sample (50 µl) was incubated for 30 min at 35 °C in assay buffer (50 mM HEPES-KOH, pH 7.5, 20 mM KCl and 4 mM $MgCl_2$) containing (a) $V_{max}$ assay; 12 mM UDP-Glc, 10 mM Fruc 6-P and 40 mM Glc-6-P, or (b) $V_{lim}$ assay; 4 mM UDP-glucose, 2 mM Fru-6-P, 8 mM Glc-6-P and 5 mM $KH_2PO_4$. The reaction was stopped by heating at 95 °C for 5 min followed by centrifugation at 13200 rpm for 5 min. To remove the non-reacted phosphates, 5 M KOH was added to the supernatant and incubated at 95 °C for 20 min. Following cooling, anthrone reagent (0.14% anthrone in 14.6 M $H_2SO_4$) was added to the sample and incubated at 40 °C for 20 min. The absorbance was measured at 620 nm using the POLARstar OMEGA spectrophotometer and the sucrose content determined using a standard curve with 0-200 nmol sucrose.



### 3.2.5 Analysis of differentially expressed sucrose biosynthesis genes

#### 3.2.5.1 Total RNA isolation and cDNA synthesis

Stem tissues harvested from the groups of four biological replicates of each variety were ground to powder in liquid nitrogen prior to RNA extractions. RNA from the ground tissues was extracted using the SV 96 Total RNA isolation system (#Z3105, Promega, USA), following the manufacturer's instructions.

Extracted RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA) at 260 nm and electrophoresed on a 3 % agarose gel to verify its integrity. Approximately 500 ng of RNA from each sample was reverse-transcribed into cDNA using the High Capacity cDNA synthesis Kit (#4368814, Life Technologies, USA) following the manufacturer's instructions. The synthesized cDNA was stored at -20 °C until further use. Before expression analysis, each experimental sample was diluted 1:4 with nuclease-free water (BIO-37080 Bioline Water, 18.2MΩ PCR Grade).

### 3.2.5.2 Relative quantification of sucrose biosynthesis genes by Real-Time PCR



The mRNA sequences of sorghum genes coding for sucrose synthases, sucrose phosphate synthases, invertases and sucrose transporters and the housekeeping genes, (β-actin, ubiquitin and 18S RNA), were obtained from the NCBI database (http://blast.ncbi.nlm.nih.gov/BLAST).

Gene-specific primers were designed so that they would bind to all variants of the target genes, and in areas that do not have secondary structure. The primers were designed across the intron/exon boundary using PerlPrimer (http://perlprimer.sourceforge.net/) software.

Secondary structure analysis was performed using Oligo Analyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx) to check for primer dimers.

Reactions were carried out on ABI 7900HT Real Time PCR system with SYBR® Green PCR Mastermix (#4367650, Life Technologies, USA), following the manufacturer's instructions. The thermal cycling conditions are stipulated in Table 3.2. The primer sequences, expected product size and annealing temperatures given in Table 3.3.

**Table 3.2:** qPCR thermal cycling parameters

| Step | Temperature (°C) | Time (sec) | Number of cycles |
|---|---|---|---|
| **Hold** | 95 | 600 | 1 |
| **Denature** | 95 | 15 | 40 |
| **Anneal/Extend** | 60 | 15 | 40 |

**Table 3.3:** Sorghum sucrose biosynthesis gene primer pairs used for gene expression studies

| Gene | Accession number | Primer sequence | Product size | Annealing temperature |
|---|---|---|---|---|
| **18S RNA** | Sb03g017560 | FP5'CCTTGAAACAACAACGATTA'3<br>RP5'CTGTGTCTAGGACCAGTA '3 | 136 | 56°C |
| **B-actin** | X79378 | FP5'CCTTACCGACTACCTCAT'3<br>RP5'GTGGATATTAGGAAGGATCTAT'3 | 329 | 53°C |
| **Ubiquitin** | Sb04g031060 | FP5'GCCAAGATTCAGGATAAG'3<br>RP5'CACATTGGCTGATTACA'3 | 326 | 51°C |
| **SUT** | Sb01g045720 | FP5'GTGCTCATCTGCATTGCTGT'3<br>RP5'CCACAAACAATTGGCACAAG'3 | 303 | 54°C |
| **SPS** | Sb05g007310 | FP5'GCAAACCTTACGCTGATACTG'3<br>RP5'CTTGTGGTGCTTAGGGTAGG'3 | 141 | 55°C |
| **Susy** | Sb01g033060 | FP5' ATGGTATTCTCCGCAAGTGG'3<br>RP5' CCTGCGATTTCTTGGAATGT'3 | 346 | 52°C |
| **Inv** | Sb04g000620 | FP5'CATCGTTGCAGGGTATCCC'3<br>RP5'GTAGTCGATGGTGATGCCG'3 | 134 | 56°C |

FP-forward primer RP-reverse primer

PCR products were detected by monitoring the increase in fluorescence of the SYBR Green dye during the extension phase of each cycle when the SYBR Green dye binds to double-stranded DNA. The results obtained for the different cDNAs were normalized against the expression levels of three stable housekeeping genes: sorghum β-actin, ubiquitin and 18S RNA. The expression of the three housekeeping genes was averaged using a multiple reference gene normalization method adapted from Hellemans *et al.,* (2007)

### 3.2.6 Statistical analysis



All experiments were performed three times, independently. Results are reported as the mean ± standard error (SE) of four independent determinants. For statistical analysis, a two-way analysis of variance (ANOVA) test was used to compare the effect of drought stress on soluble sugar content, starch content, enzymatic activity and gene expression for all data. Means were compared according to the Student-T lsd (least significant differences) at 5% level of significance, using the statistical software SAS version 9.3 (SAS, 2012) statistical software. The data analysis, for the relative mean fold expression level and standard error of each of the genes was determined for the control and drought-stressed groups by reference to the sample with lowest expression for each gene, using SDS v2.3 software (Life Technologies, USA) and qBase+ produced by BioGazelle (Ibraheem *et al.,* 2011).

## 3.3 Results

### 3.3.1 Sugar metabolism in the basal stem with stress induction

Stress induction elevated the total soluble sugar levels (brix) in the basal stems of all four varieties. Figure 3.1 (A) shows that ICV213 presented the most significant difference in sugar content with a 35 % increase in stressed plants compared to the watered. The other varieties showed no significant differences between stressed and watered plants. Measurement of individual soluble sugars (Figure 3.1 B-D) revealed a general pattern across the different varieties, where the sucrose levels were significantly decreased by stress with the exception of S35, although the levels were very low. Glucose and fructose concentrations were significantly increased. The decline in sucrose concentration was prominent in ICSB73 variety with a 50 % decrease (Figure 3.1 B). Fructose and glucose accumulation under stressed conditions was the most significant in ICSV213 with a 50% increase recorded (Figure 3.1 C and D). Fructose was the prominent sugar produced in the stems of all well watered varieties with the exception of variety S35. Figure 3.2 indicates that water deprivation affected the starch concentration in all four varieties under study. Starch levels under watered conditions were highest for ICSB338 and similar for ICSB73, S35 and ICSV213. Stress significantly reduced the concentration levels of starch in all four varieties. Variety ICSB338 displayed the most prominent reduction in starch concentration relative to other varieties with, a 60% decrease under stressed conditions.

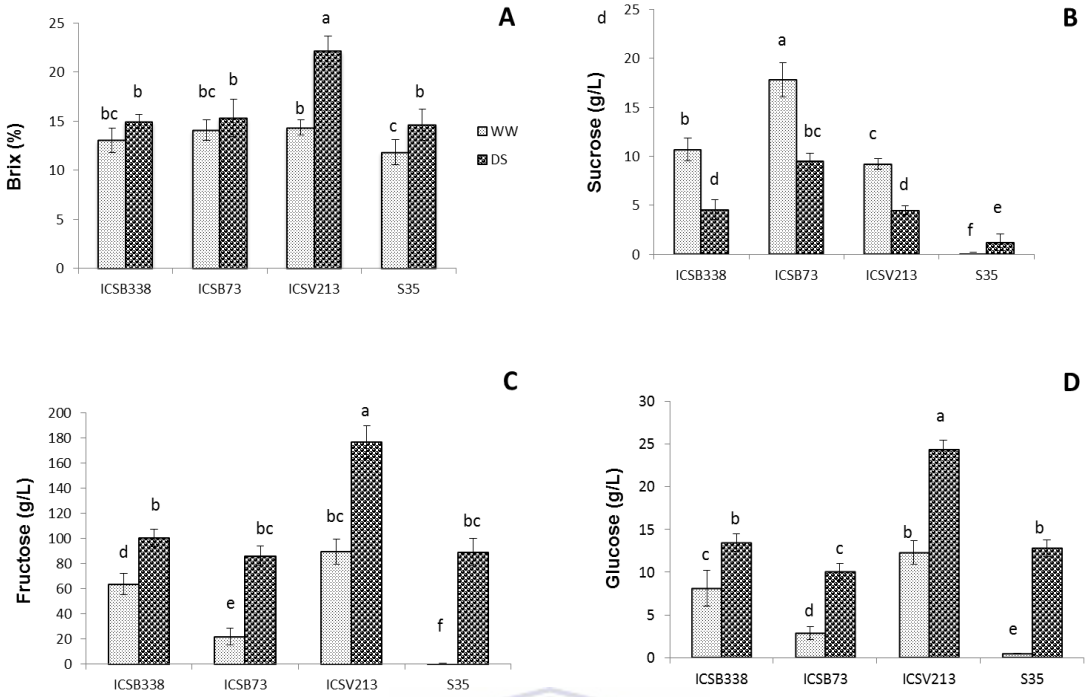


**Figure 3.1:** Shows (A) brix; (B) sucrose; (C) fructose; (D) glucose accumulation in the stem of 80 day old ICSB338, ICSB73; ICSV213 and S35 sorghum plants subjected to 10 days of water deficit. Each data point is an average of 4 biological samples. The error bars represent the mean standard error obtained from the SAS (Version 9.3) analysis. Well watered (WW); drought stressed (DS)

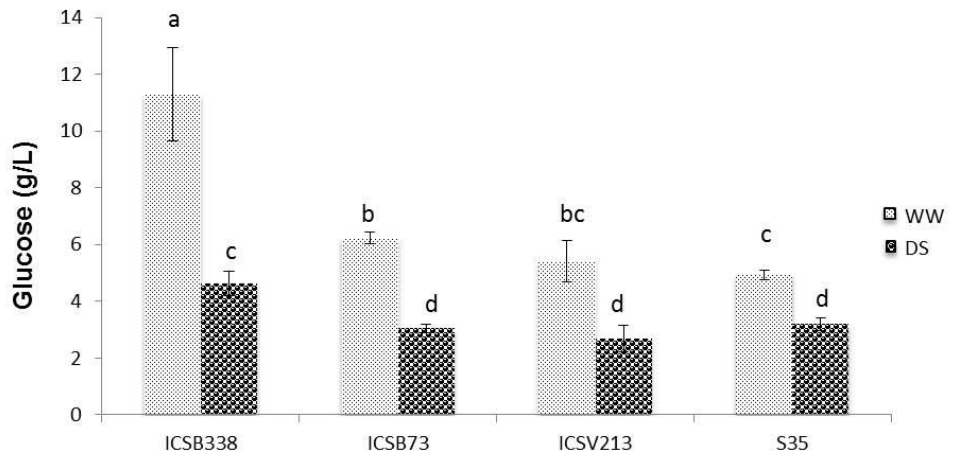


**Figure 3.2:** Starch content in the stem of 80 day old ICSB338; ICSB73; ICSV213 and S35 sorghum plants subjected to 10 days of water deficit. Each data point is an average of 4 samples. The error bars represent the mean standard error obtained from the SAS (Version 9.3) analysis. Well watered (WW); drought stressed (DS)

### 3.3.2 Sucrose biosynthesis enzyme activity assays

SPS activity under $V_{lim}$ (limiting substrate) and $V_{max}$ (excess substrate) assay conditions are shown in Table 3.4. Under both assay conditions, SPS activity was significantly increased in varieties ICSB338, ICSV213 and S35 with stress induction. Drought-induced hyperosmotic stress had insignificant effect on SPS activity for the ICSB73 variety when compared to the other varieties. This is demonstrated by the 4% and 15% change observed under $V_{lim}$ and $V_{max}$ conditions compared to the above 40% activity increase detected in the other varieties.

**Table 3.4:** SPS activity in mature sorghum stems under limiting ($V_{lim}$) and maximal ($V_{max}$) conditions. Activity expressed in pmol/mg protein/min. Mean ± SE, n=4

| Variety | $V_{lim}$ | $V_{max}$ |
|---|---|---|
| ICSB338 | 71.5 ± 1.2 $_b$ | 135.5 ± 0.2 $_b$ |
| ICSB338-ds | 128.0 ± 1.2 $_a$ | 249.4 ± 2.3 $_a$ |
| ICSB73 | 29.7 ± 0.7 $_e$ | 32.8 ± 0.1 $_e$ |
| ICSB73-ds | 31.0 ± 0.9 $_d$ | 38.9 ± 0.5 $_e$ |
| ICSV213 | 17.9 ± 0.1 $_f$ | 31.2 ± 0.8 $_e$ |
| ICSV213-ds | 59.2 ± 0.2 $_c$ | 72.0 ± 1.1 $_c$ |
| S35 | 7.5 ± 0.03 $_g$ | 8.20 ± 0.2 $_f$ |
| S35-ds | 34.0 ± 0.7 $_a$ | 37.8 ± 1.0 $_d$ |

**ds-** drought stress



As shown in Table 3.5, ICSB338 Susy activity favored synthesis under both watered and stressed conditions although there was a decrease in synthesis by 11% under stress as compared to watered conditions. Sucrose synthesis was favoured under watered conditions in variety ICSB73. With stress induction however, the synthesis levels declined resulting in sucrose breakdown being more favorable with a 1.8 fold difference compared to sucrose synthesis. ICSV213 Susy activity was directed towards sucrose synthesis under both growth conditions. Activity was enhanced with stress induction with the variety displaying a 2-fold increase in activity. In the S35 variety, sucrose synthesis was favored under both watering regimes. There was however a 44% decline in synthesis under stressed conditions.

**Table 3.5:** Susy activity in mature sorghum stems in the breakdown and synthesis direction. Activity expressed in pmol/mg protein/min. Mean ± SE, n = 4

| Variety | Sucrose breakdown | Sucrose synthesis |
|---|---|---|
| ICSB338 | 6.5 ± 0.3 $_{b}$ | 14.1 ± 0.2 $_{d}$ |
| ICSB338-ds | 7.4 ± 0.7 $_{b}$ | 12.5 ± 0.2 $_{c}$ |
| ICSB73 | 4.1 ± 0.2 $_{b}$ | 11.1 ± 0.1 $_{d}$ |
| ICSB73-ds | 6.5 ± 0.4 $_{c}$ | 3.6 ± 0.04 $_{b}$ |
| ICSV213 | 3.1 ± 0.6 $_{c}$ | 11.9 ± 0.2 $_{d}$ |
| ICSV213-ds | 3.7 ± 0.7 $_{c}$ | 23.6 ± 0.2 $_{e}$ |
| S35 | 4.5 ± 0.7 $_{c}$ | 27.0 ± 0.8 $_{c}$ |
| S35-ds | 8.7 ± 0.7 $_{a}$ | 15.0 ± 0.8 $_{a}$ |

**ds-** drought stress



Soluble acid invertase activity was triggered by stress induction with all four varieties displaying increased activity in stressed plants compared to the watered (Table 3.6). Drought-induced hyperosmotic stress had less effect on the acid invertase activity in variety ICSV213 when compared to the other varieties. This is demonstrated by the 18% change observed under stressed conditions compared to the above 50% activity increase detected in other varieties. A similar pattern was observed for ICSV213 neutral invertase, which had the least significant change compared to the above 50% activity increase detected in other varieties under stressed conditions.

**Table 3.6:** Acid invertase activity in mature sorghum stems. Activity is expressed in pmol/mg protein/min. Mean ± SE, n = 4

| Variety | Watered | Stressed |
|---|---|---|
| ICSB338 | 18.7 ± 0.4 $_{f}$ | 42.1 ± 1.3 $_{e}$ |
| ICSB73 | 11.3 ± 0.3 $_{g}$ | 66.8 ± 2.5 $_{d}$ |
| ICSV213 | 77.3 ± 0.4 $_{c}$ | 94.3 ± 0.7 $_{b}$ |
| S35 | 20.9 ± 0.6 $_{f}$ | 170.1 ± 2.6 $_{a}$ |

**Table 3.7:** Neutral invertase activity in mature sorghum stems. Activity expressed in pmol/mg protein/min. Mean ± SE, n = 4

| Variety | Watered | Stressed |
|---|---|---|
| ICSB338 | 14.3 ± 0.2 $_{f}$ | 39.0 ± 0.5 $_{d}$ |
| ICSB73 | 7.8 ± 0.2 $_{g}$ | 60.2 ± 1.1 $_{c}$ |
| ICSV213 | 43.4 ± 1.3 $_{c}$ | 83.2 ± 0.5 $_{b}$ |
| S35 | 18.7 ± 0.5 $_{e}$ | 165.5 ± 1.1 $_{a}$ |

### 3.3.3 Differential gene expression in sorghum stem tissues during drought induction

Figure 3.3 shows the low gene expression levels detected for invertase in varieties ICSB338, S35 and ICSB73 (Figure 3.3 A). Moreover these varieties ICSB338, ICSB73 and S35 displayed insignificant changes in expression levels with stress induction. Interestingly, an 11-fold increase in ICSV213 invertase expression levels was observed in the stressed plants compared to the watered.

An increase in sucrose phosphate synthase (SPS) expression levels relative to controls were observed in stressed plants of ICSB73, ICSV213 and S35, at 3.7, 4.5 and 2-fold increases respectively (Figure 3.3 B). ICSB338 displayed lowest levels of SPS expression relative to the three varieties reported. Stress induction further repressed the low expression observed for the ICSB338 SPS.



A decrease in sucrose synthase expression levels relative to watered plants was observed in ICSB338 and ICSV213 whereas; stress induction triggered increases in expression for ICSB73 and S35 (Figure 3.3 C). ICSB73 displayed the most prominent change in gene expression with an 8-fold increase observed under stress conditions.

The expression levels of sucrose transporters were regulated by stress (Figure 3.3 D). All four varieties displayed an increase in expression levels of the sucrose transporters in stressed plants relative to the watered.ICSV213 and S35 displayed a significant 8 and 3-fold change under stress, whereas changes in expression levels of ICSB338 and ICSB73 sucrose transporters

were insignificant. The melting curves of housekeeping genes (Figure 3.4) and sucrose biosynthesis genes (Figure 3.5) were used to generate the data presented in Figure 3.3

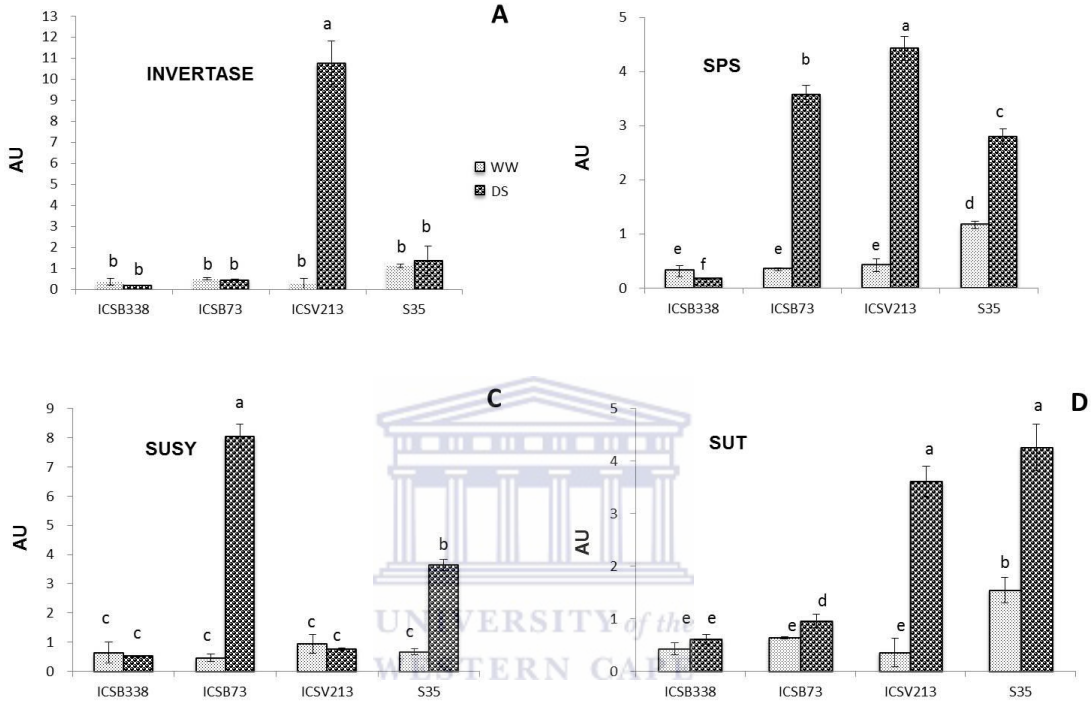


**Figure 3.3:** Quantitative PCR analysis of (A) invertase, (B) sucrose phosphate synthase, (C) sucrose synthase and (D) sucrose transporter genes during well watered and drought stress treatments of sorghum varieties. All values were normalized to the relative expression levels of 18S RNA, β-actin and ubiquitin genes using qBase+ (BioGazelle) analysis. AU (Arbitrary Units) represents fold change in expression relative to the lowest value obtained for each gene. Well watered (WW); drought stressed (DS)

## 3.4 Discussion

Plants have the ability of storing carbohydrates in stem tissue when production from the source is greater than whole-plant demand (Slewinski, 2012). Under adverse environmental conditions when photosynthesis is inhibited, these reserves become essential for yield stability in grain crops (Liu *et al.,* 2004). Sugar accumulation has been demonstrated as one of the defense mechanisms plants acquire in response to stressed conditions in order to facilitate the stability of cell structures and the maintenance of osmotic balance for plant metabolic activities to operate effectively. Therefore, understanding the molecular mechanisms that regulate sugar transport and allocation is vital for creating and implementing strategies to optimize whole-plant carbohydrate partitioning for increased productivity under stress.



Sucrose is a major sugar translocated in higher plants for plant development. Under stressed environmental conditions, its concentration is determined by several factors that include the rate of remobilization of the stem reserves, and the rate of sucrose hydrolysis and export to sink tissues (Xu *et al.,* 2008). In the present study, four sorghum varieties were evaluated for their sugar producing capacity under stressed conditions. In particular, sucrose production and its underlying molecular mechanisms were the key focus of the study. This was to assess whether regulation at the molecular level contributed to the role of sucrose as an osmoprotectant under stressed conditions.

Results indicated an overall increase in the total soluble sugar content under stress in all four varieties as indicated by brix measurements though ICSB338 and ICSB73 were not statistically significant (Figure 3.1A). The stem starch and sucrose concentrations were decreased (Figure 3.1 B and, Figure 3.2) whereas the concentrations of the hexoses (glucose and fructose) were significantly increased in response to drought stress (Figure 3.1 C-D). Therefore the high brix values could be attributed to the accumulation of hexoses under stress. Similar results have been observed in several plant species under drought conditions including soybean (*Glycine max L. Merr*.) (Liu *et al.,* 2004) and pigeonpea (Lawlor and Cornic, 2002) leaves. Collectively, these results indicate that there are insufficient carbon sources entering primary metabolism from photosynthesis to result in the net increase of sucrose. The increase in hexose concentration under drought could be attributed to starch and/or sucrose degradation. Starch serves as the main carbohydrate stored in most plants that can be mobilized to provide soluble sugars. Its metabolism is very sensitive to changes in the environment, with drought stress generally leading to the decline in starch content (Krasensky and Jonak, 2012). This study on sorghum corroborates these observations. However, the higher levels of fructose produced in stressed plants of all varieties was an indicator that fructans were the main storage carbohydrates, remobilized under stress. This is supported by Pilon-Smits *et al.,* (1995) who stated that Poacea plants (wheat, barley, sorghum etc.) are prominent members of the fructan flora and accumulate fructans as their main carbohydrate storage form. Fructans are soluble linear or branched β-2, 1- or 6-linked fructosyl-oligosaccharides that are derived from

sucrose and synthesized in the vacuole (Chalmers *et al.,* 2005; Van Laere and Van Ende, 2002).

These sugars are hydrolyzed to fructose under unfavorable growth conditions when the overall supply of photosynthate is reduced (Wardlaw and Willenbrink, 2000). In wheat cultivars grown under drought stressed conditions, fructans were reported to be the key sugars within the water-soluble carbohydrate (WSC) fractions, with suggestive roles in osmoregulation and drought tolerance (Gaudet *et al.,* 2011; Ende and Esawe, 2014). Other evidence of the involvement of fructans in drought response was shown in transgenic tobacco, potato and sugar beet plants. These plants, which accumulated fructans, showed enhanced tolerance when compared to watered plants (Pilon-Smits *et al.,* 1999; Knip *et al.,* 2006). Therefore, this study is in agreement with the current in the field and provides new information on sorghum varieties.



To gain a better understanding on the underlying mechanisms attributing to the observed accumulation of hexoses and depletion of starch and sucrose with stress induction, the sucrose biosynthesis enzyme activities in response to stress induction were examined. Of the key sucrose metabolizing enzymes investigated, sucrose phosphate synthase and invertases were significantly regulated by stress. The activity of the sucrose hydrolyzing enzymes (acid and neutral invertases) was increased under stress in three of the four varieties. These results could possibly explain the hexose accumulation observed under stress.

They are further supported by observations made by Wardlaw and Willenbrink (2000) who reported that there was an increase in hexose accumulation in response to water stress, which was accompanied by a rise in invertase activity in wheat plants.

An increase in acid invertase activity was also reported by Trouverie *et al.* (2003) in drought-stressed maize leaves, which coincided with the accumulation of glucose and fructose. The effect of stress on sucrose biosynthesis in sorghum plants was also characterized by the statistically significant increases in SPS activity in all four varieties under study. These observations were comparable to previous reports for a $C_4$ resurrection plant *Sporobolus stapfianus* (Whittaker *et al.,* 2007) and grapevines (*Vitis vinifera cv. Moscatel*) (Maroco *et al.,* 2002) during dehydration. The present results did not indicate a positive correlation between sucrose accumulation and SPS activity in the stressed plants. It is therefore possible that some of the glucose and fructose produced from sucrose hydrolysis by the invertase enyzme was re-synthesized to sucrose by SPS.



The decrease in the hexose to sucrose ratio however indicated that although SPS was up regulated by stress, its activity was below a threshold level necessary for sucrose to accumulate significantly. This is supported by the observed higher invertase activity compared to SPS in all sorghum varieties under stressed conditions. The activity of sucrose synthase in both the synthesis and hydrolysis direction was, however, low compared to the two enzymes previously mentioned. The change in activity levels of

Susy under stressed relative to watered conditions was also insignificant. Yang *et al.* (2004) reported similar observations on sucrose-metabolizing enzymes of wheat stems subjected to water stress at anthesis. These results are an indication that the rate of sucrose synthesis or hydrolysis was dependent on the activity of SPS and invertase. Therefore, these two enzymes contributed to the changes in sugar concentrations under stressed conditions. They are a further indication of the flexibility in metabolism by sorghum to cope with different drought stressed conditions. These observations are also speculative of other abiotic stresses including salt stress.

Although carbon flux in plants is regulated at multiple levels, transcriptional regulation is considered to be one of the major regulatory forms in controlling metabolic alteration in plants (Xue *et al.,* 2008). Thus, transcriptomics serves as a valuable tool in establishing the interactions between genes involved in stress response mechanisms. The regulation of the sucrose biosynthesis enzymes at transcript level was therefore investigated. Quantitative expression analysis using RT-PCR showed a general increase in expression levels of genes involved in sucrose biosynthesis in stressed sorghum plants. The expression levels of the invertase gene were regulated by stress induction, with a significant up regulation evident in variety ICSV213. There was evidently a link between changes in invertase expression and the shift in sucrose to hexose accumulation under stress in this variety. Increase in the expression levels of invertase have previously been reported in the vegetative organs of stressed maize plants (Kim *et al.,* 2000), which resulted in an increase in osmotic pressure that led to drought resistance. The significantly low invertase expression levels in relation to the

high enzyme activity under stressed conditions for ICSB338 and ICSB73 suggests that there are other factors that could attributed to the high enzyme activity, including substrate concentration and specificity.

Gene expression levels of sucrose phosphate synthase were up regulated by stress in three of the four sorghum varieties. This concurs with previous observations in wheat leaves subjected to drought stress (Xue *et al.,* 2008). A comparison of the gene expression levels between SPS and invertase indicated that SPS was more up regulated compared to the invertase. This observation demonstrated a disparity to the regulatory patterns observed at enzyme level where the invertases were predominantly up-regulated compared to SPS. This suggested that the expression levels of the SPS transcripts were not sufficient to increase SPS expression at protein level. Protein activity is often independent of transcript abundance and post-translational activity can increase or decrease protein activity, especially of enzymes.

Sucrose synthase mRNA levels were up regulated by stress in two of the four sorghum varieties. This is in contrast to the insignificant regulation observed at protein levels. Schafleitner *et al.* (2007) previously reported on the up-regulation of two *Arabidopsis* sucrose synthase genes under drought stress conditions. The failure of these sorghum varieties to accumulate Susy protein in proportion to the amount of transcripts could be attributed to inefficient translation and other post-translational events including protein modification and stability. Similar observations were made by Talierco and Chourey

(1989) in maize seedlings where under anoxic stress sucrose synthase transcripts accumulated, but not protein. Sugar levels in plants are also dependent on the transportation activity of sugar transporters. The shift in carbon allocation from sucrose towards hexoses reported under stressed conditions could therefore be attributed to the regulation of these transporters.

This study focused in particular on the regulation of sucrose biosynthesis transporters under stress. Sucrose transporters play a major role in modulating sucrose export to sink tissues for cellular energy demands in the plant during stress and osmoprotection (Ibraheem e*t al.,* 2011). The regulation of the sucrose transporter by stress induction was indicated by changes observed in expression in all four sorghum varieties. Of the four varieties, two (ICSV213 and S35) demonstrated significant up regulation of the mRNA levels of the transporter. The down-regulation of sucrose transporters has been demonstrated in stressed plants correlating with sucrose accumulation (Xue *et al.,* 2008; Noiraud *et al.,* 2000). This has been linked to the decreased metabolic demands. In this study however, these transporters were up-regulated. It is postulated that this was for the purpose of transporting sucrose to sink tissues where it would be readily available for hydrolysis by invertases to the hexoses necessary for, in this instance, grain filling at anthesis even under these adverse conditions.

The observed changes in sugar levels with stress inductions was an indication that carbohydrate metabolism is amongst the defense mechanisms these sorghum varieties use to protect themselves against environmental stresses. Hexose accumulation confirmed stem reserve mobilization, thus highlighting the significant role of plant stems under stress when photosynthesis is compromised.

Stem reserves are a powerful resource for grain filling and therefore sugar mobilization demonstrated their role at anthesis in the sorghum varieties subjected to stressed conditions. The increase in sucrose to hexose ratio indicated that hexoses could be involved in osmotic adjustment, and therefore their accumulation could be considered a strategy for stress adaptation by these varieties. Hexose accumulation also suggests that sucrose was used by these varieties as a carbon source for heterotrophic growth rather than an osmoprotectant. Hexose accumulation accompanied by an increase in invertase expression indicated that the induction of this enzyme was associated with drought-induced hyperosmotic stress. The corresponding increased SPS expression was an indication that the high invertase activity prevented most but not all sucrose accumulation. However there was a critical threshold of invertase activity at which high concentrations of sucrose did not accumulate. The difference in sugar levels observed across the varieties suggested the difference in response levels to stress amongst the varieties. These major differences could be attributed to differences in regulation of the invertase and SPS genes. However, results indicated that sucrose biosynthesis genes were regulated by decrease in osmotic potential rather than changes in the sugar concentrations themselves.

Therefore, the expression of these genes was independent of the osmoticum effect suggesting an involvement of a signal transduction mechanism distinct from the regulating sucrose metabolizing enzyme activities. The differential stress-responsive mechanism of these genes could therefore represent part of a general response to the allocation of carbohydrates during acclimation period. Interestingly, variety ICSV213 displayed a positive correlation between the hexose accumulation, sucrose depletion and the up regulated activity and transcript levels of invertase. These results suggested (i) a possible role of invertase in osmotic adjustment, and therefore a possible protection against dehydration in this variety.



And (ii), that the ICSV213 sucrose biosynthesis genes are potential targets for metabolic engineering to improve stem soluble sugar concentrations in sorghum under adverse conditions. Using sugar content as a basis for characterization of drought tolerance, this variety would be considered the most tolerant based on the levels of its sugar content under stress compared to the other three varieties studied. Future studies are required to examine the role of cellular localization or developmental stages on the expression levels of these genes under stress. Gene expression studies of other sucrose-linked biosynthesis pathways together with expression correlation analysis studies should also be carried out to investigate the significance of co-regulation on sugar content under stress. Bioinformatics approaches would also aid in the investigation of cross links between the sugar biosynthesis and stress-related pathways. These investigations would aid in the elucidation of gene regulatory networks for controlling sucrose metabolism in sorghum under stress.

# CHAPTER 4

## Investigating changes in the *Sorghum bicolor (L) Moench* stem proteome in response to drought stress


### Abstract

Sorghum is a drought-tolerant cereal crop, which is an important staple food in many arid regions of the world. Abiotic stress-related studies have demonstrated the importance of sorghum as a model plant for gaining a better understanding of the various molecular mechanisms plants adopt in response to stress. In this study, a comparative proteomic analysis on drought stress responses in two sorghum varieties (ICSB338 and ICSV213) was conducted. These varieties were selected based on their contrasting stress responses at the mRNA level. Western blotting analysis, using a known stress responsive protein Hsp70, indicated that the 10-day water deficit growth period was sufficient to induce stress responses at proteome level amongst the sorghum varieties. Through two-dimensional gel electrophoresis and mass spectrometry, differential protein expression patterns were observed between ICSB338 and ICSV213. Of the twenty six proteins selected for identification, fourteen were positively annotated. These spots represented proteins with functions in carbohydrate metabolism (57.2%), amino acid metabolism (14.3%), disease/defense (14.3%), protein synthesis (7.1%), and signal transduction (7.1%). Two proteins, which were previously confirmed as stress-related proteins (isoflavone reductase homologue IRL and glutathione S-transferase), were up-regulated in ICSV213.

These results demonstrate that stress-induction activates regulatory mechanisms of response in various metabolic pathways. These mechanisms ultimately contribute to the variation in stress responses, in particular amongst these two varieties. There is also an indication that the proteins up-regulated in variety ICSV213 could be considered for transgenic studies towards stress-tolerant sorghum varieties.

## 4.1 Introduction

Plant stems play a major role in improving yield stability in crops, by providing an alternative source when photosynthetic capacity is reduced during periods of adverse environmental conditions such as cold, salinity and drought (Slewinski, 2012). Therefore, the understanding of the regulatory mechanisms of plant stems towards abiotic stress responses could contribute to increases in crop productivity under adverse environmental conditions. Stress-regulated proteins have been identified in stems of various plants including wheat and rice via proteomic studies (Ali and Komatsu, 2006; Song *et al.,* 2011; Bazargani *et al.,* 2011). These identified proteins are involved in diverse cellular functions including carbohydrate, nitrogen and energy metabolism, ROS scavenging, signal transduction, and RNA and protein processing (Nam *et al.,* 2012). A large number of stress-related proteins reported in literature have however been identified through transcriptomic approaches, and not via proteomics. Furthermore, several studies have proven that changes in gene expression at transcript level often do not correlate with the protein level (Yan *et al.,* 2005).

Therefore, in creating stress tolerant plants, more research in elucidating plant response to stress induction is important for the identification of stress-related proteins. Such research is crucial as protein accumulation under stress could be correlated to plant physiological responses, thus contributing to stress tolerance levels (Kosova *et al*., 2011).

Stress-related studies on sorghum have made proteomics an increasingly important and effective approach in identifying stress-related proteins (Ngara *et al.,* 2012; Swami *et al.,* 2011). Despite these developments, little is known about the underlying regulatory mechanisms of stem reserve utilization under drought-induced stress in sorghum. Therefore, studies on sink-source interactions upon stress conditions at protein levels could significantly contribute to our understanding of physiological mechanisms (such as osmolyte accumulation) which aid to plant stress tolerance. These studies could also lead to the identification of molecular markers whose change in abundance could be linked with changes in physiological parameters used for categorizing a plant's level of stress response. This study is, therefore, aimed at identifying candidate proteins for stress tolerance in sorghum varieties using proteomic approaches.

## 4.2 Materials and methods

### 4.2.1 Stress treatment and protein extraction from sorghum stems

Two sorghum varieties ICSB338 and ICSV213 were used as sources of plant material. The sorghum plants were obtained from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) India and grown under conditions previously described in Section 2.2.1. Crude protein extracts were prepared from 4 biological replicated of each of the basal stem tissues and flag leaf tissues, and ground in liquid nitrogen using pestle and mortar. The extraction buffer used consisted of 100 mM Hepes-KOH (pH 7.5), 2 mM EDTA, 5 mM DTT, 4 mM $MgCl_2$, 10% glycerol and 2% PVPP. Extracts were centrifuged for 4 min at 12000 rpm at 4°C. Crude protein extracts were transferred to Sephadex G-50 spin columns pre-equilibrated in extraction buffer and centrifuged for 2 min at 4000 rpm at 4 °C. The supernatant containing soluble protein fraction was collected and stored at -20°C until further use for protein quantification and electrophoresis.



### 4.2.2 Protein quantification

The concentration of all protein extracts was determined using a modified Bradford Assay (Bradford, 1976), as previously described by Ndimba *et al.*, (2003). Bovine serum albumin (BSA) standards were prepared in triplicates from a 1 mg/ml BSA stock solution in a bottom-flat micro-titer plate as indicated in Table 3.1 (Section 3.2.4.2). Protein extracts were prepared in triplicates by mixing 10 µl of unknown protein sample with a 190 µl of diluted Bradford reagent.

The same volume of the diluted Bradford reagent was added to 10 µl of the standards to make up a total volume of 200 µl. The flat-bottom micro-titer plate containing standards and protein extracts was incubated for 5 min at room temperature. Absorbance was measured at 595 nm in a POLARstar OMEGA micro-titer plate reader (BMG LABTECH GmbH, Germany) using Bradford reagent as a blank. BSA standards were used to derive a standard curve from which concentrations of all the unknown protein extract samples were extrapolated.

### 4.2.3 SDS-PAGE and Immunoblotting for Heat shock protein 70 (Hsp70)



Flag leaf (20 µg) and stem (60 µg) proteins were separated in a 12% resolving and 5% stacking gel at 120V using a Mini-PROTEAN® 3 Electrophoresis Cell (BIO-RAD) (Laemmli, 1970) using a PowerPac™ Universal Power supply (BIO-RAD). Constituents of the resolving and stacking gels are listed in Appendix B. Prior to electrophoresis, 2x SDS sample loading buffer was added to the protein extracts were denatured , followed by the boiling of the samples on a VWR digital heat block (TROEMNER USA) at 95°C for 10 minutes. After heating the samples were pulse spun in a centrifuge and loaded on to the gels. A high molecular weight marker (PageRuler™ Unstained Protein Ladder Thermo Scientific, United Kingdom) was used for molecular mass determination. After separation, the gels were stained with three different concentrations of Coomassie brilliant blue stain (Appendix C). The gel was immersed in CBB stain I, heated for 1 minute in a microwave and incubated for 1 hour with shaking at room temperature.

The CBB solution I was discarded and the staining procedure repeated with CBB II and CBB III, respectively. After staining, the gel was immersed in de-staining solution at room temperature with shaking until the protein bands were clearly visible. For the western blots, a high range blot marker (pre-stained SDS-PAGE Standards, BIO-RAD) was applied to the gel.

After gel electrophoresis of the flag leaf extracts, the proteins were blotted onto polyvinylidene difluoride (PVDF) membrane (Hybond-P PVDF membrane, GE Healthcare) at 25 V for 10 minutes using a semi-dryblot (BIO-RAD). The blocking of non-specific binding and the subsequent immuno-reactions were carried out as described by Ngara *et al*. (2012). The membrane was incubated with the primary antibody [human HeLa cells anti-Hsp70/Hsc70 monoclonal antibody raised in mouse (Stressgen Bioreagents Corp., Victoria, Canada)] diluted 1:1000 in 2.5% (w/v) blocking solution (Appendix I) for 1 hour. The membrane was washed three times with TBST [TBS containing 0.1% (v/v) Tween 20] for 10 minutes per wash. The membrane was then washed with 0.5% (v/v) blocking solution for 10 minutes and incubated with the secondary antibody [goat anti-mouse IgG (H & L) horseradish peroxidase conjugate (Invitrogen Corp., Carlsbad, CA, USA)] diluted 1:2000 in 2.5% (w/v) blocking solution for 1 hour. The membrane was washed three times in TBST, for 15 minutes per wash. Heat shock proteins were detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL, USA) according to the manufacturer's instructions.

The PVDF membrane was exposed and automatically developed using the UVP Imaging System (UVP Bio-Spectrum Imaging System (Upland, CA, USA).

### 4.2.4 Two Dimensional (2D) SDS-PAGE

#### 4.2.4.1 Sample preparation

Protein samples for 2D SDS-PAGE were prepared by mixing 300 µg of each of the stem protein extracts with 1.25 µl of 40% (w/v) BioLyte (3/10) ampholytes (BIO-RAD) and 50% (w/v) DTT. This was made up to a final volume of 125 µl using urea buffer (9 M urea, 2 M thiourea and 4% 3 [(Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The rehydration solution of each protein sample was briefly vortexed, pulse spun and pipetted on to an Immobiline™ re-swelling tray (GE Healthcare, Amersham, UK). Seven cm, pH 4-7 ReadyStrip (BIO-RAD) IPG strips were placed on the samples, with the gel side of the strip in contact with the protein sample. The strips were then covered with mineral oil. The strips were left to re-hydrate passively overnight at room temperature.



#### 4.2.4.2 Isoelectric focusing (IEF) of the IPG Strips

Following rehydration, IPG strips were briefly rinsed with distilled water to remove unabsorbed protein sample and carefully blotted with moist filter paper. The strips were then placed gel side up on the focusing platform of an Ettan™ IPGphor II™ (GE Healthcare).

Distilled water moistened wicks were placed at the extreme ends of both the anodic and cathodic ends of the IPG strips to collect excess salts and impurities from the sample during focusing. The IPG strips were then covered with mineral oil to avoid sample evaporation and carbon dioxide absorption during focusing. Isoelectric focusing was performed in a three-phase stepwise program at 20°C as indicated in Table 4.1.

**Table 4.1:** IEF conditions for 7cm (pH 4-7) IPG strips

| Step | Voltage (V) | Time (hrs)/Volts hours (Vhrs) |
|---|---|---|
| 1 | 250 | 0.15 |
| 2 | 4000 | 1 |
| 3 | 4000 | 12000 |



### 4.2.4.3 IPG Equilibration for Second Dimension

Following the completion of IEF, the IPG strips were equilibrated with 1 X SDS PAGE running buffer. The focused IPG strips were incubated gel-side up in reswelling tray channels containing 2.5 ml equilibration buffer I (6M urea, 2% SDS, 0,375M Tris-HCl (pH 8.8); 20% glycerol and 2% DTT) for 20 minutes followed by equilibration buffer II (6M urea, 2% SDS, 0,375 M Tris-HCl (pH 8.8), 20% glycerol and 2, 5% iodoacetamide) for another 20 minutes with gentle agitation at room temperature.

After equilibration, the strips were briefly immersed in 1X SDS PAGE running buffer and subsequently used on second- dimension SDS-PAGE.

#### 4.2.4.4 2DE preparation and running

For the second dimension electrophoresis, a 12% (v/v) resolving gel solution was prepared as indicated in Appendix B. The resolving gel solution was poured into the cast plates according to the instruction manual and each gel was overlaid with 1 ml of 100% isopropanol. The gels were left to polymerize for 1 hour at room temperature. After polymerization, the isopropanol overlay was rinsed off with distilled water and gel surfaces were blotted dry with filter paper. Equilibrated IPG strips were gently rinsed with 1X SDS-PAGE running buffer and placed on top of the mini format 12% SDS-PAGE resolving gels with the plastic backing against the spacer plate. Three microlitres of PageRuler$^{TM}$ unstained protein ladder (Fermentas Life Sciences, Ontario, Canada) were spotted onto a small piece of filter paper, air-dried and placed at the anodic side of each IPG strip. The IPG strips were then overlaid with 1 ml of 0.5% (w/v) molten agarose prepared in 1X SDS-PAGE running buffer containing a tint of bromophenol blue. The bromophenol blue dye was used as a migration-tracking dye during electrophoresis. Electrophoresis was carried out using the Mini-PROTEAN® gel tanks (BIO-RAD) at 120 V until the bromophenol dye reached the bottom of the glass plates. After the second dimension, gels were stained with CBB as described in Section 4.2.3.

### 4.2.5 Comparative 2D SDS-PAGE analysis

Comparative analysis of 2D gels was done using PDQuest Advanced 2D Analysis Software, version 8.0.1 build 055 (BIO-RAD). 2D gels were initially imaged using the Molecular Imager PharosFX Plus System (BIO-RAD) and then analyzed according to the PDQuest$^{TM}$ Advanced 2D Analysis Software user manual (BIO-RAD). PDQuest experiments were created with each treatment group having three technical replicates. Prior to differential protein expression analysis across treatment groups of each experiment, spots were manually edited using the group consensus tool to obtain spot expression consensus in all three biological replicates per treatment group. Differentially expressed protein spots were qualitative (present/absent spots), quantitative (showing at least a 2-fold expression change) and/or Student's *t*-test (95% significance level) significant spots. Well-resolved protein spots were manually picked using pipette tips, for identification using mass spectrometry.



### 4.2.6 Mass Spectrometry (MS)

#### 4.2.6.1 Protein spot excision and tryptic digestion from 2D gels

The protein spots were excised from the gel using 20 µl pipette tips. Gel pieces were washed twice for 5 minutes using 50 mM ammonium bicarbonate to get rid of any dust that might be in the tube in addition to washing away gel buffers. This was followed by destaining for 30 minutes using 50 mM ammonium bicarbonate and acetonitrile at a ratio of 1:1, with occasional vortexing.

The gel pieces were dehydrated with 100 µl acetonitrile for 5 minutes, and then completely desiccated using the Speed Vac SC100 (ThermoSavant, Waltham, MA, USA). Proteins were in-gel digested overnight at 37°C with approximately 20 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) dissolved in 50 mM ammonium bicarbonate. After at least 18 hours, trypsin was inactivated by quenching with 5 µl of 2% trifluoroacetic acid (TFA). This brought the pH of the tryptic digest to approximately pH 2. The supernatants were desalted using C-18 Zip-Tips, according to manufactures instructions (ZTC 185096, Merck, Darmstadt, Germany).



#### 4.2.6.2 Protein identification using MALDI-TOF MS

MALDI-TOF MS and LIFT MS/MS were performed using an UltrafleXtreme MALDI TOF/TOF system (Bruker Daltonics) with instrument control through Flex Control 3.4. Approximately 0.5 µl of each digest was spotted onto a PAC II HCCA pre-spotted anchor chip MALDI target plate for peptide mass fingerprinting. Peptides were ionized with a 337 nm laser and spectra were acquired in reflector positive mode at a 28 kV using 500 laser shots per spectrum with a scan range of m/z = 700 – 4000 Da. Spectra were internally calibrated using PAC II peptide calibration standard (Bruker Daltonics). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 to 4000 Da. Peptide spectra of accumulated 3,000 shots were automatically processed using Biotools 3.2 software (Bruker Daltonics). Database interrogation was performed with the MASCOT algorithm using the SwissProt database on a Bio-tools 3.2 workstation.

The search parameters were: taxonomy - other green plants; enzyme - trypsin; missed cleavages - 1; fixed modification - carbamidomethyl (C); variable modification - oxidation (M); precursor tolerance - 50 ppm; fragment tolerance - 0.7 Da. Candidate protein matches with molecular weight search (MOWSE) score greater than 55 were considered as positive identifications.

## 4.3 Results and Discussion

### 4.3.1 Hsp 70 expression patterns in sorghum flag leaves following drought stress

To establish whether the 10-day water deficit period was sufficient to induce a hyperosmotic effect at protein level, the expression levels of a known stress responsive protein, Hsp70, were investigated. Four biological replicates of each of the watered and stressed flag leaf samples of both ICSB338 and ICSV213 sorghum varieties were used. Figure 4.1 demonstrates immunoblots of Hsp70 in watered and stressed plants of varieties ICSB338 and ICSV213. In both varieties, a single protein band with a relative molecular weight of 70 kDa was reactive to the Hsp70 antibody used. Enhanced levels of Hsp70 were evident in the treated sorghum plants compared to the watered plants in both varieties (Figure 4.1).

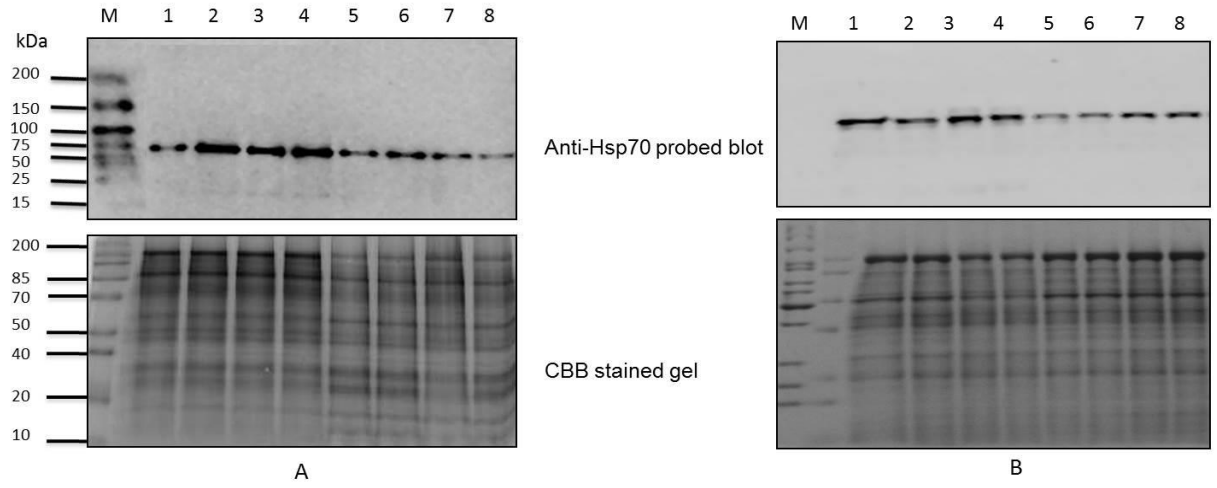


**Figure 4.1:** Western blot analysis of Hsp70 expression patterns on (A) ICSB338 and (B) ICSV213 sorghum flag leaf protein extracts. Lane M - molecular weight marker. Lanes 1-4 represents independent biological replicate protein extracts from stressed samples. Lanes 5-8 represents protein extracts from watered sorghum plants



### 4.3.2 1D protein profiles of sorghum stem proteins following drought stress

1D gel electrophoresis was used to evaluate the quality and quantity of the stem protein extracts prior to 2D gel electrophoresis. Figure 4.2 shows CBB stained 1D of stem proteome profiles of the two sorghum varieties. From these profiles it was observed that the quality of the stem protein extracts was good; showing no visible signs of streaking or protein degradation. The sample loading was also uniform. The biological replicates (Lane 1-4; and Lane 5-8) within an experiment (Figure 4.2 A and B) showed high similarity in terms of protein expression, abundance and banding patterns. This suggests that protein preparation was reproducible between independent extractions.

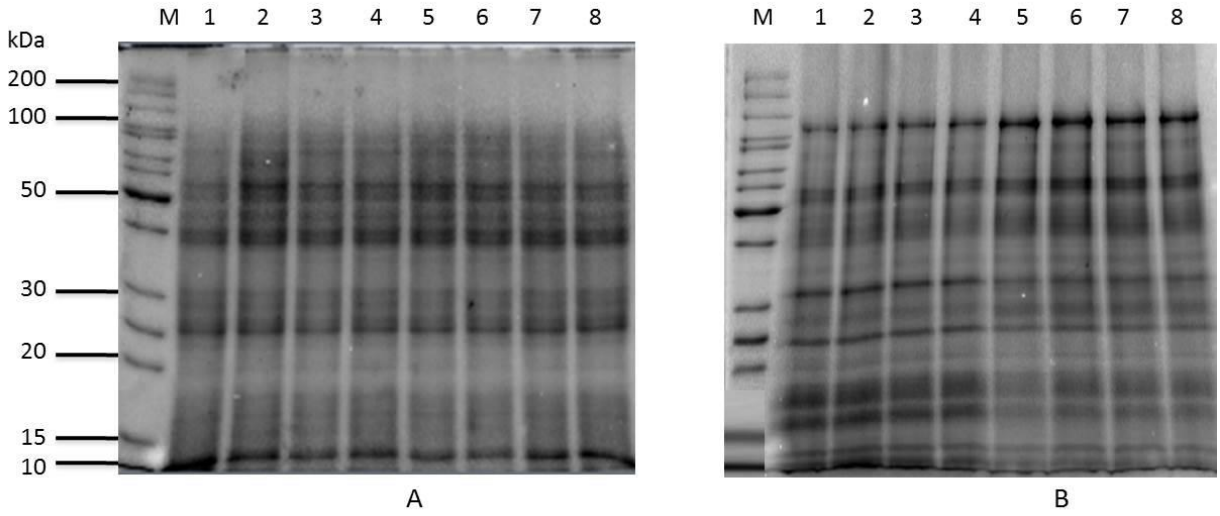


**Figure 4.2:** One dimensional gel electrophoresis analysis of total stem protein of sorghum ICSB338 (A) and ICSV213 (B) plants following drought stress. Lane M is the molecular weight marker. Lanes 1-4 represent stem extracts from watered sorghum plants from independent biological replicates. Lanes 5-8 represent stem extracts from stressed sorghum plants. These gels are representatives of 3 1D gels



### 4.3.3 2D gels of sorghum stem proteins of ICSB338 and ICSV213

Three of the four biological replicates of stem protein extracts, from each treatment of each variety, were randomly selected for 2DE analysis. A total of 300 μg protein sample was loaded onto IPG strips. For all samples, IPG strips of pH range 4-7 were used. This was the range in which most soluble proteins were confined. Figures 4.3 and 4.4 illustrate representative 2D gels of stem tissues in both watered (A) and stressed (B) samples of ICSB338 and ICSV213 sorghum varieties respectively. In general, ICSB338 and ICSV213 varieties demonstrated uniform protein profiles. However, differences in protein expression patterns were observed.

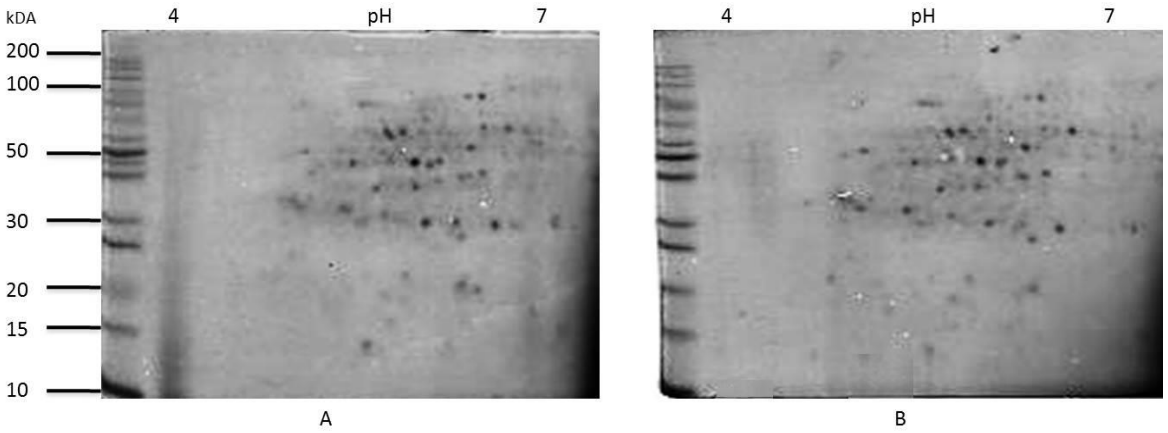


**Figure 4.3:** 2D gel electrophoresis of stem protein extracts of ICSB338 sorghum variety on pH 4-7 IPG strip. An approximate 300 µg of total soluble protein was loaded onto a 12% SDS-PAGE gel. (A) watered (B) stressed. These gels are representatives of 3 2D gels

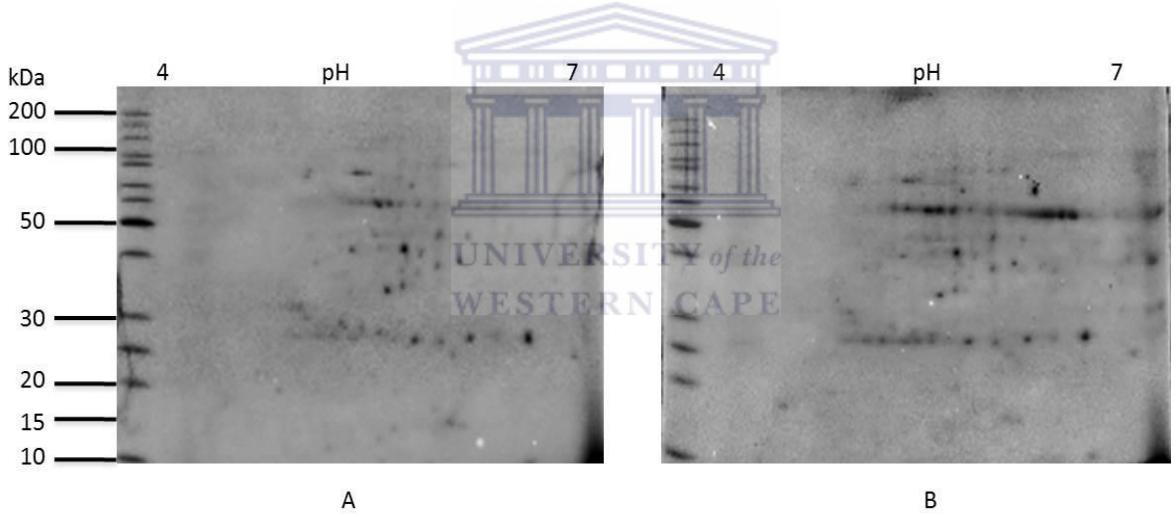




**Figure 4.4:** Two dimensional gel electrophoresis of stem protein extracts of ICSV213 sorghum variety on pH 4-7 IPG strip. An approximate 300 µg of total soluble protein was loaded onto a 12% SDS-PAGE gel. (A) watered (B) stressed. These gels are representatives of 3 2D gels

### 4.3.4 Comparative analysis of stress-responsive proteins of ICSB338 and ICSV 213

The stem proteome of both watered and stressed plants of ICSB338 and ICSV213 varieties were compared using the PDQuest™ software. Figure 4.5 and 4.6 represent biological replicate gels used for each treatment group of each variety respectively. The master gel of each experiment, automatically generated by the software, includes all spots that are reproducibly present in all biological replicate gels per treatment group. The proteins spots were detected as up/down regulated, or common between the treatment groups. They were also categorized by the software as qualitative, quantitative or statistically significant. Table 4.2 gives a summary of the PDQuest™ results per treatment group (control and drought treatment) per sorghum variety.



#### 4.3.4.1 Qualitative differential protein expression analysis set

Qualitative spots are the number of spots that were detected in either the watered or stressed groups but not in both. In this experiment, only one qualitative spot following stress was observed in ICSB338 sorghum stem proteome (Table 4.2). This spot was induced by stress and thus only present in the stressed ICSB338 stem proteome.

#### 4.3.4.2 Quantitative differential protein expression analysis set

The quantitative spots are those showing at least 2-fold expression change following treatment, and are either up or down regulated. A total of 9 and 14 protein spots showed at least a 2-fold change in abundance between the control and treatment groups of ICSB338 and ICSV213 stem proteomes, respectively. In variety ICSB338, the abundance of 7 spots increased, while those of 2 spots decreased, following stress treatment. Of the 14 quantitative protein spots of ICSV213, the abundance of 4 spots increased, while those of 10 spots decreased, following treatment.

#### 4.3.4.3 Student's *t*-test differential protein expression analysis set

The Student's *t*-test determines the differentially expressed spots at 95% significance level. The abundance of 7 ICSB338 stem proteins and 3 ICSV213 stem proteins were shown to be significantly different between the control and treatment groups of each variety, respectively. Of these, the abundance of 5 spots increased in ICSB338 whilst 1 spot was decreased and 1 induced. Of the 3 spots differentially expressed in ICSV213, 1 spot was decreased and 2 induced following treatment.

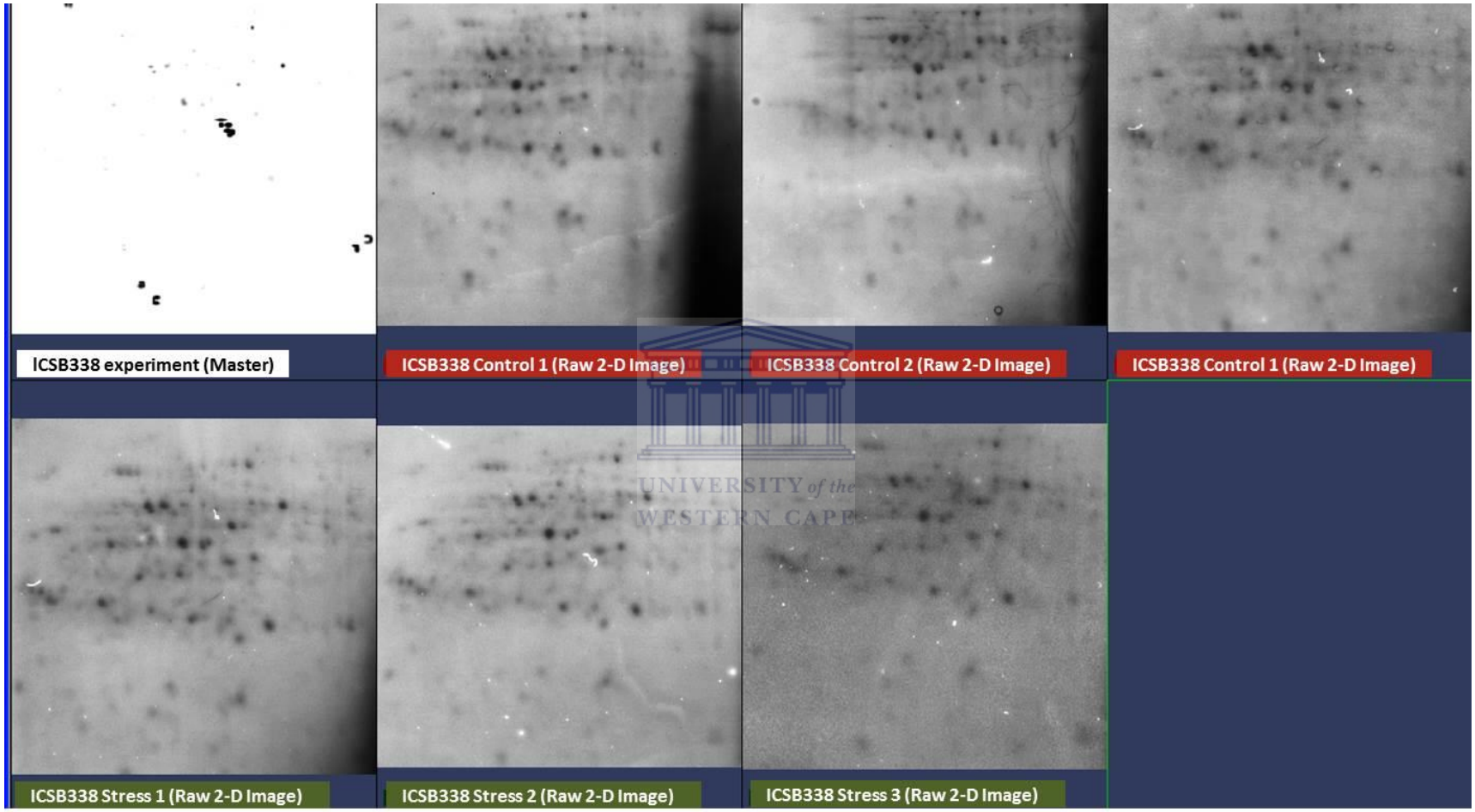




**Figure 4.5: PDQuest ™ analysis gels of ICSB338 sorghum stem proteome.** Three biological replicates for each treatment group (watered and drought stressed) are shown. The master gel was automatically created by the analysis software and it contains spots that are reproducibly expressed in each group




**Figure 4.6:** PDQuest TM analysis gels of ICSV213 sorghum stem proteome. Three biological replicates for each treatment group (control and drought stressed) are shown. The master gel was automatically created by the analysis software and it contains spots that are reproducibly expressed in each group

**Table 4.2:** A summary of comparative proteomics of drought stress responsive stem proteins of ICSB338 and ICSV213 sorghum varieties

| Sorghum Variety | Total spots | | Qualitative spots | Quantitative spots | Students's *t*-test spots of significance |
|---|---|---|---|---|---|
| | Watered | Treatment | | | |
| ICBS 338 | 143 | 66 | 1 [1 up] | 9 [2 down; 7 up] | 7 [1 induced; 5 up; 1 down] |
| ICSV 213 | 211 | 135 | 0 | 14 [10 down; 4 up] | 3 [2 induced; 1 down] |

The total spots are the total reproducible spots amongst the biological replicate gels of each treatment group per sorghum variety. Qualitative spots are the number of spots that are only present in either control or drought treatments. The quantitative spots are those showing at least 2-fold expression changes following treatment. The Student's *t*-test determines differentially expressed spots at a 95% significance level. **Induced:** spots present only in the drought treatment group, **up:** spots with increased abundances after drought stress, **down:** spots with decreased abundance after drought stress

### 4.3.5 Landmarking drought stress responsive protein spots in the stem proteome of the two sorghum varieties

A stem proteome reference map was established for landmarking the differential protein expression patterns in response to stress induction in the two sorghum varieties under study. Figure 4.7 indicates a combination of drought stress responsive and non-responsive spots from both sorghum varieties amongst the 26 spots that were picked for MALDI-TOF MS and MALDI-TOF TOF MS analysis. Table 4.3 summarizes proteins annotated by MS analysis, and Table 4.4 and 4.5 the number of stress-responsive and non-responsive proteins between ICSB338 and ICSV213 sorghum varieties. Of the 26 proteins selected amongst the two varieties, 23 spots were responsive and 3 spots non-responsive to drought stress, respectively. Of these responsive spots, the abundances of 16 and 19 spots changed between the watered and stressed groups of ICSB338 and ICSV213, respectively (Table 4.4 and 4.5). Of the 19 responsive proteins of ICSV213, 14 were up-regulated and 5 down-regulated. For ICS338, 7 were up-regulated whilst 9 were down-regulated by stress. Twelve protein spots (2; 7; 8; 9; 10; 11; 13; 14; 15; 16; 17; 20) were responsive for both varieties. Of these, spot 9, which does not match any protein on the database, was down regulated in both ICSB338 and ICSV213. A majority of the spots identified on the database were differentially regulated in ICSB338 and ICSV213 (Table 4.4 and 4.5) following stress induction.

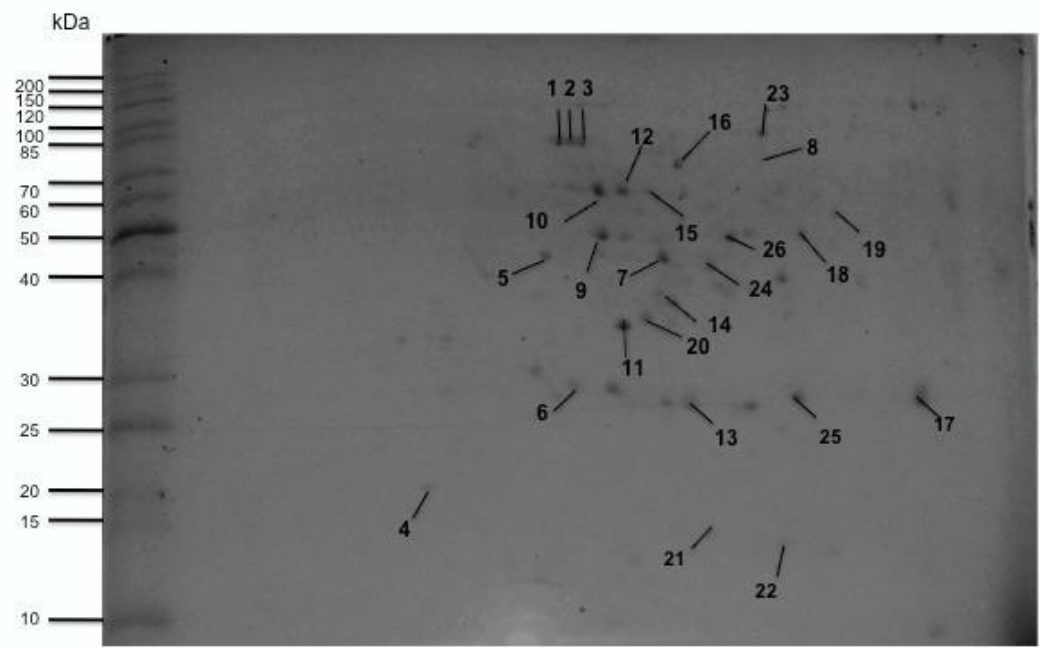


**Figure 4.7:** A representative CBB stained 2D gel of the sorghum stem proteome showing spots picked for mass spectrometry. Protein spots that were up or down regulated, induced or repressed, annotated or non-annotated are represented in the gel

**Table 4.3:** List of sorghum stem proteins identified by MALDI-TOF MS and MALDI-TOF-TOF MS

| Spot [a)] | Best Protein Match | Plant Species | Accession [b)] | MOWSE score [c)] | Theo,MW/pI [d)] | Exp.MW/pI [e)] | Location [f)] |
|---|---|---|---|---|---|---|---|
| | **Carbohydrate metabolism** | | | | | | |
| 13 | Triosephosphate isomerase | *Zea mays* | P12863 | 112 | 27/5.5 | 27/5.5 | cytoplasm |
| 12 | Enolase 1 | *Zea mays* | P26301 | 76 | 48/5.2 | 48/5.2 | cytoplasm |
| 15 | Enolase 1 | *Zea mays* | P26301 | 155 | 48/5.2 | 48/5.2 | cytoplasm |
| 16 | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | *Zea mays* | P30792 | 94 | 60/5.3 | 60/5.3 | cytoplasm |
| 21 | RuBisCO, large subunit | *Aethionema cordifolium* | A4QJC3 | 135 | 53/6.1 | 53/6.0 | chloroplast |
| 7 | Alpha-1,4-glucan-protein synthase | *Zea mays* | P80607 | 108 | 41/5.8 | 41/5.8 | n.d |
| 24 | Alpha-1,4-glucan-protein synthase | *Zea mays* | P80607 | 108 | 41/5.8 | 41/5.8 | n.d |
| 26 | Phosphoenolpyruvate carboxylase 3 | *Sorghum bicolor* | P15804 | 61 | 108/5.9 | 108/5.9 | cytoplasm |
| | **Amino acid metabolism** | | | | | | |
| 23 | 5-methyltetradydropteroyltriglutamate-homocysteine methyltransferase | *Catharanthus roseus* | Q42699 | 94 | 84/6.1 | 85/6.1 | cytoplasm |
| 18 | Adenosylhomocysteinase | *Triticum aestivum* | P32112 | 147 | 53/5.6 | 54/5.6 | n.d |

| Spot [a] | Best Protein Match | Plant Species | Accession No [b] | MOWSE score [c] | Theo,MW/pI [d] | Exp.MW/pI [e] | Location [f] |
|---|---|---|---|---|---|---|---|
| | **Protein synthesis** | | | | | | |
| 22 | Eukaryotic translation initiation factor 5A | *Zea mays* | P80639 | 58 | 175/5.6 | 177/5.6 | n.d |
| | **Signaling** | | | | | | |
| 6 | 14-3-3-like protein 2 (fragments) | *Pseudotsuga menziesii* | P85939 | 77 | n.d | 77/4.2 | n.d |
| | **Stress/Defence** | | | | | | |
| 20 | Isoflavone reductase homolog IRL | *Zea mays* | P52580 | 79 | 32/5.7 | 33/5.7 | cytoplasm |
| 25 | Glutathione S-transferase | *Zea mays* | P12653 | 97 | 24/5.5 | 24/5.4 | cytoplasm |
| | **Spots with no significant matches** | | | | | | |
| | 1,2,3,4,5,8,9,10,11,14,17,19 | | | | | | |



a) Spot number as indicated on the 2D gel image (Fig 4.7)
b) Accession number in the SwissProt database
c) Mascot score
d) Theoretical Mr (kDa) and pI obtained from SwissProt database
e) Experimental Mr (kDa) and pI obtained from SwissProt database
f) Subcellular localization
g) n.d - not defined

**Table 4.4:** Sorghum stem protein spots differentially expressed under drought stress conditions identified by MALDI-TOF MS

| Spot [a)] | Best Protein Match | Accession [b)] | ICSB338 | ICSV213 |
|---|---|---|---|---|
| | **Carbohydrate metabolism** | | | |
| 13 | Triosephosphate isomerase | P12863 | up [c;e)] | down [e)] |
| 12 | Enolase 1 | P26301 | * | up [c,e)] |
| 15 | Enolase 1 | P26301 | down [d)] | up [e)] |
| 16 | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | P30792 | down [e)] | up [d;e)] |
| 21 | RuBisCO, large subunit | A4QJC3 | * | up [e)] |
| 7 | Alpha-1,4-glucan-protein synthase | P80607 | up [d;e)] | down [e)] |
| 24 | Alpha-1,4-glucan-protein synthase | P80607 | * | - |
| 26 | Phosphoenolpyruvate carboxylase 3 | P15804 | down [d)] | - |
| | **Amino acid metabolism** | | | |
| 23 | 5-methyltetradydropteroyltriglutamate-homocysteine methyltransferase | Q42699 | - | up [c)] |
| 18 | Adenosylhomocysteinase | P32112 | * | up [e)] |
| | **Protein synthesis** | | | |
| 22 | Eukaryotic translation initiation factor 5A | P80639 | up [d;e)] | - |
| | **Signaling** | | | |
| 6 | 14-3-3-like protein 2 (fragments) | P85939 | * | up [d)] |
| | **Stress/Defence** | | | |
| 20 | Isoflavone reductase homolog IRL | P52580 | down [d)] | up [d)] |
| 25 | Glutathione S-transferase | P12653 | - | up [d)] |

**Table 4.5:** Unidentified sorghum stem protein spots differentially expressed under drought stress conditions

| Spots with no significant matches | ICSB338 | ICSV213 |
|---|---|---|
| 1 | down [d] | - |
| 2 | up [d] | down [d] |
| 3 | * | - |
| 4 | * | up [e] |
| 5 | * | - |
| 8 | down [d] | up [d] |
| 9 | down [d] | down [d] |
| 10 | down [d] | up [e] |
| 11 | down [d;e] | up [c] |
| 14 | up [d] | down [e] |
| 17 | up [c;d] | down [d] |
| 19 | up [c;d] | * |



a) Spot number as indicated on the 2D gel image (Fig 4.7)
b) Accession number in the SwissProt database
c) Spots only present in either control or treatment group
d) Spots showing at least a 2-fold expression change following drought induction
e) Differential expression as determined by the student's t-test at 95% significance level

* Spot not present either in control or treatment groups of ICB338 or ICSV213

up: increased expression following stress induction

down: decreased expression following stress induction

-no significant change in abundance between control and treatment groups

### 4.3.6 Biological significance of the differential protein expression between the two sorghum varieties after drought treatment

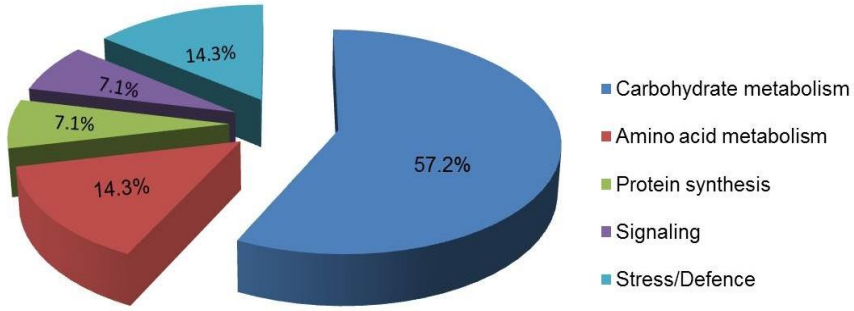


**Figure 4.8:** Functional annotation of the identified proteins classified by biological function. Proteins were grouped into functional categories using data available on the UniProt database (www.uniprot.org) and according to Bevan *et al.,* (1998)



The proteins identified by mass spectrometry were grouped into fundamental biological processes (Table 4.3 and Figure 4.8) according to Bevan *et al.,* (1998). This served the purpose of speculating on the phenotypic implications of the differential expression patterns observed in the two sorghum varieties under study.

#### 4.3.6.1 Carbohydrate metabolism

Of the 8 proteins identified to have putative functions in carbohydrate metabolism (Table 4.3 and Figure 4.8), a total of 5 and 6 were stress responsive in ICSB338 and ICSV213 sorghum varieties, respectively. Six protein spots involved in glycolysis and/or gluconeogenesis were responsive to stress (Table 4.4). These included spot 12 and 15 (Enolase 1); spot 13 (triosephosphate isomerase); spot 16 (2,3-biphosphoglycerate-

independent phosphoglycerate mutase)(iPGAM) and, spot 7 and 24 (Alpha-1, 4-glucan-protein synthase). Under stress conditions, plant metabolic pathways are disturbed. As a result, in order to maintain homeostasis under these conditions, plants are required to strengthen their resistance mechanisms such as ion transport, reactive oxygen species (ROS) scavenging and osmolyte synthesis (Yan *et al.,* 2005). These processes require energy, which is supplied by glycolysis, an energy-producing pathway. Therefore, the regulation of these glycolysis proteins modulates energy supply in plant cells.

Cabiscol *et al.,* (2000) proposed that the inactivation of these proteins would slow down glycolysis and consequently arrest energy metabolism under stress conditions, leading to the inhibition of ROS scavenging. Increased expression levels of triosephosphate isomerase in response to stress have been reported previously in rice, maize and poplar trees (Yan *et al.,* 2005; Riccardi *et al.,* 1998). These findings concur with the observations made for the ICSB338 sorghum variety. The repression of this protein in ICSV213 following stress treatment is unknown. Plomion *et al.* (2006) also reported on the repressed expression levels of this protein in poplar trees following drought stress. The up regulation of enolase 1 and Ipgam proteins following stress was observed in ICSV213.

Previous reports on rice roots, transgenic rice, maize and Arabidopsis also demonstrated increased expression levels of these proteins following stress treatments (Yan *et al.,* 2005; Nam et al., 2012; Riccardi *et al.,* 1998; Ndimba *et al.,* 2005). Both proteins were repressed in ICSB338 under treatment. Similar observations were previously made in poplar trees subjected to drought and heat stress (Plomion *et al.,* 2006; Ferreira *et al.,* 2006).

Alpha-1, 4-glucan-protein synthase is involved in cell wall synthesis. Previous stress-related studies demonstrated the regulation of this protein under adverse environmental conditions. Increases in expression levels of alpha-1, 4-glucan-protein synthase following stress induction have been demonstrated in rice roots and leaves as observed in ICSB338 (Lee *et al.,* 2010). The repression of this protein was reported in ICSV213 with similar observations previously made by Plomion *et al.,* (2006). Differential regulation of this protein between the two varieties could be attributed to which metabolic pathway is more prioritized in each plant under stress. ICSV213 promotes glycolysis to meet energy demands of the plant under stress, hence the down-regulation of this protein. On the other hand, ICSB338 promotes gluconeogenesis for cell wall synthesis. The role of cell walls in stress response is however not well documented.

Two spots involved in the carboxylation phase of the Calvin cycle were identified. These included spot 21 (RuBisCo large unit) and 26 (phosphoenolpyruvate carboxylase 3 (PEPC). The RuBisCo large subunit spot was responsive only in ICSV213 with increased abundance following stress treatment (Table 4.4). PEPC abundance was reduced in response to stress treatment in variety ICSB338. Both proteins are important in the Calvin cycle for $CO_2$ assimilation, and therefore essential for photosynthesis efficiency (Shi *et al.,* 2014). As a bifunctional protein, RuBisCo has the capacity of competitively using $CO_2$ or $O_2$ depending on the physiological state of the plant (Tolbert, 1997). Under stress exposure, plants are more prone to photorespiration, a wasteful process involved in the release rather than the fixation of carbon dioxide, which results in an overall reduction in plant productivity.



Several studies have reported on oxygenase activity of RuBisCo catalyzing the first step of photorespiration (Moreno and Spreitzer, 1999; Duff *et al.,* 2000). Plants subjected to abiotic stresses have been reported to show enhanced respiration, initiated by enhance oxygenase activity of RuBiSco (Sivakumar *et al.,* 2000; Sivakumar *et al.,* 2002). Therefore, increased abundance levels of RuBisCo following stress in ICSV213 might be attributed to enhanced photorespiration. Abundance levels for the ICSB338 RuBisCo remained unaltered following stress. It should be noted that the molecular weight of RuBiSco predicted by MS/MS analysis was not reflective of the weight observed for this spot (21) in the 2D-SDS gel, as a Mr lower than predicted was observed.

This could be attributed to protein degradation. Feller *et al.,* (2008) has reported on the rapid degradation of RuBisCo fragments of the large subunit under abiotic stresses, a finding that supports our observations.

PEPC was modulated by stress induction in ICSB338; indicating the involvement of carbon fixation for photosynthesis under stressed conditions. The reduced levels of PEPC abundance was however reflective of the reduced carbon fixation, thus reduced photosynthesis efficiency. A decline in PEPC abundance levels has been reported in the leaves and nodules of chick-pea (*Cicer arietinum L.)* subjected to salt treatment (Soussi *et al.,* 1998); and cucumber cotyledons under leaf senescence (Chen *et al.,* 2000). The molecular weight of PEPC predicted by MS/MS analysis was not reflective of the weight observed for this spot (26) in the gel. This could be attributed to proteolysis, as was reported by Pladys and Vance (1993) on alfafa (*Medicago sativa)* nodules during senescence. Another possible explanation is the occurrence of different PEPC isoforms in nature, which show differential expression in response to environmental stress (Sánchez *et al.,* 2006). Immunoblots used in both studies supported our speculations on the observed discrepancies for this protein.

#### 4.3.7.2 Amino acid metabolism

Hyperosmotic stresses have been shown to cause changes in the pool of free amino acids essential in plant metabolism (Wang *et al.,* 1999). Here we found two amino acid biosynthesis-related proteins whose cellular levels are affected by drought treatments. The abundances of 5-methyltetra-hydropteroyltriglutamatehomocysteine methyltransferase (MHMT) (spot 23) and adenosylhomocysteinase (spot 18) were increased in ICSB338 following stress treatments (Table 4.5).

No significant changes in MHMT were detected in ICSV213 with stress induction, whereas adenosylhomocysteinase was not detected in either watered or stressed ICSV213 plants. MHMT and adenosylhomocysteinase are involved in the pathway of the synthesis of betaine, an osmoprotectant involved in plant metabolic adjustment under unfavourable growth conditions. Increases in expression levels of MHMT following stress induction have also been reported in Arabidopsis (Ndimba *et al.,* 2005). Li *et al.* (2011) reported increased levels of adenosylhomocysteinase following stress treatments on *Suaeda salsa*, a $C_3$ plant. The different expression patterns of these proteins involved in the synthesis of betaine suggest that the synthesis of betaine is regulated by drought stress, and that betaine accumulation is triggered in ICSB338 with stress induction.

#### 4.3.7.3 Stress/Defense

The glutathione S transferase (GST) (spot 25) was increased in ICSV213 with stress induction. Drought stress induces oxidative damage at cellular level, which results in the accumulation of ROS (Chaves *et al.,* 2009; Valliyodan and Nguyen, 2006). To protect themselves against these toxic oxygen intermediates, plants have antioxidant enzymes, such as superoxide dismutases, catalases, ascorbate peroxidases (APX), glutathione S-transferases (GST) and glutathione peroxidases (GPX) (Roxas *et al.,* 2000; Salekdeh *et al.,* 2002; Ndimba *et al.,* 2005; Haluskova *et al.,* 2009; Gomez-Garay *et al.,* 2013) that catalyze the scavenging of ROS. GST activity is dependent upon the availability of reduced glutathione. This protein conjugates glutathione to products of oxidative stress (Edwards *et al.,* 2000). Increased levels of GST following stress have also been reported in tomato (*Solanum lycopersicum*) leaves (Csiszar *et al.,* 2014); barley root tips (Haluskova *et al.,* 2009) and in different rice genotypes (Kumar *et al.,* 2013).



The isoflavone reductase homologue (IRL) (spot 20) was decreased in ICSB338 and increased in ICSV213 in response to water stress. This protein is a ROS scavenging enzyme involved in a glutathione-independent mechanism (Alam *et al.,* 2010). It accumulates in response to oxidative stress, which is correlated with drought stress occurrence. Increased levels of isoflavone reductase in response to oxidative stress have been demonstrated in rice suspension-culture cells treated with oxidants, over-expressing IRL (Kim *et al.,* 2010).

Their results confirm that rice transgenic plants confer a strong resistance to oxidative stresses. The down-regulation of isoflavone reductase in sorghum variety ICSB338 could be attributed to its suppression by the presence of tripeptide glutathione (GSH), which is a ROS quenching chemical. The increased levels of GST and IRL in ICSV213 under drought stress indicate that these proteins may act as down regulators to prevent excess ROS during oxidative stress

#### 4.3.7.4 Other drought stress responsive proteins

Other drought stress responsive proteins observed (Table 4.4 and 4.5) include eukaryotic translation elongation factor 5A (spot 22), 14-3-3 like protein 2 (fragment) (spot 6) and several unidentified proteins (spots 1; 2; 4; 8; 9; 10; 11; 14; 17; 19). Of these spots 22 had increased abundance following stress in ICSV213 but no significant changes were observed in ICSB338.



Plant eIF5A proteins are highly conserved and are involved in multiple biological processes, including protein synthesis and regulation, translation elongation, mRNA turnover and programmed cell death (Wang and Krishnaswamy, 2012). Furthermore, elF5A confers abiotic stress resistance. An increased accumulation of eukaryotic translation initiation factors indicates profound cellular reorganization leading to programmed cell death (PCD) under stress treatments. Therefore, a change in elF5A expression levels with stress induction is related to the cell requirements for new

proteins and RNA synthesis (Kosavá *et al.,* 2011). Ndimba *et al.* (2005) previously reported on the increase in abundance of this protein, with stress induction.

The 14-3-3 like protein (spot 6) levels were increased following stress induction in ICSB338 sorghum variety. This protein is highly conserved and plays a major role in protein-protein interactions. It mediates signal transduction pathways including various metabolic processes, hormone cross-talks, gene transcription, protein modification and stress responses (Porcel *et al.,* 2006; Takahashi *et al.,* 2007;Sun *et al.,* 2011). In this study, higher protein abundance of spot 6 (14-3-3 like protein) was observed in ICSB338 suggesting this protein might play an essential role in drought stress response. The over expression of a maize ZmGF14-6 gene and OsGF14c in rice conferred drought resistance indicating the involvement of 14-3-3 protein in drought stress response in plant (Shi *et al.,* 2014).



Amongst the unidentified drought stress responsive proteins, a total of two (2; 9) and seven (1; 8; 9; 11; 14; 17) had reduced abundances in ICSV213 and ICSB338 respectively (Table 4.4). Of these, the abundance of spot 9 was reduced in both varieties. Seven spots (4; 8; 10; 11; 14; 17; 19) and one spot (2) had increased abundances following stress induction in ICSV213 and ICSB338 respectively. The differential regulation in protein abundance between these two varieties serves as a possible indication of important roles of these proteins in stress tolerance.

## 4.4 General Discussion

Investigation of the underlying mechanisms of stress adaptation in plants is crucial for improving stress tolerance. Most studies on stress adaptation have however been focused mainly on changes in gene expression, while far less information is available on their protein counterparts. Recently, there has been a marked increase in the number of reports on the proteomic studies of plants exposed to various abiotic stresses (Toorchi and Kholgi, 2014; Wang *et al.,* 2013; Ngara *et al*., 2012; Swami *et al.,* 2011) .

These studies have identified a number of proteins differentially regulated by such stresses. However, the responses of these identified proteins vary from species to species. As a result, there is still a need to analyze a much larger set of stress-related proteins in more plant species. A comparative proteomics approach was applied to analyze the effects of drought induced hyperosmotic stress on the stem proteome during the early stages of anthesis. We compared the proteome pattern of the sorghum variety ICSB338 with ICSV213. Flag leaf is used as an index when making selections for drought stress tolerance due to its sensitivity (Boureima *et al.,* 2012); hence it was used in this study. Hsp70 is a known stress responsive protein and is expressed in response to abiotic stresses (Wang *et al.,* 2004; Ngara *et al.,* 2012). Following water stress, Hsp70 abundance levels increased in the flag leaf tissues for both sorghum varieties under study. These observations point out that Hsp70 immunoblotting is a good experimental indication of stress levels in stressed plants.

The observation made from the comparative analysis of 2D gels was that ICSV213 sorghum variety had more stress inducible protein spots compared to ICSB338 (Table 4.4). This is a probable indication that ICSV213 expresses more gene products, which are directly involved in stress tolerance. The results provide evidence that water deficit caused a redirection in sorghum metabolic processes in order to increase the stem reserve remobilization efficiency, and to a greater extent in ICSV213. In addition, the predominance of stress related proteins in ICSV213 highlights the importance of managing ROS and oxidative stress to protect stem cells, and to sustain stem reserve remobilization under drought conditions (Bazargani *et al.,* 2011).



These results provide new insights towards gaining a better understanding of molecular responses of sorghum to drought stress through regulation of proteins associated with stem reserve remobilization. The biological roles of the stress responsive proteins identified suggest that these proteins may be important targets for the genetic improvement of crops to drought stress. Therefore, these proteins need to be further investigated using transgenic approaches so as to determine whether increased expression provides a better and more advantageous sorghum variety and whether they can be applied as markers for selection and breeding programmes. Further work is also needed to identify the remaining candidates whose identities were not successfully established in this study with MS/MS approaches. The development of efficient methods to maximize protein visualization and resolution has become an important goal in applications of 2-DE for proteome analysis.

Poor detection of low-abundant proteins can be resolved by the use of: (i) more sensitive staining protocols; (ii) protocols optimized for the extraction and solubilization of proteins based on cell type and subcellular compartmentation; and (iii) very narrow (1 pH unit) gradients (Plomion *et al.,* 2006). Non-gel based approaches, such as liquid chromatography and multidimensional liquid chromatography coupled with electrospray ionization ion trap tandem mass spectrometry should also be incorporated into gel-based techniques as these provide closely related but distinct information about proteins (Monteoliva and Albar, 2004).

Various studies have reported on many proteins that are resolved into multiple spots on 2-D gels (Ngara *et al.,* 2012; Feller *et al.,* 2008; Rose *et al.,* 2004). As a result, there are discrepancies observed between experimentally and theoretically determined isoelectric points (pI) and molecular weights (Mr), as was reported for the carbohydrate metabolism proteins in this study. These discrepancies are attributed to isoforms displaying different signal or target sequences, resulting in shifts in pI and Mr (Plomion *et al.,* 2006). Also, *in vivo* proteolysis or in vitro protein degradation during sample preparation could contribute to these variations. Therefore, studies on protein posttranslational modifications, as well as protein interactions could also contribute in discriminating whether variations in plant proteomes, are naturally occurring (result from cellular processes) or artificial. These studies will also help in detailed protein functional characterization and to better understand the processes of plant stress acclimation and stress tolerance acquisition.

# CHAPTER 5

## CONCLUSION AND FUTURE OUTLOOK

Drought is among the major limitations to the sustainability of crop productivity in arid and semi-arid regions globally. Therefore, the development of agricultural crops with increased resistance to drought stress is required to mitigate the rise in cost of limited food resources, and to keep pace with the exponential need for renewable energy fuels. The success in developing crops with enhanced tolerance to drought stress depends upon a basic understanding of the physiological, biochemical and gene regulatory responses towards stress. Plants have acquired a variety of whole-plant protection mechanisms in response to abiotic stresses. One effective mechanism plants use to reduce damage from drought stress is the accumulation of metabolites that act as osmoprotectants. Metabolite accumulation in response to stress has been attributed to gene regulation. Therefore, the engineering of osmoprotectant biosynthesis pathways for improved stress tolerance becomes largely dependent upon the regulatory mechanisms of stress-related genes. The use of approaches, which combine physiological, metabolic and molecular aspects of drought stress tolerance, is proving useful in bridging the knowledge gap between the gene expression studies and plant physiology under environmental stress conditions. Different ‘omics’ approaches are being used to understand the various processes of molecular networks in response to drought, amongst other abiotic stresses.

They allow us to obtain a holistic view of how plants respond to a variety of abiotic stress conditions. This is due to the ability to look at stress response on a number of different cellular levels. In a continued effort to identify stress-related genes, this study explored ‘omics’ approaches to investigate the potential role of sugar-biosynthesis genes, as well as other novel mechanisms, in conferring drought tolerance in sorghum varieties.

Chapter 2 explored the response mechanisms in sorghum varieties at a physiological level. The results obtained revealed that sorghum plants undergo a series of changes at physiological and biochemical levels with the onset of stress. The reduction of the photosynthetic capacity during the period of stress was an indicator that stem reserves serve as an alternative energy source under stress conditions. The significant role of these stem reserves in osmotic adjustment under stress was highlighted by the accumulation of several metabolites, which confirmed stem reserve mobilization. It was observed that among the accumulated metabolites, soluble sugars play a major role in maintaining homeostasis and modulating plant development under adverse growth conditions. These results also suggested that sugar signaling pathways interact with stress-related pathways to modulate metabolic plant responses. For this reason the study was extended to the role of sucrose, a major photoassimilate and osmoprotectant, in stress tolerance through regulatory mechanisms of the sucrose biosynthesis pathway

Chapter 3 focused on the regulation of sucrose biosynthesis genes under stressed conditions. The differential regulation, at protein and transcript level, under the different growth conditions highlighted the significant role of these genes in sorghum stress response. In linking the expression levels with sucrose levels among the sorghum varieties, it was established that expression was regulated in response to two types of stresses: water deficit or osmoticum (sugar concentration) stress. Where a relationship was established between sugar levels and gene expression, it could be suggested that gene regulation was in response to the influx in sugar concentrations, and therefore more related to carbohydrate partitioning for the maintenance of the grain filling capacity under stressed conditions. The non-existent pattern observed between sugar levels and gene expression in some of the sorghum varieties indicated that gene regulation was linked directly to responses towards water deficit. Therefore, other candidate genes and/or isoforms of the carbohydrate metabolic pathway could have contributory roles in regulating sugar levels under stressed conditions in these varieties.



For this reason, a comparative proteomic analysis on the regulation of carbohydrate metabolism components in response to stress was investigated in Chapter 4, using the most stress responsive and least stress response sorghum varieties based on the expression studies (Chapter 3). Apart from the sucrose metabolism proteins that were influenced by either water or sugar stress, differential regulation was also observed amongst other proteins coding for carbohydrate metabolism and other metabolic functions. The distinct regulatory patterns observed between these varieties confirmed the possible role of other metabolic pathways to stress response.

From these results, combined together with the physiology and gene expression work, we could conclusively say that variety ICSV213 had the enhanced tolerance towards drought-induced stress. Furthermore we could conclude that the role of soluble sugars strengthens the foundation on which the genetic engineering of crop plants for regulated sucrose biosynthesis under drought conditions can be used to improve stress tolerance in stress susceptible sorghum varieties. The value of the sucrose pathway for drought tolerance can however be judged only through transgenics or by evidence of solid field performance. The role of soluble sugars in plant protection cannot however be limited to the modification of the sucrose biosynthesis pathway. Therefore, efforts in identifying molecular signaling components that contribute to sorghum function in response to stress are mandatory. A starting point would be to conduct further studies at both transcriptional and post-translational levels in order to fully elucidate regulatory mechanisms of the identified proteins, as well as to characterize their roles in sorghum varieties under stress.

## REFERENCES

Abbas A, Ansumali S (2010) Global potential of rice husk as a renewable feedstock for ethanol biofuel production. Bioenergy Research 3: 328-334

Abramson M, Shoseyov O, Shani Z (2010) Plant cell wall reconstruction toward improved lignocellulosic production and processability. Plant Science 178: 61-72

Ahmed CB, Rouina BB, Sensoy S, Boukhris M, Abdallah FB (2009) Changes in gas exchange proline accumulation and antioxidative enzyme activities in three olive cultivars under contrasting water availability regimes. Environmental and Experimental Botany 67: 345-352

Alam I, Sharmin SA, Kim K, Yang JK, Choi MS, Lee B (2010) Proteome analysis of soybean roots subjected to short-term drought stress. Plant Soil 333: 491-505



Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant, Cell and Environment 24: 1337-1344

Ali GM, Komatsu S (2006) Proteomic analysis of rice leaf sheath during drought stress. Journal of Proteome 5: 396-403

Amin F, Bhatti HN, Asgher M (2010) Partial purification and characterization of an acid invertase from *Saccharum Officinarum L*. Pakistan Journal of Botany 42: 2531-2540

Amudha J, Balasubramani G (2011) Recent molecular advances to combat abiotic stress tolerance in crop plants. Biotechnology and Molecular Biology Review 6: 31-58

Anjum SA, Xie X, Wang L, Saleem MF, Man C, Lei W (2011) Morphological, physiological and biochemical responses of plants to drought stress. African Journal of Agricultural Research 6: 2026-2032

Aoki N, Scofield GN, Wang XD, Patrick CE, Furbank (2004) Expression and localisation analysis of the wheat sucrose transporter *TaSUT1* in vegetative tissues. Planta 219: 176-184

Arnon D (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiology 24: 1-15

Audilakshmi S, Mall AK, Swarnalatha M, Seetharama, N (2010) Inheritance of sugar concentration in stalk (brix), sucrose content, stalk and juice yield in sorghum. Biomass and Bioenergy 34: 813-820

Barchet GLH, Dauwe R, Guy RD, Schrieder WR, Soolanayakanahallay RY, Campbell MM, Mansfield SD (2012) Investigating the drought-stress response of hybrid poplar genotypes by metabolite profiling. Tree Physiology 00: 1-17

Barker L, Kuhn C, Weise A, Schulz A, Gebhardt C, Hirner B, Hellman H, Ward JM, Frommer, WB (2000) SUT2 , a Putative sucrose sensor in sieve elements. The Plant Cell 12: 1153-1164

Barratt, DHP, Barber L, Kruger NJ, Smith AM, Wang TL, Martin C (2001) Multiple, distinct isoforms of sucrose synthase in pea. Plant Physiology 127: 655-664

Barrs HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficits in leaves. Australian Journal of Biological Science 15: 413-428

Barth I, Meyer S, Sauer N (2003) PmSUC3 : Characterization of a SUT2 / SUC3-type sucrose transporter from *Plantago major*. The Plant Cell 15: 1375-1385

Baxter CJ, Foyer CH, Turner J, Rolfe SA, Quick WP (2003) Elevated sucrose-phosphate synthase activity in transgenic tobacco sustains photosynthesis in older leaves and alters development. Journal of Experimental Botany 54: 1813-1820

Bazargani MM, Sarhadi E, Bushehri AS, Matros A, Mock H, Naghavi M, Hajihoseini V, Ehdaie B, Salekdeh GH (2011) A proteomics view on the role of drought-induced senescence and oxidative stress defence in enhanced stem reserves remobilization in wheat. Journal of Proteomics 74: 1959-1973

Bevan M, Bancroft I, Bent E, Love K,Goodman H, Dean C, Bergkamp R, Dirkse W, Van Staveren M, Stiekema W,Drost L, Ridley P, Hudson SA, Patel K, Murphy G, Piffanelli P, Wedler H, Wedler E, Wambutt R, Weitzenegger T, Pohl TM, Terryn N, Gielen J, Villarroel R, De Clerck R, Van Montagu M, A. Lecharny A, Auborg S, Gy I, Kreis M, Lao N, Kavanagh T,Hempel S, Kotter P, Entian KD, Rieger M, Schaeffer M, Funk B, Mueller-Auer S, Silvey M, James R, Montfort A, Pons A, Puigdomenech P, Douka A, Voukelatou E, Milioni D, Hatzopoulos P, Piravandi E, Obermaier B, Hilbert H, Du¨ sterho¨A, Moores T, Jones JDG, Eneva T, Palme K, Benes V, Rechman S, Ansorge W,

Cooke R, Berger C, Delseny M, Voet M, Volckaert G, Mewes HW, Klosterman S, Schueller S, Chalwatzis N (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of Arabidopsis thaliana. Nature 391: 485-488

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidant oxidative damage and oxygen deprivation stress. Annual Botany 91: 179-194

Bohnert HJ, Gong Q, Li P, Ma S (2006) Unraveling abiotic stress tolerance mechanisms-getting genomics going. Current Opinion in Plant Biology 9: 180-188

Boureima S, Oukarroum A, Diouf M, Cisse N, Van Damme P (2012) Screening for drought tolerance in mutant germplasm of sesame (*Sesamum indicum*) probing by chlorophyll a fluorescence. Environmental and Experimental Botany 81: 37-43

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254

Bucio JL, Nieto-Jacobo MF, Ramirez-Rodriguez V, Herrera-Estrella L (2000) Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Science 160: 1-13

Burger Y, Schaffer A (2007) The contribution of sucrose metabolism enzymes to sucrose accumulation in *Cucumis melo.* Journal of American Society for Horticultural Science 132: 704-712

Bogyo M, Rudd PM (2013) New technologies and their impact on ‘‘omics’’ research Current Opinion in Chemical Biology 17: 1-3

Cabiscol E, Piulats E, Echave P, Herrero E, Ros J (2000) Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. The Journal of Biological Chemistry 275: 27393-27398

Calvino M, Messing J (2011) Sweet sorghum as a model system for bioenergy crops. Current Opinion in Biotechnology 23:1-7

Capell T, Christou P (2004) Progress in plant metabolic engineering. Current Opinion in Biotechnology 15: 148-154

Carlson SJ, Chourey, PS, Helentjaris T, Datta R (2002) Gene expression studies on developing kernels of maize sucrose synthase (Susy) mutants show evidence for a third Susy gene. Plant Molecular Biology 49: 15-29

Carpentier SB, Coemas B, Podevin N, Laukens K, Witters E, Matsumura H, Terauchi R, Swennen R, Panis B (2008) Functional genomics in a non-model: transcriptomics or proteomics? Plant Physiology 133:117-130

Castleden CK, Aoki N, Gillespie VJ, Macrae EA, Quick WP, Buchner P, Foyer CH, Furbank RT, Lunn JE (2004) Evolution and Function of the Sucrose-Phosphate Synthase Gene Families in Wheat and Other Grasses. Plant Physiology 135: 1753-1764

Chalmers J, Cummings AL, Cao Y, Forster J, Spangenberg G (2005) Molecular genetic of fructan metabolism in perennial ryegrass. Plant Biotechnology 3: 459-474

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annuals of Botany 103: 551-560

Chaves MM, Oliveira MM (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. Journal of Experimental Botany 55: 2365-2384

Chaves MM, Maroco JP,Pereira JS (2003) Understanding plant responses to drought-from genes to the whole plant. Functional Plant Biology 30:239-264

Chaves MM (1991) Effects of water deficits on carbon assimilation. Journal of Experimental Botany 42: 1-16



Chen Z, Walker R, Acheson RM, Técsi L, Wingler A, Lea PJ, Leegood RC (2000) Are isocitra lyase and phospho*enol*pyruvate carboxykinase Involved in glucogenesis during senescence of barley leaves and cucumber cotyledons? Plant Cell Physiology 41: 960-967

Chengappa S, Guilleroux M, Phillips W, Shields R (1999) Transgenic tomato plants with decreased sucrose synthase are unaltered in starch and sugar accumulation in the fruit. Plant Molecular Biology 40:213-221

Cho J, Kim H, Kim C, Hahn T, Jeon J (2011) Identification and characterization of the duplicate rice sucrose synthase genes *OsSUS5* and *OsSUS7* which are associated with the plasma membrane. Molecules and Cells 31: 553-561

Ciereszko I, Kleczkowski LA (2002) Glucose and mannose regulate the expression of a major sucrose synthase gene in Arabidopsis via hexokinase-dependent mechanisms. Plant Physiology and Biochemistry 40: 907-911

Cornic G (2000) Drought stress inhibits photosynthesis by decreasing stomatal aperture -not by affecting ATP synthesis. Trends in Plant Science 5: 187-188

Cramer GR, Urano K, Delrot S, Pezzotti M, Shinozaki K (2011) Effects of abiotic stress on plants : a systems biology perspective. BMC Plant Biology 11: 163-177

Csiszar J, Horvath E, Vary Z, Galle A, Bela K, Brunner S (2014) Glutathione transferase supergene family in tomato: Salt stress-regulated expression of representative genes from distinct GST classes in plants primed with salicylic acid. Plant Physiology and Biochemistry 78: 15-26

Cushman JC, Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Current Opinion in Plant Biotechnology 3: 117-124

DaCosta M, Huang B (2006) Changes in carbon partitioning and accumulation patterns during drought and recovery for colonial bentgrass, creeping bentgrass, and velvet bentgrass. Journal of American Society of Horticultural Science 131: 484-490

Delfine S, Tognettir R, Loreto F, Alvino A (2002) Physiological and growth responses to water stress in field grown bell pepper (*Capsicum annuum,* L.). Journal of Horticultural Science and Biotechnology 77: 697-704

Delhaize E, Ryan PR, Randall PJ (1993) Aluminium tolerance in wheat (*Triticum aestivum* L.) II. Aluminium-stimulated excretion of maic acid from root apices. Plant Physiology 103: 695-702

Dejardin A, Sokolov LM, Kleczkowsi LA (1999) Sugar/osmoticum levels modulate differential abscicic acid-independent expression of two stress-responsive sucrose synthase genes in *Arabidopsis*. Biochemical Journal 344: 503-509



de Oliveria Neto CF, da Silva Lobato AK, Goncalves-Vidigal MC, da Costa RCL, dos Santos Filho BG, Alves GAR, de Mello e Silva Maia WJ, Cruz FJR, Neves HKB, dos Santos Lopes MJ (2009) Carbon compound and chlorophyll contnes in sorghum submitted to water deficit during three growth stages. Journal of Food, Agriculture and Environment 7: 588-593

Djilianov D, Georgievia T, Moyankova D, Atanassov A, Shinozaki K, Smeekens SCM, Verma DPS, Murata V (2005) Improved abiotic stress tolerance in plants by accumulation of osmoprotectants-gene transfer approach. Biotechnology and Biotechnology 19:63-71

Diouf D (2011) Recent advances in cowpea [*Vigna unguiculata* (L.) Walp.] research for genetic improvement. African Journal of Biotechnology 10: 2803-2810

Dinant S, Lemoine R. (2010) The phloem pathway : New issues and old debates. Comptes Rendus Biologies 333: 307-319

Dixon RA, Gang DR, Charlton AJ, Fiehn O, Kuiper HA, Reynolds TL, Tjeerdema RS, Jeffery EH, German JB, Ridley WP, Sieber JN (2006) Application of metabolomics in agriculture. Journal of Agricultural and Food Chemistry 54: 8984-8994

Doubnerova V, Ryslava H (2011) What can enzymes of $C_4$ photosynthesis do for $C_3$ plants under stress? Plant Science 180: 575-583

Duff AP, Andrews TJ, Curmi PMG (2000) The Transition between the open and closed states of rubisco is triggered by the inter-phosphate distance of the bound bisphosphate. Journal of Molecular Biology 298: 903-916



Edwards GE, Franceschi VR, Voznesenskaya EV (2004) Single-cell $C_4$ photosynthesis versus the dual-cell (kranz) paradigm. Annual Review in Plant Biology 55:173-196

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione S-transferase enzymes with multiple functions in sickness and in health. Trends in Plant Science 5: 193-198

Elmore A J, Shi X, Gorence NJ, Li X, Jin H, Wang F, Zhang X (2008) Spatial distribution of agricultural residue from rice for potential biofuel production in China. Biomass and Bioenergy 32: 22-27

ElSayed AI, Rafudeen MS, Golldack D (2014) Physiological aspects of raffinose family oligosaccharides (RFOs) in plants: Protection against abiotic stress. Plant Biology 16: 1-8

ElSayed AI, Boulila N, Rafudeen MS (2013) Investigation into the expression of sucrose transporters and sucrose phosphate synthase mRNA in different plant species. Agricultural Research 2: 31-42

Endler A, Meyer S, Schelbert S, Schneider T, Weschke W, Peters SW, Keller F, Baginsky S, Martinoia E, Schmidt UG (2006) Identification of a vacuolar sucrose transporter in barley and Arabidopsis mesophyll cells by a tonoplast proteomic approach. Plant Physiology 141: 196-207

Feller U, Anders I, Demirevska K (2008) Degradation of rubisco and other chloroplast proteins under abiotic stress. General Application in Plant Physiology 34: 5-8

Ferreira S, Hjerno K, Larsen M, Wingsle G, Larsen P, Fey S, Roepstorff P, Pais MS (2006) Proteome profiling of *Populus euphratica* Oliv. upon heat stress. Annals of Botany 98: 361-377

Fischer RAT, Edmeades GO (2010) Breeding and cereal yield progress. Crop Science 50: 85-98

Flexas J, Bota K, Loreto F, Cornic G, Sharkey TD (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in $C_3$ plants. Plant Biology 6: 269-279

Flexas J, Bota J, Escalona JM, Sampol B, Medrano H (2002) Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. Functional Plant Biology 29:461-471

Furbank RT, Scofield GN, Hirose T, Wag X, Patrick JW, Officer CE (2001) Cellular localisation and function of a sucrose transporter *OsSUT1* in developing rice grains. Australian Journal of Plant Physiology 28: 1187-1196

Gao D, Gao Q, Xu H, Ma F, Zhao C, Liu J (2009) Physiological responses to gradual drought stress in the diploid hybrid Pinus densata and its two parental species. Trees 23: 717-728

Gaudet DA, Wang Y, Frick M, Puchalski B, PennikетC, Ouellet T, Robert L, Singh J, Laroche A (2011) Low temperature induced defence gene expression in winter wheat in relation to resistance to snow moulds and wheat diseases. Plant Science 180: 99-110



Gavaghan CL, Li JV, Hadfield ST, Hole S, Nicholson JK, Wilson ID, Howe PWA, Stanely PD, Holmes E (2011) Application of NMR-based metabolomics to the investigation of salt stress in maize (*Zea mays*). Phytochemical Analysis 2011: 214-224

George EF, Hall MA, De Klerk G (2008) The components of plant tissue culture media II: organic additions, osmotic and pH effects, and support systems. Plant Propagation by Tissue Culture pp 115-173

Ghobadi M, Taherabadi S, Ghobadi M, Mohammadi G, Jalali-Honarmand S (2013) Antioxidant capacity, photosynthetic characteristics and water relations of sunflower

(*Helianthus annus* L.) cultivars in response to drought stress. Industrial Crops and Products 50: 29-38

Gibon Y, Usadel B, Blaesing OE, Kamlage B, Hoehne M, Trethewey RN, Stitt M (2006) Intergration of metabolite with transcript and enzyme activity profiling during diurnal cycles in *Arabidopsis* rosettes. Genome Biology 7: 1-23

Gibson S (2005) Control of plant development and gene expression by sugar signaling. Current Opinion in Plant Biology 8: 93-102

Gill PK, Sharma AD, Singh P, Bhullar SS (2001) Effect of various abiotic stresses on the growth, soluble sugars and water relations of sorghum seedlings grown in light and darkness. Bulgarian Journal of Plant Physiology 27: 72-84

Glassop D, Roessner U, Bacic A, Bonnet GD (2007) Changes in the sugarcane metabolome with stem development. Are they related to sucrose accumulation? Plant Cell Physiology 48:573-584

Gomez-Garay A, Lopez JA, Camafeita E, Bueno MA, Pintos B (2013) Proteomic perspective of Quercus suber somatic embryogenesis. Journal of Proteomics 93: 314-325

Gordon AJ, Minchin FR, James CL, Komina O (1999) Sucrose synthase in legume nodules is essential for nitrogen fixation. Plant Physiology 120:867-877

Gupta AK, Kaur N (2005) Sugar signalling and expression in relation to carbohydrate metabolism under abiotic stresses in plants. Journal of Bioscience 30: 761-776

Hajirezaei M, Bornke F, Piesker M, Takahata Y, Lerchi J, Kirakosyan A, Sonnewald U (2003) Decreased sucrose content triggers starch breakdown and respiration in stored potato tubers ( *Solanum tuberosum*) 54: 477-488

Haluskova L, Valentovicova K, Huttova J, Mistrik I, Tamss L (2009) Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiology and Biochemistry 47: 1069-1074

Hamad SAR, Ali OAM (2014) Physiological and biochemical studies on drought tolerance of wheat plants by applications of amino acids and yeast extract. Annals of Agricultural Science 59: 133-145

Hanjra MA, Qureshi ME (2010) Global water crisis and future food security in an era of climate change. Food Policy 35: 365-377

Hayward SAL (2014) Application of functional 'Omics' in environmental stress physiology : insights, limitations, and future challenges. Current Opinion in Insect Science 5: 1-7

He H, Chincinska I, Hackel, A, Grimm B, Kuhn C (2008) Phloem mobility and stability of sucrose transporter transcript. The Open Plant Science Journal 2: 1-14

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesopele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8: 1-14

Heuer B (1994) Osmoregulatory role of proline in water and salt-stressed plants. In Handbook of Plant and Crop Stress pp 363-381

Hirai MY, Klein M, Yano M, Dayan B, Yamazaki Y, Nakamura Y, Kitayama NM, Suzuki H, Sakurai N, Shibata D, Tokuhisa J, Reichelt M, Papenbrock J, Saito K (2005) Elucidation of gene-to-gene and metabolite-to-gene networks in *Arabidopsis* by integration of metabolomics and transcriptomics. The Journal of Biological Chemistry 280: 25590-25595

Hochberg U, Degu A, Toubiana D, Gendler T, Nikoloski Z, Rachmilevitch S, Fait A (2013 Metabolite profiling and network analysis reveal coordinated changes in grapevine water stress response. BMC Plant Biology 13: 184-218

Huang L, Bocock PN, Davis JM, Koch KE (2007) Regulation of invertase: a 'suite' transcriptional and post-transcriptional mechanisms. Functional Plant Biology 34: 499-507



Huang W, Wang A, Wang L, Sung H (2003) Expression and characterization of sweet potato invertase in pichia pastoris. Journal of Agriculture and Food Chemistry 51: 1494-1499

Huber SC, Huber JL (1996) Role and regulation of sucrose phosphate synthase in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology 47: 431-444

Huber SC, Akazawa T (1986) A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. Plant Physiology 81: 1008-1013

Huseynova IM (2012) Photosynthetic characteristics and enzymatic antioxidant capacity of leaves from wheat cultivars exposed to drought. Biochimica et Biophysica Acta 1817: 1516-1523

Ibraheem O, Dealtry G, Roux S, Bradley G, Box PO, Elizabeth P, Africa S (2011) The effect of drought and salinity on the expressional levels of sucrose transporters in rice (*Oryza sativa Nipponbare* ) cultivar plants. Plant Omics Journal 4: 68-74

Ibraheem O, Hove RM, Bradley G (2008) Sucrose assimilation and the role of sucrose transporters in plant wound response. African Journal of Biotechnology *7*: 4850-4855

Intergovernmental Panel on Climate Change (2007) http://www.ipcc.ch. Accessed 25 October 2014

Iraqi D, Le VQ, Lamhamedi MS,Tremblay FM (2005) Sucrose utilization during somatic embryo development in black spruce : involvement of apoplastic invertase in the tissue and of extracellular invertase in the medium. Journal of Plant Physiology 162: 115-124

Ishimaru K, Hirotsu N, Kashiwagi T, Madoka Y, Nagasuga K, Ono K, Ohsugi R (2008) Overexpression of a maize *SPS* gene improves yield characters of potato under field conditions. Plant Production Science 11: 104-107

Ishimaru K, Hirose T, Aoki N, Ono K, Yamamoto S, Wu J, Saji S, Baba T, Ugaki M, Matsumoto, Ohsugi (2001) Antisense expression of a rice sucrose transporter OsSUT1 in rice (*Oryza sativa* L.). Plant Cell Physiology 42: 1181-1185

Kang B, Xiong Y, Williams DS, Pozueta-Romero D, Chourey PS (2009) Miniature1 - encoded cell wall invertase is essential for assembly and function of wall-in-growth in the maize. Plant Physiology 151: 1366-1276

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL (2004) Exploring the temperature-stress metabolome of Arabidopsis. Plant Physiology 136: 4159-4168

Karp A, Richter GM (2011) Meeting the challenge of food and energy security. Journal of Experimental Botany 62: 3263-3271

Khalili A, Akbari N, Chaichi MR (2008) Limited irrigation and phosphorus fertilizer effects on yield and yield components of grain sorghum (*Sorghum bicolor L.var.Kimia*). Journal of Agricultural and Environmental Science 3:697-702



Khan S, Hanjra MA (2009) Footprints of water and energy inputs in food production - Global perspectives. Food Policy 34: 130-140

Kholova J, Hash C, Kumar PL, Yadav RS, Kocova M, Vadez V (2010) Terminal drought-tolerant pearl millet [*Pennesetum glaucum* (L.) R.Br.] have high leaf ABA and limit transpiration at high vapour pressure deficit. Journal of Experimental Botany 61: 1431-1440

Khun C, Franceschi VR, Schulz, Lemoine R, Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. Science 275: 1298-1301

Kim SG, Kim ST, Wang Y, Kim S, Lee CH, Kim K, Kim J, Lee SY, Kang KY (2010) Overexpression of rice isoflavone reductase-like gene (*OsIRL*) confers tolerance to reactive oxygen species. Physiologia Plantarum 138: 1-9

Kim J, Mahe Bragneon J, Prioul J (2000) A maize vacuolar invertase, *IVR2*, is induced by water stress. Organ/tissue specificity and duirnal modulation of expression. Plant Physiology 124: 71-84

Klann EM, Hall B, Bennett AB (1996) Antisense acid invertase (*TIV1*) gene alters soluble sugar composition and size in transgenic tomato fruit. Plant Physiology 112: 1321-1330

Knipp G, Honermeier (2006) Effect of water stress on proline accumulation of genetically modified potatoes (*Solanum tuberosum* L.) generating fructans. Journal of Plant Physiology 163: 392-397



Koch, K (2004) Sucrose metabolism : regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current Opinion in Plant Biology 7: 35-246

Kosova K, Vitamvas P, Prasil T, Renaut J (2011) Plant proteome changes under abiotic stress-contribution of proteomics studies to understanding plant stress response. Journal of Proteomics 74: 1301-1322

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Corted D, Shulaev V, Mittler R (2008) Ascorbate peroxidise 1 plays a key role in the response of Arabidopsis thaliana to stress combination. The Journal of Biological Chemistry 283: 34197-34203

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. Journal of Experimental Botany 63: 159-1608

Krishnan P, Kruger NJ, Ratcliffe RG (2005) Metabolite fingerprinting and profiling in plants using NMR. Journal of Experimental Botany 56: 255-265

Kudoyarova G, Veselova S, Hartung W, Farhutdinov R, Veselov D, Sharipova G (2011) Involvement of root ABA and hydraulic conductivity in the control of water relations in wheat plants exposed to increased evaporative demand. Planta 233: 87-94

Kuhn C, Grof CPL (2010) Sucrose transporters of higher plants. Current Opinion of Plant Biology 13: 288-298

Kumar S, Asif MH, Chakrabarty D, Tripathi RD, Dubey RS, Trivedi PK (2013) Expression of a rice Lamda class of glutathione S-transferanse, *OsGSTL2*, in Arabidopsis provides tolerance to heavy metal and other abiotic stresses. Journal of Hazardous Material. 248: 228-247

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lalonde S, Wipf D, Frommer WB (2004) Transport mechanisms of carbon and nitrogen between source. Annual Review in Plant Biology 55: 341-372

Lalonde S, Tegeder M, Frommer WB, Patrick JW (2003) Phloem loading and unloading of sugars and amino acids. Plant Cell and Environmental 26: 37-56

Langenkamper G, Fung RWM, Newcomb RD, Atkinson RG, Gardner RC, MacRae EA (2002) Sucrose phosphate synthase genes in plants belong to three different families. Journal of Molecular Evolution 54: 322-332

Langridge P, Fleury D (2011) Making the most of 'omics' for crop breeding. Trends in Biotechnology 29: 33-40

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficit in higher plants. Plant, Cell and Environment 25: 275-294

Lawrence CJ, Walbot V (2007) Translational genomics for bioenergy production from fuelstock grasses : maize as the model species. The Plant Cell 19: 2091-2094

Lee K, Bae DW, Kim SH, Han HJ, Liu X, Park HC, Lim CO, Lee SY, Chung WS (2010) Comparative proteomic analysis of the short-term responses of rice roots and leaves to cadium. Journal of Plant Physiology 167: 161-168

Lemoine R (2000) Sucrose transporters in plants : update on function and structure. Biochimica et Biophysica Acta 1465: 246-262

Li W, Zhang C, Lu Q, Wen X, Lu C (2011) The combined effect of salt stress and heat shock on proteome profiling in *Suaeda sala.* Journal of Plant Physiology 168: 1743-1752

Li CY, Shi JX, Weiss D, Goldschmidt EE (2003) Sugars regulate sucrose transporter gene expression in citrus. Biochemical and Biophysical Research Communications 306:402-407

Liu C, Huang L, Chang C, Sung H (2006) Purification and characterization of soluble invertases from suspension-cultured bamboo (*Bambusa edulis*) cells. Food Chemistry 96: 621-631

Liu F, Jensen C, Andersen MN (2004) Pod et Related to Photosynthetic Rate and Endogenous ABA in Soybeans Subjected to Different Water Regimes and Exogenous ABA and BA at Early Reproductive Stages. Annals of Botany 94: 405-411

Liu S, Chen S, Chen S, Liang X, Guo Z (2003) Responses of proline content and activity of antioxidant enzymes in warm-season turfgrasses to soil drought stress. Acta Horticulturae Sinica 30: 303-306



Lu Y, Lam H, Pi E, Zhan Q, Tsai S, Wang C, Kwan Y, Ngai S (2013) Comparative metabolomics in *Glycine max* and *Glycine soja* under salt stress to reveal the phenotypes of their offspring. Journal of Agricultural and Food Chemistry 61: 8711-8721

Lunn JE (2007) Compartmentation in plant metabolism. Journal of Experimental Botany 58: 35-47

Lunn JE, Macrae E (2003) New complexities in the synthesis of sucrose. Current Opinion in Plant Biology 6: 208-214

Lutfiyya LL, Xu N, Ordine RLD, Morrell JA, Miller PW, Duff SMG (2007) Phylogenetic and expression analysis of sucrose phosphate synthase isozymes in plants. Journal of Plant Physiology 164: 923-933

MacGregor EA (2002) Possible structure and active site residues of starch, glycogen, and gucrose synthases. Journal of Protein Chemistry 21: 297-306

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: An overview. Archives of Biochemistry and Biophysics 444: 139-158

Manivannan P, Jaleel CA, Somasundaram R, Panneerselvam R (2008) Osmoregulation and antioxidant metabolism in drought-stressed *Helianthus annus* under triadimefon drenching. Comptes Rendus Biologies 331: 418-425

Massacci A, Battistelli A, Loreto F (1996) Effect of drought stress on photosynthetic characteristics, growth and sugar accumulation of field-grown sweet sorghum. Australian Journal of Plant Physiology 23: 331-340

McCutchan H, Shackel KA (1992) Stem water potential as a sensitive Indicator of water stress in prune tress (Prunus domestica L. cv. French). Journal of the American Society for Horticultural Science 117: 607-611

Misic D, Siler B, Zivkovic JN, Simonovic A, Maksimovic V, Budimir S, Janosevic D, Durickovic M, Nikolic M (2012) Contribution of inorganic cations and organic compounds to osmotic adjustment in root cultures of two *Centarium* species differing in tolerance to salt stress. Plant Cell Tissue and Organ Culture 108: 389-400

Moghaddam MRB, Van den Ende (2013) Sugars and plant innate immunity. Journal of Experimental Botany 63: 3989-3998

Mohammadkhani N, Heidari R (2008) Drought-induced accumulation of soluble sugars and proline in two maize varieties. World Applied Sciences Journal 3: 448-453

Monteoliva L, Albar JP (2004) Differential proteomics: An overview of gel and non-gel based approaches. Briefings in Functional Genomics and Proteomics 3: 220-239

Moreno J, Spreitzer RJ (1999) Chloroplast-encoded large subunit affects stability and stress-induced turnover of ribulose-1,5-bisphosphate carboxylase/ oxygenase. The Journal of Biological Chemistry 274: 26789-26793

Moroco JP, Rodrigues ML, Lopes C, Chaves MM (2002) Limitation to leaf photosynthesis in field-grown grapevine under drought-metabolic and modelling approaches. Functional Plant Biology 29: 451-459

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annual Review of Plant Biology 59: 651-681

Munns R (2002) Comparative physiology of salt and water stress. Plant, Cell and Environment 25: 239-250

Murray SC, Sharma A, Rooney WL, Klein PE, Mullet JE, Mitchell SE, Kresovich S (2008) Genetic improvement of sorghum as a biofuel feedstock : QTL for stem sugar and grain non-structural carbohydrates. Crop Science 48:2165-2179

Mutava RN, Prasad PVV, Tuinstra MR, Kofoid KD, Yu J (2011) Characterization of sorghum genotypes for traits related to drought tolerance. Field Crops Research 123:10-18

Nam MH, Huh SM, Kim KM, Park WJ, Seo JB, Cho K, Kim DY, Kim BG, Yooh IN (2012) Comparative proteomic analysis of early salt stress-responsive proteins in roots of SnRK2 transgenic rice. Proteome Science 10:25-44

Namich AAM (2007) Response of cotton cultivar Gza 80 to application of glycine betaine under drought conditions. Minufiya Journal of Agricultural Research 32: 1637-1651

Nasim W, Ahmad A, Wajid A, Akhtar J, Muhammad D (2011) Nitrogen effects on growth and development of sunflower hybrids under agro-climatic conditions of multan. Pakistan Journal of Botany 43: 2083-2092



Nayyar H, Gupta D (2006) Differential sensitivity of $C_3$ and $C_4$ plants to water deficit stress: association with oxidative stress and antioxidants. Environmental and Experimental Botany 58: 106-113

Ndimande GS (2007) Comparative analysis of differential gene expression in the culms of sorghum. MSc Thesis Stellenbosch University pp 1-117

Ndimba BK, Chivasa S, Simon WJ, Slabas AR (2005) Identification of *Arabidopsis* salt and osmotic stress responsive proteins using two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 5: 4185-4196

Ndimba BK, Chivasa S, Hamilton JM, Simon WJ, Slaba AR (2003) Proteomic analysis of changes in extracellular matrix of Arabidopsis cell suspension cultures induced by fungal elicitors. Proteomics 3: 1047-1059

Ngara R, Ndimba R, Borch-Jensen J, Jensen ON, Ndimba B (2012) Identification and profiling of salinity-stress responsive proteins in *Sorghum bicolor* seedlings. Journal of Proteomics 75: 4139-4150

Niu G, Xu W, Rodriguez D, Sun Y (2012) Growth and physiological responses of maize and sorghum genotypes to salt stress. International Scholarly Research Network Agronomy 2012: 1-12

Noiraud N, Delrot S, Lemoine R (2000) The sucrose transporter of celery: identification and expression during salt stress. Plant Physiology 122: 1447-1456

Nookaraju A, Upadhyaya CP, Pandey SK, Eun K, Jin S, Keun S, Won S (2010) Molecular approaches for enhancing sweetness in fruits and vegetables. Scientia Horticulrae 127: 1-15

Ogbaga CC, Stepien P, Johnson GN (2014) Sorghum (*Sorghum bicolor*) varieties adopt strongly contrasting strategies in response to drought. Physiologia Plantarum 152: 389-401

Okamura M, Aoki N, Hirose T, Yonekura M, Ohto C, Ohsugi R (2011) Tissue specificity and diurnal change in gene expression of the sucrose phosphate synthase gene family in rice. Plant Science 181: 159-166

Park JY, Canam T, Kang KY, Ellis DD, Mansfield SD (2008) Over-expression of an arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development. Transgenic Research 17: 181-92

Park JY, Canam T, Kang KY, Ellis DD, Mansfield SD (2008) Over- expression of an Arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development. Transgenic Research 17: 181-192

Park OK (2004) Proteomic studies in plants. Journal of Biochemistry and Molecular Biology 37: 133-138

Passarinho JAP, Lamosa P, Baeta JP, Santos H, Ricardo CPP (2006) Annual changes in concentrations of minerals and organic compounds of *Querucus suber* leaves. Plant Physiology 127: 100-110



Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, Haberer G, Hellsten U, Mitros T, Poliakov A, Schmutz J, Spannagl M, Tang H, Wang X, Wicker T, Bharti AK, Chapman J, Feltus FA, Gowik U, Grigoriev IV, Lyons E, Maher CA, Martis M, Narechania A, Otillar RP, Penning BW, Salamova A A, Wang Y, Zhang L, Carpita NC, Freeling M, Gingle AR, Hash CT, Keller B, Klein P, Kresovich S, McCann MC, Ming R, Peterson DG, Mehboobur R, Ware D, Westhoff P, Mayer KF, Messing J, Rokhsar DS (2009) The *Sorghum bicolor* genome and the diversification of grasses. Nature 457: 551-556

Pilon-Smits EAH, Terry N, Sears T, Dun KV (1999) Enhanced drought resistance in fructan-producing sugar beet. Plant Physiology and Biochemistry 37: 313-317

Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeuken MJW (1995) Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiology 107: 125-130

Pinheiro C, Chaves MM, Ricardo CP (2001) Alterations in carbon and nitrogen metabolism induced by water deficit in the stems and leaves of *Lupinus albus* L. Journal of Experimental Botany 52: 1063-1070

Pladys D, Vance CP (1993) Proteolysis during development and senescence of effective and plant gene-controlled ineffective alfafa nodules. Plant Physiology 103: 379-384



Plomion C, Lalanne C, Claverol S, Meddour H, Kohler A, Bogeat-Triboulot M, Barre A, Le Provost G, Dumazet H, Jacob D, Bastien C, Dreyer E, de Daruvar A, Guehl J, Schitter J, Martin F, Bonneu, M (2006) Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. Proteomic 6: 6509-6527

Porcel R, Aroca R, Azcón R, Ruiz-Lozano JM (2006) PIP aquaporin expression in arbscular mycorrhizal *Gycine max* and Lactuca sativa plants in relation to drought stress tolerance. Plant Molecular Biology 60: 389-404

Qazi HA, Paranjpe S, Bhargava S (2012) Stem sugar accumulation in sweet sorghum-Activity and expression of sucrose metabolizing enzymes and sucrose transporters. Journal of Plant Physiology 169: 605-613

Qi X, Wu Z, Li J, Mo X, Wu S, Chu J, Wu P (2007) AtCYT-INV1, a neutral invertase, is involved in osmotic stress-induced inhibition on lateral root growth in *Arabidopsis*. Plant Molecular Biology 64: 575-587

Quintero, JA, Montoya MI, Sanchez OJ, Giraldo OH, Cardona CA (2008) Fuel ethanol production from sugarcane and corn : Comparative analysis for a Colombian case. Energy 33:385-399

Rabilloud T (2002) Two-dimensional gel electrophoresis in proteomics: old fashioned, but it still climbs up the mountains. Proteomics 2: 3-10



Rae AL, Perroux JM, Grof CPL (2005) Sucrose partitioning between vascular bundles and storage parenchyma in the sugarcane stem: a potential role for the ShSUT1 sucrose transporter. Planta 220: 817-825

Rao GP, Srivastava S, Guota PS, Sharma SR, Singh A, Singh S, Singh M, Marcone C (2008) Detection of sugarcane grassy shoot phytoplasma infecting sugarcane in India and its phylogenetic relationships to closely related phytoplasmas. Sugar Tech 10: 74-80

Rathinasabapathi B (2000) Metabolic engineering for stress tolerance: Installing osmoprotectant synthesis pathways. Annuals of Botany 86: 709-716

Reddy BVS, Ramesh S, Kumar A, Wani SP, Ortiz R, Ceballos H, Sreedevi TK (2008) Bio-fuel crops research for energy security and rural development in developing countries. Bioenergy Research 1: 248-258

Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology 161: 1189-1202

Reimholz R, Geier M, Haake V, Deitng U, Krause KP, Sonnewald U, Stitt M (1997) Potato plants contain multiple forms of sucrose phosphate synthase , which differ in their tissue distributions, their levels during development, and their responses to low temperature. Plant, Cell and Environment 20: 291-305



Reismeier JW, Willimitzer L, Frommer WB (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. The EMBO Journal 11: 4705-4713

Reismeier JW, Hirner B, Frommer WB (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. The Plant Cell 5:1591-1598

Riccardi F, Gazeau P, de Vienne D, Zivy M (1998) Protein changes in response to progressive water deficit in maize. Plant Physiology 117: 1253-1263

Rizal G, Karki S (2011) Alcohol dehydrogenase (ADH) activity in soybean (*Glycine max* [L.] Merr.) under flooding stress. Electronic Journal of Plant Breeding 2: 50-57

Roitsch T (1999) Source-sink regulation by sugar and stress. Current Opinion in Biology 2: 198-206

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annual Reviews in Plant Biology 57: 675-709

Rolland N, Ferro M, Seigneurin-Berny D, Garin J, Douce R, Joyard J (2003) Proteomics of chloroplast envelope membranes. Photosynthesis Research 78: 205-230

Rong-hua LI, Peio-pol G, Baumz M, Grando S, Ceccarelli S (2006) Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. Agricultural Sciences in China 5: 751-757



Rontein D, Basset G, Hanson AD (2002) Metabolic engineering of osmoprotectant accumulation in plants. Metabolic Engineering 4: 49-56

Rooney WL, Blumenthal J, Bean B, Mullet JE (2007) Designing sorghum as a dedicated bioenergy feedstock. Biofuels Bioproducts and Biorefining 1: 147-157

Rosa M, Prado C, Podaza G, Interdonato R, Gonzalez JA, Hilal M, Prado FE (2009) Soluble sugars-metabolism, sensing and abiotic stress. Plant Signal and Behavior 4: 388-393

Rose JK, Bashir S, Giovannoni JJ, Jahn MM, Saravanan RS (2004) Tackling the plant proteome: practical approaches, hurdles and experimental tools. Plant Journal 39: 715-733

Rossouw D, Kossmann J, Botha FC, Groenewald J (2010) Reducing neutral invertase activity in the culm tissues of transgenic sugarcane plants results in a decrease in respiration and sucrose cycling and an increase in the sucrose to hexose ratio. Functional Plant Biology 37: 22-31

Roxas VP, Lodhi SA, Garrett DK, Mahan JR, Allen RD (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. Plant Cell Physiology 41: 1229-1234

Ruan Y, Liwellyn DJ, Furbank RT (2003) Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. The Plant Cell 15: 952-964



Sage RF (2004) The evolution of $C_4$ photosynthesis. New Phytologist 161: 341-370

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazle B, Bennet J (2002) Proteomics analysis of rice leaves during drought stress and recovery. Proteomics 2: 1131-1145

Sanchez R, Flores A, Cejudo FJ (2006) *Arabidopsis* phospho*enol*pyruvate carboxylase genes encode immunologically unrelated polypeptides and are differentially expressed in response to drought and salt stress. Plant 223: 901-909

SAS Institute, Inc. (2012) SAS Version 9.2 64 bit. SAS Institute Inc, SAS Campus Drive,Cary, North Carolina

Sauer N (2007) Molecular physiology of higher plant sucrose transporters. FEBS Letters 581: 2309-2317

Schafer WE, Rowher JM, Botha FC (2004) Protein-level expression and localization of sucrose synthase in the sugarcane culm. Physiologia Plantarum 121:187-195

Schafleitner R, Rosales ROG, Gaudin A, Aliaga CAA, Martinez GN, Marca LRT, Bolivar LA, Delgado FM, Simon R, Bonierbale M (2007) Capturing candidate drought tolerance traits in two native Andean potato clones by transcription profiling of field grown plants under water. Plant Physiology and Biochemistry 45: 673-690

Scofield GN, Hirose T, Aoki N, Furbank RT (2007) Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. Journal of Experimental Botany 58: 3155-3169



Seki M, Umezawa T, Urano K, Shinozaki K (2007) Regulatory metabolic networks in drought stress responses. Current Opinion in Plant Biology 10: 296-302

Shakoor N, Nair R, Crasta O, Morris G, Feltus A, Kresovich S (2014) A Sorghum bicolor expression atlas reveals dynamic genotype-specific expression profiles for vegetative tissues of grain, sweet and bioenergy sorghums. Biomedical Central Plant Biology 14: 35-49

Sheen J, Zhou L, Jang J (1999) Sugars as signalling molecules. Current Opinion in Plant Biology 2: 410-418

Shi H, Ye T, Chan Z (2014) Comparative proteomic responses of two bermudagrass (*Cynodon dactylon* (L). Pers.) varieties contrasting in drought stress resistance. Plant Physiology and Biochemistry 82: 218-228

Shulaev V, Cortes D, Miller G, Mittler R (2008) Metabolomics for plant stress response. Physiologia Plantarum 132: 199-208

Sircelj H, Tausz M, Grill D, Batic F (2007) Detecting different levels of drought stress in apple trees (*Mallus domestica Borkh*) with selected biochemical and physiological parameters. Scientia Horticulturae 113: 362-369

Sivakumar P, Sharmila P, Jain V, Saradhi PP (2002) Sugars have potential to curtail oxygenase activity of Rubisco. Biochemical and Biophysical Research Communications 298: 247-250

Sivakumar P, Sharmila P, Saradhi P (2000) Proline alleviates salt-stress-induced enhancement in ribulose-1,5-bisphosphate oxygenase activity. Biochemical and Biophysical Research Communications 279: 512-515

Slewinski TL (2012) Non-structural carbohydrate partitioning in grass stems : a target to increase yield stability, stress tolerance, and biofuel production. Journal of Experimental Botany 63: 4647-4670

Slewinski TL, Braun DM (2010) Current perspectives on the regulation of whole-plant carbohydrate partitioning 178: 341-349

Slewinski TL, Meeley R, Braun DM (2009) *Sucrose transporter1* functions in phloem loading in maize leaves. Journal of Experimental Botany 60: 881-892

Smeekens S (1998) Sugar regulation of gene expression in plants. Current Opinion in Plant Biology 1: 230-234

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell and Environment 30: 1126-1149

Song Y, Zhang C, Ge W, Zhang Y, Burlingame AL. Guo Y (2011) Identification of NaCl stress-responsive apoplastic proteins in rice shoot stems by 2D-DIGE. Journal of Proteomics 74: 1045-1067



Soussi M, Ocana A, Lluch C (1998) Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-peas (*Cicer arietinum* L.). Journal of Experimental Botany 49: 1329-1337

Sturm A, Tang G-Q (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in Plant Science 4: 40-407

Sun G, Xie F, Zhang B (2011) Transcriptome-wide identification and stress properties of the 14-3-3 gene family in cotton (*Gossypium hirsutum* L.). Functional and Integrative Genomics 11: 627-636

Swami AK, Alam SI, Sengupta N, Sarin R (2011) Differential proteomic analysis of salt stress response in *Sorghum bicolor* leaves. Environmental and Experimental Botany 71: 321-328

Taji T, Ohsumi C, Iuchi S, Seki M, Masuga M, Kobayashi K, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidposis thaliana*. The Plant Journal 29: 417-426

Takahashi Y, Kinoshita T, Shimazaki K (2007) Protein phosphorylation and binding of a 14-3-3 protein in *Vicia* guard cells in response to ABA. Plant Cell Physiology 48: 1182-1191

Taliercio EW, Chourey PS (1989) Post-transcriptional control of sucrose synthase in anaerobic seedlings of maize. Plant Physiology 90: 135-1364

Tanase K, Yamaki S (2000) Purification and characterization of two sucrose synthase iIsoforms from japanese pear fruit. Plant Cell Physiology 41: 408-414

Tang G, Luscher, Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early plant development and sucrose partitioning. The Plant Cell 11: 177-189

Tardieu F, Tuberosa R (2010) Dissection and modelling of abiotic stress tolerance in plants. Current Opinion in Plant Biology 13: 206-212

Tarpley L, Vietor DM (2007) Compartmentation of sucrose during radial transfer in mature sorghum culm. Biomedical Central Plant Biology 7: 33-42

Thiellement H, Zivy M, Plomion C (2002) Combining proteomic and genetic studies in plants. Journal of Chromatography 78: 137-149

Tolbert NE (1997) The C2 oxidative photosynthetic carbon cycle. Annual Reviews in Plant Physiology 48: 1-25

Toorchi M, Kholgi M (2014) Proteomic analysis of salt-responsive proteins in canola leaves. International Journal of Biosciences 5: 68-76

Trouverie J, Thevenot C, Rocher J, Sotta B, Prioul J (2003) The role of absisic acid in the response of a specific vacuolar invertase to water stress in the adult maize leaf. Journal of Experimental Botany 54: 2177-2186



Truernit E (2001) The importance of sucrose transporters. Plant Current Biology 11: 169-171

Truernit E, Sauer N (1995) The promoter of the Arabidopsis thaliana SUC 2 sucrose-$H^+$ symporter gene directs expression of beta-glucoronidase to the phloem: evidence for phloem loading and unloading by SUC2. Planta 196: 564-570

Turgeon R, Wolf S (2009) Phloem transport: cellular pathways and molecular trafficking. Annual Reviews in Plant Biology 60: 207-221

Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Current Opinion in Biotechnology 17: 113-122

Unger M, Dreyer M, Specker S, Laug S, Pelzing M, Neusub C, Holzgrabe U, Bringmann G (2004) Analytical characterisation of crude extracts from an african *Ancistrocladus* species using high-performance liquid chromatography and capillary electrophoresis coupled to ion trap mass spectrometry. Phytochemical Analysis 15: 21-26

Urano K, Kurihara Y, Seki M, Shinozako K (2010) 'Omics' analyses of regulatory networks in plant abiotic stress responses. Current Opinion in Plant Biology 13: 132-138

Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Current Opinion in Plant Biology 9: 1-7



Van den Ende W, El-Esawe (2014) Sucrose signalling pathways leading to fructan and anthocyanin accumulation: A dual function in abiotic and biotic stress response? Environmental and Experimental Botany 108: 4-13

Van Laere A, Van den Ende W (2002) Inulin metabolism in dicots: chicory as a model system. Plant, Cell and Environment 25: 803-813

Vinocur B, Altman A (2005) Recent Advances in engineering plant tolerance to abiotic stress: achievements and limitations. Current Opinion in Biotechnology 16: 123-132

Waclawovsky AJ, Sato PM, Lembke CG, Moore PH, Souza GM (2010) Sugarcane for bioenergy production: an assessment of yield and regulation of sucrose content. Plant Biotechnology Journal 8: 263-276

Wang X, Chang L, Wang B, Wang B, Li P, Wang L, Yi X, Huang Q, Peng M, Guo A (2013) Comparative proteomics of *Thellungiella halophila* leaves from plants subjected to salinity reveals the importance of chloroplastic starch and soluble sugars in halophyte salt tolerance. Molecular and Cellular Proteomics 12: 2174-2195

Wang A, Krishnaswamy S (2012) Eukaryotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement. Molecular Plant Pathology 13: 795-803

Wang J, Roe B, Macmil SM Yu Q, Murray JE, Tang H, Chen CM Najar F, Willey G, Bowers J, Van Sluys M, Rokhsar DS, Hudson ME, Moose SP, Paterson AH, Ming R (2010) Microcollinearity between autopolyploid sugarcane and diploid sorghum genomes. Biomedical Central Genomics 11: 261-278

Wang W, Basia Vinocur B, Oded Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science 9: 244-252

Wang H, Lee P, Liu L, Su J (1999) Effect of sorbitol induced osmotic stress on the changes of carbohydrate and free amino acid pools in sweet potato cell suspension cultures. Botabical Bulletin of Academia Sinica 40: 219-225

Wardlaw IF, Willenbrink J (2000) Mobilization of fructan reserves and changes in enzyme activities in wheat stems correlate with water stress during kernel filling. New Phytologist 148: 413-422

Weise A, Barker L, Kühn C, Lalonde S, Buschmann H, Frommer WB, Ward JM (2000) A new subfamily of sucrose transporters , SUT4, with low affinity / high capacity localized in enucleate sieve elements of plants. The Plant Cell 12: 1345-1355

Whittaker A, Martinelli T, Farrant JM, Bochicchio A, Vazzana C (2007) Sucrose phosphate synthase activity and the co-ordination of carbon partitioning during sucrose and amino accumulation in desiccation-tolerant leaf material of the $C_4$ resurrection plant *Sporobolus stapifianus* during dehydration. Journal of Experimental Botany 58: 3775-3787

Widodo JHP, Newbigin E, Tester M, Bacic A, Roessner U (2009) Metabolic responses to salt stress of barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. Journal of Experimental Botany 60: 4089-4103

Wind J, Smeekens S, Hnason J (2010) Sucrose: metabolite and signaling molecule. Phytochemistry 71: 1610-1614

Wingler A, Quick WP, Bungard RA,Bailey KJ, Lea PJ, Leegood RC (1999) The role of photorespiration during drought stress: an analysis utilizing barely mutants with reduced activities of photorespiratory enzymes. Plant, Cell and Environment 22: 361-373

Winter H, Huber SC (2000) Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. Critical Reviews in Plant Sciences 19: 31-67

Xi J, Wang W, Li S, Zhou X, Yue L, Fan J, Hao D (2006) Polyethylene glycol fractionation improved detection of low-abundance proteins by two-dimensional electrophoresis analysis of plant proteome. Phytochemistry 67: 2341-2348

Xue G, McIntrye CL, Jenkins CLD, Glassop D (2008) Molecular dissection of variation in carbohydrate metabolism related to water-soluble carbohydrate accumulation in stems of wheat. Plant Physiology 146: 441-454

Yan S, Tang Z, Su W, Sun W (2005) Proteomic analysis of salt-stress responsive proteins in rice root. Proteomics 5: 235-244

Yang Z, Zhang L, Diao F, Huang M, Wu N (2004) Sucrose regulates elongation of carrot somatic embryo radicles as a signal molecule. Plant Molecular Biology 54: 441-459

Yonekura M, Aoki N, Hirose T, Onai K, Ishiura M, Okamura M, Ohsugi R, Ohto C (2013) The promoter activities of sucrose phosphate synthase genes in rice, *OsSPS1* and *OsSPS11*, are controlled by light and circadian clock, but not by sucrose. Frontiers in Plant Science 4: 1-8

Ytterberg AJ, Jensen ON (2010) Modification-specific proteomics in plant biology. Journal of Proteomics 73: 2249-2266

Yu X, Wang X, Zhang W, Qian T, Tang G, Guo Y, Zheng C (2008) Antisense suppression of an acid invertase genes (*MAI1*) in muskmelon alters plant growth and fruit development. Journal of Experimental Botany 59: 2969-2977

Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis. Cell 126: 636-644

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annual Reviews in Plant Biology 53: 247-273

Zhuang J, Zhang J, Hou X, Wang F, Xiong A(2014) Transcriptomics, proteomics, metabolomics and functional genomic approaches for the study of abiotic stress in vegetable crops. Critical Reviews in Plant Science 33: 225-237

# APPENDICES: Chemicals, stock solutions, buffers and melting curves

## **Appendix A:** List of chemicals used in the study

| Chemical | Supplier | Catalogue No. |
|---|---|---|
| Acetone | Merck | SAAR1022040LC |
| Acetonitrile | Merck | 1.00030.2500 |
| Ammonium hydrogen bicarbonate | Merck | 1.01131.5000 |
| 40% acrylamide/Bis Solution, 37.5:1(2.6% C) | BIO-RAD | 161-0148 |
| Adenosine 5'-triphosphate | Sigma | A2383 |
| Agarose D-1 LE | Whitehead Scientific | H101119 |
| Amyloglucosidase | Sigma | A7095 |
| Ammonium Persulfate | BIO-RAD | 161-0700 |
| 100x Bio-Lyte 3/10 Ampholyte | BIO-RAD | 163-2094 |
| Anthrone reagent | Sigma | 10740 |
| BIO-RAD Protein assay dye reagent concentrate | BIO-RAD | 500-0006 |
| Bovine Serum Albumin (BSA) Fraction V | Roche | 10 735 078 001 |
| Bromophenol blue sodium salt | Sigma | B5525 |
| Calcium nitrate | LabChem | QF3Q631208 |
| 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) | Sigma | C3023 |
| Chloroform | Merck | K36315455 |
| Coomassie® brilliant blue (CBB) R-250 | BIO-RAD | 161-0400 |
| Dithiothreitol (DTT) Cleland's reagent | Fermentas | R0861 |
| Sodium dihydrogen phosphate | Sigma | 71505 |

| | | |
|---|---|---|
| Glacial acetic acid | Merck | SAAR1021020LC |
| Ethanol | Kimix | 52/12/67 |
| Ethylenediaminetetracetic acid | Merck | SAAR2236020EM |
| Fructose | Merck | SAAR2475000EM |
| Fructose-6-phosphate disodium salt hydrate | Sigma | F3627 |
| Glucose | LabChem | 003352 |
| Glucose-6-phosphate Dehydrogenase | Sigma | G2921 |
| Glucose-6-phosphate disodium salt hydrate | Sigma | G7879 |
| Glycerol | Merck | 2676520LC |
| Glycine | BIO-RAD | 161-0724 |
| 4-(2-Hydroxyethyl) piperazin-1-ethanosulfonic acid | Sigma | H0887 |
| Hydrochloric acid | Merck | 306 30 40 LP |
| Iodoacetamide | BIO-RAD | 163-2109 |
| Magnesium chloride | Merck | 4122980EM |
| Methanol | Merck | SAAR4164080LC |
| Mineral Oil | GE HealthCare | 17-1335-01 |
| Nicotinamide adenine dinucleotide reduced dipotassium salt | Sigma | N4505 |
| Nicotinamide adenine dinucleotide | Sigma | N7004 |
| PageRulerTM unstained protein ladder | Fermentas | SM0661 |
| Phosphoglucomutase | Sigma | P3397 |
| Phospho(enol) pyruvate | Sigma | P7252 |
| Polyvinylpolypyrrolidone | Sigma | 77627 |

| | | |
|---|---|---|
| Potassium chloride | Sigma | 60132 |
| Potassium dihydrogen phosphate | LabChem | 1310402 |
| Potassium hydroxide pellets | Merck | 1.05033.0500 |
| Protease inhibitor | Sigma | P2714 |
| Pyruvate kinase/ Lactic Dehydrogenase | Sigma | P0294 |
| Sephadex 25 | Sigma | G2580 |
| Sodium acetae | Merck | 582098EM |
| Sodium chloride | Merck | 5822300EM |
| Sodium dodecyl sulfate (SDS) | BIO-RAD | 161-0302 |
| Sodium pyrophosphate | Sigma | P8010 |
| Sulphuric acid | Kimix | A1076/07 |
| Sucrose | Merck | SAAR5881500EM |
| N,N,N',N'-Tetramethylethylenediamine (TEMED) | Sigma | T9281 |
| Thiourea | Sigma | T8656 |
| Trifluoroacetic acid (TFA | Merck | 8.08260.0100 |
| Tris (hydroxymethyl)-aminomethane | BIO-RAD | 161-0719 |
| Trypsin | Promega | V5111 |
| Urea | Sigma | U0631 |
| Uridine 5'- diphosphate disodium salt hydrate | Sigma | 94330 |
| Uridine 5'- diphosphate-D-glucose | Sigma | 94335 |

**Appendix B:** Preparation of 12% resolving and 5% stacking gels for SDS-PAGE

| Constituents | 12% resolving (ml) | 5% stacking (ml) |
|---|---|---|
| Distilled water | 2.15 | 1.85 |
| 40% Acrylamide | 1.5 | 0.315 |
| 1.5M Tris-HCl pH 8.8 | 1.25 | - |
| 0.5 M Tris-HCl pH 6.8 | - | 0.315 |
| 10% SDS | 0.05 | 0.025 |
| 10% APS | 0.05 | 0.025 |
| TEMED | 0.004 | 0.0025 |



**Appendix C:** General Stock Solutions and Buffers

**80% Acetone:** 80 (v/v) in distilled water

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

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

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

**1 mg/ml BSA stock solution:** 1 mg/ml BSA in urea buffer

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

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

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

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

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

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

**Equilibration base buffer I:** 6M urea, 2% SDS, 0.375M Tris-HCL (pH 8.8); 20% glycerol and 2% DTT

**Equilibration base buffer II:** 6M urea, 2% SDS, 0.375 M Tris-HCL (pH 8.8), 20% glycerol and 2. 5% iodoacetamide

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



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

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

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

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

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

**Urea buffer:** 9 M urea, 2 M thiourea and 4% 3 [(Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

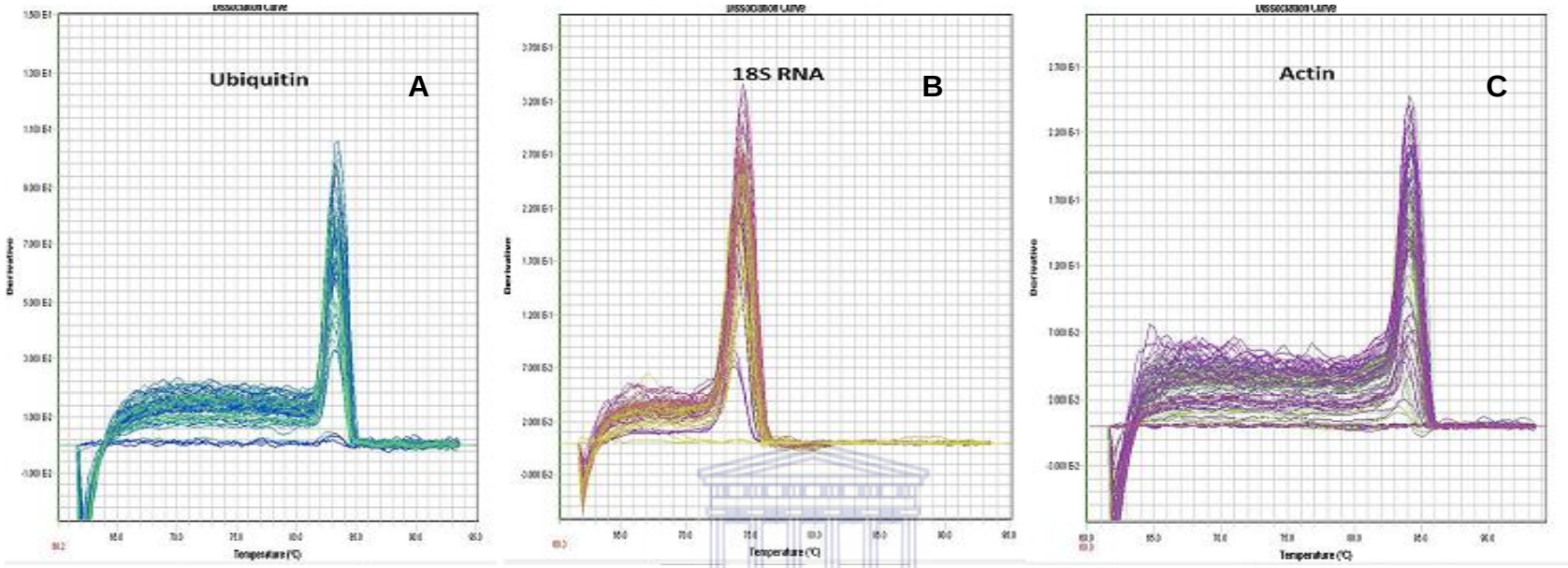


**Figure A:** Melt curve analysis of sorghum genes indicating single products for housekeeping genes: (A) ubiquitin, (B) 18S RNA and (C) actin

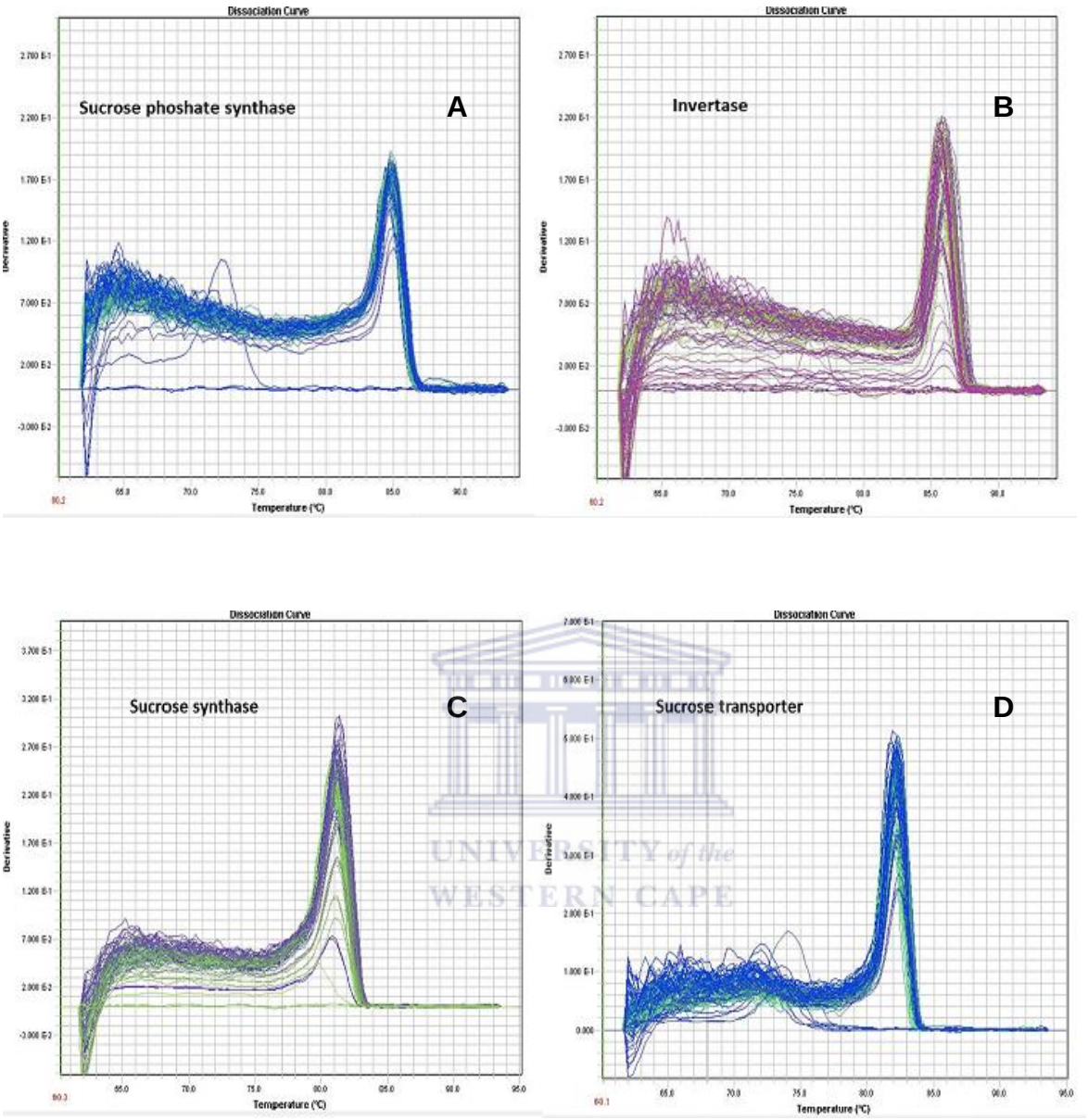


**Figure B:** Melt curve analysis of sorghum genes indicating single products for sucrose biosynthesis genes.(A) sucrose phosphate synthase, (B) soluble Invertase, (C) sucrose synthase and (D) sucrose transporter