Molecular Responses of Sorghum Cell Suspension Cultures to High Temperature Stress

Mamosa Gloria Ngcala

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Supervisor: Dr. Rudo Ngara

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DECLARATION

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ABSTRACT

High temperatures and frequent drought episodes limit plant growth and development. Ultimately, low crop yields are realised and food insecurity follows. Therefore, there is a need to develop crops that can tolerate extreme temperatures as part of adapting to the global climate change. Sorghum (*Sorghum bicolor* (L) Moench), a naturally drought tolerant crop, which survives in hot and dry environments was used in this study. The aim of the study was to evaluate the molecular responses of ICSB 338 sorghum cell suspension cultures to high temperature stress. ICSB 338 sorghum cell suspension cultures were exposed to heat stress at 35 and 40ºC for 72 hours. Analysis of the cells' metabolic activity indicated that the cells could survive at both temperatures for 72 hours. However, when Arabidopsis cell suspension cultures were exposed to 40ºC for the same period, a significant decrease in cell viability was observed. These results suggest that sorghum is more heat tolerant than Arabidopsis. The proline and glycine betaine content of ICSB 338 cell cultures was determined, following heat stress treatment at 40ºC for 72 hours. A decrease in proline content was observed during the stress treatment period. On the other hand, glycine betaine was not detectable at all in the cell culture during the entire stress treatment period. Furthermore, a western blotting experiment was performed to detect the expression pattern of HSP70 in sorghum and Arabidopsis cells in response to heat stress. The results indicated that HSP70 was highly expressed at 40ºC in both cell lines. In addition to the metabolic and biochemical changes that occur in plant cells in response to heat stress, protein and gene expression is also altered. Secreted proteins were extracted from the ICSB 338 culture medium, quantified and gel electrophoresed. Furthermore, the differential protein expression analysis of the extracellular matrix (ECM) proteins, following heat stress at 40ºC was conducted using isobaric tags for relative and absolute quantitation (iTRAQ) technology. A total of 290 proteins were positively identified. Of these, 231 (80%) were predicted to contain a signal peptide whereas 59 (20%) did not.

This indicates that most proteins were targeted for secretion via the classical secretory pathway into the ECM. Of the 290 proteins, 105 were responsive to heat stress with putative functions in metabolism (31%), disease/defence (30%), protein destination and storage (21%), signal transduction (6%) and energy (3%), while 9% had unclear classifications. However, most of the identified proteins (69%) were uncharacterised, possibly indicating their novelty in heat stress response. The expression analysis of ten target heat stress responsive genes from the proteomic dataset and other heat shock marker genes was conducted using quantitative real time-polymerase chain reaction in a time-course experiment (qRT-PCR). For all the genes analysed, differential expression patterns were observed in response to the heat stress. The observed gene and protein expressional changes indicate that sorghum is responsive to heat stress. The knowledge gained could be applied in breeding programmes for the development of heat tolerant crops to alleviate food insecurity in hot and arid regions.

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DEDICATION

This Masters dissertation is wholeheartedly dedicated to my beloved mother, Mofokeng Violet Mapapo. Being a single parent is not easy, but you have always been with your daughters, through thick and thin. Your courageous spirit and hardwork always inspire me to do better in life. I truly appreciate your unconditional love, endless support and prayers. Thank you mamzo.

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1D	One-dimensional
2D	Two dimensional
2,4-D	2,4-dichlorophenoxyacetic acid
bp	base pairs
BSA	Bovine serum albumin
CBB	Coomassie Brilliant blue
CF	Culture filtrate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
cDNA	Complementary deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol Cleland's reagent
ECM	Extracellular matrix
GO	Gene ontology
HILIC	Hydrophilic interaction chromatography
HSP	Heat shock protein
iTRAQ	isobaric Tags for Relative and Absolute Quantitation
kDa	kilo Dalton
LC	Liquid chromatography
MS	Mass spectrometry
MSMO	Murashige and Skoog Basal Medium with Minimal Organics
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MUDPIT	Multidimensional protein identification technology
MW	Molecular weight

NAA	1-Naphthalenacetic acid
NCBI	National Centre for Biotechnology Information
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TSP	Total soluble protein
v/v	volume to volume
w/v	weight to volume

CHAPTER 1

LITERATURE REVIEW

1.1 High Temperature Stress

High temperature stress is defined as an increase in temperature beyond the threshold level for a certain period, which is enough to cause irreversible changes in plant growth and development (Essemine *et al*., 2010). According to Wahid and co-workers, if the temperature is 10-15ºC above the ambient, it is considered a heat stress (Wahid *et al*., 2007). It is estimated that the global mean surface temperature will rise in the range of 1.8 to 4.0°C by the year 2100 (IPCC, 2007). These temperature increases are likely to pose a serious threat to agricultural crop productivity and food security, worldwide.

1.2 Effects of High Temperature on Plants

Crop production is constrained by both biotic and abiotic factors. Amongst the abiotic constraints, high temperature and drought are some of the most important plant growth limiting factors (Gong *et al*., 2015; Casaretto *et al*., 2016). High temperatures affect plant processes such as seed germination, respiration, photosynthesis and protein synthesis (Wahid *et al*., 2007). The chronic and acute exposure of plants to high temperatures, especially during pollination and reproductive stages of development, leads to reduced crop yield (Hatfield & Prueger, 2015). Hartfield and Prueger investigated the effect of extreme temperatures on different growth stages of maize (*Zea mays*). A significant difference in the total vegetative dry weight at extreme temperature was observed, ultimately resulting in low grain yield (Hatfield & Prueger, 2015).

Figure 1.1 below shows some of the general effects of high temperature stress on plants. However, the overall effects depend on the plant species and the developmental stage, the intensity of temperature, period of exposure and the rate at which the temperature rises (Gong *et al*., 2015). For example, an extreme temperature in broccoli (*Brassica oleracea* L.) is an optimum temperature for maize (Hatfield & Prueger, 2015). The minimum growth temperatures of broccoli and maize are 5°C and 10°C, optimum are 15°C and 25°C, and maximum 25°C and 38°C, respectively (Hatfield & Prueger, 2015). This example illustrates that a favourable temperature for one species may be limiting to another.

(Source: Hasanuzzaman *et al*., 2013)

Figure 1.1: General effects of high temperature stress on plants.

During normal growth processes at optimal temperature conditions, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, are produced by cellular processes in various plant organelles and compartments. These include the mitochondria, chloroplast, peroxisome, apoplast and plasma membrane (Ahmad *et al*., 2008). Apart from being by-products of photosynthesis (Szymańska *et al*., 2017), at low levels, ROS are also useful signalling molecules in plants (Zandalinas *et al*., 2018). However, when temperatures are elevated, the production of ROS increases. This results in an imbalance between their production and detoxification by various antioxidant systems (Ahmad *et al*., 2008; Szymanska *et al*., 2017). The excess accumulation of ROS results in oxidative stress (Essemine *et al*., 2010), which damages lipids, membranes, proteins and nucleic acids (Apel & Hirt, 2004). Consequently, cellular homeostasis is disrupted, subsequently affecting plant growth and development. Furthermore, during heat stress, plants lose water due to increased transpiration to cool their leaves via stomatal openings (Rizhsky *et al*., 2002). This process ultimately results in dehydration, which in turn impedes physiological processes such photosynthesis and respiration (Hirt & Shinozaki, 2004). Ultimately, plant growth and development is reduced.

1.3 Plant Responses to High Temperature Stress

Plants have developed various morphological, physiological, biochemical and molecular mechanisms in order to adapt and grow under stressful conditions (Howarth & Ougham, 1993; Zandalinas *et al*., 2018). These mechanisms may render some plant species heat tolerant. Heat tolerance is defined as the ability of plants to grow and produce an economic yield under increased temperatures (Wahid *et al*., 2007). However, the level of heat tolerance varies with the intensity of the temperature stress, the extent at which the increase in temperature occurs and the plant species (Hasanuzzaman *et al*., 2013; Szymanska *et al*., 2017). As such, there is a great variation in heat response within and between species, thus

providing opportunities to improve heat stress tolerance in crops through molecular breeding and genetic engineering.

At extremely high temperatures, protein denaturation and aggregation, and increased membrane fluidity may occur (Wahid *et al*., 2007). As a result, cellular organisation collapses and cell death may occur within minutes of exposure to severe temperature (Wahid *et al*., 2007). The molecular responses of plants towards the stress imposed involve the perception of stimuli by cells through different sensors. The sensors subsequently activate signalling pathways, which involve secondary messengers, plant hormones, transcriptional regulators and signal transducers (Gilroy *et al*., 2014). These signals coordinate the regulation and expression of stress-related genes involved in adaptive stress responses (Wang *et al*., 2004; Hirt & Shinozaki, 2004). Some of the stress-related genes expressed include those involved in the synthesis of enzymes that detoxify ROS (Mittler & Blumwald, 2015), heat-shock proteins (HSPs) and osmolytes (Howarth & Ougham, 1993; Wang *et al.*, 2004). The activities of the proteins and metabolites produced subsequently contribute towards stress response (Casaretto *et al*., 2016).

1.3.1 ROS Scavenging

The harmful effects of accumulated ROS in plants can be mitigated by an enhanced accumulation and activity of enzymatic and non-enzymatic antioxidants (Hasanuzzaman *et al*., 2013; Omari & Nhiri, 2015). Enzymatic antioxidants include superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase, while non-enzymatic antioxidants include tocopherol, ascorbic acid, glutathione and phenolic compounds (Ahmad *et al*., 2008). Enzymatic antioxidants act sequentially and simultaneously to detoxify ROS into less harmful compounds (Omari & Nhiri, 2015) thus alleviating the negative effects of oxidative

stress. It has also been suggested that there is a correlation between stress tolerance and the presence of an effective antioxidant system in plants (Omari & Nhiri, 2015).

In a study conducted by Casaretto and co-workers, wild type maize plants and transgenics with an *OsMYB55* gene were simultaneously exposed to heat stress at 40°C and drought for five days (Casaretto *et al*., 2016). *OsMYB55* is a heat shock factor, which activates genes involved in heat stress tolerance. Evaluation of physiological and growth parameters of the plants following stress indicated a significant decrease in dry biomass, stem diameter, plant height and chlorophyll content in wild type maize plants compared to the transgenics. The reduced leaf injury observed in *OsMYB55* lines suggests that the transgenics might have increased cytomembrane stability and enhanced ROS scavenging systems under high-temperature conditions (Casaretto *et al*., 2016). In addition, Wang *et al*. (2015) also observed an alleviation in oxidative damage in wheat (*Triticum aestivum*) cultivars in response to heat stress. After exposing the wheat cultivars to a 35°C high temperature stress for 5 days, high activities of catalase, superoxide dismutase and glutathione reductase were observed. From the results obtained, Wang and co-workers proposed that the activity of the antioxidant enzymes contributed to the thermo-tolerance of the wheat cultivars (Wang *et al*., 2015).

1.3.2 Heat Shock Proteins

Heat shock proteins (HSP) are a unique set of highly conserved proteins that are induced by heat and other abiotic stresses such as drought and salinity (Carper *et al*., 1987). The synthesis of HSPs is a necessary step towards heat acclimation in plants (Timperio *et al*., 2008). HSPs are encoded by several heat shock genes including *HSP60, HSP70*, *HSP90* and *HSP101* (Hsu *et al*., 2010). The expression of these genes is activated by the heat shock transcription factors, which are important in the regulation of protein interactions, signalling pathways and defence responses to heat stress (Wang *et al*., 2018). In their study, Howarth

and Ougham (1993) reported the synthesis of HSPs in sorghum (*Sorghum bicolor*) seedlings at different temperatures. At the optimal growth temperature of 35°C, there was normal protein synthesis. When the temperature was increased by 5°C to 40°C, HSP synthesis was induced and the synthesis of normal cellular proteins continued. At 45°C and above, a full spectrum of HSPs was expressed and the synthesis of non-HSPs was inhibited, thus making HSPs predominant (Howarth & Ougham, 1993). Wang *et al*. (2015) also observed an increase in the synthesis of HSPs, particularly HSP70, in wheat cultivars in response to high temperature stress. An increase in the abundance of HSP70 in these cultivars might have contributed towards the protection of plants against heat stress damage (Wang, *et al*., 2015).

In a recent study, an increase in the expression of HSPs in radish (*Raphanus sativus* L., 2n = 2x = 18) taproot was also observed, following heat stress treatment at 40°C for 2, 6, 12, 24 and 48 hours (Wang *et al*., 2018). The heat stress treatment resulted in reduced cellular damage. Overall, these results suggest that HSPs play an important role in protecting plants against heat stress and maintaining cellular homeostasis by re-establishing normal protein conformation (Wang *et al*., 2004, Wang *et al*., 2018).

1.3.3 Accumulation of Compatible Osmolytes

Osmolytes are low molecular weight, highly soluble compounds that are usually nontoxic at high cellular concentrations (Wahid *et al*., 2007; Ashraf & Foolad, 2007). These compounds accumulate in various plants under different abiotic stresses such as extreme temperatures, salinity and drought (Ashraf & Foolad, 2007). Examples of osmolytes include sugars and sugar alcohols, glycine betaine and proline (Wang *et al*., 2004). Their general function is to protect plants and cellular components from stress through cellular osmotic adjustment, ROS detoxification and in maintaining membrane integrity, as well as protein and enzyme stability (Bohnert & Jensen, 1996). The accumulation of compatible osmolytes is a key adaptive

mechanisms in many plants against abiotic stresses (Sakamoto & Murata, 2002; Wahid *et al*., 2007). For the purpose of this study, only two types of osmolytes; glycine betaine and proline, will be discussed.

1.3.3.1 Glycine Betaine

Glycine betaine is an amphoteric quaternary amine, which plays an important role as a compatible osmolyte in plants under different abiotic stresses, such as high temperature, drought and salinity (Sakamoto & Murata, 2002; Wang *et al*., 2004). Glycine betaine naturally accumulates in barley (*Hordeum vulgare*), wheat and sorghum in response to abiotic stresses (Yang *et al*., 2003). This osmolyte is mainly found in the chloroplast, where it protects the thylakoid membrane against oxidative damage, thereby maintaining high photosynthetic efficiency (Ashraf & Foolad, 2007). A review on the genetic engineering of glycine betaine synthesis in plants under heat stress, reported that glycine betaine protects some enzymes and protein complexes from destabilization (Sakamoto & Murata, 2002). Glycine betaine thus increases the resistance of some plants to high temperature stress (Sakamoto & Murata, 2002).

The synthesis and accumulation of glycine betaine under stress conditions however, differs between plant species (Ashraf & Foolad, 2007). Plants such as rice (*Oryza sativa*), mustard (*Brassica nigra*), Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*), do not naturally produce glycine betaine (Wahid *et al*., 2007). However, due to genetic engineering, the introduction of glycine betaine-biosynthetic pathways into species that are naturally deficient of this osmolyte has been made possible (Sakamoto & Murata, 2002). For example, Alia and co-workers transformed Arabidopsis with the *codA* gene, which is involved in the biosynthesis of glycine betaine (Alia *et al*., 1998). These transgenic plants accumulated glycine betaine, which enhanced their tolerance to high temperatures ranging

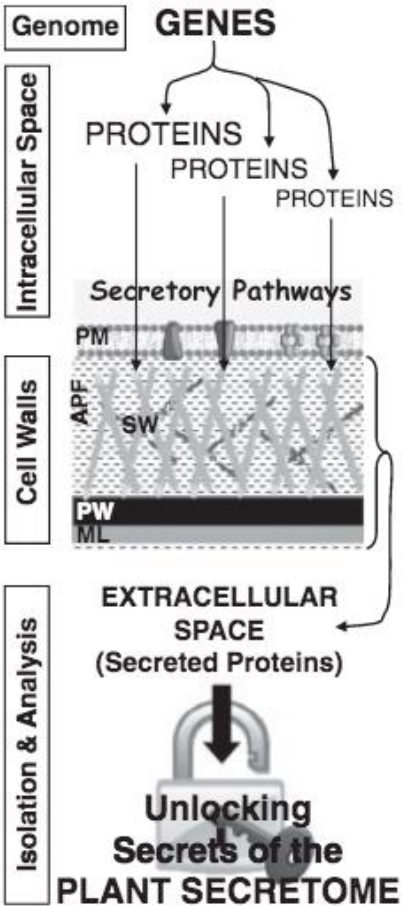
between 30 and 40°C during imbibition and seed germination, and in the growth of young seedlings (Alia *et al*., 1998).

1.3.3.2 Proline

Proline is an amino acid, that widely occurs in higher plants and accumulates in response to both biotic and abiotic stresses (Kishor *et al*., 2005; Rejeb *et al*., 2014). Proline functions as an osmolyte for osmotic adjustment. Additionally, this amino acid is responsible for stabilizing sub-cellular structures such as membranes and proteins and scavenging free radicals under stress conditions (Ashraf & Foolad, 2007). After a stress period, proline is degraded in the mitochondria through the sequential action of proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase (Rejeb *et al*., 2014). In addition to the biochemical changes that occur in response to heat stress, it is also important to study protein expressional changes that occur in plants.

1.4 Proteomics and Secretome Analysis

Proteomics is defined as the analysis of a complete set of proteins, which is expressed by a cell, tissue or organism (Stastna & Van Eyk, 2012). Proteomic approaches are also important for secretome analysis. This is due to the fact that secreted proteins are usually expressed in low quantities by specialized cell types (Jung *et al*., 2008). The secretome is a subset of proteins that are secreted by a cell, tissue, organ or organism into the extracellular matrix (ECM) at any given time (Agrawal *et al.*, 2010; Alexandersson *et al*., 2013). These proteins are secreted through classical and unclassical secretory pathways, involving constitutive and regulated secretory organelles (Agrawal *et al*., 2010). Figure 1.2 below shows the secretion of proteins from the cell into the ECM.



(Source: Agrawal *et al*., 2010)

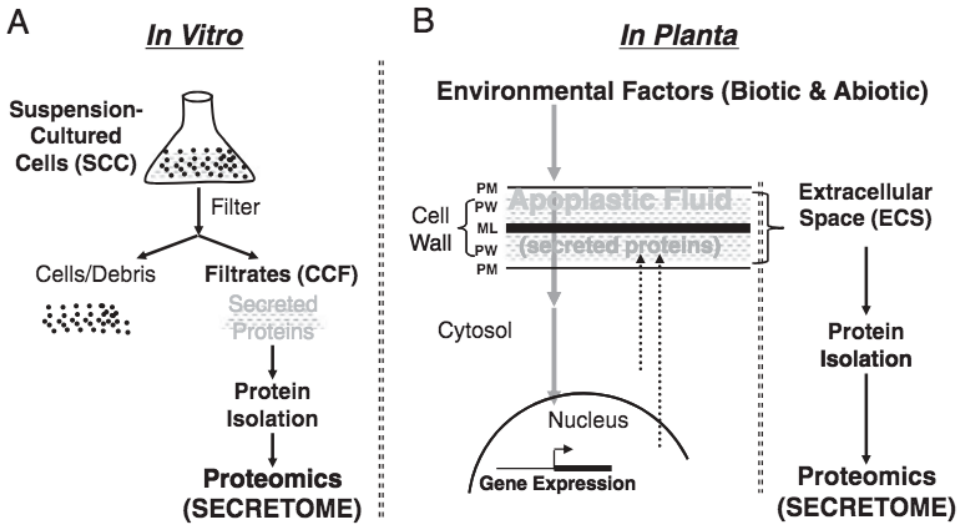
Figure 1.2: Protein secretion into the extracellular space.

Proteins secreted via the classical pathway often possess an N-terminal signal peptide, which targets them to the ECM. The N-terminal signal peptide mediates the secretion of proteins into the endoplasmic reticulum (ER) lumen, where it is cleaved off. After signal peptide cleavage, proteins are folded and transported by vesicles to the Golgi apparatus, where chemical protein modifications may occur. The proteins are eventually secreted from the cell into the ECM (Krause *et al*., 2013; Lehtonen *et al*., 2014). On the other hand, non-classical proteins lack the signal peptide for uptake into the ER and are therefore, referred to as

leaderless secretory proteins (Krause *et al*., 2013). These proteins are transported into the ECM with or without the assistance of vesicles across the plasma membrane (Lehtonen *et al*., 2014).

1.4.2 The Role of Secreted Proteins in Plants

Secreted proteins are involved in a wide range of processes such as the maintenance of cell structure, biogenesis, regulation of the external environment and in signalling and defence mechanisms against abiotic stresses (Jung *et al*., 2008, Gupta *et al*., 2011; Alexandersson *et al*., 2013). Given the importance of secretome in plant development, a number of studies have analysed the plant secretome in response to pathogen attack (Lehtonen *et al*., 2014; Kim *et al*., 2014), osmotic (Ngara *et al*., 2018) and high temperature (Echevarría-Zomeño *et al*., 2016) stresses, among others. These studies have either used the *in planta* or the *in vitro* systems as sources of the secreted proteins as illustrated in Figure 1.3.



(Source: Agrawal *et al*., 2010)

Figure 1.3: The *in vitro* and *in planta* methods used in secretome studies.

1.4.3 The *in planta* System

The *in planta* system involves the isolation of secreted proteins from the apoplastic fluid (APF) of plant cells (Agrawal *et al*., 2010). Although this experimental system provides a natural environment for secretome studies, it does not always yield pure secreted protein fractions (Jung *et al*., 2008). This is due to the complexity in structural organization of cells and the difficulty in extracting the APF without cellular damage (Agrawal *et al*., 2010). However, the *in planta* system is still useful since cell suspension cultures do not provide a natural environment for the cells and some physiological relevant treatments are difficult to apply in cell cultures (Alexandersson *et al*., 2013).

1.4.4 The *in vitro* System

Plant cell suspension cultures are an example of an *in vitro* system. Cell cultures are mostly used as sources of secreted proteins because they are easy to handle and maintain (Agrawal *et al*., 2010; Gupta *et al.*, 2011). Cell suspension cultures are defined as a group of undifferentiated cells that are grown in a liquid medium (Chawla, 2009). These cell cultures are established from friable callus masses. Callus consists of a population of undifferentiated cells that arise from explants such as shoots or roots (Lehtonen *et al*., 2014). Cell suspension cultures are maintained under suitable growth conditions with agitation until a desired cell density is reached. During growth, cell suspension cultures continuously secrete proteins into the culture medium. These proteins are easily extracted from the culture medium through filtration without cell disruption (Agrawal *et al*., 2010). The culture filtrate may further be centrifuged to remove cellular debri from the secreted protein fractions (Alexandersson *et al*., 2013).

The secretome analyses of important cereals such as rice (Cho *et al*., 2009) and sorghum (Ngara & Ndimba, 2011; Ngara *et al*., 2018) have been conducted. In their study, Cho and

co-workers identified a total of 555 unique protein sequences from the secreted protein fractions using multidimensional protein identification technology (MUDPIT) analysis (Cho *et al*., 2009). Of these proteins, only 154 (27.7%) proteins had a predicted signal peptide. The identified proteins had putative functions in stress response, metabolism, protein modification, transport, cell death, development and signal transduction. However, about 10 (8%) proteins had unknown functions. Moreover, the conserved domains and families of the proteins were also identified so as to further understand the proteins functions (Cho *et al*., 2009). The data obtained in the study suggests that the identified rice secreted proteins play important roles in a wide range of cellular functions.

A recent study on mapping and characterisation of the sorghum cell suspension culture secretome in response to osmotic stress was conducted (Ngara *et al*., 2018). Using the isobaric tag for relative and absolute quantitation (iTRAQ) analysis, 179 secreted proteins were positively identified in a White sorghum cell line (Ngara *et al*., 2018). Of these proteins, 129 (72%) had a signal peptide and 92 (51%) were responsive to sorbitol-induced osmotic stress. The osmotic-stress responsive proteins were functionally grouped into glycosyl-hydrolases/glycosidases, cell wall modifying enzymes, proteases and redox proteins. In an earlier study on the same sorghum cell culture line, only 14 proteins were positively identified using two dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry (MALDI-TOF-TOF MS) (Ngara & Ndimba, 2011). Using bioinformatics tools, the identified proteins were assigned with putative functions in cell wall metabolism, signalling and defence related processes as well as in normal physiological processes during plant growth and development (Ngara & Ndimba, 2011). Collectively, these studies give us some insight on the composition of sorghum secreted proteins. However, since sorghum has a wide genetic diversity and gene pool (Motlhaodi *et al*., 2017), it is important to broaden the coverage of the secretome map of

sorghum as well as understand their functions under normal conditions and in stress response. The current study used an ICSB 338 sorghum cell suspension culture to identify proteins secreted into the ECM in response to high temperature stress.

1.4 Why Sorghum?

Sorghum is a naturally drought tolerant (Bibi *et al*., 2012; Sutka *et al*., 2016) C_4 photosynthetic crop, which is mostly cultivated in the tropical areas, worldwide (Taylor 2003). It is ranked the world's fifth most important cereal after wheat, maize, rice and barley (*Hordeum vulgare*) (Amelework *et al*., 2015; Ramatoulaye *et al*., 2016). Sorghum can grow and produce high yields under hot and dry conditions, where other important cereals such as wheat and maize fail (Taylor, 2003, Sutka *et al*., 2016). The extensive deep-penetrating root system contributes to sorghum's drought tolerance (National Research Council, 1996). In addition, there are other mechanisms used by this crop to withstand drought conditions. For example, under stress conditions, sorghum conserves moisture through the reduction of transpiration by rolling its leaves and closing the stomata. Furthermore, it can reduce its metabolic processes and retreat into near dormancy until the period of rainfall starts again (National Research Council, 1996). Figure 1.4 below shows a sorghum crop under cultivation.

(Source: http://naturalagsolutionsllc.com)

Figure 1.4: Sorghum crop under cultivation.

Sorghum is grown for food, animal feed, fibre and fuel production (FAO, 2012). It is the primary staple food for over 500 million people in the world, mainly in African countries (Wu *et al*., 2014). Due to its remarkable ability of growing in hot and dry areas, sorghum is a potentially good model system in plant stress studies (Ngara & Ndimba, 2014). Furthermore, Taylor (2003), projects sorghum as an important driver of economic development in Africa. It is therefore, important to put more focus on fundamental and applied research on this crop so as to improve food security.

1.5 Aim, Objectives and Significance of the Study

The aim of this study was to evaluate the molecular responses of sorghum cell suspension cultures to high temperature stress.

The objectives were:

i. to analyse the changes in osmolyte content and cell metabolic activity in ICSB 338 sorghum cell suspension cultures, following high temperature stress,
ii. to map the ICSB 338 sorghum cell suspension culture secretome and identify the differentially expressed proteins in response to high temperature stress using iTRAQ, and
iii. to validate the expression of target genes in response to high temperature stress using quantitative real-time polymerase chain reaction.

The current research study was significant in identifying heat stress responsive genes in sorghum. The information obtained would also serve as fundamental knowledge in molecular responses of plants to heat stress. Furthermore, this knowledge could be applied in plant breeding programmes aimed at developing high temperature stress tolerant plants with high yield, thus improve food security in the changing climate.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material

Sorghum and Arabidopsis cell suspension cultures were used in this study. The ICSB 338 sorghum seeds used to establish callus were obtained from Agricultural and Research Council in South Africa. ICSB 338 sorghum cell suspension cultures were initiated from callus masses previously established in our research group (Ramulifho, 2017). ICSB 338 is a salt sensitive sorghum cell line (Satish *et al*., 2016), and also sensitive to drought. Arabidopsis (*Arabidopsis thaliana* var Erecta) suspension cultures were maintained as described (May & Leaver, 1993).

2.2 Plant Tissue Culture Methods

2.2.1 Maintenance of Sorghum Callus Masses

ICSB 338 callus masses were maintained on sorghum callus medium [4.4 g/L Murashige and Skoog Basal Salt with minimal organics (MSMO) medium; 3% (w/v) sucrose; adjusted to pH 5.8 using 1 M NaOH; 0.8% (w/v) bacteriological agar]. The medium was supplemented with plant growth hormones; 2.5 mg/L 1-naphthaleneacetic acid (NAA) and 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The growth hormones were prepared by dissolving 2.5 mg of NAA in 100 μL of 1 M NaOH, while 3 mg of 2,4-D were dissolved in 100 μL of absolute ethanol, before making up to the respective volumes to 1 mL with distilled water.

Sorghum callus was maintained in culture by aseptically transferring six pea-sized easily breakable (friable) callus from 4 week-old mature calli into each Petri dish, containing fresh sorghum callus medium. The Petri dishes were sealed with parafilm and incubated under dark conditions in a Labcon growth chamber (Lab design Engineering, Maraisburg, South Africa)

at 27°C. Callus growth was visually assessed over a 4-week period. Only friable callus masses were sub-cultured every 4 weeks and maintained in culture for use in the initiation of cell suspension cultures.

2.2.2 Initiation and Maintenance of Cell Suspension Cultures

2.2.2.1 Sorghum Cell Suspension Cultures

ICSB 338 sorghum cell suspension cultures were initiated from 4-week old friable calli. About five friable callus masses (each with a fresh weight of approximately 0.5 g) were transferred into 250 mL Erlenmeyer flasks, containing 50 mL of sorghum cell suspension culture medium [4.4 g/L MSMO medium; 3% (w/v) sucrose; 2.5 mg/L NAA; 3 mg/L 2,4-D, adjusted to pH 5.8 using 1 M NaOH]. At least four biological replicate cell suspension cultures were prepared. The flasks were incubated in a shaking incubator (Already Enterprise Inc., Taipei, Taiwan) at 27°C, under dark conditions with agitation at 130 rpm for 4 days. After 4 days, the culture medium was topped up to 100 mL, and the cell cultures were further incubated until they were 10-12 days old. The sorghum cell suspension cultures were maintained in culture by transferring 30 mL of the 10-12 day old cultures into 70 mL of fresh medium.

2.2.2.2 Arabidopsis Cell Suspension Cultures

Arabidopsis cell suspension cultures were grown in MSMO medium [4.43 g/L MSMO medium; 3% (w/v) sucrose; 0.5 mg/mL NAA and 0.05 mg/L kinetin growth hormones adjusted to pH 5.7 using 1 M KOH]. Cell cultures were incubated in a shaking incubator at 22°C under dark conditions with agitation at 130 rpm for 3-4 days prior to stress treatment. The Arabidopsis cell cultures were sub-cultured every seven days by transferring 10 mL of old cultures into 90 mL fresh medium.

2.3 Heat Stress Treatment

A preliminary heat stress treatment experiment was conducted on the sorghum cell suspension cultures in order to establish the optimal temperature and duration of treatment to use. Eight-day old ICSB 338 sorghum cell suspension cultures, growing at the mid-log phase (Ramulifho, 2017) were incubated under dark conditions in separate shaking incubators as follows: control cell cultures were incubated at 27°C, while heat stress treatment cultures were incubated at either 35 or 40°C for 72 hours. In order to reduce technical variation in the experiment, each 8-day old mother culture was subdivided into 50 mL each for the control and the heat stress treatment. Three such biological replicate cultures were prepared for each treatment. During the 72-hour incubation period, the cell suspension cultures were aseptically sampled for the determination of cell viability and protein extraction. For Arabidopsis, the cell suspension cultures were maintained in culture at 22°C for four days prior to heat stress treatment. On day four, three biological replicates of Arabidopsis cell suspension cultures were exposed to a 40°C heat stress treatment for 72 hours.

2.4 Cell Viability Estimations Using the MTT Assay

The viability of the sorghum cell suspension cultures during the 72 hour heat stress treatment period was determined using an MTT [3-(4,5-dimethylthiazolyl2)-2,5-diphenyltetrazolium bromide] assay as described by Ngara (2009). Three biological replicate 8-day old ICSB 338 cell suspension cultures were prepared for the control and heat stress treatment groups at 35 and 40°C. From each treatment group, 150 µL of the cell culture was sampled into 1.5 mL Eppendorf tubes at 0, 24, 48 and 72 hours. Two technical replicates were also prepared at each time point.

Fifty microlitres of a 5 mg/mL MTT stock solution was added to the cell aliquots and the tubes were incubated with gentle shaking for 30 minutes at room temperature. Thereafter, the

cells were left to settle on a flat bench-top for 5 minutes before discarding the supernatant. One millilitre of 100% (v/v) dimethyl sulfoxide (DMSO) was added to all tubes and the cells were further incubated for 10 minutes with gentle shaking. After incubation, the MTT treated cells were left to settle on flat bench-top for 5 minutes. The supernatant was collected and its absorbance measured at 490 nm on a spectrophotometer, using DMSO as a blank solution. For Arabidopsis, three biological replicates of 4-day old cell suspension cultures, control and heat stressed samples were prepared. Cell viability was measured at 0, 24, 48 and 72 hours as described above.

2.5 Protein Extraction from ICSB 338 Sorghum and Arabidopsis Cell Suspension Cultures

Eight-day old ICSB 338 sorghum cell suspension cultures were heat stressed at 35 and 40°C, while the four-day old Arabidopsis cell suspension cultures were treated at 40°C for 72 hours as described in Section 2.3. ICSB 338 sorghum cell cultures control samples were incubated at 27°C, and 22°C for Arabidopsis cell cultures for the same duration. Three biological replicates per treatment group were prepared. Prior to protein extraction, the cells and culture medium were separated by filtration through four layers of sterile Miracloth (Merck, Darmstadt, Germany). The cells were briefly washed with sterile distilled water by filtration and immediately stored at -80°C for use in subsequent protein extraction experiments.

2.5.1 Total Soluble Proteins Extraction from ICSB 338 Sorghum and Arabidopsis Cells

ICSB 338 sorghum and Arabidopsis cells previously stored at -80°C were ground to a fine powder using sterile pestle and mortar. Approximately 1 g of the ground material was transferred into 2 mL Eppendorf tubes, and 1 mL of 10% (w/v) trichloroacetic acid (TCA) was added. The mixture was briefly vortexed and placed on ice while preparing other samples. Thereafter, the homogenate was centrifuged at 9 400 × g for 10 minutes. The pellet

was washed three times with 1 mL of 80% (v/v) ice-cold acetone with centrifugation for 10 minutes per wash. The third acetone wash was followed by air-drying the pellet for 5 minutes and re-suspension in 1 mL extraction buffer [9 M urea, 2 M thiourea and 4% (w/v) 3-[3-(cholamidopropyl)dimethylammonio]-1propanesulfonate (CHAPS)]. The total soluble proteins (TSP) were extracted with vigorous vortexing overnight at room temperature. Thereafter, the homogenate was centrifuged at 15 000 × *g* for 10 minutes and the supernatant containing the TSP extracts was collected. The protein extracts were stored at -20°C for use in subsequent protein quantification, gel electrophoresis and western blotting experiments.

2.5.2 Culture Filtrate Protein Extraction from ICSB 338 Sorghum Cells

The filtered culture medium (Section 2.5), now called the culture filtrate (CF), was centrifuged at 3000 rpm for 10 minutes. The supernatant was carefully collected and mixed with four volumes of 100% (v/v) acetone to precipitate the CF proteins, overnight at -20°C. After protein precipitation, the samples were centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The protein pellet was washed three times with 1 mL of 80% (v/v) ice-cold acetone by centrifuging at 15 000 × *g* for 10 minutes per wash. The pellet was air-dried for 5 minutes and then re-suspended in appropriate volumes of extraction buffer with vigorous vortexing overnight. The solubilised protein samples were centrifuged at 15 000 × *g* for 10 minutes and the supernatant containing CF proteins was collected. The protein extracts were stored at -20°C for use in protein quantification, gel electrophoresis and isobaric tags for relative and absolute quantitation (iTRAQ) experiments.

2.6 Protein Quantification

The protein concentration of CF and TSP extracts was quantified using the Bradford assay (Bradford, 1976) with minor modifications as previously described (Ngara, 2009). Protein standard solutions were prepared in duplicates using a 5 mg/mL bovine serum albumin

(BSA) stock solution as indicated in Appendix 1-Table 1. Protein extracts were also prepared in duplicates in 2 mL plastic cuvettes by adding 10 µL of protein sample, 10 µL of 0.1 M HCl and 80 µL of distilled water. In both the standard solutions and protein samples, 900 µL of a 1:4 diluted Bio-Rad Protein Assay Dye Reagent Concentrate (BIO-RAD, Hercules, California, USA) was added, mixed well and incubated at room temperature for 5 minutes. Thereafter, the absorbance was measured at 595 nm on a spectrophotometer, using the 0 mg/mL BSA standard solution as a blank. The BSA standard solutions were used to plot a standard curve for estimating the concentrations of unknown protein samples.

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

The ICSB 338 sorghum cell suspension culture CF and TSP protein extracts were electrophoresed on 1D SDS-PAGE as previously described (Laemmli, 1970). A 12% (v/v) and 5% (v/v) resolving and stacking gel, respectively were prepared as indicated in Appendix 1-Table 2. All protein samples were mixed with a protein sample buffer at a ratio of 1:1 in 1.5 mL Eppendorf tubes. The samples were pulse vortexed and incubated at 100°C for 5 minutes prior to loading on the gels. Five microliters of the broadrange Unstained Protein Ladder (New England Biolabs, Hertfordshire, UK) and appropriate concentrations of CF and TSP protein samples were loaded on the gel. Electrophoresis was carried out in electrode running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS] initially at 100 V for 30 minutes, and then at 150 V until the bromophenol blue tracking dye reached the bottom of the gels.

2.8 CBB Staining of 1D SDS-PAGE Gels

After the electrophoretic run, the protein gels were sequentially stained with three Coomassie Brilliant Blue (CBB) R-250 staining solutions prepared from a 1.25% (w/v) CBB stock solution as follows: CBB stain I [0.025% (w/v) CBB R-250, 10% (v/v) glacial acid, 25%

(v/v) propan-2-ol], CBB stain II [0.003% (w/v) CBB R-250, 10% (v/v) glacial acetic acid, 10% (v/v) propan-2-ol] and CBB stain III [0.003% (w/v) CBB R-250, 10% (v/v) glacial acetic acid]. The gels were successively stained in the three CBB stains I, II and III for 30 minutes each, with gentle shaking at room temperature. Thereafter, the gels were immersed in a destaining solution [10% (v/v) acetic acid and 1% (v/v) glycerol] until the protein bands were visibly clear against the background of the gel. Gel images were captured using the Molecular Imager Gel Doc™ XR+ with Image Lab ™ Software version 5.2.1 (BIO-RAD).

2.9 Acetone Precipitation of Protein Extracts

For protein expression and western blotting analyses, only the control and heat stress treatment sorghum samples at 40°C were used. Four biological replicate CF and TSP protein extracts per treatment group were precipitated overnight at -20°C with 80% (v/v) acetone. Thereafter, the samples were centrifuged at 9 400 × *g* for 10 minutes, and the pellets were washed twice with 200 µL of 80% (v/v) acetone. A hundred microliters of 80% (v/v) acetone was added to the protein pellets and stored at -20°C until shipment to Durham University, UK, for iTRAQ analysis and western blotting experiments.

2.10 Osmolyte Content Analysis

2.10.1 Sample Preparation for Osmolyte Analysis

ICSB 338 sorghum cell suspension cultures were heat stressed at 40°C for 72 hours as described in Section 2.3. Control samples were incubated at 27°C for the duration of the experiment. For the determination of osmolyte content analysis, sorghum cell suspension cultures were harvested at 48 and 72 hours following the heat stress treatment and four biological replicates were used. The cell suspension cultures were filtered through four layers of sterile Miracloth (Merck). A 100 mg of cells was weighed directly into 1.5 mL Eppendorf

tubes and immediately stored at -80°C. To prepare for osmolyte analysis, 125 μL of 0.25 N HCl was added into each tube containing the cells. The samples were incubated at 60°C for 5 minutes on a heat block and centrifuged at 9 400 × *g* for 5 minutes. The supernatant was collected into new 1.5 mL Eppendorf tubes and stored at -20°C until shipment to Durham University for proline and glycine betaine osmolyte content analysis.

2.10.2 Proline Content Analysis

The analysis of proline was conducted using the Hydrophilic Interaction Chromatography (HILIC) Liquid Chromatography-Mass Spectrometry (LC-MS) as previously described (Prinsen *et al.*, 2016). The chromatographic separation of the cell extract was carried out on an Acquity UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm particle size) (Waters, Manchester, UK). The column was maintained at 30°C and 2 μL of the sample (diluted 1:10), was injected. Optimal chromatographic separation was attained at a flow rate of 200 μL/minute using a gradient with solvent A (10 mM ammonium formate, 0.15% (v/v) formic acid in 85% (v/v) acetonitrile) and solvent B (10 mM ammonium formate, 0.15% (v/v) formic acid in water). The initial conditions were 100% solvent A. After 6 minutes, a gradient started for 0.1 minute (6.0-6.1 minutes) and solvent A was decreased to 94.1% and solvent B increased to 5.9%. From 6.1 to 10 minutes solvent A was set at 82.4% and solvent B was set at 17.6% and from 10 to 12 minutes, solvent A was set at 70.6% and solvent B was set at 29.4%. Then the column was equilibrated for 6 minutes in the initial conditions. The total run time was 18 minutes including column equilibration. The column was coupled to the QTRAP 6500 mass spectrometer (AB Sciex, Redwood city, USA) for proline identification. A multiple reaction monitoring (MRM) positive mode was used for proline analysis, with the transition of 116→70.

2.10.3 Glycine Betaine Content Analysis

The analysis of glycine betaine was conducted using the HILIC LC-MS as previously described (Prinsen *et al.*, 2016). The chromatographic separation of the osmolyte samples was carried out on an Acquity UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm particle size) (Waters). The column was maintained at 30°C and 2 μL of the sample (diluted 1:10), was injected. Optimal chromatographic separation was achieved at a flow rate of 200 μL/minute (Ascentis HILIC) using a gradient with solvent A (10 mM ammonium formate, 0.15% (v/v) formic acid in 85% (v/v) acetonitrile) and solvent B (10 mM ammonium formate, 0.15% (v/v) formic acid in water). The gradient for solvent A was started at 100% and held for 2 minutes and solvent B was ramped to 100% at 5 minutes. Solvent A was then held for 5 minutes then equilibrated at 100% for 5 minutes. The analysis was conducted by MRM using QTRAP 6500 hybrid triple-quadrupole mass spectrometer (AB Sciex) for glycine betaine identification and quantification. The transitions from MRF were as follows: ES+ 118→58, 118→59.

2.11 Heat Shock Protein 70 (HSP70) Western Blotting

For the HSP70 western blotting analysis, both the heat stressed and untreated ICSB 338 and Arabidopsis samples were used. ICSB 338 sorghum samples were heat stressed at 40°C for 48 and 72 hours while the Arabidopsis samples were treated at 40°C for 24 and 48 hours. The ICSB 338 sorghum protein pellets (Section 2.9) were centrifuged at 15 000 × *g* for 10 minutes to discard the acetone and briefly air-dried. The pellets were then re-suspended in 50-100 μL of extraction buffer depending on their sizes, vortexed for 2 hours and centrifuged at 15 000 × *g* for 10 minutes. The TSP contents of the clarified supernatants were quantified using the Bradford assay (Section 2.6). Ten micrograms of both the control and heat stressed sorghum and Arabidopsis TSP samples were subsequently electrophoresed on a 12% (v/v)

SDS polyacrylamide gel (Section 2.7) before transferring the proteins onto nitrocellulose membranes.

2.11.1 Transfer of Proteins from the Gel onto the Nitrocellulose Membrane

The electrophoresed proteins were transferred from the gel onto a nitrocellulose membrane (GE Healthcare Life Science, Freiburg, Germany) using a Mini-Trans Blot electrophoretic cell (BIO-RAD), according to the manufacturer's instructions. During assembling the transblot sandwich, the air bubbles between the gel and the membrane were carefully rolled out. The protein transfer was carried out using transfer buffer [25 mM Tris, 192 M glycine and 20% (v/v) ethanol] at 70 V for 2 hours at 4°C.

2.11.2 Immunoprobing of the Nitrocellulose Membrane Using Antibodies

After protein transfer, the nitrocellulose membrane was stained with Ponceau S stain [0.1% (w/v) Ponceau in 10% (v/v) acetic acid] for a few minutes until the protein bands were visible. Thereafter, the membrane was rinsed once with Tris-buffered saline containing Tween 20 (TBS-T) [100 mM Tris HCl, pH 8.0, 1.5 mM NaCl, 0.1% (v/v) Tween 20] for 5 minutes. The membrane was then incubated in a blocking solution [0.5% (w/v) BSA in TBS-T], overnight at 4°C and subsequently rinsed three times for 15, 5 and 5 minutes per wash. Thereafter, the membrane was incubated with the primary HSP70 antibody (AS08 371) (Agrisera, Vannas, Sweden) diluted 1:3 000 in a blocking solution with shaking at room temperature for 1 hour. The membrane was then washed three times with TBS-T for 15, 5 and 5 minutes per successive wash. The membrane was subsequently incubated with an anti-rabbit IgG (whole molecule) F (ab′)$_2$ fragment-Cy3 secondary antibody (Amersham Life Science, Steinheim, Germany) diluted 1:20 000 in a blocking solution with shaking at room temperature for 1 hour. The membrane was subsequently washed three times with TBS-T for

15, 5 and 5 minutes per wash, scanned and imaged using the Typhoon 9400 variable mode imager (Amersham Biosciences) to detect the presence of HSP70.

2.12 isobaric Tags for Relative and Absolute Quantitation (iTRAQ) Analysis

The ICSB 338 sorghum CF protein samples (Section 2.9) were analysed using iTRAQ as previously described by Smith *et al.* (2015) with minor modifications. Due to the high cost of the iTRAQ reagents, for this Masters dissertation, only the heat stressed CF samples at 40°C for 72 hours and the controls were analysed. The acetone precipitated CF protein pellets (Section 2.9) were centrifuged at 15 000 × *g* for 5 minutes prior to discarding the supernatant. Thereafter, the protein pellets were briefly air-dried, resuspended in 100 µL of extraction buffer and vortexed. The samples were centrifuged at 15 000 × *g* for 10 minutes and the supernatant containing CF proteins was collected. The protein samples were subsequently quantified using the Bradford assay (Section 2.6) and electrophoresed on a 12% (v/v) SDS polyacrylamide gel (Section 2.7) for quality and quantity checks.

2.12.1 Protein Sample Labelling

Protein concentrations of 12.5 µg for each of the control and 40°C heat stressed ICSB 338 sorghum CF samples were prepared for iTRAQ labelling. Four biological replicate samples of each of the treatment groups were used. The protein samples were precipitated with 80% (v/v) acetone overnight at -20°C and centrifuged at 15 000 × *g* for 10 minutes. The air-dried pellets were resolubilised using an iTRAQ Reagent-Multiplex Buffer Kit (AB Sciex) according to the manufacturer's instructions. Briefly, 2.5 µL of the denaturant was added to the pellets and incubated at 60°C for 1 hour. Thereafter, 47.5 µL of the dissolution buffer was added and the samples were vortexed for 20 minutes and subsequently centrifuged at 15 000 × *g* for 10 minutes. The supernatant was collected and mixed with 1 µL of the reducing agent. The samples were then incubated at 60°C for 1 hour and alkylated with 0.5 µL of the cysteine

blocking agent, briefly vortexed and incubated at room temperature for 10 minutes prior to digestion with trypsin (Promega, Madison, USA). Trypsin digestion was done overnight at 37°C. The digested peptides were vacuum-dried, resuspended in MilliQ water before adjusting the pH of each sample to 7.5 using dissolution buffer and pH strips 4.5-10.0 (Sigma). All samples were subsequently labelled with the iTRAQ Reagent-8PLEX Multiplex kit (AB-Sciex) according to manufacturer's instructions. The four control samples were labelled with isobaric tags 113, 114, 115 and 116, while the heat stressed samples were labelled with tags 117, 118, 119 and 121.

2.12.2 iTRAQ Sample Clean-up

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

2.12.3 LC-MS/MS Analysis

The peptides from 5 µg protein were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Triple TOF 6600 (AB Sciex) mass spectrometer linked to an Eksigent 425 LC system via a Sciex Nanospray III source. Samples were loaded on to YMC Triart C18 capillary guard, × 3 mm (HiChrom Ltd, Reading, UK) trap column and online chromatographic separation performed over 85 minutes on a YMC TriArt C18 1/32", 3 µm, 150 × 0.3 mm capillary column (HiChrom Ltd) with a linear gradient of 0-80% acetonitrile 0.1% formic acid at a flow rate of 5 µL/minute. Applied Biosystems Analyst software version 1.1 was used to acquire all MS and MS/MS data switching between the survey scan (400-600 *m/z* for 250 milliseconds) and product ion scans. Top 30 ions in the range of +2 to +4 charge state, TIC >500 counts were selected for fragmentation, with a rolling exclusion of 15 seconds after first occurrence. The MS acquisition settings were; high sensitivity product-ion MS/MS for 50 milliseconds each, mass window 100-1500 *m/z*, cycle time of 1.8 seconds, and collision energy adjusted for iTRAQ reagent use. Analyst TF 1.7.1 instrument control and data processing software (AB Sciex) was used to acquire spectrometer data.

2.12.4 Mass Spectra Data Analysis

Mass spectra data were analysed as previously described by Smith *et al.* (2015) with minor modifications. ProteinPilot™ software 5.0.1 version 4895 software, incorporating the Paragon™ Algorithm 5.0.1.0.4874, (AB Sciex) was used for data analysis against the UniProt protein sequences of *Sorghum bicolor* only (downloaded in May 2018). MS and MS/MS tolerances were set to 0.15 and 0.1 Da, respectively, and analysis and search parameters were set as: iTRAQ 8-plex labelling, trypsin digestion with allowance for a single missed cleavage, and only two amino acid modifications. No bias correction was applied to the quantitative data as this is a secretome experiment. A minimum threshold of 1.3 (95%

confidence) was set for each peptide identified and a minimum protein score threshold of 2.0 (99% confidence) was set for protein identification. All proteins identified on the basis of a single peptide were filtered out of the dataset. The abundance of each protein in all samples was calculated as a ratio to the 113-tagged sample. Averages of the ratios for each protein across the 4 replicates in control and in heat stressed were calculated. The fold-change in protein expression was denoted by the ratio of control average to heat stressed average.

2.12.5 Bioinformatics Analysis

The bioinformatic analyses of the mass spectrometry identified proteins was conducted using a variety of publicly available databases. The Gene Ontology (GO) analysis of proteins, their molecular weights and the presence of signal peptides were determined using data available on the UniProt (www.uniprot.org) database. The conserved domains and family names of the proteins were determined using the InterPro (https://www.ebi.ac.uk/interpro/protein/) database. All the identified proteins were analysed according to their fold-changes using a MapMan software 3.5.1R2 (https://mapman.gabipd.org/mapman).

2.13 Gene Expression Analysis

For gene expression analysis experiments, ICSB 338 sorghum cell suspension cultures were grown and maintained at 30°C at Durham University. Cell cultures were exposed to heat stress at 40°C and harvested after 0, 24, 48 and 72 hours of stress treatment. Four biological replicates were prepared for each treatment group and the cell samples were stored at -80°C for use in total RNA extraction.

2.13.1 Total RNA Extraction

Total RNA was extracted from ICSB 338 sorghum control and heat stressed samples using the Spectrum^{TM} Plant Total RNA Kit (Sigma, Missouri, USA) according to manufacturer's

instructions. Briefly, 500 μL of Lysis solution/2-mercaptoethanol was added to 100 mg of ground cell samples and vigorously vortexed for 30 seconds. The samples were incubated at 56°C for 3 minutes prior to centrifugation at 15 000 × *g* for 3 minutes. The lysate supernatant was pipetted into a filtration column and then centrifuged at 15 000 × *g* for 1 minute to remove residual cell debri. Thereafter, 500 μL of the binding solution was added into the lysate and briefly vortexed to mix.

The mixture was centrifuged at 15 000 × *g* for 1 minute to bind RNA. The bound RNA was washed with 300 μL of wash solution 1 by centrifugation at 15 000 × *g* for 1 minute. The flow-through was discarded and 80 μL of a DNase 1 and DNase digestion buffer mixture was added into the column before incubation at room temperature for 15 minutes. To remove the digested DNA, 500 μL of wash solution 1 was passed through the binding column twice and centrifuged at 15 000 × *g* for 1 minute. The third column wash was done using 500 μL of the wash solution 2 with centrifugation at 15 000 × *g* for 30 seconds. The column was subsequently dried by centrifuging at 15 000 × *g* for 1 minute and then transferred to a clean 2 mL Eppendorf tube. Thereafter, 50 μL of the elution solution was directly pipetted onto the centre of the binding matrix inside the column. The tubes were incubated at room temperature for 10 minutes and then centrifuged for 1 minute at 15 000 × *g* to collect the purified RNA. The concentration of total RNA extracted was determined using a Nano-Drop® ND 1000 spectrophotometer (NanoDrop-Technologies, Inc. Willington, USA) using sterile MilliQ water as a blank.

2.13.2 RNA Agarose Gel Electrophoresis

A 1.2% (w/v) RNA agarose gel was prepared in MOPS buffer [20 mM MOPS pH 7.0, 2 mM sodium acetate pH 7.0, 1 M (ethylenedinitrilo)-tetraacetic acid (EDTA) pH 8.0]. RNA

samples were prepared by adding 5 µL of RNA loading buffer [63.7% (v/v) formamide, 1.14 M formaldehyde, 6.4% (v/v) MOPS, 50 µg/mL ethidium bromide] to 300 ng of RNA and incubating at 65°C for 10 minutes. The mixture was then pulse centrifuged, loaded on the RNA agarose gel and run at 50 V for 20 minutes. The gel image was captured using the Ingenius Bio-Imager (SynGene, Cambridge, UK).

2.13.3 Complementary Deoxy-ribonucleic Acid (cDNA) Synthesis

cDNA was synthesised from the total RNA samples using the GoScript™ Reverse Transcriptase System (Promega) according to manufacturer's instructions. Briefly, 1 µg of total RNA and 1 µL of Oligo(dT) were mixed with nuclease free water to a total volume of 11.8 µL and incubated at 70°C for 5 minutes. The mixture was chilled on ice water for at least 5 minutes, pulse centrifuged at 15 000 × *g* and stored on ice whilst preparing the reverse transcriptase mix. A total volume of 8.2 µL of the reverse transcriptase mixture was prepared by adding 1 µL of GoScript™ 5X Reaction buffer, 1.8 µL of 1.5-5.0 mM $MgCl_2$, 1 µL of 0.5 mM dNTPs, 0.4 µL of Recombinant RNasin® Ribonuclease inhibitor and 1 µL of GoScript™ Reverse Transcriptase. The reverse transcription mix was mixed with 11.8 µL of the RNA mixture, annealed at 25°C for 5 minutes, extended at 42°C for 1 hour before inactivating the reverse transcriptase at 70°C for 15 minutes. The cDNA was diluted to a ratio of 1:20 with sterile MilliQ water and stored at -20°C for use in polymerase chain reaction (PCR) analysis.

2.13.4 Primer Design for the Heat Stress Responsive Target Genes

A list of ten heat stress responsive ICSB 338 CF proteins with the highest magnitude of fold-change were selected for validation by gene expression analysis. Primers were subsequently designed for their respective target genes using the Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) on the National Centre for Biotechnology

Information (NCBI) database. The following parameters were set during the primer design process: organism is *Sorghum bicolor*, none preference for exon junction span, GC clamp of 1 and a PCR product size of 70-150 for the minimum and maximum length, respectively. Additionally, two HSP marker genes and two reference control genes were used. The gene accession numbers, names and their corresponding primer sequences are shown in Table 2.1.

Table 2.1: List of primer sequences of sorghum heat-stress target genes used in qRT-PCR.

Accession no.	Gene ID	Protein name	Forward Primer (5'-3')	Reverse Primer (5'-3')
C5XHX2	SORBI_3003G427700	Uncharacterised protein	GAGCTTCCAGATCGACGGAG	TGAACATGAACATGGCAGGC
C5XHP7	SORBI_3003G419300	Uncharacterised protein	GGTGATCATCGGAGGGTTCC	TCGGTGTACCCTACCTAGCC
C5Y2R8	SORBI_3005G126200	Uncharacterised protein	CGGTTCCATCGGAAGTCCTC	CATGCAGTCTTCAGCGCATC
C5Z8T4	SORBI_3010G246600	Xyloglucan endotransglucosylase/hydrolase	GGCACTTCAATTCCTCGCAC	TTCGTTCGTTTTGTTCGGGC
C5XB39	SORBI_3002G055700	Uncharacterised protein	GAGGATCACACCGAGACCAC	GTTGTTTTTGGCTGTCCGGC
C5YVR0	SORBI_3009G093200	Superoxide dismutase	GAAACTTGGCTGGGCCATTG	CAGCGCCTTCTGCATTCATC
C5X578	SORBI_3002G128000	Uncharacterised protein	TGCTATTCAAACCGCCATCG	TAAGGTTGCGACACTTTTGC
C5WVG9	SORBI_3001G324800	Cysteine proteinase inhibitor	ACTCCTCCCTGCCTCTATGG	CAGCGACTTGCAGTGAAACC
C5XHP9	SORBI_3003G419500	Uncharacterised protein	TCAACAGAGCTCGGTGAAATC	TGCATGCGTCCAAAAATTCG
C5XGM0	SORBI_3003G126800	Cysteine proteinase inhibitor	TTGCTTGCGAGCAGTGTATG	TGGAGCTCGCATGTTCACTC
HSP70	Sb03g039360	Heat shock protein 70	TCATATCGTTGCCTTTCGTGTTG	CACTTGATTCTCTTCGTACAGTTTG
HSP90	Sb07g028270	Heat shock protein 90	GCTGGATGCGTGTGTTATCG	TGAAAGACAGCAGGATAAACGG
*Ref. Sb910	Sb03g038910	Uncharacterised protein	TCCTGAAGCATCTTTCCCTCC	ACAGCCTGATTAGTTGGGGG
*Ref. EIF4A	Sb04g003390	Eukaryotic initiation factor-4A	GATGAGATGCTCTCCCGTGG	TGATCTCTAGGGCCTCTGGG

*Reference control genes

2.13.5. Polymerase Chain Reaction for Primer Testing

Conventional PCR analysis was used to test for primer specificity. A reaction mixture containing 4% (v/v) cDNA, 10 mM dNTPs, 50 mM $MgCl_2$, 10 μM forward and reverse primers, and 0.4% (v/v) Taq polymerase was prepared. The mixture was topped up to 25 μL with MilliQ water, vortexed to mix and then placed in the PCR machine. The PCR conditions are summarised in Table 2.2 below.

Table 2.2: Thermal Cycling Conditions for PCR.

Step	Temperature (°C)	Time (Seconds)	Cycles
Initial denaturation	95	60	1
Denaturation	94	30	40
Annealing	56	60	
Elongation	72	60	

Upon the completion of the PCR runs, the amplicons were electrophoresed on a 3.5% (w/v) agarose gel in TAE Agarose DNA gel buffer [40 mM Tris acetate, 1 mM 0.5 M EDTA, 20 mM (v/v) glacial acid] containing 0.5 μg/mL of ethidium bromide. A 25 bp DNA marker HyperLadder™ (BIOLINE, London UK) and PCR products were electrophoresed at 120 V for 10-20 minutes. The gels were visualised using the Ingenius Bio-Imager (SynGene).

2.13.6 Quantitative Real Time-Polymerase Chain Reaction

The expression patterns of the *HSP70*, *HSP90* and the 10 sorghum genes listed in Table 2.1 were analysed by quantitative real time-polymerase chain reaction (qRT-PCR) analysis using the SensiFAST^{TM} SYBR No-ROX Kit (BIOLINE). Two reference control genes, *EIF4A* and *Sb910* were used. Three technical replicates qRT-PCR reaction mixtures were prepared for each of the four biological replicate cDNA templates. The samples contained 5 μL of the 1:20 diluted cDNA, 10 μL of SensiFAST^{TM} SYBR No-ROX Kit (BIOLINE), 10 μM forward and reverse primers and 3.4 μL of MilliQ water made up to a total volume of 20 μL. The qRT-PCR was performed using a Rotor-Gene 3000 (Corbett research, Sydney, Australia) using the thermal cycling conditions shown in Table 2.3. The fold-change was used to analyse the expression of each gene across all time points using REST2009 software version 2.0.13 (QIAGEN).

Table 2.3: Thermal Cycling Conditions for qRT-PCR.

Step	**Temperature (°C)**	**Time (Seconds)**	**Cycles**
Initial denaturation	95	90	1
Denaturation	95	10	45
Annealing	56	15	
Elongation	72	25	

CHAPTER 3

THE PROTEOMIC MAPPING OF SECRETED PROTEINS OF AN ICSB 338 SORGHUM CELL SUSPENSION CULTURE

3.1 Introduction

Proteins are secreted by plant cells into the extracellular matrix (ECM) under normal and stress conditions. This protein secretion is mediated by an N-terminal signal peptide, which directs proteins into the ECM (Lehtonen *et al*., 2014). However, there have been reports of leaderless proteins, that are secreted into the ECM without the assistance of signal peptides (Jung *et al*., 2008; Gupta *et al*., 2011). The ECM itself comprises of an extracellular fluid, which plays a role in facilitating the movement of substances to and from cells as well as in communication within and between cells (Agrawal *et al*., 2010). In the ECM, secreted proteins are involved in biological and physiological processes such as cell-to-cell communication, apoptosis, signalling, and response to biotic and abiotic stresses (Agrawal *et al*., 2010; Gupta *et al*., 2011). Understanding the identities of proteins present in enriched fractions of the ECM is an important step towards unravelling their diverse functional roles, including during abiotic stress response.

Secreted proteins also referred to as the secretome, may be separated and analysed using gel-based and/or gel-free proteomic approaches. Gel-based techniques, which include one (1D) and two dimensional (2D) gel electrophoresis are usually coupled with mass spectrometry for protein identification. On the other hand, gel-free techniques include multi-dimensional protein identification technology (MUDPIT) and isobaric tags for relative and absolute quantitation (iTRAQ) (Agrawal *et al*., 2010). The study of the secretome is carried out using *in vivo* (whole plant) and/or *in vitro* (cell suspension culture) systems. Although the whole plant systems provide tissue-specific secreted protein fractions, cell suspension cultures are a

preferred source of secreted proteins mainly due to their easy maintenance and handling (Gupta *et al*., 2011).

Various studies have been conducted on the secretome mapping of plants using cell suspension cultures. These include among others; studies on rice (*Oryza sativa*) (Cho *et al*., 2009), where a total of 555 proteins were identified, moss plants (*Physcomitrella patens*) (Lehtonen *et al*., 2014) with 72 proteins identified and 773 proteins identified in chickpea (*Cicer arietinum* L) (Gupta *et al*., 2011). The first reported gel-based proteomic mapping study of sorghum secreted proteins was conducted by Ngara and Ndimba (2011). In the study, only 14 proteins were positively identified from the culture filtrate of a White sorghum cell suspension culture line. Using bioinformatics tools, the identified proteins were assigned with putative functions in cell wall metabolism, physiological processes during plant growth and development, as well as signalling and defence related processes (Ngara & Ndimba, 2011).

More recently, 179 secreted proteins were identified from the same White sorghum cell culture system under control conditions using MUDPIT as part of an iTRAQ experiment (Ramulifho, 2017; Ngara *et al*., 2018). Of these proteins, 129 (72%) possessed a predicted signal peptide (Ngara *et al*., 2018). Indeed, these studies (Ngara & Ndimba, 2011; Ramulifho, 2017; Ngara *et al*., 2018) have given us some insight into the composition of sorghum secreted proteins. However, since sorghum has a wide genetic diversity and gene pool (Motlhaodi *et al*., 2017), it is important to broaden the coverage of proteomic map of sorghum secreted proteins as well as their functions under normal conditions and in stress response. The aim of this chapter was to identify and functionally annotate the secreted proteins of an ICSB 338 sorghum cell suspension culture using proteomic and bioinformatic tools.

3.2 Maintenance of ICSB 338 Callus Masses and Cell Suspension Cultures

ICSB 338 callus masses were maintained on sorghum callus medium at 27°C and sub-cultured after every four weeks (Section 2.2.1). Figure 3.1A shows 4-week old mature callus masses prior to sub-culturing. Only friable (easily breakable) calli were used to establish cell suspension cultures in liquid medium at 27°C (Section 2.2.2). Figure 3.1B shows a fine ICSB 338 sorghum cell suspension cultures prior to sub-culturing.

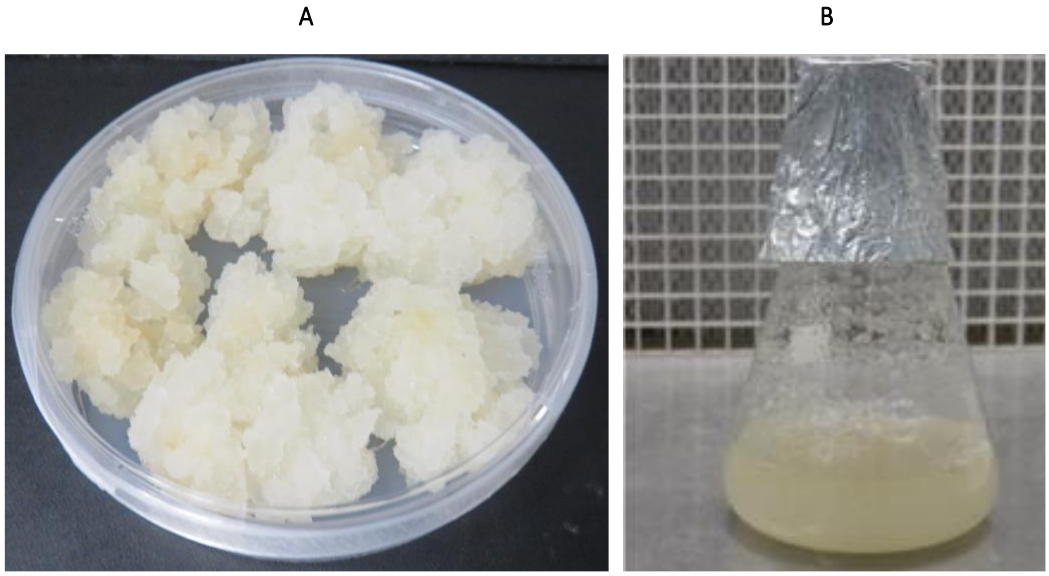


Figure 3.1: Sorghum callus and cell suspension cultures. (A) shows 4-week-old ICSB 338 callus masses, while (B) shows a 12-day old ICSB 338 cell suspension culture.

3.3 Cell Viability Test using the MTT Assay

Preliminary heat stress experiments were conducted to establish the most appropriate stress treatment temperature and duration for the sorghum cell cultures. The ICSB 338 sorghum cell suspension cultures were exposed to high temperatures of 35 and 40°C, and cell viability was

estimated using an MTT [3-(4,5-dimethylthiazolyl2)-2,5-diphenyltetrazolium bromide] assay. The control cells were maintained at 27°C. The MTT assay detects the metabolic activity of cells. Viable cells convert the MTT reagent into a purple-coloured formazan product with an absorbance maximum near 570 nm (Berridge *et al*., 2005).

The cell viability test was performed using three biological replicate 8-day old ICSB 338 cell suspension cultures. In order to reduce technical variation within the experiment, each mother culture was subdivided into two 50 ml sub-cultures for the control and heat stressed samples. From each flask per treatment group, cell viability was estimated at 0, 24, 48 and 72 hours. The results obtained are shown in Figure 3.2. Statistical analysis was performed using the Mann-Whitney test, $p < 0.05$, and there was no significant difference between the control and the heat stressed samples at all-time points. Therefore, ICSB 338 sorghum cells were viable at both 35 and 40°C following 72 hours of heat stress in comparison with the controls.

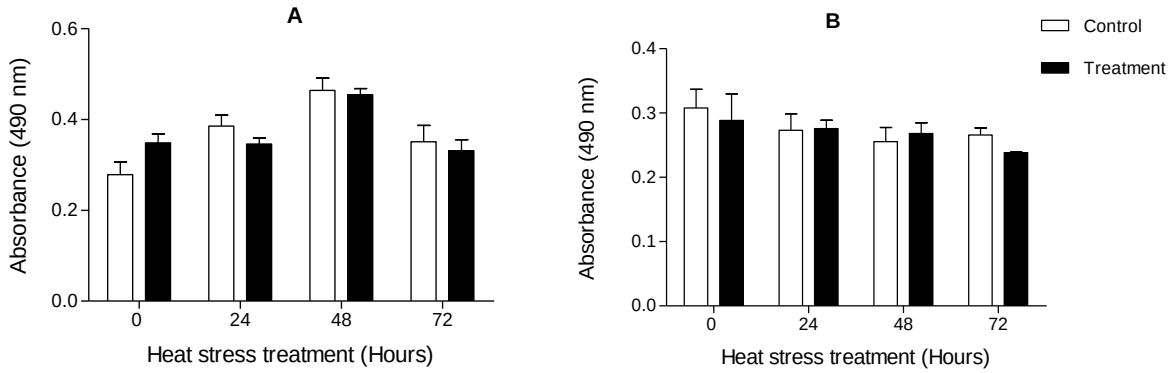


Figure 3.2: Cell viability of sorghum cell suspension cultures following heat stress treatment. ICSB 338 sorghum cell suspension cultures were exposed to 35 and 40°C heat stress treatment for 72 hours. Control cells were maintained at 27°C. The viability of the cells was estimated using an MTT assay. (A) shows the cell viability between the control and 35°C treated cell samples, while (B) shows the viability between control and 40°C treated cells. Data presented as mean ± SD (*n = 3*). Statistical analysis was performed using the Mann-Whitney test. No significant difference in the cell viability between control and treatment means at each time point at $p \leq 0.05$ was observed.

To validate the heat tolerance of sorghum, the cell viability results of ICSB 338 were compared with those of Arabidopsis. Arabidopsis cell suspension cultures were maintained in culture at 22°C post sub-culturing. Four days later (corresponding to the mid-log phase), the cell cultures were exposed to heat stress treatment at 40°C for 72 hours. From each flask per treatment group, cell viability was estimated at 0, 24, 48 and 72 hours using MTT assay on three biological replicates. Figure 3.3 shows the viability of Arabidopsis cells at different time points following the heat stress treatment. A statistically significant gradual decline in cell viability was observed between the control and treated cells across all time points. The results suggest that Arabidopsis is heat sensitive, while sorghum is heat tolerant.

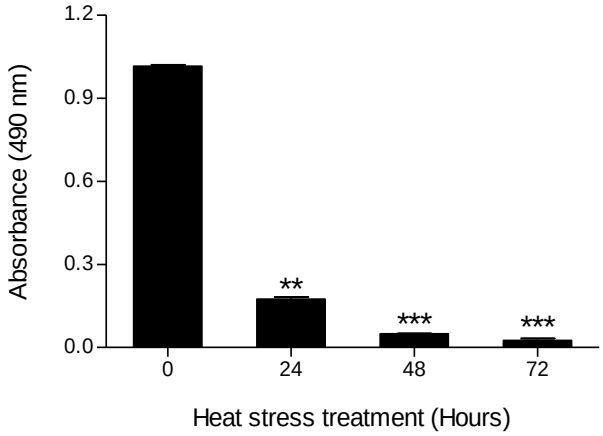


Figure 3.3: Cell viability of Arabidopsis cell suspension cultures following heat stress treatment. Arabidopsis cell suspension cultures were grown in culture at 22°C. Four days post sub-culturing, cell suspension cultures were exposed to heat stress treatment at 40°C for 72 hours. The viability of the cells was estimated using an MTT assay. Error bars represent means ± SD (*n = 3*). Statistical analysis was performed using the Mann-Whitney test. Two and three asterisks above the error bars indicate statistically significant differences between the control and heat stress treatment means at each time point at $p \leq 0.01$ and 0.001, respectively.

3.4 1D Gel Analysis of the ICSB 338 Cell Suspension CF Proteome

After a 72-hour high-temperature stress treatment at either 35 or 40°C, secreted proteins present in the culture medium were extracted as described in Section 2.5. The control and heat stressed protein samples were separated on 1D SDS-PAGE to assess the quality of the extracts. Figure 3.4 illustrates the CF protein profiles of ICSB 338 control and heat stressed samples. It was observed that the protein profiles of the control and 35°C heat stress-treated groups were almost similar except at around the 25 kDa region as indicated by the red arrow in Figure 3.4A. An increase in temperature from 35 to 40°C resulted in highly distinct protein expression profiles between the control and the heat stressed samples. This differential protein expression pattern is highlighted by the red arrows in Figure 3.4B. Based on these

results, the 40°C heat treatment threshold was selected for use in subsequent experiments including osmolyte content analyses, western blotting and the CF proteome mapping and differential protein and gene expression analyses following heat stress.

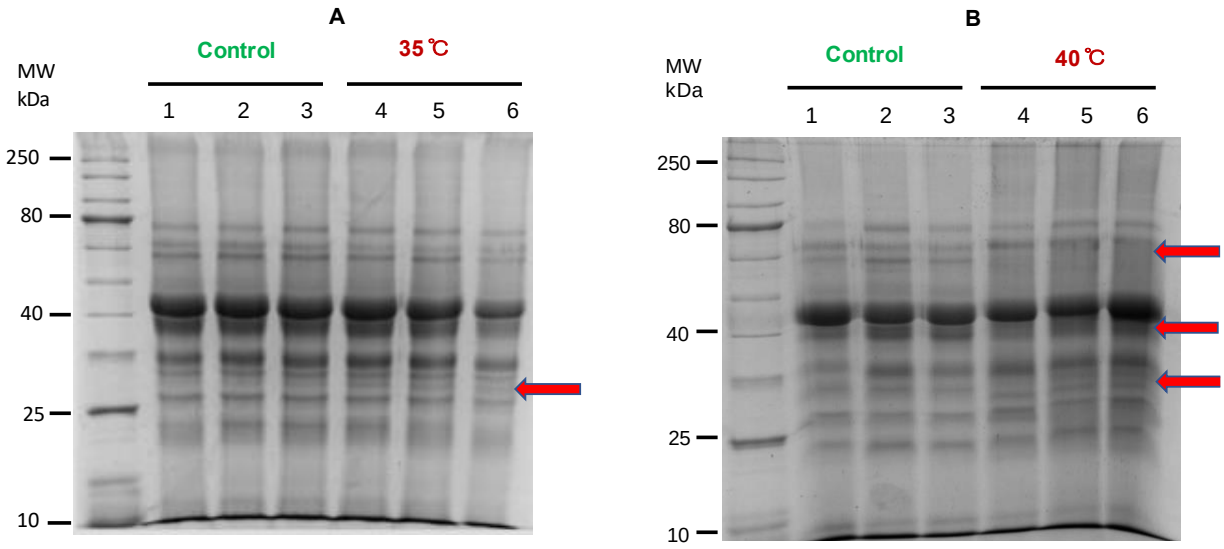


Figure 3.4:1D SDS-PAGE analysis of ICSB 338 culture filtrate proteins following heat stress. (A) shows the CBB stained protein profiles between the control and at 35°C, while (B) shows that at 40°C following 72 hours of heat treatment. In both gels, the MW lane represents the molecular weight markers in kDa. Lanes 1-3 show the controls at 27°C, while lanes 4-6 show the heat stressed samples at the respective high temperatures. The red arrows point out the protein expression differences between the control and high temperature stress treatment conditions.

3.5 Osmolyte Content Analysis following Heat Stress

Osmolytes play an important role in protecting plant cells when exposed to abiotic stresses. In this study, the proline and glycine betaine content was determined in the ICSB 338 sorghum cell suspension cultures following exposure to high temperature stress at 40°C for 48 and 72 hours. It was observed that the proline content of the cells decreased with an

increase in temperature treatment (Figure 3.5). Furthermore, this decrease in proline content was significantly different at 72 hours according to Mann-Whitney statistical test $p \leq 0.05$. Surprisingly, negative values were obtained for glycine betaine content analysis in the same samples (results not shown). The results signify undetectable glycine betaine levels in both the heat treated and untreated ICSB 338 cell suspension cultures.

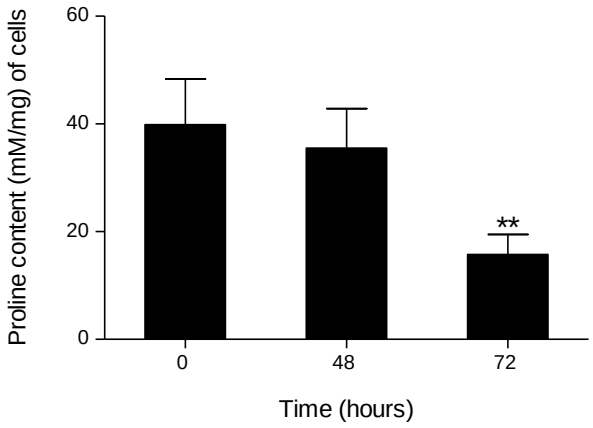


Figure 3.5: Proline content of sorghum cells following heat stress treatment. The ICSB 338 cell suspension cultures were exposed to heat stress at 40°C for 72 hours. The control cells were maintained at 27°C for the duration of the experiment. Samples for the proline content analysis were collected at 0, 48 and 72 hours following heat stress and was analysed using the HILIC LC-MS method. Error bars represent means ± SD ($n = 4$). Statistical analysis was performed using the Mann-Whitney test. The asterisk above the error bars indicates that there is a significant difference in the proline content between control and treatment means at $p \leq 0.05$.

3.6 HSP70 Western Blotting Analysis

Heat shock protein 70 (HSP70) is a cytoplasmic protein which is highly expressed under heat stress (Carper *et al*., 1987). This protein acts as a chaperone where it folds proteins in response to heat stress. Total soluble proteins (TSP) were extracted from Arabidopsis and

ICSB 338 sorghum cell suspension cultures (controls) grown at 22°C and 27°C, respectively, and heat stressed samples at 40°C. The TSP samples were separated on a 12% (v/v) SDS-PAGE gel prior to blotting on a nitrocellulose membrane. Figure 3.6 shows the HSP70 western blotting analysis results of the TSP from Arabidopsis and ICSB 338 control and heat stressed samples. The red arrow indicates the expression of HSP70 in sorghum and Arabidopsis control and heat stressed samples.

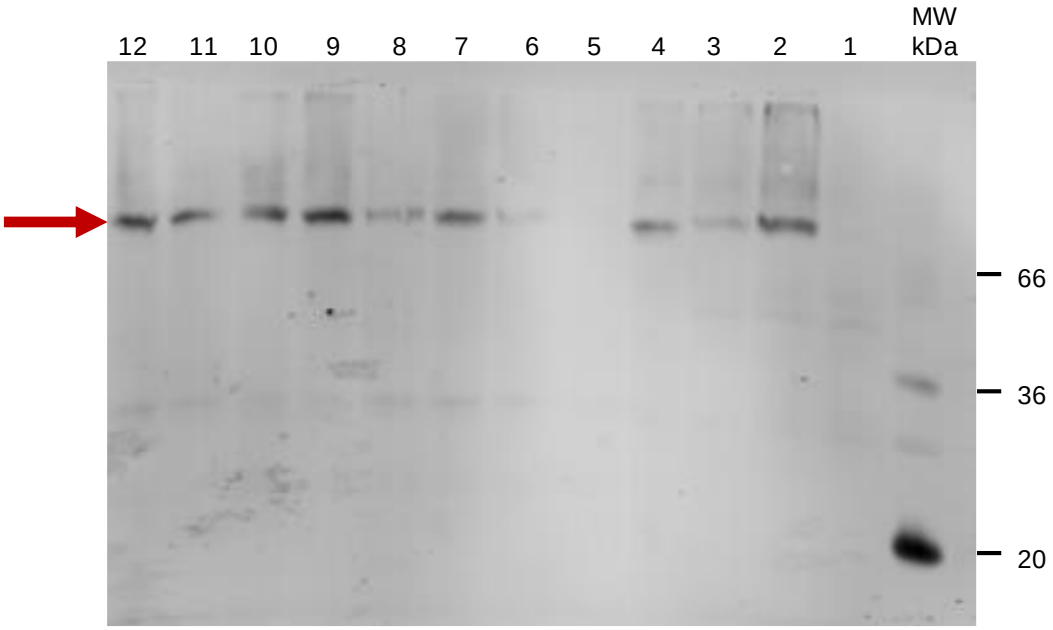


Figure 3.6: HSP70 western blotting analysis in sorghum and Arabidopsis TSP samples following heat stress. The TSP samples were separated on a 12% (v/v) SDS-PAGE gel prior to transferring onto nitrocellulose membrane. Lane 1 represents Arabidopsis TSP control at 22℃. Lanes 2-3: Arabidopsis TSP at 40℃ for 24 and 48 hours, respectively. Lane 4: ICSB 338 control at 23℃. Lanes 5-6: ICSB 338 control at 27℃ for 48 hours. Lanes 7-8: ICSB 338 control 27℃ for 72 hours. Lanes 9-10: ICSB 338 heat treated TSP at 40℃ for 48 hours. Lanes 11-12: ICSB 338 heat stressed TSP at 40℃ for 72 hours. Lane MW is the molecular weight marker protein in kDa. The arrow shows the expression of HSP70 in sorghum and Arabidopsis control and heat treated samples.

3.7 MUDPIT Analysis of ICSB 338 CF Control Proteins

The ICSB 338 CF protein extracts under control conditions and after exposure to 40°C for 72 hours were acetone precipitated and shipped to Durham University for iTRAQ analysis (Section 2.9). The CF mapping exercise was performed using MUDPIT analysis, which was part of the iTRAQ experiment. The samples were digested with trypsin and analysed by LC-MS/MS. The mass spectrometry data was analysed using the ProteinPilot software against the *Sorghum bicolor* only sequences on the UniProt database downloaded in May 2018. The raw MS dataset was filtered out to retain only proteins that were identified on the basis of at least two peptides. All proteins were considered positively identified at a ≥ 95% statistical confidence threshold. In this study, a total number of 290 CF proteins were positively identified in the ICSB 338 sorghum cell suspension culture under control conditions. This dataset thus constitutes the CF proteome map of this sorghum cell line. The complete list is shown in Table 3.1 and Appendix 2-Table 1.

The theoretical molecular weights (MW) of all the identified CF proteins was estimated using data available on the UniProt database (www.uniprot.org). The MW of the secreted proteins ranged between 11 and 114 kDa (Table 3.1). From the 290 positively identified secreted proteins, 231 (80%) were predicted to contain a signal peptide, whereas 59 (20%) did not (Table 3.1). This indicates that most proteins were targeted for secretion via the classical secretory pathway. The majority of the identified proteins (69%) were uncharacterised. The largest group of proteins with known functions were peroxidases (16%), followed by carboxypeptidases (2%), cysteine proteinase inhibitors (2%) and purple acid phosphatases (2%). Other proteins included alpha galactosidase, superoxide dismutase, xyloglucan endotransglucosylase, protein disulfide-isomerase, pectinesterase, alpha-mannosidase, beta-hexosaminidase, endoglucanase, glyceraldehyde-3-phosphate dehydrogenase, dirigent

protein, aldose 1-epimerase, dihydrolixyl dehydrogenase, fructose-bisphosphate aldolase, nucleoside diphosphate kinase and pectin acetylesterase in very low proportions (Table 3.1).

Table 3.1: List of culture filtrate proteins identified from non-stressed ICSB 338 sorghum cell suspension cultures using MUDPIT and database searches.

Pro. No.[a]	Accession no. [b]	Protein name	Score[c]	% Cov[d]	Seq Pep[e]	Theor MW (kDa)[f]	SP[g]	GO analysis[h]			Conserved domains & family name[i]
								P	F	C	
1	C5Y8G7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G224500	102.2	64.05	370	37.53	+	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Aspartic pepdidase domain Peptidase A1 family
2	C5Y397	Alpha mannosidase OS=*Sorghum bicolor* GN=SORBI_3005G132400	52.48	33.07	1019	114.28	+	Carbohydrate metabolic activity	Catalytic activity	Plant-type cell wall	Glycosidase hydrolase domain Family none predicted
3	C5XYP5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G233700	48.3	35.08	784	84.26	+	Carbohydrate metabolic process	Hydrolyse activity, O-hydrolyzing glycosyl compounds	None predicted	Glycoside hydrolase domain and family
4	C5Z484	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G163000	44.9	51.8	529	57.56	+	Oxidation-reduction process	Catalytic activity	Nucleus	FAD-binding domain Oestrogen receptor family
5	C5Y360	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011300	40.4	62.8	328	34.43	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase, plant domain Plant peroxidase family
6	C5XQV7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085900	38.5	23.71	928	102.51	+	Metabolic process	Catalytic activity	None predicted	Glycoside hydrolase family3, N-terminal domain Glycoside hydrolase family31
7	A0A1W0W7T8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G132400	36.6	59.25	319	33.37	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase plant domain Plant peroxidase family
8	C5Y8Y2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G235600	36.51	31.68	767	80.91	+	Carbohydrate metabolic activity	Hydrolyse activity, O-hydrolyzing glycosyl compounds	None predicted	Glycoside hydrolase family3 C-terminal domain Family none predicted
10	A0A1B6PLA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G104300	33.94	44.35	584	62.06	+	Proteolysis	Glutathione hydrolase activity	None predicted	Domain none predicted Gamma-glutamyltranspeptidase family
11	A0A1B6QCB0	Alpha-amylase OS=*Sorghum bicolor* GN=SORBI_3002G190500	33.34	54.12	412	45.1	+	Carbohydrate metabolic process	Alpha-amylase activity	None predicted	Alpha-amylase, C-terminal-beta sheet domain Alpha-amylase, plant family
12	A0A1B6QI05	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089100	31.6	45.73	261	67.20	+	Carbohydrate metabolic process	Hydrolyse activity, O-hydrolyzing glycosyl compounds	None predicted	Glycoside hydrolase family 3 C terminal domain Family none predicted

13	C5Z8N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G118900	28.48	38.9	473	47.82	+	None predicted	None predicted	None predicted	FAS1 domain Fasciclin-like arabinogalactan protein family
15	C5X5K6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416700	26.58	44.73	313	32.41	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase plant domain Plant peroxidase family
16	C5WXC7	Alpha galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	25.4	38.97	426	46.96	+	Metabolic process	Catalytic activity	Plant-type cell wall	Alpha galactosidase A, C-terminal beta-sandwich domain Glycoside hydrolase, family 27
17	A0A1B6PKE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G056300	25.4	41.87	534	57.24	+	Oxidation-reduction process	Catalytic activity	None predicted	FAD-binding, type 2 domain Family none predicted
18	C5XB38	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055600	25.24	54.72	307	33.65	+	Carbohydrate metabolic activity	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain Family none predicted
19	C5WN99	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G261600	24.66	37.02	516	57.33	+	Carbohydrate metabolic activity	Hydrolyse activity, O-hydrolyzing glycosyl compounds	Cytoplasm	Glycoside hydrolase, family 5 domain Fascin family
20	A0A1B6PD27	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3008G113000	24.66	42.44	483	54,508	+	Dephosphorylation	Acid phosphatase activity	None predicted	Purple acid phosphatase, N-terminal domain Purple acid phosphatase-like family
21	C5Z240	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G003100	24.1	35.25	590	65.56	+	Oxidation-reduction process	Oxidoreductase activity	None predicted	Ascorbate oxidase homologue, first cupredoxin domain Family none predicted
22	A0A1B6Q838	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422200	24.1	51.19	336	34,95	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Domain none predicted Glycoside hydrolase family 17
23	C5XHP8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419400	23.84	44.36	417	44,74	+	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family
24	C5Z475	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G162000	23.84	62.15	325	34,43	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
25	C5WXD7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G209300	23.69	58.22	225	24.42	+	None predicted	None predicted	None predicted	Domain none predicted Uncharacterised protein family, basic secretory protein
26	A0A1Z5R915	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3007G091100	23.28	22.1	638	70.24	-	Dephosphorylation	Hydrolase activity	None predicted	Purple acid phosphatase, N-terminal domain Family none predicted
27	C5WYQ4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G360400	23.08	43.98	332	34.75	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family

28	C5Y1P4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G099000	22.68	51.92	312	34.38	+	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family, catalytic domain Family none predicted
29	C5XWE5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G197600	22.63	17.93	753	81.75	+	Lipid metabolic process	Phosphoric diester hydrolase activity	None predicted	Glycerophosphodiester phosphodiesterase domain Family none predicted
30	C5XIK1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G432700	21.7	40.17	463	48.75	+	None predicted	None predicted	None predicted	Bulb-type lectin domain Family none predicted
31	C5YRS3	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3008G037000	21.49	24.85	491	53.30	-	Dephosphorylation	Hydrolase activity	None predicted	Purple acid phosphatase, N-terminal domain Family none predicted
32	C5YK12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G100600	20.99	36	200	19.92	+	Electron transport chain	Electron transfer activity	Integral component of membrane	Phytocyanin domain Family none predicted
33	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600	20.92	43.84	333	35.66	+	Negative regulation of catalytic activity	Enzyme inhibitor activity	None predicted	Leucine-rich repeat-containing N-terminal, plant type domain Family none predicted
34	A0A1B6PLT5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G133000	20.6	34.47	380	40.48	+	None predicted	None predicted	None predicted	Domain of unknown function DUF642 Family none predicted
35	A0A1B6QEI0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G317600	20.45	63.69	179	18.69	-	None predicted	None predicted	None predicted	Domain none predicted RidA family
36	C5XB39	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055700	20.42	46.91	307	33.60	+	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain Family none predicted
37	A0A194YQ33	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G166700	20.2	20.48	581	64.68	+	Hydrolyse activity, acting on glycosyl bonds	Hydrolyse activity, O-hydrolyzing glycosyl compounds	None predicted	Glycoside hydrolase, family 32 N-terminal domain Glycoside hydrolase, family 32
38	A0A1B6Q6M7	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G327700	20.1	48.87	133	14.89	+	Negative regulation of peptidase activity	Peptidase inhibitor activity	None predicted	Cystatin domain Cystatin family
39	C5Z469	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G161600	20	54.46	314	33.21	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
40	C5XHT5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G423500	19.43	45.43	317	33.39	-	Carbohydrate metabolic process	Hydrolyse activity, O-hydrolyzing glycosyl compounds	Anchored component of plasma membrane	Domain none predicted Glycoside hydrolase family 17
41	C5YA35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G260300	19.4	18.63	467	50.21	+	Proteolysis involved in cellular protein catabolic process	Hydrolase activity	Extracellular region	Peptidase C1A, papain C-terminal Peptidase C1 family
42	C5XQ74	Uncharacterized protein OS=*Sorghum bicolor*	19.08	28.76	518	53.53	-	Protein catabolic process	Hydrolase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family

		GN=SORBI_3003G208800									
43	C5YBE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132400	19	42.12	273	28.56	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase, family 19, catalytic domain Glycoside hydrolase, family 19
44	C5XCE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G351400	18.78	30.04	233	24.57	+	None predicted	None predicted	None predicted	Domain none predicted Thaumatin family
45	C5Z483	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G162900	18.2	33.08	526	57.69	+	Oxidation-reduction process	Catalytic activity	None predicted	FAD-binding oxidase, N-terminal domain Family none predicted
46	C5YIX7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G078800	17.96	30.57	494	52.08	+	Carbohydrate metabolic process	Hydrolyse activity, O-hydrolyzing glycosyl compounds	Integral component of membrane	X8 domain Glycoside hydrolase family 17
47	C5XHP9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419500	17.73	36.96	414	43.89	+	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Xylanase inhibitor, N-terminal Aspartic peptidase A1 family
48	C5YJ56	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G214700	17.67	33.21	262	28.06	+	None predicted	None predicted	None predicted	FAS1 domain Family none predicted
49	C5XHP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419300	17.63	24.36	431	44.95	-	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Xylanase inhibitor, N-terminal Aspartic peptidase A1 family
50	C5X040	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G080300	17.47	39.22	334	35.26	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
51	C5Y675	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G064200	17.31	35.42	415	44.46	+	Proteolysis	Aspartic-type endopeptidase activity Hydrolase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family
53	C5YQU5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G146700	16.83	37.58	330	34.31	+	Carbohydrate metabolic process	Hydrolyse activity, O-hydrolyzing glycosyl compounds	Anchored component of plasma membrane	Domain none predicted Glycoside hydrolase family 17
54	C5XV25	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G333600	16.71	22.59	540	58.61	+	None predicted	Hydrolyse activity, acting on glycosyl bonds	Plant-type cell wall	Domain none predicted Glycoside hydrolase, family 79
55	A0A1Z5S979	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3001G348900	16.6	38.15	464	49.74	+	Proteolysis	Carboxypeptidase activity	None predicted	Domain none predicted Peptidase S10, serine carboxypeptidase family
56	A0A1B6QJE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G170700	16.44	22.61	774	79.06	+	Proteolysis	Hydrolase activity	None predicted	Peptidase S8/S53 domain Peptidase S8, subtilisin-related family
57	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor*	16.15	47.56	225	23.75	+	None predicted	Nutrient reservoir activity	Extracellular region	Cupin 1 domain Germin family

		GN=SORBI_3001G129700									
58	C5YVR0	Superoxide dismutase OS=*Sorghum bicolor* GN=SORBI_3009G093200	16.13	53.65	283	25.36	-	Superoxide metabolic process	Oxidoreductase activity	None predicted	Manganese/iron superoxide dismutase, C and N terminal domain Manganese/iron superoxide dismutase family
59	A0A1B6PNL9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G242000	16.09	16.38	464	50.39	+	Proteolysis	Peptidase activity	None predicted	Peptidase C1A, papain C-terminal domain Peptidase C1A family
60	C5XHF1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G136200	15.91	52.51	219	23.04	+	Plasmodesmata-mediated intercellular transport	Nutrient reservoir activity	Extracellular region	Cupin 1 domain Germin family
61	C5X5L7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3002G417800	15.85	24.36	431	47.15	+	Carbohydrate metabolic process	Catalytic activity	Plant-type cell wall	Domain none predicted Glycoside hydrolase, family 27
62	A0A1W0VUE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G227400	15.77	15.26	839	92.59	-	Carbohydrate metabolic process	Catalytic activity	Plant-type cell wall	Glycoside hydrolase family 31, N-terminal domain Glycoside hydrolase family 31
63	C5YYX0	Beta-hexosaminidase OS=*Sorghum bicolor* GN=SORBI_3009G017500	15.64	16.85	546	60.66	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Cytosol	Glycoside hydrolase family 20, catalytic domain Beta-hexoaminidase family
64	C5Z0N9	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055300	15.32	22.59	363	38.28	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
65	C5Z6U2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G210000	15.06	50.98	153	17.20	-	None predicted	Protein binding	None predicted	Ubiquitin domain and family
66	C5WS35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G081100	14.58	18.85	764	78.51	+	Proteolysis	Hydrolase activity	None predicted	Peptidase S8/S53 domain Peptidase S8, subtilisin-related family
67	C5WW86	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G196700	14.49	21.76	510	56.06	+	Proteolysis	Serine-type peptidase activity	None predicted	Domain none predicted Peptidase S28 family
68	A0A1B6QHZ6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089000	14.47	17.92	703	76.19	-	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Membrane	Glycoside hydrolase, family 3, N-terminal Family none predicted
69	A0A1W0W7I8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391900	14.44	20	555	57.58	+	Response to oxidative stress	Peroxidase activity	None predicted	Secretory peroxidase domain Plant peroxidase family
70	C5XYY5	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3004G105100	14.08	44.92	325	33.56	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
71	C5X4N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255600	14.07	22.57	483	52.42	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-	Anchored component of plasma membrane	X8 domain Glycoside hydrolase family 17

									glycosyl compounds		
72	C5XI24	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G140700	13.93	28.94	349	38.09	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
73	C5Y7T1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G086000	13.79	16.3	460	49.85	+	None predicted	Hydrolase activity	None predicted	Domain none predicted Nucleoside phosphatase GDA/CD39 family
74	C5WVG9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324800	13.69	57.04	135	14.38	+	Negative regulation of cysteine-type endopeptidase activity	Cysteine-type endopeptidase inhibitor activity	None predicted	Cystatin domain and family
75	A0A1W0W7U5	Carboxypeptidase (Fragment) OS=*Sorghum bicolor* GN=SORBI_3002G401200	13.67	26.74	460	49.77	-	Proteolysis	Carboxypeptidase activity	None predicted	Domain none predicted Peptidase S10, serine carboxypeptidase family
76	C5XIY1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152100	13.37	23.53	357	37.72	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
77	C5X5H5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G268200	13.15	14.16	572	62.76	-	Carbohydrate metabolic process	Alpha-L-fucosidase activity	None predicted	Domain none predicted Glycoside hydrolase, family 29
78	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	12.78	10.4	654	69.79	-	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Carbohydrate binding domain Glycoside hydrolase, family 9
80	C5WU08	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G025400	12.09	36.36	231	24.71	-	Metabolic process	Catalytic activity	None predicted	Fumarylacetoacetase-like, C-terminal domain Family none predicted
81	C5YB25	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3006G277600	12.09	30.55	347	36.26	+	Response to oxidative stress	Peroxidase activity	Plant-type cell wall	Secretory peroxidase domain Plant peroxidase family
82	A0A1B6QFT1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G392000	12.01	15.3	379	40.53	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
83	A0A1B6Q515	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G238500	12.01	23.03	482	51.76	-	Carbohydrate metabolic process	Catalytic activity	None predicted	Lactate/malate dehydrogenase, N-terminal domain Malate dehydrogenase, type 1 family
84	C5X3C1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391300	11.88	20.6	369	39.09	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
85	C5Y2P0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G122300	11.87	31.69	142	15.40	+	None predicted	None predicted	None predicted	Domain and family none predicted
86	C5Z6U1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G209900	11.82	13.37	516	57.79	+	None predicted	Catalytic activity	None predicted	Phospholipase D-like domain Family none predicted

87	C5XX52	Glyceraldehyde-3-phosphate dehydrogenase OS=Sorghum bicolor GN=SORBI_3004G205100	11.8	21.66	337	36.35	-	Oxidation-reduction process	Oxidoreductase activity	None predicted	Glyceraldehyde 3-phosphate dehydrogenase catalytic domain Glyceraldehyde/Erythrose phosphate dehydrogenase family
88	A0A1W0W2E3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050900	11.61	15.92	377	41.43	+	None predicted	Protein binding	None predicted	Leucine-rich repeat-containing N-terminal, plant-type domain Family none predicted
89	A0A1Z5S9P3	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G379400	11.53	29.6	375	39.89	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
90	C5WP48	Alpha-mannosidase OS=*Sorghum bicolor* GN=SORBI_3001G268700	11.43	9.335	1007	111.61	+	Metabolic process	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Glycoside hydrolase family 38, N-terminal domain Family none predicted
91	C5WPY8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G277000	11.37	17.46	338	36.09	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
92	C5XD22	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G356800	11.28	16.33	398	42.91	+	None predicted	Hydrolase activity, acting on ester bonds	None predicted	GDSL lipase/esterase domain Family none predicted
93	C5X1L1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G247900	11.19	22.26	310	33.80	+	Carbohydrate metabolic process	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Glycoside hydrolase family 18, catalytic domain 2S globulin family
94	C5XX83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G208700	11.08	25.19	270	28.43	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase, family 19, catalytic domain Glycoside hydrolase, family, 19
95	A0A1B6P5R2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G009600	10.91	46.2	158	16.56	-	Response to wounding	Serine-type endopeptidase inhibitor activity	None predicted	Domain none predicted Proteinase inhibitor I13, potato inhibitor I family
97	C5Z0P5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G055900	10.11	22.01	418	44.504	-	None predicted	None predicted	None predicted	FAS1 domain Fasciclin-like arabinogalactan protein family
98	A0A1Z5RER0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G186300	10.09	15.15	429	43.87	+	None predicted	None predicted	None predicted	FAS1 domain Fasciclin-like arabinogalactan protein family
99	C5X022	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G525000	10.07	24.19	463	49.22	+	Carbohydrate metabolic process	Hydrolase activity	Extracellular region	Domain none predicted Glycoside hydrolase, family 28
100	A0A194YU12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G341200	10.04	12.12	495	53.04	-	Oxidation-reduction process	Oxidoreductase activity	Peroxisome	FAD/NAD(P)-binding domain Pyridine nucleotide-disulphide oxidoredutase. Class I family
101	C5Y2R8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G126200	10.04	24.7	247	26.58	+	Cell surface receptor signaling pathway	Transmembrane receptor protein serine/threonine kinase activity	Plasma membrane	Leucine-rich repeat-containing N-terminal, plant-type domain Family none predicted
102	C5YYK3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G014700	10.01	29.8	302	31.95	-	None predicted	None predicted	None predicted	Gnk2-homologous domain Family none predicted

103	C5X0X1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G528100	10	21.62	333	33.98	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
104	A0A1B6PTQ9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G204700	9.88	20.84	451	48.35	+	Carbohydrate metabolic process	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Domain none predicted Glycoside hydrolase, family 28
106	A0A1W0VY92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205900	9.76	13.91	381	39.37	+	None predicted	Hydrolase activity, acting on ester bonds	None predicted	GDSL lipase/esterase-like, plant domain Family none predicted
107	C5WY32	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G061900	9.66	16.36	495	53.62	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Integral component of membrane	X8 domain Glycoside hydrolase family 17
108	C5XL59	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G024700	9.65	24.63	406	41.94	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
109	C5WXN2	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3001G348800	9.54	26.51	464	50.61	+	Proteolysis	Hydrolase activity	None predicted	Domain none predicted Peptidase S10, serine carboxypeptidase family
110	C5WSX6	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3001G013800	9.49	9.242	541	59.77	+	Dephosphorylation	Hydrolase activity	None predicted	Purple acid phosphatase, N-terminal domain Purple acid phosphatase-like family
111	A0A1Z5RHN3	Dirigent protein OS=*Sorghum bicolor* GN=SORBI_3005G101500	9.43	33.51	188	19.49	+	None predicted	None predicted	None predicted	Domain none predicted Dirigent protein family
112	C5YVJ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G232100	9.4	37.96	245	24.90	+	None predicted	None predicted	None predicted	FAS1 domain Family none predicted
113	A0A1B6Q537	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G244600	9.35	19.23	338	35.27	-	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Glycoside hydrolase, family 18, catalytic domain Family none predicted
114	C5WSE5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G300400	9.29	18.97	290	31.85	+	Sexual reproduction	None predicted	Extracellular region	Expansin, cellulose-binding-like domain Expansin/LoI pI family
115	A0A1W0W3H0	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3002G123100	9.25	16.76	358	39.70	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Alpha galactose A, C-terminal beta-sandwich domain Glycoside hydrolase, family 27
116	C5WPH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G131100	9.21	7.649	706	75.94	+	None predicted	Catalytic activity	None predicted	Glucose/Sorbosone dehydrogenase domain Family none predicted
117	C5XDR4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G217200	9.06	16.53	363	39.26	+	Immune response	Hydrolase activity	Extracellular space	Peptidase C1A, papain C-terminal Peptidase C1A family

118	A0A1Z5R0P5	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G033300	9.04	14.71	333	36,28	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
119	C5WU20	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G026800	9.02	13.26	475	51.61	+	Carbohydrate metabolic process	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Domain none predicted Glycoside hydrolase, family 28
120	C5WPY7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G277500	8.85	8.264	605	66.92	+	Regulation of defense response	Protein kinase activity	Integral component of membrane	Protein kinase domain Family none predicted
121	C5YQ75	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3008G010500	8.76	44.21	328	34.73	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
122	C5XAQ6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G326000	8.54	16.07	448	46.42	+	Proteolysis	Hydrolase activity	None predicted	peptidase A1 family domain Aspartic peptidase A1 family
123	C5XY68	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3004G224700	8.52	14.32	475	52.45	+	Proteolysis	Peptidase activity	Vacuole	Domain none predicted Peptidase S10, serine carboxypeptidase family
124	C5X3C6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391800	8.5	15.25	387	41.72	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
125	C5WSY5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G014700	8.44	16.15	483	51.21	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Anchored component of plasma membrane	X8 domain Glycoside hydrolase family 17
126	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	8.39	32.31	229	23.88	+	None predicted	None predicted	None predicted	Domain none predicted Thaumatin family
127	C5Z8T4	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246600	8.26	15.28	288	31.49	+	Metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Extracellular region	Glycoside hydrolase family 16 domain Xyloglucan endotransglucosylase/hydrolase family
128	C5XHR8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G421700	8.25	19.88	332	34.56	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Anchored component of plasma membrane	Domain none predicted Glycoside hydrolase family 17
129	C5XIY0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152000	8.23	24.73	364	37.65	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
130	C5XC95	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G345800	8.19	26.84	190	18.84	+	Electron transport chain	Electron transfer activity	Anchored component of plasma membrane	Phytocyanin domain and family
131	C5Z6D9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G079100	8.09	18.92	407	40.67	+	None predicted	None predicted	None predicted	LysM domain Family none predicted

132	C5Z864	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G232500	8.01	22.36	331	35.81	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
133	C5X780	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G007200	7.96	20.77	207	21.52	+	Electron transport chain	Electron transfer activity	Anchored component of plasma membrane	Phytocyanin domain and family
134	A0A1B6QIM7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G123300	7.96	10.12	504	56.50	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Domain none predicted Glycoside hydrolase family 1
135	C5Z4E5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044900	7.95	11.99	367	39.46	+	None predicted	Hydrolase activity, acting on ester bonds	None predicted	GDSL lipase/esterase domain Family none predicted
136	C5XQW7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G087300	7.86	18.06	288	32.60	+	DNA catabolic process	Hydrolase activity, acting on ester bonds	None predicted	Domain none predicted S1/P1 nuclease family
137	C5XHX2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G427700	7.85	34.38	224	23.24	+	Plasmodesmata-mediated intercellular transport	Nutrient reservoir activity	Extracellular region	Cupin 1 domain Germin family
138	C5Y7R1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G084300	7.84	19.23	286	31.10	-	Carbohydrate metabolic process	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Glycoside hydrolase family 18, catalytic domain 2S globulin family
140	A0A1B6QEG2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G315800	7.67	13.52	355	38.92	+	Regulation of catalytic activity	Cysteine-type endopeptidase activity	None predicted	Peptidase C1A, propeptide domain Peptidase C1A family
141	C5WN51	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G119000	7.39	38.28	128	13.55	+	Electron transport chain	Electron transfer activity	Anchored component of plasma membrane	Phytocyanin domain and family
142	C5WRN5	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G444500	7.36	23.15	337	36.21	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
143	C5WX83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G058300	7.19	22.59	394	42.57	+	None predicted	None predicted	None predicted	Leucine-rich repeat-containing N-terminal, plant type domain Family none predicted
144	C5XRC3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G374100	7.13	6.999	843	93.85	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Plant-type cell wall	Glycoside hydrolase 35, catalytic domain Glycoside hydrolase family 35
145	C5YBF0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132500	7.1	41.94	279	28.98	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase, family 19, catalytic domain Glycoside hydrolase family 19
146	C5XAS9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G329000	6.96	8.065	682	74.29	+	Protein phosphorylation	Protein kinase activity	Plasma membrane	Gnk2-homologous domain Family none predicted
148	A0A1Z5R3E0	Uncharacterized protein	6.88	18.26	356	37.58	+	Carbohydrate	Chitinase	None predicted	Glycoside hydrolase, family 19,

		OS=*Sorghum bicolor* GN=SORBI_3009G130100						metabolic process	activity		catalytic domain Glycoside hydrolase family 19
149	C6JSB7	Peroxidase OS=*Sorghum bicolor* GN=Sb0246s002010	6.64	49.69	320	33.77	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
150	A0A1B6PQR2	Protein disulfide-isomerase OS=*Sorghum bicolor* GN=SORBI_3005G074400	6.61	7.961	515	57.31	+	Protein folding in endoplasmic reticulum	Protein disulfide isomerase activity	Endoplasmic reticulum lumen	Disulfide isomerase domain Protein disulfide isomerase family
151	C5Y9T3	Aldose 1-epimerase OS=*Sorghum bicolor* GN=SORBI_3006G105200	6.55	11.65	369	39.87	+	Carbohydrate metabolic process	Catalytic activity	None predicted	Domain none predicted Aldose 1-/Glucose-6-phosphate 1-epimerase family
152	C5XLM4	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3003G314400	6.54	6.723	476	54.05	+	Dephosphorylation	Hydrolase activity	Plant-type cell wall	Purple acid phosphatase, N-terminal domain Purple acid phosphatase-like family
153	C5Z1X3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G268400	6.5	7.394	541	56.51	+	Proteolysis	Hydrolase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family
154	C5XJT8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G156400	6.45	12.5	368	40.13	+	Protein binding	Protein disulfide isomerase activity	Endoplasmic reticulum	Disulphide isomerase domain Family none predicted
155	C5XIH4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G430100	6.38	8.987	523	57.68	+	Metabolic process	Catalytic activity	None predicted	Phosphoesterase domain Family none predicted
156	A0A1Z5RIA3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G126100	6.36	19.46	221	23.69	-	None predicted	Protein binding	None predicted	Leucine-rich repeat-containing N-terminal, plant type domain Family none predicted
157	C5Y5U9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177500	6.35	23.78	307	33.36	+	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain Family none predicted
158	A0A1Z5RHM3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G098700	6.21	32.35	306	33.49	+	Carbohydrate metabolic process	None predicted	None predicted	Glycoside hydrolase family 18, catalytic domain Family none predicted
159	A0A1B6QN00	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G360500	6.12	12.46	337	35.97	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
160	C5WZU7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G516000	6.04	19.84	257	27.75	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase, family 19, catalytic domain Glycoside hydrolase family 19
161	C5WX01	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G050100	6.02	17.17	233	24.27	+	None predicted	Nutrient reservoir activity	Extracellular region	Cupin 1 domain Germin family
162	C5YZI6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G032800	6.02	10.18	334	35.06	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
163	A0A1B6PNV2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G255600	6.01	5.76	625	68.30	-	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Glycosyl hydrolase family 32, N-terminal domain Glycosyl hydrolase, family 32

164	C5YLE3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G002800	6	7.674	430	45.70	+	Lipid metabolic process	Phosphoric diester hydrolase activity	None predicted	Phosphatidylinositol-specific phospholipase C, X domain Family none predicted
165	A0A1W0W1X3	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3002G024400	6	11.92	478	52.33	+	Proteolysis	Carboxypeptidase activity	None predicted	Domain none predicted Peptidase S10, serine carboxypeptidase family
166	C5YD83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G031200	6	16.82	220	24.31	+	None predicted	None predicted	None predicted	Thioredoxin-like superfamily domain Family none predicted
167	C5XYB7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G229500	6	12.58	310	32.12	+	None predicted	None predicted	None predicted	Domain none predicted Protein EXORDIUM-like family
168	C5XPK9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205600	5.98	5.873	613	65.66	+	None predicted	Protein binding	None predicted	Domain and family none predicted
169	A0A1B6QJR7	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G189000	5.93	11.97	401	43.29	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
170	C5WWH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G333400	5.83	31.84	179	19.10	+	None predicted	None predicted	Extracellular region	CAP domain Cysteine-rich secretory protein, allergin V5/Tpx-1-related family
171	C5YV55	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G084300	5.77	8.914	359	38.95	+	Nucleoside metabolic process	Catalytic activity	None predicted	Nucleoside phosphorylase domain Family none predicted
172	A0A1B6QC86	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G189100	5.7	6.658	766	83.49	-	None predicted	Glucan endo-1,3-beta-glucanase activity, C-3 substituted reducing group	None predicted	Domain none predicted Endo-1,3(4)-beta-glucanase family
174	C5YCI1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G154300	5.66	12.61	230	23.94	+	Negative regulation of catalytic activity	Endopeptidase inhibitor activity	None predicted	Domain none predicted Proteinase inhibitor I3, Kunitz legume family
175	A0A1Z5S8J9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G300800	5.61	11.38	325	34.84	-	Sexual reproduction	None predicted	Extracellular region	Expansin, cellulose-binding-like domain Expansin/Lol pI
176	C5XQS6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G082600	5.57	15.53	206	21.35	-	None predicted	Calcium ion binding	None predicted	EF-hand domain Family none predicted
177	C5YNA1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G172100	5.51	8.602	372	40.26	+	Proteolysis	Hydrolase activity	Lysosome	Peptidase C1A, papain C-terminal domain Peptidase C1A family
178	A0A1B6QN59	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G366800	5.45	4.791	814	92.11	+	None predicted	None predicted	None predicted	Alpha-N-acetylglucosaminidase, N-terminal domain Alpha-N-acetylglucosaminidase family
179	A0A1B6QG28	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor*	5.45	36.14	166	16.98	-	Superoxide metabolic process	Oxidoreductase activity	None predicted	Superoxide dismutase, copper/zink binding domain Superoxide dismutase (Cu/Zn)

		GN=SORBI_3002G407900									superoxide dismutase copper chaperone family
180	C5XIT6	Pectinesterase OS=*Sorghum bicolor* GN=SORBI_3003G148400	5.39	6.771	576	61.54	+	Pectin catabolic process	Pectinesterase activity	Cell wall	Pectinesterase, catalytic domain Family none predicted
181	C5X6P6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G140300	5.34	22.09	172	17.42	+	Electron transport chain	Electron transfer activity	Anchored component of plasma membrane	Phytocyanin domain and family
182	C5X5T3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G130700	5.09	13.42	380	40.60	+	Proteolysis involved in cellular catabolic process	Peptidase activity	Extracellular space	Peptidase C1A, papain C-terminal domain Peptidase C1A family
183	C5Z6Y0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G088700	4.7	9.763	338	34.48	+	None predicted	None predicted	None predicted	Domain none predicted Protein EXORDIUM-like family
184	A0A1B6Q0N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G005800	4.65	9.091	275	30.02	+	Cellular response to stimulus	None predicted	Anchored component of plasma membrane	Domain none predicted DREPP family
185	C5XCL8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G070200	4.49	13.81	210	20.94	+	None predicted	None predicted	None predicted	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain Family none predicted
186	A0A1Z5RAM1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G188100	4.43	7.107	605	65.41	+	Protein phosphorylation	Protein kinase activity	Integral component of membrane	Protein kinase domain Family none predicted
187	A0A1W0VX32	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G127100	4.41	11.63	344	35.93	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
188	C5YC92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G018100	4.29	10.48	229	24.98	+	None predicted	Nutrient reservoir activity	Extracellular region	Cupin 1 domain Germin family
189	C5XG67	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G400400	4.25	14.19	155	16.18	+	Negative regulation of peptidase activity	Protease binding	None predicted	Cystatin domain and family
190	C5XCI9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G068000	4.19	6.714	700	73.04	+	None predicted	Catalytic activity	Integral component of membrane	Glucose/Sorbone dehydrogenase domain Family none predicted
191	C5X8J1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G301700	4.18	5.882	510	54.34	+	None predicted	None predicted	None predicted	Amidase signature domain Family none predicted
192	C5XIY9	Dihydrolipoyl dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3003G152900	4.17	6.746	504	52.96	+	Oxidation-reduction process	Dihydrolipoyl dehydrogenase activity	Cell	FAD/NAD(P)-binding domain Dihydrolipoyl dehydrogenase family
193	C5X9Y2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G314800	4.15	5.109	548	60.50	+	Oxidation-reduction process	Oxidoreductase activity	Plant-type cell wall	Multicopper oxidase, type 3 domain Family none predicted
194	A0A1Z5RDM9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132100	4.11	19.4	232	24.56	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase family, 19, catalytic domain Glycoside hydrolase family, 19

195	C5YYA0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G145500	4.08	12.54	319	34.53	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
196	C5XN41	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G330300	4.05	16.85	184	20.50	-	None predicted	Calcium ion binding	None predicted	EF-hand domain Family none predicted
197	C5WY51	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G063500	4.05	7.806	474	50.76	+	None predicted	None predicted	None predicted	FAS1 domain Family none predicted
198	C5YH35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G177500	4.04	12.26	318	33.42	+	None predicted	None predicted	None predicted	Domain none predicted Thaumatin family
199	A0A1B6QGB6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416800	4.03	10.25	322	33.61	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
200	C5WT90	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G173300	4.03	6.044	364	41.42	+	Plant-type cell wall biogenesis	Intramolecular transferase activity	Cytosol	Domain none predicted Reversibly glycosylated polypeptide family
201	C5Z1Z7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G270800	4.03	7.513	386	40.47	+	None predicted	Hydrolase activity, acting on ester bonds	None predicted	GDSL lipase/esterase domain Family none predicted
202	C5Y9W4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G108400	4.02	9.579	261	29.65	-	None predicted	Protein domain specific binding	None predicted	14-3-3 protein domain and family
203	C5Z0N8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055100	4.01	12.32	357	37.89	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
204	A0A1B6PTZ2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G217900	4.01	7.539	451	50.12	-	None predicted	None predicted	None predicted	Neprosin domain Family none predicted
205	C5YM54	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G151300	4.01	10.55	218	22.73	+	None predicted	Nutrient reservoir activity	Extracellular region	Cupin 1 domain Germin family
206	C5WNS8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G123100	4	6.173	567	62.84	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Integral component of membrane	Domain none predicted Glycoside hydrolase family 1
207	A0A194YN27	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G064900	4	3.474	662	71.54	-	None predicted	None predicted	None predicted	Uncharacterised conserved protein UCP015417, vWA domain and family
208	C5YBF1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132700	4	13.24	272	28.77	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase, family 19, catalytic domain Glycoside hydrolase family 19
209	C5WN09	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G113800	4	3.115	963	102.26	+	Protein phosphorylation	Protein kinase activity	Integral component of membrane	Protein kinase domain Family none predicted
211	C5Z861	Uncharacterized protein OS=*Sorghum bicolor*	4	8.993	278	26.92	+	Electron transport chain	Electron transfer activity	Anchored component of	Phytocyanin domain and family

		GN=SORBI_3010G231900								plasma membrane	
212	C5XFH6	Fructose-bisphosphate aldolase OS=*Sorghum bicolor* GN=SORBI_3003G393900	4	8.169	355	38.56	-	Glycolytic process	Catalytic activity	None predicted	Domain none predicted Fructose-bisphosphate aldolase, class-I
213	C5Z8S3	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G245400	4	9.687	320	34.26	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
214	C5YU74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G068000	4	19.08	152	16.35	-	None predicted	None predicted	None predicted	Domain and family none predicted
215	C5XAQ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G326100	4	8.989	445	45.87	+	Proteolysis	Hydrolase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family
216	C5X972|	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3002G306900	4	15.33	150	16.92	-	Phosphorylation	Kinase activity	Intracellular	Nucleoside diphosphate kinase-like domain Nucleoside diphosphate kinase family
217	A0A1Z5R4X8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G048100	4	8.108	222	25.13	+	None predicted	None predicted	None predicted	Alginate lyase 2 domain Family none predicted
218	A0A1W0VZJ3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G294800	4	11.46	253	26.62	-	Electron transport chain	Electron transfer activity	Aleurone grain membrane	Phytocyanin domain and family
219	A0A1B6Q242	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085300	4	10.1	99	10.58	+	Negative regulation of peptidase activity	Peptidase inhibitor activity	Extracellular region	Proteinase inhibitor I12, Bowman-Birk domain Family none predicted
220	A0A1B6PHX9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G150800	4	7.595	316	33.29	-	None predicted	None predicted	None predicted	Domain and family none predicted
221	A0A1B6QD45	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G237000	3.93	15.57	334	33.67	-	None predicted	None predicted	None predicted	FAS 1 domain Fasciclin-like arabinogalactan protein family
222	A0A1B6QQP5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G515500	3.93	6.571	350	37.09	+	None predicted	protein self-association	None predicted	LysM domain Family none predicted
223	C5Z8E7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G110800	3.9	17.12	146	15.12	+	Electron transport chain	Electron transfer activity	Anchored component of plasma membrane	Phytocyanin domain and family
224	C5XIY2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152200	3.89	13.21	371	39.02	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
225	C5XGM0	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G126800	3.85	11.67	240	26.48	+	Negative regulation of peptidase activity	Peptidase inhibitor activity	None predicted	Cystatin domain and family
226	C5XL53	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3003G024000	3.82	3.618	608	66.06	+	None predicted	None predicted	None predicted	Domain none predicted Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A

227	C5YBP8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G009000	3.69	4.624	519	55.73	-	None predicted	None predicted	None predicted	VWA-Hint protein, Vwaint domain Family none predicted
229	A0A1W0W0W5	Pectin acetylesterase OS=*Sorghum bicolor* GN=SORBI_3003G384700	3.62	5	420	46.06	-	Cell wall organization	Hydrolase activity	Extracellular region	Domain none predicted Pectinacetylesterase/NOTUM family
230	C5XQ07	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G072300	3.58	8.197	305	32.58	-	Metabolic process	Catalytic activity	Cytosol	Domain none predicted Triosephosphate isomerase family
231	A0A194YL19	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G236500	3.57	14.47	152	15.41	+	None predicted	None predicted	None predicted	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain Family none predicted
232	A0A1W0W1H5	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3003G440900	3.53	5.23	631	71.52	-	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	Glycoside hydrolase family 32, N-terminal domain Glycoside hydrolase, family 32
233	C5YBA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G004300	3.53	12.15	214	22.83	+	Negative regulation of catalytic activity	Enzyme inhibitor activity	None predicted	Pectinesterase inhibitor protein domain Family none predicted
234	C5XQP2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G078400	3.52	8.055	509	52.20	+	Proteolysis	Peptidase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family
235	C5XLV5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G030700	3.51	7.66	470	51.47	-	Metabolic process	Catalytic activity	Integral component of membrane	Domain none predicted Type I phosphodiesterase/nucleotide pyrophosphatase/phosphate transferase
236	C5WRH5	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3001G295200	3.44	16.78	149	16.57	-	Nucleoside diphosphate phosphorylation	Nucleoside diphosphate kinase activity	Intracellular	Nucleoside diphosphate kinase-like domain Nucleoside diphosphate kinase family
237	C5Z4N3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G168200	3.42	9.701	268	28.86	+	Dephosphorylation	Acid phosphatase activity	None predicted	Domain none predicted Acid phosphatase, class B-like family
238	C5X750	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G003800	3.39	9.278	194	19.25	+	Electron transport chain	Electron transfer activity	Integral component of membrane	Phytocyanin domain and family
239	C5WWQ2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G342600	3.38	3.938	584	64.78	-	Cellular oxidant detoxification	Thioredoxin-disulfide reductase activity	Cytoplasm	Thioredoxin domain Family none predicted
240	C5WVM2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G330500	3.32	14.18	282	29.63	+	None predicted	None predicted	None predicted	Leucine-rich repeat-containing N-terminal, plant type domain Family none predicted
241	C5Z471	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G161800	3.29	19.45	329	34.32	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
242	C5X733	Uncharacterized protein	3.29	3.838	469	49.46	+	None predicted	None predicted	None predicted	Domain and family none predicted

		OS=*Sorghum bicolor* GN=SORBI_3002G002100									
243	C5Y7Z1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G215900	3.27	16.07	112	11.64	+	Negative regulation of endopeptidase activity	serine-type endopeptidase inhibitor activity	Extracellular region	Proteinase inhibitor I12, Bowman-Birk domain Family none predicted
244	A0A1Z5RC44	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G213300	3.24	2.712	885	96.35	-	Lipid metabolic process	Phosphoric diester hydrolase activity	Integral component of membrane	Glycerophosphodiester phosphodiesterase domain Family none predicted
245	A0A1W0W1V5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G016400	3.21	5.244	553	59.43	+	Oxidation-reduction activity	Oxidoreductase activity	Plant-type cell wall	Ascorbate oxidase homologue, first cupredoxin domain Family none predicted
246	A0A1Z5S8P9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324700	3.09	12.2	205	21.90	-	Negative regulation of peptidase activity	Peptidase inhibitor activity	Integral component of membrane	Cystatin domain and family
247	A0A1Z5RDF6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G119700	3.01	9.231	260	28.80	+	Lipid metabolic process	Phosphoric diester hydrolase activity	Anchored component of plasma membrane	Phosphatidylinositol-specific phospholipase C, X domain Family none predicted
248	C5YY92	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G144600	2.97	13.35	322	34.55	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
249	C5WSG0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301600	2.95	13.83	282	30.85	+	Sexual reproduction	None predicted	Extracellular region	Expansin cellulose-like binding domain Expansin/Lol pI family
250	C5X578	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G128000	2.95	8.178	269	28.66	+	None predicted	None predicted	None predicted	Sialate O-acetylesterase domain Family none predicted
252	C5XQD5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G214900	2.84	4.783	690	70.96	+	Protein phosphorylation	Protein kinase activity	Integral component of membrane	Protein kinase domain Family none predicted
253	A0A1Z5R5E6	Non-specific lipid-transfer protein OS=*Sorghum bicolor* GN=SORBI_3008G030900	2.82	19.33	119	11.54	+	Lipid transport	Lipid binding	None predicted	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain Plant lipid transfer protein/Par allergen family
254	C5YBE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132300	2.79	9.774	266	28.28	+	Carbohydrate metabolic process	Chitinase activity	None predicted	Glycoside hydrolase family 19, catalytic domain Glycoside hydrolase, family 19
255	C5Y4W3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G160700	2.79	8.225	231	23.52	+	Negative regulation of catalytic activity	Enzyme inhibitor activity	None predicted	Pectinesterase inhibitor protein domain Family none predicted
256	A0A1B6PJ93	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G227300	2.77	27.22	180	17.44	+	Electron transport chain	Electron transfer activity	None predicted	Phytocyanin domain and family
257	C5XKY2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G308300	2.76	6.371	518	57.34	+	Proteolysis	Serine-type peptidase activity	Vacuole	Domain none predicted Peptidase S28 family
258	C5YBF3	Uncharacterized protein OS=*Sorghum bicolor*	2.68	12.66	379	40.44	+	None predicted	None predicted	None predicted	Domain of unknown function DUF642

		GN=SORBI_3006G132900									Family none predicted
259	A0A1B6Q6G6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G314800	2.64	6.126	506	53.49	+	Proteolysis	Peptidase activity	None predicted	Peptidase family A1 domain Aspartic peptidase A1 family
260	A0A1B6PEC9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G178800	2.61	3.525	766	84.41	-	Cellular amino acid biosynthetic process	Zinc ion binding	None predicted	Cobalamin-independent methionine synthase MetE, N-terminal domain Cobalamin-independent methionine synthase family
261	A0A1W0W287	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050100	2.58	5.725	262	26.60	-	None predicted	None predicted	None predicted	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain Family none predicted
262	C5YYF4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G009800	2.54	39.73	73	7.97	-	Response to wounding	Serine-type endopeptidase inhibitor activity	None predicted	Domain none predicted Proteinase inhibitor I13, potato inhibitor I
263	C5YHR8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3007G192300	2.52	5.655	336	36.48	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
264	A0A1W0W2G8	Thioredoxin OS=*Sorghum bicolor* GN=SORBI_3002G057900	2.51	16.95	118	12.90	+	Oxidation-reduction process	Protein disulfide oxidoreductase activity	Cell	Thioredoxin domain and family
266	C5WP98	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G414000	2.37	3.093	873	93.45	+	Protein phosphorylation	Protein kinase activity	Integral component of membrane	Protein kinase domain Family none predicted
268	C5X4M5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255000	2.37	7.547	265	25.95	+	None predicted	None predicted	None predicted	DOMON domain Family none predicted
269	C5XKG0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G016800	2.36	3.968	378	40.27	+	None predicted	Hydrolase activity, acting on ester bonds	None predicted	GDSL lipase/esterase domain Family none predicted
272	C5YZJ2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G033400	2.31	7.101	338	36.06	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
273	C5WPH2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G130400	2.28	14.2	169	17.49	+	None predicted	None predicted	None predicted	Domain none predicted Protein of unknown function DUF538
275	C5XP10	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3003G345700	2.26	7.066	467	51.91	+	Proteolysis involved in cellular protein catabolic process	Peptidase activity	Vacuole	Domain none predicted Peptidase S10, serine carboxypeptidase
276	A0A1Z5S3X9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G020500	2.25	3.286	852	91.36	+	Protein phosphorylation	Protein kinase activity	Integral component of membrane	Protein kinase domain S-receptor-like serine/threonine-protein kinase family
279	A0A1B6QEM0	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3002G324100	2.17	7.524	319	34.80	+	Carbohydrate metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	Cell wall	Xyloglucan endo-transglucosylase C-terminal domain Xyloglucan endotransglucosylase/hydrolase family
281	C5WQD8	Uncharacterized protein	2.16	24.11	224	22.77	+	None predicted	None predicted	None predicted	Domain none predicted

		OS=*Sorghum bicolor* GN=SORBI_3001G145600									Thaumatin family
284	A0A194YMM6	Glyceraldehyde-3-phosphate dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3010G262500	2.11	15.43	337	36.53	-	Oxidation-reduction process	Oxidoreductase activity	None predicted	Glyceraldehyde 3-phosphate dehydrogenase, catalytic domain Glyceraldehyde/Erythrose phosphate dehydrogenase family
286	C5Y1M2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G097400	2.09	10.4	375	40.57	+	Catabolic process	Hydrolase activity	None predicted	Alpha/beta hydrolase fold-3 domain Family none predicted
287	C5XEB7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G226000	2.09	6.436	404	41.27	+	Carbohydrate metabolic process	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Domain none predicted Glycoside hydrolase, family 28
288	C5Y981	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G094000	2.09	8.017	237	25.01	-	Negative regulation of catalytic activity	Enzyme inhibitor activity	None predicted	Pectinesterase inhibitor domain Pla a 1-like family
299	A0A1Z5S810	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3001G267600	2.02	6.789	383	38.67	+	None predicted	None predicted	None predicted	Domain none predicted Thaumatin family
305	A0A1Z5S985	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G353501	2.01	16.00	175	16.61	+	None predicted	None predicted	None predicted	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain Family none predicted
318	A0A194YGY2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G027000	2	26.46	446	48,05	-	Glycolytic process	Phosphopyruvate hydratase activity	Phosphopyruvate hydratase complex	Enolase, N-terminal domain Enolase-like superfamily
321	C5WN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G119100	2	27.91	129	13,54	+	Electron transport chain	Electron transport activity	Anchored component of plasma membrane	Phytocyanin domain Phytocyanin family
322	C5X1U2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G390300	2	32.21	149	16,83	-	None predicted	Calcium ion binding	None predicted	EF-hand domain Family none predicted
323	A0A1Z5RIM0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G145201	2	9.39	266	29.34	-	None predicted	Protein domain specific binding	None predicted	14-3-3 domain 14-3-3 protein family
324	A0A1B6QN96	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3001G371900	2	16.45	152	15,09	-	Oxidation-reduction process	Oxidoreductase activity	None predicted	Superoxide dismutase, copper/zinc binding domain Superoxide dismutase (Cu/Zn) / superoxide dismutase copper chaperone family
329	A0A1Z5RGB0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3006G243600	2	7.48	321	34,22	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
330	C5YI12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G065900	2	6.19	226	24,65	+	None predicted	Nutrient reservoir activity	Cell wall	Cupin 1 domain Germin family
364	C5XL87	Uncharacterized protein OS=*Sorghum bicolor*	2	6.897	261	26,46	+	Electron transport chain	Electron transport activity	Anchored component of	Phytocyanin domain Phytocyanin family

		GN=SORBI_3003G027200								plasma membrane	
365	C5X385	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G387100	2	8.738	206	21,33	+	None predicted	Hydrolase activity, hydrolyzing O-glycosyl compounds	Anchored component of plasma membrane	X8 domain Family none predicted
373	A0A194YKY7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G225300	1.85	58.24	273	30,52	-	None predicted	None predicted	None predicted	Ubiquitin domain and family
374	A0A194YMI7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G036800	1.83	5.112	489	53,363	+	Metabolic process	Hydrolase activity, hydrolyzing O-glycosyl compounds	None predicted	X8 domain Glycoside hydrolase family 17
384	C5XMN8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G042200	1.3	3.627	579	61,73	+	None predicted	Protein binding	None predicted	Domain and family none predicted
391	C5X3C2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391400	1.1	12.88	365	39,130	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
395	C5WRN4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G444400	0.99	4.819	332	34,86	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
398	C5X216	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G090400	0.91	9.619	499	54,10	-	Carbohydrate metabolic process	hydrolase activity, acting on glycosyl bonds	Extracellular region	Domain none predicted Glycoside hydrolase, family 28
400	A0A1W0W5X4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G258300	0.86	8.136	381	40,61	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
403	C5Y359	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011200	0.75	43.82	340	35,67	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
423	C5YT19	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G191400	0.44	4.076	736	79,04	-	None predicted	Catalytic activity	Integral component of membrane	Glucose/Sorbosone dehydrogenase domain Family none predicted
429	C5XYY9	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3004G105800	0.35	7.788	321	34,21	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
433	A0A194YNU6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3004G106100	0.31	10.12	326	34,11	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
442	C5WSF9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301500	0.24	11.35	282	30,89	+	Sexual reproduction	None predicted	Extracellular region	Expansin, cellulose-binding-like domain Expansin/Lol pI family
456	C5Z8S2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G245300	0.16	5.363	317	33,85	+	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain Plant peroxidase family
478	C5XHS1	Uncharacterized protein	0.07	3.858	337	35,69	+	Carbohydrate	Hydrolase	Anchored	Domain none predicted

	OS=*Sorghum bicolor* GN=SORBI_3003G422000	metabolic process	activity, hydrolyzing O-glycosyl compounds	component of plasma membrane	Glycoside hydrolase family 17	

[a] Protein number assigned in ProteinPilot software.

[b] Protein accession numbers obtained from the UniProt database searches against sequences of *Sorghum bicolor* only.

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

[d] Percentage coverage as determined by the number of amino acids of sequenced peptides against the total length of the protein with a threshold of at least 95% confidence interval.

[e] Sequence peptide is the number of peptide sequences that were sequenced and yielded protein identity.

[f] Theoretical molecular weight (kDa) as obtained from UniProt (http://www.uniprot.org).

[g] Signal peptide predicted from UniProt database (http://www.uniprot.org). The positive sign (+) indicates the presence of the signal peptide and the negative sign (-) indicates the absence of signal peptide.

[h] Gene ontology analysis as predicted by the UniProt database (https://www.ebi.ac.uk/QuickGO/annotations?gene). P is the Biological Process, F is the Molecular Function and C is the Cellular Component.

[I] Conserved domains and family name as predicted by InterPro database (https://www.ebi.ac.uk/interpro/protein/).

3.8 Gene Ontology Analysis of the Identified CF Proteins

The gene ontology (GO), comprising of biological process (P), molecular function (F) and cellular component (C) was analysed for the identified ICSB 338 CF proteins (290) using information available on the UniProt and InterPro databases. The results are summarised in Table 3.1 and further illustrated in Figures 3.7-3.9. About half of the 290 proteins, most of which were uncharacterised, did not have predicted cellular functions. The other half comprised of proteins with predicted cellular components. These included proteins located outside the cell in the extracellular region (25%), with the majority being secretory peroxidases (Table 3.1). Approximately 13% of the proteins were part of the membrane, that is as anchored components of the plasma membrane (6%), integral component of the membrane (6%) and 1% had unspecified membrane location (Figure 3.7). A total of 5% of the proteins were predicted to occupy the intracellular part of the cell, and these include the cytoplasm, cytosol, peroxisome, lysosome, nucleus, vacuole and endoplasmic reticulum. About 4% of the proteins were predicted to be located in the cell wall (Figure 3.7).

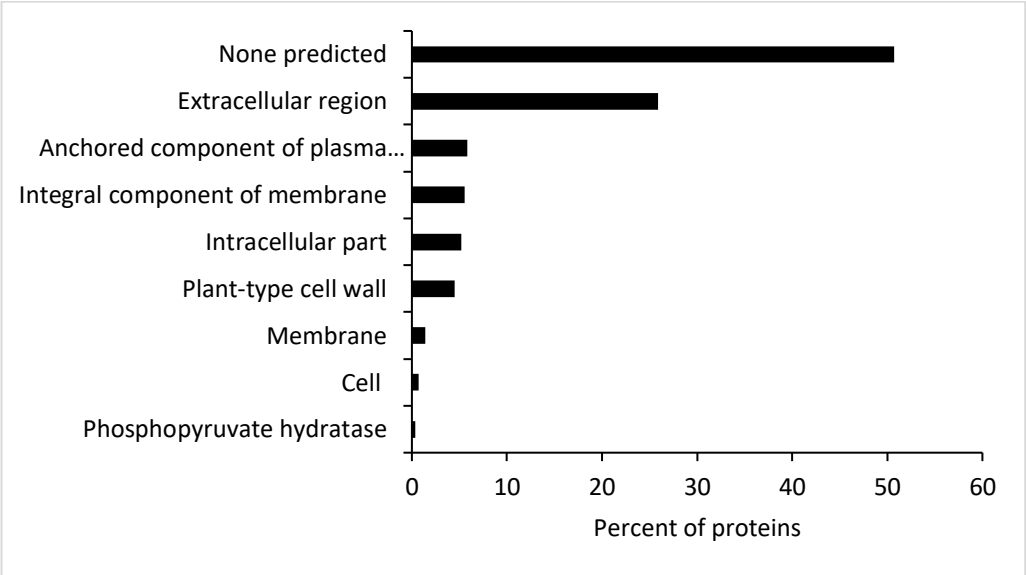


Figure 3.7: The cellular component predictions of ICSB 338 identified secreted proteins.

Unlike the cellular component, 75% of the identified secreted proteins had predicted biological processes, and only 25% did not (Figure 3.8). Among those with predicted biological processes, the majority (17%) were involved in carbohydrate metabolic processes and activities. This group was however dominated by uncharacterised proteins. About 16% of the proteins were involved in response to oxidative stress, proteolysis (9%), metabolic process (6%), electron transport chain (4%) and oxidation-reduction process (4%) (Figure 3.8). Only a small percentage of the proteins was involved in plant-type cell wall biogenesis, immune response, superoxide metabolic response, lipid transport and cellular oxidant detoxification among other processes.

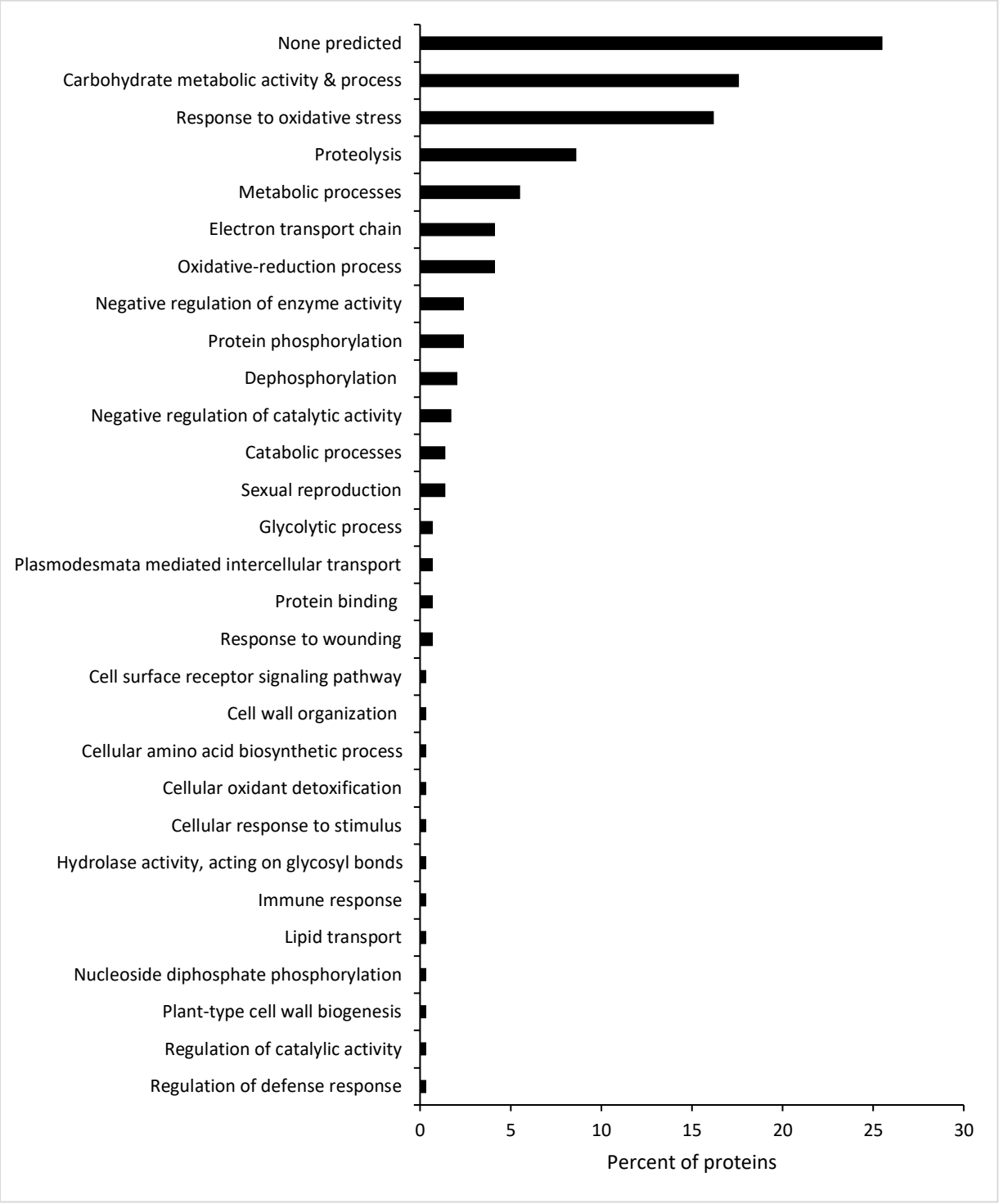


Figure 3.8: The biological process predictions of ICSB 338 identified secreted proteins.

Regarding the molecular functions, most of the identified ICSB 338 secreted proteins were involved in peroxidase activity (16%), O-hydrolyzing glycosyl compounds (10%), catalytic (7%), hydrolase (7%), chitinase (4%) and binding activities (4%). About 18% of the 290 proteins did not have any predicted molecular functions (Figure 3.9). Most of these were uncharacterised proteins.

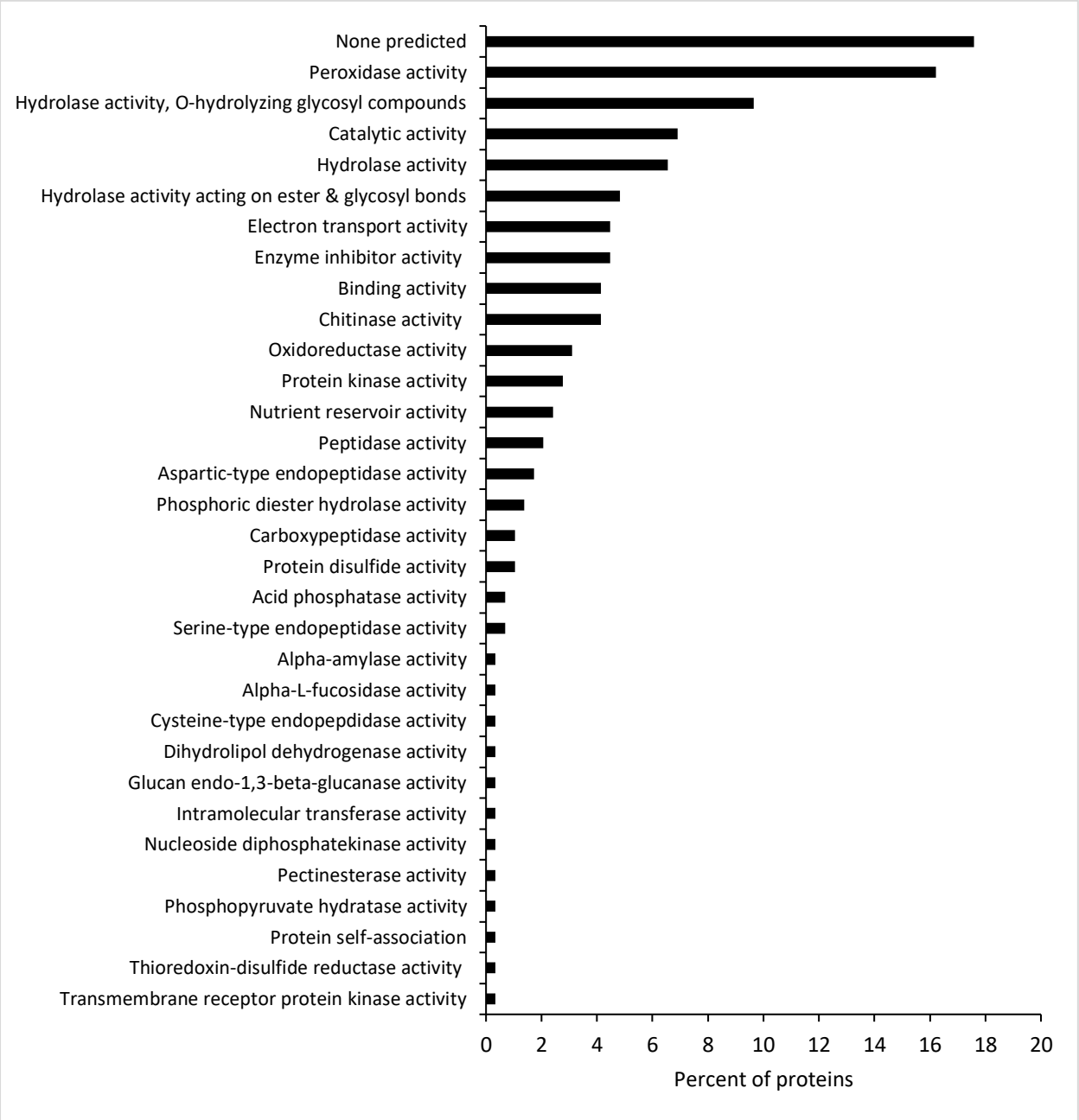


Figure 3.9: The molecular function predictions of ICSB 338 identified secreted proteins.

3.9 Conserved Domains and Family Names

The conserved domains and family names of all 290 proteins identified from the ICSB 338 cell suspension cultures were determined from the InterPro database (https://www.ebi.ac.uk/interpro/protein) (Table 3.1). Both the conserved domains and family names assist in assigning putative functions of the uncharacterised proteins. About 30% of the 290 identified proteins did not have predicted family names and most were uncharacterised. Proteins with no predicted conserved domains accounted for a total of 19% and these were also mainly dominated by uncharacterised proteins. Forty seven peroxidase proteins were identified. Amongst these, 46 possessed the secretory peroxidase domain and all 47 belonged to the plant peroxidase family. Following the plant peroxidase family, was a large group of various glycoside hydrolase families 1, 9, 17, 19, 28, 29, 31, 32, 35 and 79 (Table 3.1). Seven uncharacterised proteins had a FAS-1 domain, while four other proteins had a purple acid phosphatase N terminal domain (Table 3.1).

3.10 Discussion

In this study, an ICSB 338 sorghum cell suspension culture line was established (Figure 3.1B) and used to understand the molecular responses of sorghum towards high-temperature stresses of 35 and 40°C. Preliminary cell viability assays between the control and heat stressed samples showed that the sorghum cells could maintain viability under the heat stress conditions for 72 hours when compared to Arabidopsis (Figure 3.2-3.3). These results suggest that sorghum is more heat tolerant than Arabidopsis. Therefore, sorghum still retains its thermotolerance at cell culture level.

One dimensional gel electrophoresis of the sorghum secreted proteins was conducted. As illustrated in Figure 3.4, not much difference in the protein expression was observed between the control and samples heat stressed at 35°C (Figure 3.4A). However, when the temperature was increased to 40°C, the differences in protein expression pattern were more elaborate as indicated by the red arrows in Figure 3.4B. This result showed that there were some changes in gene and thus protein expression, and subsequently protein secretion into the ECM.

Proline and glycine betaine are compatible osmolytes, which play important roles in protecting plants from stress through cellular osmotic adjustment, ROS detoxification and maintenance of membrane integrity (Bohnert & Jensen, 1996). The HILIC LC-MS analysis of the proline and glycine betaine contents of ICSB 338 sorghum cells under control and 40°C heat stress treatment was conducted. A gradual decrease in proline content with an increase in the duration of heat stress treatment from 0, 48 and 72 hours was observed (Figure 3.5). On the other hand, glycine betaine was not detected in ICSB 338 cells under both the control and heat stress conditions (results not shown). Although sorghum is known to produce both proline and glycine betaine under stress conditions (Ashraf & Foolad, 2007), it is unclear why glycine betaine was not detected in the ICSB 338 cell samples. However, the

observed decrease in proline and lack of glycine betaine content in sorghum cell suspension cultures could possibly imply that the biosynthetic pathways of these osmolytes were not activated by the heat stress imposed (Kishor *et al*., 2005). In contrast to the observed results in the current study, proline content was increased in chickpea after exposure to high temperatures of 30, 35 and 45°C for 24 hours (Kaushal *et al*., 2011). Based on the combination of cell viability, osmolyte content and 1D gel profiles, a heat stress treatment regime at 40°C for 72 hours was selected for subsequent proteomic and gene expression workflows in Chapter 4.

The expression of HSP70 was observed in both the sorghum and Arabidopsis cell suspension cultures, following heat stress treatment. In addition, HSP70 was already expressed in sorghum cell cultures prior to exposure to heat stress, including lanes 5 and 6 in Figure 3.6. However, the expression levels increased with an increase in temperature. HSP70 family proteins are involved in folding of denatured proteins, prevention of protein aggregation and translocation of proteins across membranes, thus maintaining protein homeostasis (Hartl *et al*., 2011). These results may suggest that the expression of HSP70 contributes to the survival of sorghum under high temperatures conditions.

A proteomic mapping of the secreted proteins on ICSB 338 cell suspension cultures was performed using MUDPIT that was part of an iTRAQ experiment. A total of 290 proteins were positively identified in the CF fractions of this cell line. This protein identification supersedes the total of 14 (Ngara & Ndimba, 2014) and 179 (Ramulifho, 2017; Ngara *et al*., 2018) positively identified secreted proteins in the White sorghum cell suspension culture line. Considering the importance of secreted proteins in signalling, response to biotic and abiotic stresses, and the important role of sorghum as a model system (Ngara & Ndimba, 2014), this study will lay foundation for other sorghum secretome studies.

In the current study, most of the leaderless proteins were uncharacterised. However, the proteins are involved in a wide range of molecular functions including catalytic, oxidoreductase and enzyme inhibitor activities. Moreover, they are also involved in glycolytic, carbohydrate metabolic and oxidation-reduction processes. The majority of these proteins did not have predicted cellular components. However, some were part of the integral component of the membrane. The theoretical molecular weights (MW) of the identified CF proteins ranged between 11 and 114 kDa. Unlike the limited molecular weight range of 25 and 100 kDa which was observed in the 14 secreted proteins reported by Ngara & Ndimba (2011) using 2D-PAGE, this study presents a more diverse range of proteins, a result that is consistent with non-gel based proteomics approaches such as MUDPIT. Such gel-free technologies have much wider protein coverage as they circumvent the physical limitations of gel electrophoresis and protein solubility (Jafari *et al*., 2012).

Of the 290 identified ICSB 338 cell suspension culture secreted proteins, a large proportion of 201 proteins (69%) were uncharacterised, suggesting that their functional roles were yet to be determined. The remaining 31% comprised of peroxidases, alpha-mannosidases, purple acid phosphatases, cysteine proteinase inhibitors, carboxypeptidases, superoxide dismutase, dirigent proteins, glyceraldehyde-3-phosphate dehydrogenases, nucleoside diphosphate kinases, and thioredoxins (Table 3.1). Without a doubt an increased focus on sorghum proteomics and the subsequent functional characterisation of the proteins will continue to contribute towards the full annotation of these gene products.

Among the 31% of the characterised proteins, the largest percentage were peroxidase proteins, which accounted for 16% of the total proteins identified. These proteins form part of the extracellular region and are involved in peroxidase activity. Furthermore, their involvement in response to oxidative stress has been predicted (Table 3.1). The presence of

peroxidase proteins in cell cultures was not a surprise as these contain signal peptides, which means that they were secreted into the ECM through classical pathways. Previous secretome studies in chickpea (Gupta *et al*., 2015) and sorghum (Ngara & Ndimba, 2011; Ngara *et al*., 2018) have also identified a larger number of peroxidases in the CF fraction of the cell suspension cultures.

As shown in Table 3.1, most of the proteins identified (80%) had predicted signal peptides. The presence of N-terminal signal peptides in the CF proteins signifies that these proteins were destined for the ECM as they were secreted via classical secretory pathways (Gupta *et al*., 2011). The remaining 20% were leaderless proteins. According to Jung *et al.* (2008), the secretion of such proteins could have been due to the presence of ER/Golgi independent secretory pathway that is still poorly known. Leaderless proteins have also been identified in other secretome studies including those of rice (Jung *et al*., 2008), chickpea (Gupta *et al*., 2011) and sorghum (Ramulifho, 2017; Ngara *et al*., 2018). Overall, a large number of ICSB 338 sorghum CF proteins with putative functions in metabolism, disease/defence and protein destination and storage were identified in this study. The responses to heat stress were further analysed in Chapter 4.

CHAPTER 4

PROTEOMIC AND GENE EXPRESSION ANALYSIS IN ICSB 338 SORGHUM CELL SUSPENSION CULTURES IN RESPONSE TO HEAT STRESS

4.1 Introduction

The surface temperatures have been increasing worldwide mainly due to global warming. This increase is also coupled with a decrease in the annual rainfall in sub-tropical regions (IPCC, 2014). A combination of heat and water stresses in turn negatively affects plant growth and development. Unfortunately, plants are sessile organisms and thus cannot escape the rising temperatures in their environments (Gupta *et al*., 2015; Wang *et al*., 2018). Therefore, in order to survive, plants respond to high temperature stress through a complex network of morphological, physiological, biochemical and molecular mechanisms (Howarth & Ougham, 1993). For example, plants may activate gene expression changes, which results in the synthesis of heat shock proteins and antioxidant enzymes as protective mechanisms against damage (Zandalinas *et al*., 2018).

Apart from intracellular proteins, secreted proteins also play a role in stress responses. For example, secreted proteins are involved in a wide range of processes such as the maintenance of cell structure, biogenesis, regulation of the external environment and also signalling and defence mechanisms against abiotic stresses (Jung *et al*., 2008; Gupta *et al*., 2011), including heat. The identification and characterisation of heat stress responsive proteins in stress adaptation is thus important in the development of effective strategies to enhance thermotolerance (Wang *et al*., 2018) in crops.

Proteomics and transcriptomics studies have identified putative proteins and transcripts that are involved in plant responses to a range of abiotic stresses. For instance, a Bet v1-like protein, CaRRP1 identified in the secretome of chickpea (*Cicer arientinum*) plays an important role in dehydration stress response (Gupta *et al*., 2015). In a recent secretome study, Ngara and co-workers identified differentially expressed proteins in the extracellular matrix (ECM) of White sorghum cell suspension cultures in response to sorbitol-induced osmotic stress (Ngara *et al*., 2018). The identified osmotic-stress responsive proteins were functionally grouped into glycosyl-hydrolases/glycosidases, cell wall modifying enzymes, proteases and redox proteins. Furthermore, gene expression patterns of a few targets from the proteome results were also analysed on two sorghum varieties with distinct phenotypic responses to drought. The results revealed a differential expression pattern of six of these genes in leaf and root tissues of the two sorghum varieties (Ngara *et al*., 2018). Therefore, it is clear that secreted proteins play an important role in stress adaptive responses in plants and this knowledge could be applied in breeding programs for the development of stress tolerant crops.

In the current study, isobaric tags for relative and absolute quantitation (iTRAQ) and mass spectrometry were used to identify sorghum secreted proteins in response to heat stress. The objective of this chapter was to identify and functionally characterise the heat stress responsive proteins in the ECM fraction of ICSB 338 sorghum cell suspension cultures and validate a few target genes using quantitative real time-polymerase chain reaction (qRT-PCR) analysis.

4.2 iTRAQ Analysis of ICSB 338 Sorghum Cell Suspension Culture Secreted Proteins in Response to Heat Stress

ICSB 338 cell suspension cultures were maintained in culture as described in Section 2.2.2. Eight days after sub-culturing, the cell cultures were exposed to a heat stress treatment at 40°C for 72 hours. Control cultures were maintained at 27°C for the duration of the experiment. For each treatment group, four biological replicates were used. The culture filtrate (CF) proteins were harvested after 72 hours of heat stress treatment for protein extraction (Section 2.5.2), quantification (Section 2.6) and 1D gel electrophoresis (Section 2.7). The CF protein samples were acetone precipitated, labelled with iTRAQ reagents, digested with trypsin, fractionated and identified by LC MS/MS (Section 2.12).

A total of 290 secreted proteins were positively identified in the ECM fractions of ICSB 338 sorghum cell suspension cultures (Table 3.1). All the 290 secreted proteins were analysed according to their fold-changes using a MapMan software 3.5.1R2. Every line in the heatmap represents a group of proteins that have been expressed in response to heat stress. As shown in Figure 4.1 below, red signifies the up-regulated proteins, with the highest fold-change of 3, while green shows the down-regulated proteins in response to heat stress. All proteins with a fold-change of zero, indicated by black, showed no change in their expression post exposure to heat stress treatment. Due to a large number of proteins identified in the ECM fractions of ICSB 338 cell cultures, protein names could not be included on Figure 4.1.

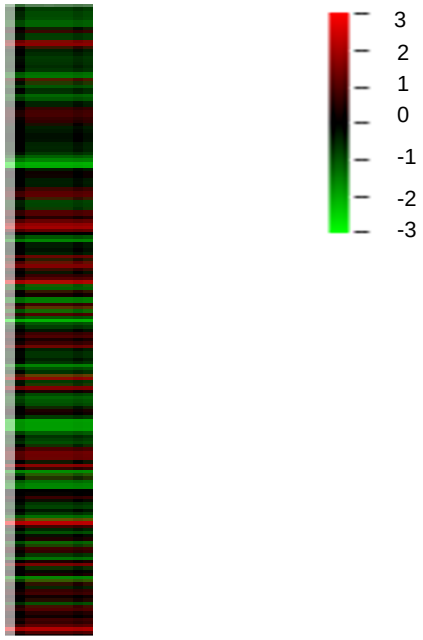


Figure 4.1: A computationally derived heatmap showing the expression patterns of ICSB 338 sorghum proteins according to fold-changes. Each line indicates a group of proteins expressed in response to a 40°C heat stress treatment. The colours represent the expression patterns of proteins. Green signifies down-regulation, black indicates no change in the level of expression, and red represents up-regulation of proteins.

Out of the 290 identified proteins, 105 were differentially expressed in response to the heat stress. Sixty five (62%) of the heat stress responsive proteins were up-regulated while 40 (38%) were down-regulated. The heat responsive proteins were functionally characterised into different functional groups according to Bevan *et al*. (1998) and other literature sources. The uncharacterised proteins were functionally grouped using their respective conserved domains and/or family names. The stress responsive proteins, their functional groupings and fold-changes are shown in Table 4.1.

Table 4.1: List of heat stress responsive secreted proteins from ECM fractions of ICSB 338 cell suspension cultures.

Prot No.[a]	Accession[b]	Protein name[c]	Ratio[d]	SD[e]	p-value[f]
Metabolism					
2	C5Y397	Alpha-mannosidase OS=*Sorghum bicolor* GN=SORBI_3005G132400	1.30	0.07	7.65E-03
11	A0A1B6QCB0	Alpha-amylase OS=*Sorghum bicolor* GN=SORBI_3002G190500	2.84	0.22	4.89E-05
12	A0A1B6QI05	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089100	1.84	0.08	3.10E-04
16	C5WXC7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	-1.29	0.06	1.88E-03
18	C5XB38	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055600	1.73	0.18	2.70E-03
30	C5XIK1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G432700	1.83	0.16	2.39E-04
36	C5XB39	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055700	2.41	0.20	3.80E-04
43	C5YBE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132400	1.86	0.18	4.21E-04
53	C5YQU5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G146700	1.60	0.16	3.66E-03
63	C5YYX0	Beta-hexosaminidase OS=*Sorghum bicolor* GN=SORBI_3009G017500	1.39	0.12	1.53E-02
71	C5X4N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255600	1.46	0.10	2.92E-03
78	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	-1.41	0.06	3.46E-03
80	C5WU08	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G025400	1.37	0.13	3.15E-03
92	C5XD22	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G356800	-2.32	0.05	2.54E-03
93	C5X1L1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G247900	1.18	0.08	4.17E-02
94	C5XX83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G208700	1.30	0.05	3.91E-04
106	A0A1W0VY92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205900	-2.31	0.08	4.37E-04
113	A0A1B6Q537	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G244600	2.02	0.47	5.86E-03
125	C5WSY5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G014700	1.25	0.08	1.61E-02
127	C5Z8T4	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246600	1.23	0.10	1.30E-03
135	C5Z4E5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044900	1.20	0.04	1.30E-03
136	C5XQW7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G087300	-1.38	0.01	5.38E-03
144	C5XRC3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G374100	1.18	0.08	1.68E-02
148	A0A1Z5R3E0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G130100	1.17	0.02	5.75E-03
157	C5Y5U9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177500	1.70	0.18	1.41E-03
163	A0A1B6PNV2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G255600	1.66	0.32	7.21E-03
201	C5Z1Z7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G270800	-1.91	0.11	2.79E-03
217	A0A1Z5R4X8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G048100	1.17	0.08	8.77E-03
233	C5YBA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G004300	1.59	0.27	6.92E-03
250	C5X578	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G128000	2.31	0.33	1.32E-02

279	A0A1B6QEM0	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3002G324100	-2.50	0.10	2.61E-02
365	C5X385	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G387100	1.38	0.20	1.34E-02
284	A0A194YMM6	Glyceraldehyde-3-phosphate dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3010G262500	-1.39	0.12	1.06E-02
Disease/Defence					
7	A0A1W0W7T8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416600	-1.97	0.04	3.81E-04
15	C5X5K6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416700	-2.17	0.02	1.34E-02
17	A0A1B6PKE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G056300	-1.23	0.10	3.88E-02
24	C5Z475	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G162000	-1.40	0.05	3.51E-02
39	C5Z469	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G161600	-1.28	0.08	1.76E-02
44	C5XCE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G351400	1.43	0.09	2.43E-02
45	C5Z483	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G162900	1.18	0.09	1.51E-02
50	C5X040	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G080300	2.06	0.27	5.18E-04
57	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G129700	-1.15	0.05	6.04E-03
58	C5YVR0	Superoxide dismutase OS=*Sorghum bicolor* GN=SORBI_3009G093200	2.35	0.38	8.83E-04
60	C5XHF1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G136200	2.08	0.24	2.38E-04
64	C5Z0N9	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055300	1.67	0.06	3.90E-04
69	A0A1W0W7I8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391900	1.34	0.07	8.26E-03
70	C5XYY5	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3004G105100	-1.17	0.04	1.68E-02
76	C5XIY1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152100	1.38	0.13	2.15E-02
81	C5YB25	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3006G277600	1.44	0.12	3.94E-03
82	A0A1B6QFT1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G392000	-1.39	0.08	2.41E-02
108	C5XL59	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G024700	-3.23	0.07	2.70E-05
121	C5YQ75	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3008G010500	-1.39	0.03	1.15E-02
126	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	1.71	0.27	4.92E-03
137	C5XHX2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G427700	2.85	0.43	4.85E-04
149	C6JSB7	Peroxidase OS=*Sorghum bicolor* GN=Sb0246s002010	-1.39	0.06	3.94E-02
154	C5XJT8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G156400	2.17	0.29	4.45E-04
166	C5YD83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G031200	2.09	0.45	7.25E-03
192	C5XIY9	Dihydrolipoyl dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3003G152900	1.61	0.22	9.55E-03
195	C5YYA0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G145500	-1.62	0.19	2.92E-02
198	C5YH35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G177500	1.31	0.17	3.34E-02
203	C5Z0N8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055100	1.44	0.18	9.41E-03
213	C5Z8S3	Peroxidase OS=*Sorghum bicolor*	-1.78	0.17	1.41E-02

		GN=SORBI_3010G245400			
245	A0A1W0W1V5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G016400	-1.24	0.08	2.54E-02
263	C5YHR8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3007G192300	1.33	0.21	3.90E-02
330	C5YI12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G065900	1.32	0.17	1.74E-02
Protein destination and storage					
10	A0A1B6PLA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G104300	1.17	0.02	5.75E-03
47	C5XHP9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419500	2.18	0.13	5.94E-05
49	C5XHP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419300	2.74	0.20	3.77E-05
51	C5Y675	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G064200	1.31	0.11	1.67E-02
55	A0A1Z5S979	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3001G348900	-1.52	0.06	1.32E-02
56	A0A1B6QJE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G170700	1.19	0.04	1.30E-03
65	C5Z6U2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G210000	1.80	0.18	1.06E-03
74	C5WVG9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324800	2.19	0.17	8.33E-05
109	C5WXN2	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3001G348800	-1.57	0.13	4.74E-02
117	C5XDR4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G217200	-1.28	0.07	1.75E-02
122	C5XAQ6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G326000	-1.32	0.12	1.40E-02
140	A0A1B6QEG2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G315800	1.18	0.09	1.51E-02
150	A0A1B6PQR2	Protein disulfide-isomerase OS=*Sorghum bicolor* GN=SORBI_3005G074400	1.61	0.21	4.12E-03
153	C5Z1X3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G268400	-2.10	0.03	5.84E-03
174	C5YCI1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G154300	1.51	0.36	4.93E-02
177	C5YNA1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G172100	1.45	0.19	1.15E-02
182	C5X5T3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G130700	1.44	0.25	2.17E-02
215	C5XAQ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G326100	-2.05	0.08	4.50E-02
219	A0A1B6Q242	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085300	-2.00	0.03	5.30E-03
225	C5XGM0	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G126800	2.16	0.42	9.39E-03
259	A0A1B6Q6G6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G314800	-2.00	0.11	2.50E-02
373	A0A194YKY7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G225300	1.49	0.29	2.32E-02
Unclear classification					
4	C5Z484	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G163000	1.44	0.17	4.89E-03
25	C5WXD7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G209300	1.46	0.22	9.96E-03
34	A0A1B6PLT5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G133000	-2.83	0.05	5.29E-04
35	A0A1B6QEI0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G317600	1.17	0.08	8.77E-03
85	C5Y2P0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G122300	1.34	0.11	6.59E-03
214	C5YU74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G068000	1.73	0.21	5.89E-03

261	A0A1W0W287	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050100	-1.52	0.10	6.43E-03
268	C5X4M5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255000	-6.40	0.10	8.97E-03
273	C5WPH2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G130400	2.34	0.35	6.69E-04
Signal transduction					
26	A0A1Z5R915	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3007G091100	-4.18	0.04	2.42E-04
33	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600	-2.55	0.06	3.77E-03
73	C5Y7T1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G086000	1.98	0.17	6.05E-04
86	C5Z6U1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G209900	1.67	0.08	4.41E-04
88	A0A1W0W2E3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050900	1.85	0.17	4.04E-04
101	C5Y2R8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G126200	2.48	0.37	6.26E-04
Energy					
141	C5WN51	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G119000	1.48	0.10	1.17E-03
211	C5Z861	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G231900	1.79	0.27	9.40E-03
238	C5X750	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G003800	-1.95	0.14	4.06E-02

[a]Protein number assigned in ProteinPilot software.

[b]Accession number obtained from TrEMBL database found in the UniProt database (http://www.uniprot.org) searches against sequences of *Sorghum bicolor* only.

[c]Protein names and functional categories according to Bevan *et al*. (1998) and other literature sources.

[d]Ratio represents the average fold-change (*n=4*). Negative values indicate down regulation.

[e]Standard deviation of the ratios of heat stressed samples (*n=4*).

[f]p-value is the probability value of the quantitative difference between the treatment and the control abundance.

4.3 Functional Categories of Differentially Expressed Secreted Proteins

The 105 differentially expressed proteins were distributed into a total of 6 functional groups as illustrated in Table 4.1 and Figure 4.2. Of the 105 differentially expressed sorghum secreted proteins, the largest number was involved in metabolism (31%), followed by disease/defence (30%) and protein destination and storage (21%). The remaining proteins were part of unclear classification (9%), signal transduction (6%) and energy (3%) categories. A brief description of proteins within each functional group is given below.

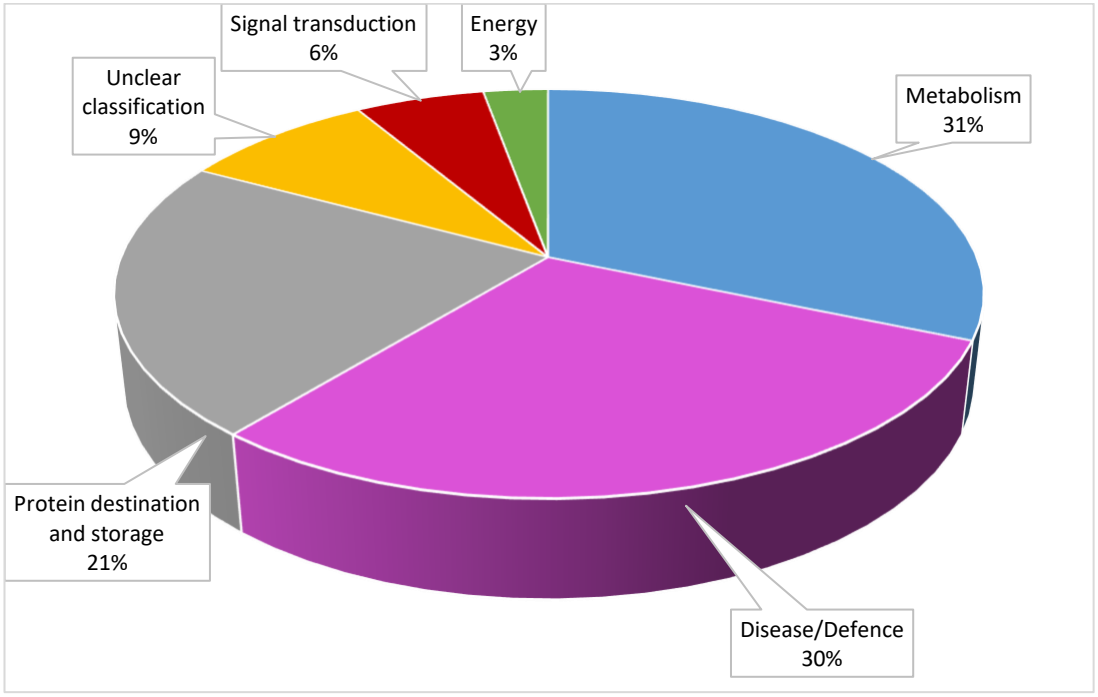


Figure 4.2: Functional categories of the ICSB 338 sorghum heat stress responsive secreted proteins.

4.3.1 Metabolism

A total of 33 (31%) of the 105 heat stress responsive secreted proteins were involved in metabolism (Figure 4.2). Most of these proteins (26) were up regulated while only 6 were down-regulated in response to heat stress treatment. The down-regulated proteins included xyloglucan endotransglucosylase/hydrolase with a fold-change magnitude of -2.50, alpha galactosidase (-1.29), endoglucanase (-1.41) and 3 uncharacterised proteins (protein numbers 92, 106 and 201) with -2.32, -2.32 and -1.91 fold-changes, respectively. Nineteen proteins that were involved in carbohydrate metabolism and 14 were uncharacterised. The characterised proteins in this functional category included alpha mannosidase (protein no. 2), alpha amylase (protein number 11), beta-hexoaminidase (protein number 63), endoglucanase (protein number 78) and xyloglucan endotransglucosylase/hydrolase (protein number 279).

The abundance of all 14 uncharacterised proteins in this functional category was increased in response to heat stress. The molecular functions of these uncharacterised proteins as predicted by GO analysis included catalytic, chitinase and enzyme inhibitor activities, the hydrolysis of O-glycosyl compounds and hydrolase activity acting on ester bonds (Table 3.1). A large number of the uncharacterised proteins belonged to glycoside hydrolase families 17 (protein numbers 53, 71 and 125) 18 (protein numbers 12, 18, 36, 43, 93, 113 and 157), 19 (protein number 94), 32 (protein number 163) and 35 (protein number 144). Only four uncharacterised proteins in this functional category (protein numbers 92, 106, 135 and 201) belonged to the GDSL lipase/esterase domain (Table 3.1). Glycoside hydrolase proteins belonging to a wide range of families were also identified in another secretome study where White sorghum cell suspension cultures were subjected to sorbitol-induced osmotic stress (Ngara *et al*., 2018).

4.3.2 Disease/defence

The disease/defence functional category had the second largest number of heat stress responsive secreted proteins with a total of 32 (30%) proteins (Table 4.1; Figure 4.2). Eighteen of these proteins were up-regulated, while 14 were down-regulated. This functional category mostly consisted of 18 peroxidases (56%) and 12 uncharacterised proteins (38%). The remaining two proteins were superoxide dismutase and dihydrolipoyl dehydrogenase (protein numbers 58 and 192), both of which were up-regulated in response to heat stress. Although some proteins were uncharacterised, according to the GO analysis results in Table 3.1, these proteins were involved in oxidation-reduction processes, protein binding, catalytic and nutrient reservoir activities. Three of the uncharacterised proteins (protein numbers 44, 126 and 198), did not have any predicted functions (Table 3.1), however, they were classified under the thaumatin family. All the thaumatin family members were up-regulated in response to heat stress. Furthermore, four uncharacterised proteins (protein numbers 57, 60, 137 and

330) involved in the nutrient reservoir activity, were members of the germin family and cupin domain. Germin-like proteins have been previously identified in a soybean (*Glycine max*) leaf tissue in response to independent stress treatments of 8% (w/v) PEG 6000 induced drought, 200 mM NaCl-induced-salinity and 200 mM abscisic acid for 0, 2, 6, 12, 24 and 48 hours (Li *et al*., 2015). The genes encoding for germin-like proteins were overexpressed upon stress treatments and this resulted in increased stress tolerance in soybean (Li *et al*., 2015).

Of the 18 peroxidases in this functional category, a total of 7 (protein numbers 50, 64, 69, 76, 81, 203 and 263) were up-regulated, while 11 (protein numbers 7, 15, 24, 39, 70, 82, 108, 121, 149, 195 and 213) were down-regulated. Contrary to these findings, all peroxidases were up-regulated in White sorghum cell suspension cultures (Ramulifho, 2017), and in soybean root tissue (Oh & Komatsu, 2015) in response to sorbitol-induced osmotic and drought stresses, respectively.

4.3.3 Protein Destination and Storage

A total of 22 (21%) of the differentially expressed proteins were involved in the protein destination and storage functional category. Of these proteins, 14 were up-regulated, while 8 were down-regulated. Seventeen proteins found in this category were uncharacterised. However, based on the GO analysis, the majority of the proteins (protein numbers 10, 47, 49, 59, 122, 153, 177, 182, 215 and 259) were involved in proteolysis (Table 3.1). Proteolytic enzymes were previously identified in cell suspension cultures of soybean in response to oxidative stress (Solomon, 1999). Two of the uncharacterised proteins (protein numbers 65 and 373) without any predicted functions belonged to the ubiquitin domain and family. A ubiquitin-like protein was also identified in a secretome study of Arabidopsis in response to pathogen infection (Oh *et al*., 2005). Out of the 22 proteins classified in the protein destination and storage functional category, only five had known functions. These included

protein disulphide isomerase (protein number 150), carboxypeptidases (protein numbers 55 and 109) and cysteine proteinase inhibitors (protein numbers 74 and 225). Both the protein disulphide-isomerase and cysteine proteinase inhibitors were up-regulated, while the carboxypeptidases were down-regulated.

4.3.4 Signal Transduction

The signal transduction functional category consisted of 6 (6%) proteins. Of these, five were uncharacterised and a purple acid phosphatase (protein number 26) was the only characterised protein in this category. All the uncharacterised proteins (protein numbers 33, 73, 86, 88, 101) were up-regulated, except for one (protein number 33), which was down-regulated by -2.58 fold (Table 4.1). Three of the uncharacterised proteins (protein numbers 33, 88 and 101) were classified as members of the leucine-rich repeat-containing N-terminal, plant type domain. According to the GO analysis, protein numbers 73 and 86 were involved in hydrolase and catalytic activities, respectively. A significant decrease in the expression of purple acid phosphatase was observed, with a fold-change of -4.18. Proteins involved in signal transduction were also identified in a recent study which investigated the protein responses in radish (*Raphanus sativus* L., 2n = 2x = 18) taproots towards high temperature stress (Wang *et al*., 2018). Three week-old radish seedlings were treated with 40°C for 0, 2, 6, 12, 24 and 48 hours. The 12 and 24 hour treatments were used for protein identification and gene expression analysis.

4.3.5 Other Functional Groups

This category consists of proteins with unclear classification and those involved in energy-related functions. Nine of the differentially expressed proteins constituting 9% of the 105 responsive proteins did not have any predicted gene ontology functions and/or lacked either conserved domains or family names were classified under the unclear classification category

(Table 4.1). Five of these proteins (protein numbers 25, 35, 85, 214 and 273) were up-regulated, while two (protein numbers 34 and 268) were down-regulated. Protein number 268 was down-regulated by a very large fold-change of -6.40. Three proteins (protein numbers 141, 211 and 238) were functionally classified under the energy category and all were uncharacterised. Protein numbers 141 and 211 were up-regulated, while protein no. 238 was down-regulated. The cellular roles played by these secreted proteins in response to heat stress are still unclear.

4.4 Gene Expression Analysis of the Heat Stress Responsive Targets

4.4.1 Total RNA Extraction from ICSB 338 Cell Suspension Cultures

ICSB 338 sorghum cell suspension cultures were exposed to a heat stress treatment of 40°C for 72 hours. The control cells were grown at 30°C. Four biological replicate cultures were prepared for each treatment group. The cell culture samples were harvested at 0, 24, 48 and 72 hours post heat stress treatment for total RNA extraction. The extracted RNA was quantified and electrophoresed on a 1.2% (w/v) agarose gel for quality checks. Figure 4.3 below shows that the total RNA extraction was successful for all biological replicates in both the control and heat stressed samples. The RNA samples were subsequently used for cDNA synthesis (Section 2.17) prior to gene expression analysis.

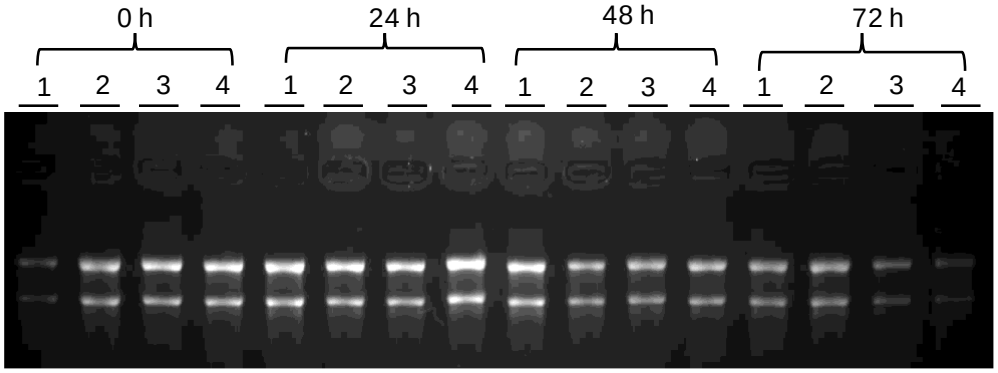


Figure 4.3: Total RNA extracts of ICSB 338 sorghum cell suspension cultures following heat stress. Cell suspension cultures were grown at 30°C. On the 8th day post sub-culture, the cells were heat stressed at 40°C for 24, 48 and 72 hours. For each treatment time point, four independent biological replicates were prepared. A total of 300 ng of RNA was loaded on a 1.2% (w/v) agarose gel.

4.5 Checking the Specificity of the Sorghum Primer Pairs

Ten ICSB 338 secreted proteins with the highest magnitude of fold-change in response to heat stress were selected for gene expression using qRT-PCR (Section 2.14: Table 2.1). The protein numbers are 36, 47, 49, 58, 74, 101, 127, 137, 225 and 250 (Table 4.1). Primers were subsequently designed for each respective target gene and two heat shock protein (HSP) marker genes using the Primer-Blast tool on the National Centre for Biotechnology Information (NCBI) database with specified parameters (Section 2.13.4). To test for the specificity of the designed primer sets, cDNA was synthesised (Section 2.13.3) from the RNA and used as template in conventional polymerase chain reactions (PCR). The resultant PCR products were electrophoresed on a 3.5% (v/v) agarose gel. Figure 4.4 below shows the desired target PCR amplicon sizes of each of the 10 primer sets. These primer sets were subsequently used for gene expression analysis using qRT-PCR.

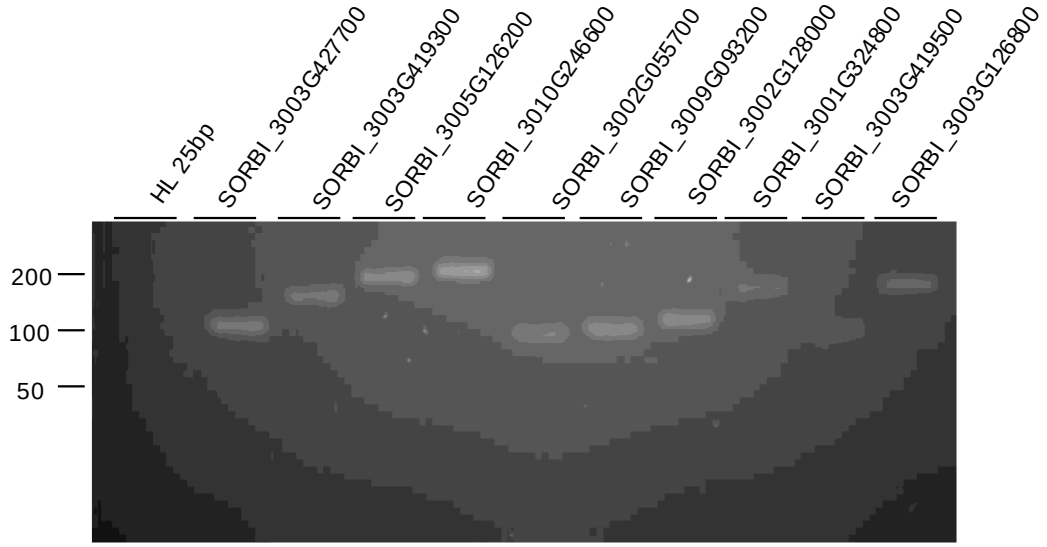


Figure 4.4: A 3.5% (w/v) agarose gel electrophoresis of PCR amplicons. cDNA was synthesised from total RNA samples and used as template for conventional PCR analysis using the 10 designed sorghum primer sets. Lane (HL 25bp) is the DNA Ladder.

4.6 Heat-Stress Induced Gene Expression in Sorghum Cell Suspension Cultures

The up- and down-regulation of the secreted proteins observed in response to heat stress (Table 4.1), could have possibly been due to a change in the expression of genes encoding for these proteins (Ngara *et al.*, 2018). For this reason, the expression of the 10 heat stress responsive proteins was validated using qRT-PCR. Furthermore, the expression patterns of two heat shock protein marker genes, *HSP70* (Sb03G039360) and *HSP90* (Sb07g028270) were also evaluated. For this analysis, two genes *Sb910* (Sb03g038910) and *EIF4A* (Sb04g003390), were used as reference control genes. Four biological replicates cDNA templates were used for each treatment group, with each qRT-PCR reaction having three technical replicates.

4.6.1 HSP70 and HSP90 Gene Expression Analysis under Heat Stress

The expression of two heat shock marker genes, *HSP70* and *HSP90* was assessed in the control and 40°C heat stressed samples over 72 hours using the REST2009 software version 2.0.13. The results are shown in Figure 4.5. There was a statistically significant increase in the expression of both HSP marker genes between the control and heat stressed samples at 24, 48 and 72 hours. However, the *HSP70* gene showed a much greater increase in expression between 0 and 24 hours, with a fold-change of six. The maximum fold-change attained in *HSP70* following 72 hours of heat stress was approximately nine, while that of *HSP90* was 3.5 in comparison with their respective controls.

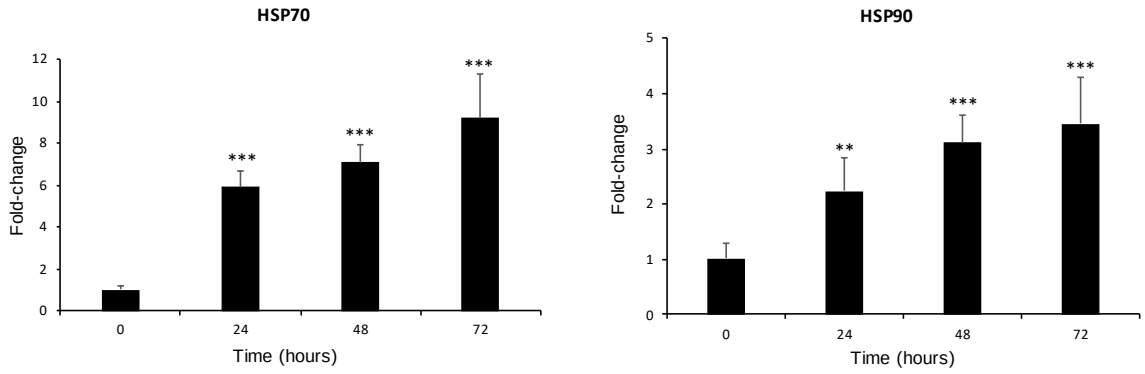


Figure 4.5: Gene expression analysis of the heat shock marker genes in response to heat stress treatment. ICSB 338 sorghum cell suspension cultures were exposed to a 40°C heat stress treatment and the control cell cultures were maintained at 30°C. The cells were harvested at 24, 48 and 72 hours post treatment. Gene expression of *HSP70* and *HSP90* was analysed using qRT-PCR and REST2009 software. Error bars represent means ± SD ($n = 4$). One, two and three asterisks above the error bars indicate statistical significant differences between the control and heat stress treatment means at each time point, $p \leq 0.05$, 0.01 and 0.001, respectively.

4.6.2 Gene Expression Analysis of the Target Sorghum Genes in Response to Heat Stress

Figure 4.6 shows the expression of the 10 target sorghum genes in response to heat stress. From the results, two general trends in gene expression were observed. Firstly, four genes (*SORBI_3003G419500, SORBI_3003G419300, SORBI_3002G128000* and *SORBI_3009G093200*) showed a gradual decrease in expression during the 72 hours of heat stress. Of these, genes *SORBI_3003G419500* and *SORBI_3003G419300* significantly decreased across all time points in response to heat stress. Although no significant difference in the expression levels of *SORBI_3009G093200* was observed between the control and the 24 hour time point, there was a significant difference at 48 and 72 hours of heat stress exposure.

Secondly, six genes (*SORBI_3001G324800, SORBI_3005G126200, SORBI_3003G427700, SORBI_3002G055700, SORBI_3000G246600* and *SORBI_300G126800*) showed an increase in gene expression during the heat stress treatment period. Of these genes, *SORBI_3005G126200, SORBI_3003G427700* and *SORBI_3002G055700* were rapidly up-regulated within the first 24 hours of heat stress. On the other hand, the expression levels of gene *SORBI_3000G246600* increased between 0 and 48 hours, and then decreased to the initial expression level at 72 hours. The change in expression at 72 hours is insignificant relative that at 48 hours.

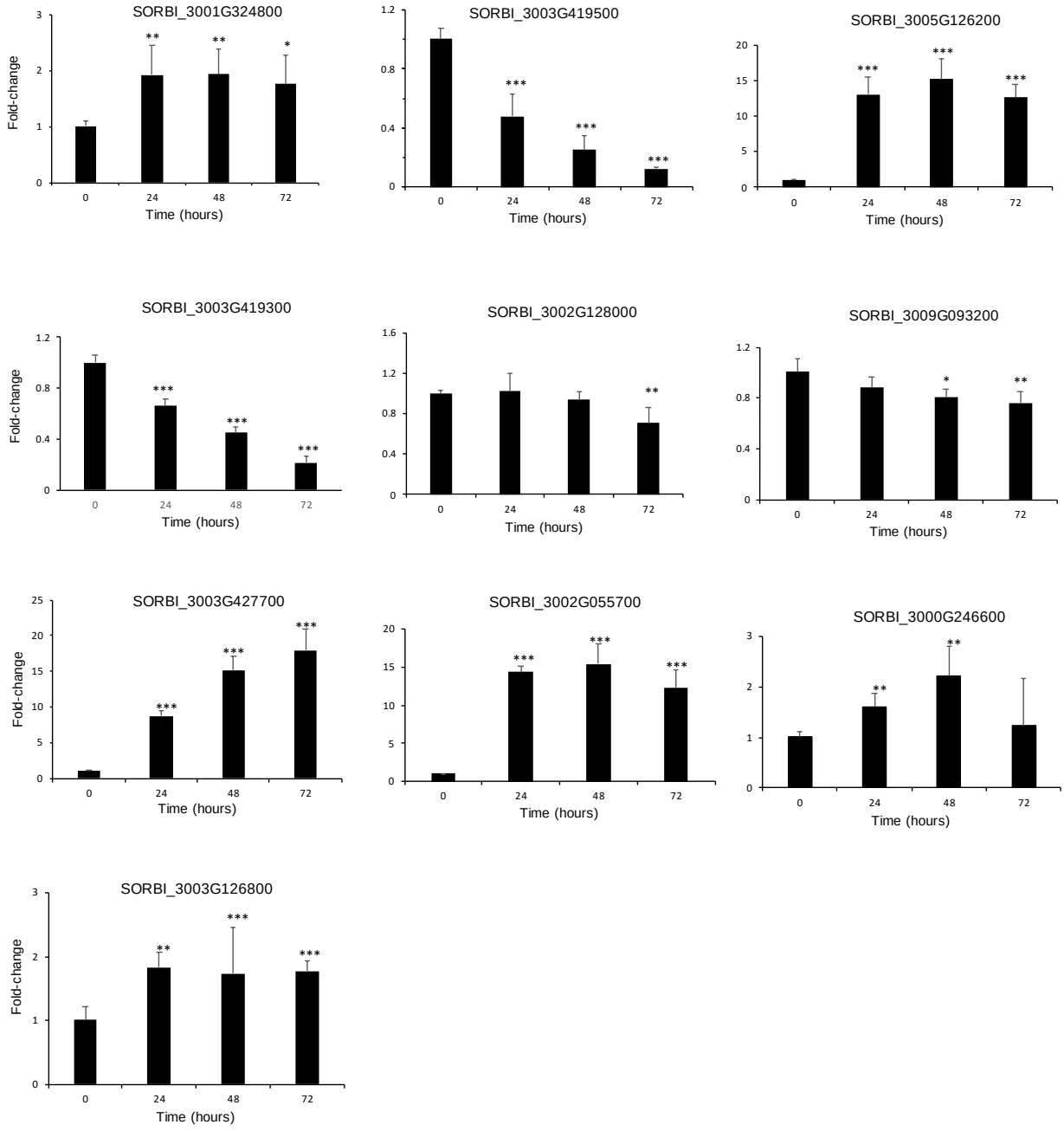


Figure 4.6: Gene expression analysis of ten sorghum genes in response to heat stress treatment. ICSB 338 sorghum control cell cultures were maintained at 30°C control. Cell cultures were exposed to a 40°C heat stress treatment and then harvested at 0, 24, 48 and 72 hours post treatment. Gene expression was analysed using qRT-PCR and REST2009 software. Error bars represent means ± SD (*n = 4*). One, two and three asterisks above the error bars indicate statistical significant differences between the control and heat stress treatment means at each time point, $p \leq 0.05$, 0.01 and 0.001, respectively.

4.6.3 Comparative Gene Expression Analysis of the Sorghum Genes under Low and High Temperature Stresses

In tropical regions, sorghum grows well under high temperatures over 30°C. Under laboratory conditions, sorghum cell suspension cultures are maintained under control temperature conditions ranging between 27-30°C. Now the question is, are the heat stress responsive genes already expressed at these temperatures where sorghum is naturally grown? To answer this question, a comparative gene expression analysis was conducted on ICSB 338 sorghum cell suspension cultures grown at low and higher temperatures of 23°C and 30°C, respectively. The analysis was conducted on the two HSP marker genes and the 10 heat stress responsive sorghum target genes. Genes *Sb910* and *EIF4A* were also used as constitutive reference control genes. The qRT-PCR data analysis was carried out using the 23°C low temperature stressed samples as the base line for gene expression. The results are shown in Figure 4.7.

The results reveal an up-regulation of eight of the ten genes at 30°C. These genes include *SORBI_3001G324800, SORBI_3003G419500, SORBI_3003G419300, SORBI_3009G093200, SORBI_3003G427700, SORBI_3002G055700, SORBI_3000G24600* and *SORBI_3003G126800*. Of particular note, genes *SORBI_3003G419500, SORBI_3003G427700, SORBI_3000G246600, SORBI_3003G126800* and *SORBI_3002G055700* showed a significant increase in expression levels with fold-changes greater than 3 (Figure 4.7). However, the expression levels of *SORBI_3005G126200, SORBI_3002G128000,* and the two HSP markers did not change between the two temperatures (Figure 4.7). Overall, the observed protein and gene expression changes following heat stress treatment shows that sorghum is responsive towards increased temperatures. The up-regulation and down-regulation of secreted proteins upon the

perception of heat stress is as a result of a change in the expression of the genes that encode these proteins.

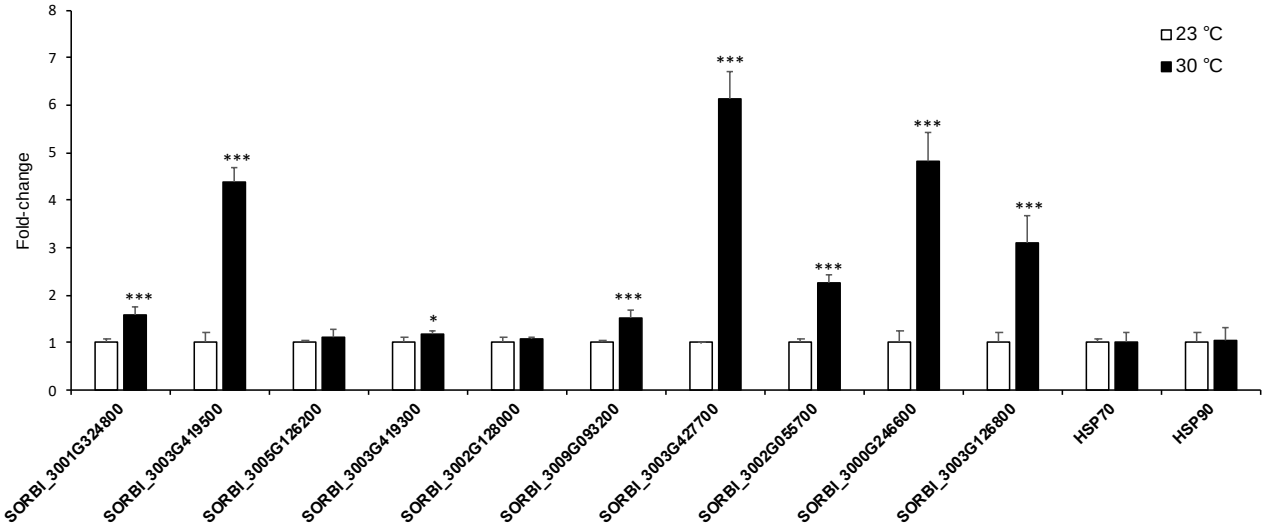


Figure 4.7: Comparative gene expression analysis in sorghum. ICSB 338 cell suspension cultures were exposed to 23 and 30°C growth temperatures for 72 hours before harvesting. Gene expression was analysed using qRT-PCR and REST2009 software. Error bars represent means ± SD *(n = 4)*. One, two and three asterisks above the error bars indicate statistical significant differences between the control and heat stress treatment means at each time point, $p \leq 0.05$, 0.01 and 0.001, respectively.

4.7 Discussion

Heat stress causes secondary effects such as osmotic and oxidative stresses (Wang *et al*., 2004). Plants respond to the effects of heat stress through a complex network of mechanisms that involve morphological, physiological, biochemical and molecular responses in order to restore cellular homeostasis (Howarth & Ougham, 1993; Bita & Gerats, 2013). Amongst the molecular responses, gene expression may be altered which may subsequently lead to changes in protein expression patterns. However, the magnitude of gene and/or protein expressional changes may differ between plant species, genotypes and the duration and intensity of stress (Szymańska *et al*., 2017). Ultimately, these responses lead to variation in the plant tolerance towards a stress factor. Since sorghum naturally survives under hot and dry conditions (Taylor, 2003), it is a good model system to study response mechanisms to a range of abiotic stresses, including heat.

In this study, ICSB 338 sorghum cell suspension cultures were used to investigate the expression patterns of secreted proteins in response to heat stress. Cell suspension cultures are particularly used in such experiments because they can be easily handled and controlled when compared to whole plants (Agrawal *et al*., 2010). Furthermore, with the cell suspension culture system, secretory proteins are produced in large amounts (Ngara *et al*., 2018) and can be easily extracted from the culture filtrate (Agrawal *et al*., 2010).

The ICSB 338 sorghum cell suspension cultures were exposed to a heat stress treatment at 40°C for 72 hours. The CF proteins, also called secreted proteins, were extracted from the culture medium and differentially expressed proteins were identified using iTRAQ and mass spectrometry. Of the 290 identified proteins, 105 were differentially expressed in response to heat stress (Table 4.1). Of these proteins, 62% were up-regulated, while 38% were down-regulated. The up and down-regulation of secreted proteins show that the imposed heat stress

on the sorghum cell suspension cultures induced a change in the secretion of proteins into the ECM. Similar differential protein changes were also observed in White sorghum culture in response to osmotic stress (Ramulifho, 2017; Ngara *et al*., 2018).

The identified ICSB 338 heat stress responsive secreted proteins were classified into different functional categories according to Bevan *et al.* (1998) and other literature sources. The largest percentage of these proteins was involved in metabolism (31%), followed by disease/defence (30%), protein destination and storage (21%), unclear classification (7%) and signal transduction (6%). Similarly, the largest number of proteins secreted in response to sorbitol-induced osmotic stress were involved in metabolism in another secretome study using White sorghum cell suspension cultures (Ramulifho, 2017).

Most proteins under the metabolism functional category were uncharacterised, and this made it difficult to understand their putative roles in heat stress response. To understand their functions, the domain and family names of the respective secreted proteins were bioinformatically predicted (Table 3.1). The uncharacterised proteins under metabolism functional category was mainly composed of proteins belonging to the glycoside hydrolase family 17, 18, 19, 32 and 35, catalytic domain. Glycoside hydrolases are a group of enzymes that catalyse the glycolytic cleavage of two or more carbohydrates or between a carbohydrate and a non-carbohydrate residue (Naumoff, 2011). Increased abundance of glycoside hydrolases was also observed in different tissues of rice including root, leaf and shoot in response to heat stress at 42°C (Sharma *et al.*, 2013). These proteins are involved in the mediation of structural changes in cell walls. This activity helps plants to survive under stressful conditions by regulating plant growth, providing a physical barrier or releasing products that trigger defence signalling (Sharma *et al*., 2013).

According to the GO analysis in Table 3.1, some of the uncharacterised proteins classified under metabolism were members of the GDSL lipase/esterase family. GDSL lipase/esterase are hydrolytic enzymes which possess a conserved catalytic triad residues; serine, histidine and aspartic acid, at the active site (Akoh *et al*., 2004; Oh *et al*., 2005). GDSL lipases have been identified in various plant species including Arabidopsis, rice and maize where they function in plant development, morphogenesis and defence response (Brick *et al*., 1995). In addition, a secretome study on the response of Arabidopsis to fungal pathogen infection (*Alternaria brassicicola*) was conducted by Oh *et al.* (2005). Their results revealed that the expression of GDSL lipase was strongly induced in Arabidopsis cell suspension cultures in response to the pathogen (Oh *et al*., 2005).

Proteins involved in the disease/defence category are important in defending plants against the negative effects of stress. For example, heat stress like many other abiotic stress factors causes oxidative stress. Oxidative stress is defined as an imbalance between the production and detoxification of ROS (Ozougwu, 2016). Plants have antioxidant systems to counteract the negative effects of oxidative stress (Omari & Nhiri, 2015). Enzymes such as superoxide dismutase, ascorbate peroxidase and catalase act simultaneously to detoxify the reactive oxygen species produced during oxidative stress (Omari & Nhiri, 2015). In the current study, peroxidases (7, 15, 24, 39, 50, 64, 69, 70, 76, 81, 82, 108, 121, 149, 195, 203, 213 and 263) were dominant in the disease/defence functional category (Table 4.1). Peroxidases are involved in the scavenging of ROS that accumulate during stress. At low levels, ROS are involved in signalling events in plants. However, when the production of ROS increases due to stress, protein denaturation, lipid peroxidation and DNA and RNA damage occur, ultimately leading to cell death (Bita & Gerats, 2013; Mittler & Blumwald, 2015). It would thus be expected that an increase in the accumulation of peroxidases would be observed so as to prevent oxidative damage caused by heat stress. On the contrary, however, a decrease in

the accumulation of most of the peroxidase proteins (61%) was observed in this study. Similar trends were also observed in an Arabidopsis secretome in response to phosphate deficiency (Tran & Plaxton, 2008). It is however, unclear why the expression levels of these peroxidases decreased in response to heat stress.

A germin protein (protein number 330) was up-regulated in response to heat stress within the disease/defence functional category. Germin proteins play important roles in signalling during the onset of growth in seed germination (Patnaik & Khurana, 2001). These glycosylated proteins possess a remarkable quality of being stable in extreme environmental conditions such as temperature and pH. Interestingly, germin proteins are also highly resistant to proteases (Patnaik & Khurana, 2001). As indicated in Table 3.1, germin proteins possess a cupin domain and they are associated with the cell walls. Their increased levels in the sorghum secretome under heat stress possibly implicates them in heat stress response.

Three of the uncharacterised proteins (protein numbers 44, 126 and 198) did not have any predicted cellular functions. However, they were classified under the thaumatin family (Table 3.1). Thaumatin is a very sweet protein, 100 000 times sweeter than sucrose (Masuda *et al*., 2011). This protein was originally isolated from katemfe fruit of *Thaumatococcus daniellii* Benth, which is native to tropical West Africa (Van der Wel & Loeve, 1972). Two positively charged amino acid residues, lysine 67 and arginine 82, are involved in the sweetness of thaumatin (Kaneko & Kitabatake, 2001). The up-regulation of thaumatin in the sorghum secretome suggests that it also plays a role in heat stress response. However, the actual mode of action is not very clear.

Twenty two of the 105 differentially expressed proteins were classified under the protein destination and storage functional category (Table 4.1). Most of these proteins were involved in proteolysis and belonged to different peptidase families (Table 3.1). The presence of these

proteins in the ICSB 338 sorghum ECM is in line with other secretome studies including sorghum (Ngara *et al*., 2018) and Arabidopsis cell (Tran & Plaxton, 2008) suspension cultures in response to sorbitol-induced osmotic stress and phosphate deficiency, respectively.

Five uncharacterised proteins and a purple acid phosphatase were categorised under the signal transduction functional category (Table 4.1). According to Zhao *et al*. (2016), the signal transduction pathway is important in the regulation of various biotic and abiotic stress responses through the activation of protein kinases such as calcium-dependent protein kinases, mitogen-activated protein kinases and hormone signalling in plants. The purple acid phosphatase protein is possibly involved in the signal transduction network that operates in the apoplast (Gupta *et al*., 2015). This protein has also been identified in the secretome analysis of chickpea in response to dehydration stress (Gupta *et al*., 2015). Amongst the uncharacterised proteins in this functional category were protein numbers 33, 73 and 101, which contained the leucine-rich repeat-containing N-terminal, plant-type domain (Table 3.1). The leucine-rich repeat in proteins is about 20-29 residues long (Kobe & Kajava, 2001). The majority of these proteins appear to be involved in protein-protein recognition processes and signal transduction pathways (Kobe & Kajava, 2001). Therefore, the presence of signal transduction-related proteins in the ECM of sorghum possibly suggests that the secretome possibly plays a role in signal transduction pathways during heat stress response.

Following the proteomic analysis of ICSB 338 sorghum cell suspension cultures, the expression of a few target heat responsive proteins was validated using gene expression analysis. There was a variety in the expression patterns of the sorghum genes in response to the heat stress treatment (Figure 4.6). An increase and decrease in the expression levels of sorghum genes was observed. The difference in the response patterns of these genes show

that sorghum cell suspension cultures were responsive to heat stress. Additionally, the observed up-regulation of *HSP70* and *HSP90* genes at 40°C for 24, 48 and 72 hours (Figure 4.5) following heat stress is indicative of the role played by these genes in heat stress response. Heat shock proteins are molecular chaperones that are highly expressed in response to a wide range of stresses including heat, drought, salinity and osmotic stresses, among others (Swindell *et al*., 2007). HSPs play a key role in re-folding proteins that have been misfolded/denatured as a result of stress and also resolve large protein aggregates that impede the normal functioning of a cell (Jacob *et al*., 2017). Furthermore, HSPs also play an important role in the induction of thermotolerance in plants (Jacob *et al*., 2017).

Heat shock proteins have been identified in other heat stressed plants including radish taproot (Wang *et al*., 2018), maize (Casaretto *et al*., 2016) and Arabidopsis (Swindell *et al*., 2007). Following five heat stress treatments for 2, 6, 12, 24 and 48 hours at 40°C, the expression levels of HSPs, particularly *HSP70*, in radish taproots were high following heat stress treatment (Wang *et al*., 2018). The expression levels of *HSP70-4* peaked at 2 hours post heat stress treatment, then decreased and remained at low expression level at 48 hours (Wang *et al*., 2018). In another study, gene expression analysis was conducted on maize leaf, stem and root tissues after heat treatment at 42°C for 5 days (Casaretto *et al*., 2016). It was observed that the expression levels of HSPs was high in all maize tissues after the heat stress treatment. The expression of *HSP70*, *HSP90* and *HSP100* in the root tissue of Arabidopsis was also high following exposure to heat stress at 38°C for 0.5, 1, 3, 6, 12 and 24 hours. However, the induction of these HSPs was very high within the first 3 hours of heat exposure than the later time points (Swindell *et al.*, 2007).

The high expression levels of sorghum genes at 30°C (Figure 4.7) also suggests that these heat response genes are already switched on during normal growth temperatures. This makes

it easy for the sorghum to survive at high temperatures treatments of 40°C where other cereal crops cannot grow. Furthermore, the survival of sorghum at both low temperatures of 23°C and high temperatures of 30°C and 40°C shows that this crop can grow in a wide range of temperature conditions. This remarkable quality could be used for the development of heat tolerant crops especially under the prevailing global warming conditions.

CHAPTER 5

GENERAL CONCLUSION AND RECOMMENDATIONS

Global warming is becoming a serious problem worldwide, due to increased surface temperatures and incidences of drought. High temperature stress negatively affects plant growth and development, ultimately leading to reduced crop yield and food insecurity. Some plants have developed morphological, physiological, biochemical and molecular mechanisms to counteract the negative effects of high temperatures. For example, sorghum is a naturally drought tolerant crop, which survives in hot and dry regions where other crops cannot. The ability of sorghum to survive under these conditions makes it a potentially good model system to study plant response mechanisms to a range of environmental stresses such as heat.

In this study, an ICSB 338 cell suspension culture line (Figure 3.1) was used to investigate the molecular responses of sorghum to heat stress. Viability results of the sorghum cell suspension cultures did not show any significant changes at 35 and 40°C during the 72 hours of heat stress treatment (Figure 3.2). On the contrary, a statistically significant decrease in cell viability was observed in Arabidopsis cell suspension cultures, following heat stress treatment at 40°C for 72 hours (Figure 3.3). These results confirmed sorghum's tolerance to high temperature stress relative to Arabidopsis.

When plants are subjected to heat stress, osmotic and oxidative stresses are induced. This results in the over-production of reactive oxygen species, which cause cellular damage and in extreme cases, cell death. Plants produce osmolytes such as proline and glycine betaine to counteract the negative effects of osmotic and oxidative stresses, thus maintaining cellular homeostasis. In this study, the concentrations of proline and glycine betaine were measured

on ICSB 338 sorghum cell suspension cultures, following heat stress treatment at 40°C for 72 hours, using a mass spectrometry-based method. A significant decrease in proline content was observed between the control and heat treated samples at 48 and 72 hours (Figure 3.5). Furthermore, glycine betaine was not detectable in all the control and heat stressed samples. Based on these sets of results, the role played by the osmolytes in heat response of sorghum cell cultures remains unclear.

In addition to the growth, metabolic and biochemical changes that occur in plant cells in response to heat stress, protein and gene expression is also altered. To investigate the protein expression changes in the sorghum cell suspension culture, both the culture filtrate (CF) and total soluble proteins (TSP) were extracted from the control and heat stressed cultures. Gel electrophoresis analysis of the CF proteins did not show much protein expressional changes between the control and the 35°C heat stressed. On the other hand, a significant difference in the protein profiles between the control and 40°C treated samples was observed (Figure 3.4).

Based on these results, the 40°C stress treatment was selected for use in subsequent protein and gene analysis experiments. A western blotting experiment was also conducted to investigate the expression of *HSP70* in sorghum and Arabidopsis control and heat stressed TSP samples (Figure 3.6). At 24 hours of heat stress, the expression of *HSP70* was induced in Arabidopsis, but this decreased after 48 hours. In sorghum, the *HSP70* was already induced in control samples grown at 27°C. An increase in the expression levels of *HSP70* in sorghum was observed at 72 hours of heat stress treatment. These results show that under normal growth conditions where sorghum is grown, the expression of *HSP70* is already high, possibly explaining why sorghum is tolerant to heat stress.

For protein identification, an iTRAQ method was used and a total of 290 secreted proteins were positively identified from the extracellular matrix (ECM) of ICSB 338 cell suspension cultures. This study extends the number of proteins obtained in the ECM of sorghum from 179 proteins identified in White sorghum to 290. Of the 290 proteins identified, 105 were responsive to heat stress (Table 4.1). All the identified secreted proteins were functionally annotated using proteomic and bioinformatic tools. Most proteins were uncharacterised, meaning that they had unknown functions. This implies that more work still needs to be done in sorghum to characterise all expressed proteins and genes with unknown functions.

The heat stress responsive proteins were classified into different functional categories according to Bevan *et al.* (1998) and other literature sources. These include metabolism, disease/defence, protein destination and storage, signal transduction and energy. Those without any predicted functions and lacked characterised domains and/or family names were under unclear classification. Some of the proteins identified in this study including peroxidases, purple acid phosphatases, glycoside hydrolases and ubiquitin among others, have been previously found in other studies (Cho *et al*., 2009, Gupta *et al*., 2015, Ngara *et al*., 2018). However, some of the proteins such as the thaumatin proteins, (protein numbers 44, 126 and 198) have not been identified in secretome studies before. Therefore, these proteins may be considered to be novel in heat response. However, their precise role in heat stress response would need to be further investigated.

The expression of ten target heat responsive proteins in sorghum was validated using gene expression analysis. The sorghum genes were either up or down-regulated in response to heat stress at 40°C for 72 hours (Figure 4.6). The expression levels of *HSP70* and *HSP90* were significantly up-regulated in response to heat stress (Figure 4.5). The results obtained in this study show that sorghum is heat stress responsive and also shows some differential

expression patterns to heat stress. The responsive mechanisms used by sorghum to tolerate heat stress could be applied in breeding programmes for the development of heat tolerant crops to ensure food security.

The results obtained in this study suggest that proline levels decrease with an increase in the period of exposure to heat stress. Therefore, measuring the concentration of proline during the initial stages of heat stress treatment such as 0, 2, 6, 12 and 24 hours is recommended to further understand the early changes that occur upon the induction of heat stress. Furthermore, the expression of the key genes involved in the biosynthetic process of this osmolyte such as pyroline-5-carboxylate synthetase and proline dehydrogenase could be analysed by qRT-PCR.

In order to further understand the response mechanisms of sorghum to heat stress, the target thermo-responsive genes that were highly up-regulated in this study such as *SORBI_3003G427700* and *SORBI_3002G055700* could be isolated and genetically cloned in heat sensitive plants such as Arabidopsis, expose them to heat stress and then observe their responses. This will help in understanding the significance of those target genes in heat stress response.

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APPENDICES

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

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

Concentration (µg)	BSA standard solution (µL)	Extraction buffer (µL)	0.1 M HCl (µL)	Distilled water (µL)
*0	0	10	10	80
5	1	9	10	80
10	2	8	10	80
20	4	6	10	80
40	8	2	10	80
50	10	-	10	80

*Blank solution

Appendix 1-Table 2 1:The preparation of resolving and stacking gels for 1D-SDS PAGE.

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

Appendix 2: The ICSB 338 Culture Filtrate (CF) Secreted Proteins

Appendix 2-Table 1: List of the ICSB 338 culture filtrate secreted proteins.

Pro No.[a]	Scor[b]	% Cov[c]	Accession[d]	Name and Species	Seq Pep[e]	Ratios of control samples[f]				Mean[g]	Rations of treated samples[h]				Mean[i]	SD[j]	Fold change[k]	p-value[l]
						113: 113	114: 113:	115: 113	116: 113		117: 113	118: 113	119 113	121 113				
1	102.2	64.05	C5Y8G7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G224500	246	1.00	1.14	1.02	0.89	1.02	0.26	0.26	0.33	0.40	0.31	0.07	0.31	2.70E-05
2	52.48	33.07	C5Y397	Alpha-mannosidase OS=*Sorghum bicolor* GN=SORBI_3005G132400	33	1.00	0.88	1.36	1.51	1.19	3.23	3.04	3.13	3.59	3.25	0.20	2.74	3.77E-05
3	48.3	35.08	C5XYP5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G233700	55	1.00	0.95	1.47	1.67	1.27	3.66	4.00	3.34	3.49	3.62	0.22	2.84	4.89E-05
4	44.9	51.8	C5Z484	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G163000	42	1.00	0.76	1.05	1.25	1.02	2.30	2.35	2.10	2.10	2.21	0.13	2.18	5.94E-05
5	40.4	62.8	C5Y360	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011300	109	1.00	0.66	0.95	1.03	0.91	1.89	1.92	1.94	2.23	2.00	0.17	2.19	8.33E-05
6	38.5	23.71	C5XQV7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085900	25	1.00	1.10	1.34	1.29	1.18	2.46	2.08	2.53	2.77	2.46	0.24	2.08	2.38E-04
7	36.6	59.25	A0A1W0W7T8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416600	57	1.00	0.97	1.15	1.31	1.11	2.08	2.13	1.76	2.13	2.03	0.16	1.83	2.39E-04
8	36.51	31.68	C5Y8Y2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G23	23	1.00	1.29	0.82	0.97	1.02	0.24	0.18	0.27	0.28	0.24	0.04	0.24	2.42E-04

				5600														
9	35.1	28.11	P04264	SWISS-PROT:P04264 Tax_Id=9606 Gene_Symbol=KRT1 Keratin, type II cytoskeletal 1	24	1.00	0.87	1.07	1.41	1.09	2.01	1.88	2.07	2.03	2.00	0.08	1.84	3.10E-04
10	33.94	37.16	A0A1B6PLA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G104300	23	1.00	0.64	1.32	1.51	1.12	2.41	2.96	2.67	2.74	2.69	0.20	2.41	3.80E-04
11	33.34	47.57	A0A1B6QCB0	Alpha-amylase OS=*Sorghum bicolor* GN=SORBI_3002G190500	27	1.00	1.27	0.95	1.06	1.07	0.60	0.51	0.52	0.54	0.54	0.04	0.51	3.81E-04
12	31.6	33.33	A0A1B6QI05	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089100	19	1.00	0.91	1.34	1.22	1.12	1.80	1.82	1.87	1.94	1.86	0.06	1.67	3.90E-04
13	29.35	38.9	C5Z8N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G118900	48	1.00	1.11	0.99	1.12	1.05	1.44	1.40	1.35	1.31	1.37	0.05	1.30	3.91E-04
14	28.48	28.16	P13645	SWISS-PROT:P13645 Tax_Id=9606 Gene_Symbol=KRT10 Keratin, type I cytoskeletal 10	16	1.00	0.74	1.11	0.92	0.94	1.74	1.77	1.53	1.92	1.74	0.17	1.85	4.04E-04
15	28.11	44.73	C5X5K6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416700	51	1.00	0.83	1.07	1.24	1.03	1.71	2.06	1.81	2.10	1.92	0.18	1.86	4.21E-04
16	26.58	38.97	C5WXC7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	26	1.00	1.33	1.03	1.27	1.16	0.37	0.58	0.53	0.52	0.50	0.08	0.43	4.37E-04
17	25.4	32.71	A0A1B6PKE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G056300	16	1.00	0.66	0.98	0.94	0.90	1.49	1.60	1.46	1.45	1.50	0.08	1.67	4.41E-04
18	25.35	54.72	C5XB38	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055600	43	1.00	0.95	1.35	1.30	1.15	2.41	2.73	2.80	2.07	2.50	0.29	2.17	4.45E-04
19	25.24	37.02	C5WN99|	Uncharacterized	21	1.00	0.77	1.36	1.55	1.17	2.62	3.09	2.54	3.28	2.88	0.30	2.47	4.69E-

				protein OS=*Sorghum bicolor* GN=SORBI_3001G261600														04
20	24.66	37.06	A0A1B6PD27	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3008G113000	14	1.00	0.89	1.67	1.69	1.31	3.11	3.42	4.34	4.09	3.74	0.43	2.85	4.85E-04
21	24.41	30	C5Z240	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G003100	27	1.00	0.96	1.26	1.35	1.14	2.17	2.75	2.06	2.41	2.35	0.27	2.06	5.18E-04
22	24.1	51.19	A0A1B6Q838	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422200	19	1.00	0.84	0.64	0.78	0.81	0.26	0.25	0.35	0.29	0.29	0.05	0.35	5.29E-04
23	24.04	44.36	C5XHP8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419400	15	1.00	0.91	1.39	1.51	1.20	2.22	2.63	2.47	2.20	2.38	0.17	1.98	6.05E-04
24	23.84	61.85	C5Z475	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G162000	36	1.00	0.98	1.58	1.62	1.30	2.99	3.79	2.70	3.38	3.22	0.37	2.48	6.26E-04
25	23.69	58.22	C5WXD7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G209300	43	1.00	0.93	1.06	0.95	0.98	1.34	1.23	1.37	1.22	1.29	0.08	1.31	6.49E-04
26	23.28	22.1	A0A1Z5R915	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3007G091100	14	1.00	0.63	0.78	1.03	0.86	1.69	2.15	1.84	2.36	2.01	0.35	2.34	6.69E-04
27	23.08	43.98	C5WYQ4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G360400	21	1.00	0.77	1.22	1.32	1.08	2.24	2.23	2.56	3.10	2.53	0.38	2.35	8.83E-04
28	22.68	51.92	C5Y1P4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G099000	17	1.00	1.15	0.77	0.78	0.93	1.79	1.49	1.83	1.55	1.66	0.18	1.80	1.06E-03
29	22.63	17.93	C5XWE5	Uncharacterized protein OS=*Sorghum*	14	1.00	0.77	0.90	1.07	0.93	1.50	1.38	1.28	1.37	1.39	0.10	1.48	1.17E-03

				bicolor GN=SORBI_3004G197600														
30	21.7	40.17	C5XIK1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G432700	17	1.00	0.94	1.01	1.07	1.00	1.26	1.20	1.16	1.18	1.20	0.04	1.19	1.30E-03
31	21.49	24.85	C5YRS3	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3008G037000	14	1.00	0.73	1.05	0.81	0.90	1.40	1.41	1.75	1.55	1.53	0.18	1.70	1.41E-03
32	20.99	36	C5YK12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G100600	24	1.00	0.94	1.31	1.41	1.16	1.84	1.93	2.04	1.74	1.89	0.11	1.62	1.56E-03
33	20.92	43.84	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G343600	18	1.00	1.04	0.90	0.99	0.98	0.75	0.84	0.77	0.70	0.77	0.06	0.78	1.88E-03
34	20.6	34.47	A0A1B6PLT5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G133000	14	1.00	0.94	0.59	0.78	0.83	0.36	0.29	0.38	0.39	0.36	0.05	0.43	2.54E-03
35	20.45	63.69	A0A1B6QEI0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G317600	19	1.00	0.96	1.34	1.57	1.22	1.99	2.38	1.89	2.14	2.10	0.18	1.73	2.70E-03
36	20.42	46.91	C5XB39	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055700	40	1.00	1.05	0.71	0.94	0.92	0.61	0.47	0.37	0.49	0.49	0.11	0.52	2.79E-03
37	20.2	20.48	A0A194YQ33	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G166700	10	1.00	0.67	0.81	0.80	0.82	1.11	1.22	1.30	1.17	1.20	0.10	1.46	2.92E-03
38	20.1	48.87	A0A1B6Q6M7	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G327700	16	1.00	1.06	1.14	1.23	1.11	1.34	1.58	1.50	1.67	1.52	0.13	1.37	3.15E-03
39	20	54.46	C5Z469	Peroxidase	20	1.00	0.96	1.03	0.80	0.95	0.74	0.60	0.70	0.65	0.67	0.06	0.71	3.46E-

				OS=*Sorghum bicolor* GN=SORBI_3010G161600														03
40	19.43	45.43	C5XHT5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G423500	18	1.00	1.27	1.32	1.65	1.31	1.93	2.40	1.97	2.08	2.09	0.16	1.60	3.66E-03
41	19.4	18.63	C5YA35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G260300	13	1.00	0.68	0.74	1.18	0.90	0.40	0.34	0.39	0.29	0.35	0.06	0.39	3.77E-03
42	19.08	28.76	C5XQ74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G208800	13	1.00	1.07	1.12	1.40	1.15	1.61	1.49	1.68	1.81	1.65	0.12	1.44	3.94E-03
43	19	42.12	C5YBE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132400	20	1.00	1.14	1.33	1.49	1.24	2.02	2.06	2.25	1.64	1.99	0.21	1.61	4.12E-03
44	18.78	30.04	C5XCE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G351400	20	1.00	0.92	1.18	1.12	1.05	1.46	1.77	1.44	1.37	1.51	0.17	1.44	4.89E-03
45	18.2	33.08	C5Z483	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G162900	15	1.00	0.79	0.80	1.36	0.99	0.43	0.31	0.47	0.37	0.39	0.07	0.40	4.89E-03
46	17.96	30.57	C5YIX7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G078800	15	1.00	0.95	1.34	1.36	1.16	1.92	1.64	2.00	2.40	1.99	0.27	1.71	4.92E-03
47	17.73	36.96	C5XHP9|	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419500	17	1.00	1.52	1.00	0.99	1.13	0.52	0.60	0.56	0.57	0.56	0.03	0.50	5.30E-03
48	17.67	33.21	C5YJ56	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G214700	11	1.00	1.18	0.86	1.00	1.01	0.73	0.74	0.72	0.73	0.73	0.01	0.72	5.38E-03
49	17.63	24.36	C5XHP7	Uncharacterized	12	1.00	0.87	0.98	1.06	0.98	1.14	1.14	1.13	1.18	1.15	0.02	1.17	5.75E-

				protein OS=*Sorghum bicolor* GN=SORBI_3003G419300														03
50	17.47	39.22	C5X040	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G080300 P	12	1.00	0.97	0.71	0.58	0.82	0.41	0.36	0.42	0.37	0.39	0.03	0.48	5.84E-03
51	17.31	35.42	C5Y675	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G064200	19	1.00	0.94	1.22	1.28	1.11	1.96	2.65	1.66	2.72	2.25	0.47	2.02	5.86E-03
52	17.08	18.78	P35527	SWISS-PROT:P35527 Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	12	1.00	0.94	1.16	0.56	0.91	1.62	1.49	1.83	1.38	1.58	0.21	1.73	5.89E-03
53	16.83	37.58	C5YQU5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G146700	22	1.00	1.25	0.95	0.64	0.96	1.60	1.48	1.79	1.43	1.58	0.17	1.64	6.00E-03
54	16.71	22.59	C5XV25	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G333600	11	1.00	1.04	1.00	1.07	1.03	0.96	0.89	0.89	0.83	0.89	0.05	0.87	6.04E-03
55	16.6	38.15	A0A1Z5S979	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3001G348900	18	1.00	0.97	0.88	1.20	1.01	0.70	0.52	0.73	0.70	0.66	0.10	0.66	6.43E-03
56	16.44	22.61	A0A1B6QJE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G170700	9	1.00	0.86	1.10	0.87	0.96	1.12	1.28	1.35	1.36	1.28	0.11	1.34	6.59E-03
57	16.15	47.56	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G129700	16	1.00	0.95	1.04	0.80	0.95	1.81	1.35	1.24	1.61	1.50	0.27	1.59	6.92E-03
58	16.13	53.65	C5YVR0	Superoxide dismutase OS=*Sorghum bicolor* GN=SORBI_3009G093200	12	1.00	0.96	1.15	1.00	1.03	1.49	1.38	1.89	2.08	1.71	0.32	1.66	7.21E-03
59	16.09	16.38	A0A1B6PNL9	Uncharacterized protein OS=*Sorghum bicolor*	28	1.00	0.78	1.30	1.63	1.18	2.01	2.01	3.02	2.81	2.46	0.45	2.09	7.25E-03

				GN=SORBI_3006G242000														
60	15.91	52.51	C5XHF1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G136200	9	1.00	0.80	1.11	1.00	0.98	1.31	1.35	1.19	1.23	1.27	0.07	1.30	7.65E-03
61	15.85	24.36	C5X5L7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3002G417800	9	1.00	1.44	1.09	1.17	1.17	1.51	1.54	1.55	1.70	1.57	0.07	1.34	8.26E-03
62	15.77	15.26	A0A1W0VUE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G227400	8	1.00	1.14	0.67	0.90	0.93	0.39	0.61	0.53	0.50	0.51	0.10	0.55	8.46E-03
63	15.64	16.85	C5YYX0	Beta-hexosaminidase OS=*Sorghum bicolor* GN=SORBI_3009G017500	8	1.00	1.02	1.04	1.09	1.04	1.25	1.30	1.10	1.22	1.22	0.08	1.17	8.77E-03
64	15.32	22.59	C5Z0N9	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055300	14	1.00	1.78	0.82	0.76	1.09	0.16	0.28	0.22	0.01	0.17	0.10	0.16	8.97E-03
65	15.06	50.98	C5Z6U2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G210000	12	1.00	0.97	0.93	0.84	0.94	1.17	1.13	1.27	1.05	1.16	0.10	1.23	9.25E-03
66	14.58	18.85	C5WS35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G081100	9	1.00	2.02	1.06	0.79	1.22	1.87	2.75	2.95	2.95	2.63	0.42	2.16	9.39E-03
67	14.49	21.76	C5WW86	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G196700	9	1.00	0.48	0.68	1.01	0.80	1.47	1.66	1.42	1.14	1.42	0.27	1.79	9.40E-03
68	14.47	17.92	A0A1B6QHZ6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089000	13	1.00	0.70	0.96	0.92	0.90	1.13	1.22	1.52	1.30	1.29	0.18	1.44	9.41E-03
69	14.44	20	A0A1W0W7I8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391900	14	1.00	1.28	0.70	1.08	1.02	1.42	1.87	1.46	1.78	1.63	0.22	1.61	9.55E-03
70	14.08	44.92	C5XYY5	Peroxidase	14	1.00	0.91	1.03	1.21	1.04	1.59	1.80	1.39	1.29	1.52	0.22	1.46	9.96E-

				OS=*Sorghum bicolor* GN=SORBI_3004G105100														03
71	14.07	22.57	C5X4N0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_3002G255600	8	1.00	0.85	1.05	0.88	0.94	0.70	0.81	0.66	0.54	0.68	0.12	0.72	1.06E-02
72	13.93	28.94	C5XI24	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G140700	8	1.00	0.66	0.83	0.85	0.83	1.17	1.26	1.03	1.40	1.21	0.19	1.45	1.15E-02
73	13.79	16.3	C5Y7T1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G086000	8	1.00	1.09	1.41	1.30	1.20	0.82	0.85	0.91	0.86	0.86	0.03	0.72	1.15E-02
74	13.69	57.04	C5WVG9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324800	18	1.00	0.88	1.00	1.43	1.08	1.55	1.62	1.51	2.03	1.68	0.22	1.56	1.23E-02
75	13.67	26.74	A0A1W0W7U5	Carboxypeptidase (Fragment) OS=*Sorghum bicolor* GN=SORBI_3002G401200	9	1.00	0.84	2.07	0.36	1.07	2.37	2.24	2.98	2.26	2.46	0.33	2.31	1.32E-02
76	13.37	23.53	C5XIY1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152100	15	1.00	0.95	1.19	1.42	1.14	0.67	0.82	0.75	0.77	0.75	0.06	0.66	1.32E-02
77	13.15	14.16	C5X5H5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G268200	6	1.00	0.98	1.10	0.92	1.00	1.20	1.25	1.65	1.40	1.38	0.20	1.38	1.34E-02
78	12.78	10.4	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	7	1.00	1.66	0.91	0.97	1.14	0.54	0.49	0.53	0.54	0.52	0.02	0.46	1.34E-02
79	12.43	20.47	P35908	SWISS-PROT:P35908 Tax_Id=9606 Gene_Symbol=KRT2 Keratin, type II cytoskeletal 2 epidermal	13	1.00	1.20	1.11	1.14	1.11	0.83	1.03	0.71	0.82	0.85	0.12	0.76	1.40E-02
80	12.09	36.36	C5WU08	Uncharacterized protein OS=*Sorghum bicolor*	6	1.00	1.62	1.48	1.47	1.39	1.00	0.84	0.83	0.45	0.78	0.17	0.56	1.41E-02

				GN=SORBI_3001G025400														
81	12.09	30.55	C5YB25	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3006G277600	7	1.00	0.88	0.97	0.95	0.95	1.02	1.19	1.20	1.07	1.12	0.09	1.18	1.51E-02
82	12.01	15.3	A0A1B6QFT1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G392000	8	1.00	0.74	1.21	0.96	0.98	1.28	1.24	1.44	1.48	1.36	0.12	1.39	1.53E-02
83	12.01	23.03	A0A1B6Q515	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G238500	6	1.00	0.88	1.13	0.85	0.97	1.14	1.21	1.31	1.17	1.21	0.08	1.25	1.61E-02
84	11.88	20.6	C5X3C1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391300	9	1.00	0.84	1.12	1.21	1.04	1.27	1.39	1.52	1.28	1.37	0.11	1.31	1.67E-02
85	11.87	31.69	C5Y2P0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G122300	6	1.00	0.99	1.07	1.18	1.06	0.94	0.94	0.88	0.87	0.91	0.04	0.86	1.68E-02
86	11.82	13.37	C5Z6U	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G209900	6	1.00	1.12	0.61	0.80	0.88	0.52	0.42	0.57	0.51	0.50	0.07	0.57	1.70E-02
87	11.8	21.66	C5XX52	Glyceraldehyde-3-phosphate dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3004G205100	7	1.00	1.06	0.86	1.01	0.98	1.43	1.17	1.12	1.45	1.29	0.17	1.32	1.74E-02
88	11.61	15.92	A0A1W0W2E3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050900	15	1.00	1.05	0.90	0.80	0.94	0.74	0.64	0.74	0.80	0.73	0.07	0.78	1.75E-02
89	11.53	29.6	A0A1Z5S9P3	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G379400	7	1.00	1.16	0.93	1.15	1.06	0.73	0.87	0.92	0.79	0.83	0.08	0.78	1.76E-02
90	11.43	9.335	C5WP48	Alpha-mannosidase OS=*Sorghum bicolor* GN=SORBI_3001G268700	9	1.00	0.82	1.02	1.08	0.98	0.81	0.79	0.83	0.80	0.81	0.02	0.82	1.97E-02
91	11.37	17.46	C5WPY8	Peroxidase	7	1.00	1.21	1.45	1.63	1.32	1.74	1.87	1.65	2.06	1.83	0.13	1.38	2.15E-

				OS=*Sorghum bicolor* GN=SORBI_3001G277000														02
92	11.28	16.33	C5XD22	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G356800	9	1.00	1.02	0.92	1.24	1.05	1.29	1.63	1.81	1.28	1.50	0.25	1.44	2.17E-02
93	11.19	22.26	C5X1L1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G247900	9	1.00	0.77	1.05	0.90	0.93	1.08	1.31	1.74	1.41	1.38	0.29	1.49	2.32E-02
94	11.08	25.19	C5XX83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G208700	27	1.00	0.83	1.01	1.25	1.02	0.81	0.64	0.79	0.71	0.74	0.08	0.72	2.41E-02
95	10.91	46.2	A0A1B6P5R2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G009600	9	1.00	0.91	1.46	1.60	1.24	1.78	1.81	1.62	1.90	1.78	0.09	1.43	2.43E-02
96	10.54	25.11	P00761	SWISS-PROT:P00761|TRYP_PIG Trypsin - Sus scrofa (Pig).	25	1.00	0.89	0.55	0.54	0.74	0.48	0.38	0.35	0.28	0.37	0.11	0.50	2.50E-02
97	10.11	22.01	C5Z0P5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G055900	10	1.00	0.86	1.09	1.04	1.00	0.83	0.70	0.89	0.81	0.81	0.08	0.81	2.54E-02
98	10.09	15.15	A0A1Z5RER0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G186300	5	1.00	1.55	0.66	0.78	1.00	0.54	0.33	0.33	0.39	0.40	0.10	0.40	2.61E-02
99	10.07	24.19	C5X022	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G525000	16	1.00	1.19	1.51	1.06	1.19	0.75	0.82	0.43	0.95	0.74	0.19	0.62	2.92E-02
100	10.04	12.12	A0A194YU12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G341200	5	1.00	1.32	0.97	1.00	1.07	1.47	1.53	1.14	1.50	1.41	0.17	1.31	3.34E-02
101	10.04	24.7	C5Y2R8	Uncharacterized protein OS=*Sorghum*	17	1.00	1.52	1.06	1.11	1.17	0.81	0.93	0.84	0.78	0.84	0.05	0.72	3.51E-02

				bicolor GN=SORBI_3005G126200														
102	10.01	29.8	C5YYK3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G014700	16	1.00	0.98	1.14	1.19	1.08	0.90	0.72	0.90	0.99	0.88	0.10	0.81	3.88E-02
103	10	21.62	C5X0X1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G528100	8	1.00	0.85	0.80	1.08	0.93	1.43	1.04	1.11	1.39	1.24	0.21	1.33	3.90E-02
104	9.88	20.84	A0A1B6PTQ9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G204700	5	1.00	1.46	1.61	1.64	1.43	0.97	0.93	1.05	1.13	1.02	0.06	0.72	3.94E-02
106	9.76	13.91	A0A1W0VY92|	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205900	6	1.00	1.88	0.98	1.10	1.24	0.83	0.66	0.66	0.40	0.64	0.14	0.51	4.06E-02
107	9.66	16.36	C5WY32	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G061900	5	1.00	1.08	1.25	1.25	1.14	1.24	1.30	1.42	1.44	1.35	0.08	1.18	4.17E-02
108	9.65	24.63	C5XL59	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G024700	16	1.00	1.50	0.61	0.79	0.97	0.59	0.40	0.44	0.48	0.48	0.08	0.49	4.50E-02
109	9.54	26.51	C5WXN2	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3001G348800	14	1.00	0.59	1.03	0.93	0.89	1.11	1.12	1.18	1.18	1.15	0.04	1.29	4.51E-02
110	9.49	9.242	C5WSX6	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3001G013800	6	1.00	0.85	0.76	1.34	0.99	0.61	0.82	0.51	0.58	0.63	0.13	0.64	4.74E-02
111	9.43	33.51	A0A1Z5RHN3	Dirigent protein OS=*Sorghum bicolor* GN=SORBI_3005G101500	5	1.00	1.08	1.55	1.14	1.19	2.11	1.17	2.04	1.86	1.80	0.36	1.51	4.93E-02
112	9.4	37.96	C5YVJ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_309G232100	21	1.00	1.31	1.08	1.31	1.18	1.46	1.33	1.32	1.45	1.39	0.06	1.18	5.38E-02

113	9.35	19.23	A0A1B6Q537	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G244600	21	1.00	1.39	0.93	1.04	1.09	0.78	0.97	0.71	0.80	0.81	0.10	0.75	5.42E-02
114	9.29	18.97	C5WSE5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G300400	5	1.00	1.25	0.76	0.82	0.96	0.74	0.69	0.69	0.71	0.71	0.02	0.74	6.50E-02
115	9.25	16.76	A0A1W0W3H0	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3002G123100	6	1.00	0.60	0.57	0.95	0.78	1.22	1.26	0.98	0.93	1.10	0.22	1.41	6.52E-02
116	9.21	7.649	C5WPH7	Uncharacterized protein OS=Sorghum bicolor OX=4558 GN=SORBI_3001G131100 PE=4 SV=1	5	1.00	1.40	1.19	1.85	1.36	0.92	0.90	0.87	1.05	0.94	0.06	0.69	6.54E-02
117	9.06	16.53	tr|C5XDR4|C5XDR4_SORBI	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G217200	5	1.00	1.14	1.04	1.13	1.08	0.96	0.99	0.93	1.05	0.98	0.04	0.91	6.59E-02
118	9.04	14.71	A0A1Z5R0P5	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G033300	5	1.00	1.95	1.69	1.85	1.62	1.02	1.19	1.21	1.13	1.14	0.05	0.70	6.81E-02
119	9.02	13.26	C5WU20	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G026800	5	1.00	0.96	0.86	0.88	0.92	0.95	1.44	1.04	1.44	1.22	0.28	1.32	7.17E-02
120	8.85	8.264	C5WPY7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G277500	5	1.00	1.46	1.13	1.14	1.18	1.04	1.03	0.88	0.76	0.93	0.11	0.78	7.28E-02
121	8.76	44.21	C5YQ75	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3008G010500	69	1.00	1.52	1.49	1.40	1.35	1.56	1.95	1.62	1.58	1.68	0.14	1.24	7.49E-02
122	8.54	16.07	C5XAQ6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G326000	5	1.00	1.15	0.93	0.88	0.99	0.87	0.94	0.68	0.76	0.81	0.12	0.82	7.66E-02
123	8.52	14.32	C5XY68	Carboxypeptidase OS=*Sorghum bicolor*	7	1.00	1.36	1.39	0.98	1.18	1.52	1.75	1.39	1.32	1.49	0.16	1.27	7.66E-02

				GN=SORBI_3004G224700														
124	8.5	15.25	C5X3C6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391800	5	1.00	1.95	1.57	1.27	1.45	0.80	1.23	1.03	0.83	0.97	0.14	0.67	8.10E-02
125	8.44	16.15	C5WSY5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G014700	6	1.00	0.92	0.73	0.92	0.89	0.80	0.69	0.78	0.78	0.76	0.05	0.86	8.16E-02
126	8.39	32.31	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	23	1.00	1.08	1.23	1.27	1.15	1.46	1.54	1.15	1.32	1.37	0.15	1.19	8.28E-02
127	8.26	15.28	C5Z8T4	Xyloglucan endotransglucosylase/ hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246600	6	1.00	1.29	1.01	1.15	1.11	0.85	0.68	1.09	0.92	0.89	0.15	0.80	8.57E-02
128	8.25	19.88	C5XHR8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G421700	8	1.00	1.22	1.22	1.33	1.19	1.44	1.59	1.20	1.41	1.41	0.13	1.18	8.66E-02
129	8.23	24.73	C5XIY0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152000	22	1.00	0.72	0.69	0.43	0.71	0.59	0.44	0.29	0.46	0.45	0.17	0.63	9.02E-02
130	8.19	26.84	C5XC95	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G345800	20	1.00	0.87	0.64	0.81	0.83	0.73	0.53	0.66	0.72	0.66	0.11	0.79	9.58E-02
131	8.09	18.92	C5Z6D9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G079100	8	1.00	1.59	0.63	0.96	1.05	0.72	0.67	0.62	0.58	0.64	0.06	0.62	9.60E-02
132	8.01	22.36	C5Z864	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G232500	5	1.00	0.68	0.99	1.29	0.99	0.74	0.73	0.78	0.74	0.75	0.02	0.75	9.99E-02
133	7.96	20.77	C5X780	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G00	6	1.00	0.88	0.76	0.81	0.86	1.02	1.10	0.84	1.10	1.02	0.14	1.18	1.03E-01

				7200														
134	7.96	10.12	A0A1B6QIM7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G123300	4	1.00	1.09	0.75	0.91	0.94	0.78	0.84	0.73	0.82	0.79	0.05	0.85	1.09E-01
135	7.95	11.99	C5Z4E5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044900	5	1.00	0.67	0.70	1.68	1.01	1.51	1.73	1.11	1.79	1.54	0.30	1.52	1.10E-01
136	7.86	18.06	C5XQW7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G087300	8	1.00	1.25	0.79	1.31	1.09	0.68	0.78	0.81	1.03	0.82	0.13	0.76	1.10E-01
137	7.85	34.38	C5XHX2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G427700	7	1.00	1.19	0.97	0.87	1.01	1.92	1.34	1.77	0.85	1.47	0.48	1.46	1.13E-01
138	7.84	19.23	C5Y7R1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G084300	4	1.00	1.52	1.36	1.17	1.26	1.92	1.38	1.56	1.42	1.57	0.19	1.24	1.15E-01
140	7.67	13.52	A0A1B6QEG2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G315800 P	6	1.00	1.30	1.02	1.13	1.11	0.92	0.94	0.84	1.11	0.95	0.10	0.85	1.16E-01
141	7.39	38.28	C5WN51	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G119000	7	1.00	0.96	0.95	1.27	1.04	1.30	0.99	1.34	1.48	1.28	0.20	1.22	1.17E-01
142	7.36	23.15	C5WRN5	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G444500	5	1.00	0.97	0.72	0.89	0.89	0.75	0.72	0.83	0.78	0.77	0.05	0.86	1.18E-01
143	7.19	22.59	C5WX83	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G058300	9	1.00	1.28	0.79	0.81	0.97	1.04	1.58	1.29	1.15	1.26	0.24	1.30	1.22E-01
144	7.13	6.999	C5XRC3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G37	4	1.00	1.31	1.43	0.99	1.18	1.36	1.51	1.28	1.44	1.40	0.08	1.19	1.22E-01

				4100														
145	7.1	41.94	C5YBF0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132500	8	1.00	1.52	0.49	0.81	0.95	0.66	0.43	0.71	0.33	0.53	0.19	0.56	1.23E-01
146	6.96	8.065	C5XAS9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G329000	4	1.00	0.60	0.76	0.55	0.73	0.98	0.83	0.89	0.97	0.92	0.09	1.26	1.24E-01
148	6.88	18.26	A0A1Z5R3E0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G130100	7	1.00	0.99	1.09	1.11	1.05	0.61	0.87	1.02	0.99	0.87	0.18	0.83	1.25E-01
149	6.64	49.69	C6JSB7	Peroxidase OS=*Sorghum bicolor* GN=Sb0246s002010	73	1.00	0.72	0.92	1.51	1.04	0.79	0.86	0.42	0.72	0.70	0.19	0.67	1.28E-01
150	6.61	7.961	A0A1B6PQR2	Protein disulfide-isomerase OS=*Sorghum bicolor* GN=SORBI_3005G074400	4	1.00	0.38	0.28	0.56	0.55	0.26	0.30	0.31	0.24	0.28	0.05	0.50	1.32E-01
151	6.55	11.65	C5Y9T3	Aldose 1-epimerase OS=*Sorghum bicolor* GN=SORBI_3006G105200	4	1.00	1.02	0.51	0.63	0.79	1.08	1.39	0.76	1.22	1.11	0.34	1.40	1.34E-01
152	6.54	6.723	C5XLM4	Purple acid phosphatase OS=*Sorghum bicolor* GN=SORBI_3003G314400	3	1.00	0.96	0.70	0.87	0.88	0.59	0.67	0.90	0.73	0.72	0.15	0.82	1.35E-01
153	6.5	7.394	C5Z1X3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G268400	3	1.00	0.72	0.80	0.99	0.88	0.74	0.79	0.68	0.78	0.75	0.06	0.85	1.36E-01
154	6.45	12.5	C5XJT8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G156400	6	1.00	1.00	0.81	0.80	0.90	0.85	0.80	0.65	0.82	0.78	0.10	0.87	1.40E-01
155	6.38	8.987	C5XIH4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G430100	3	1.00	1.31	1.32	1.11	1.18	1.74	1.14	1.36	1.48	1.43	0.21	1.21	1.42E-01
156	6.36	19.46	A0A1Z5RIA	Uncharacterized	3	1.00	1.12	0.94	1.26	1.08	1.51	1.45	1.08	1.14	1.30	0.20	1.20	1.49E-

			3	protein OS=*Sorghum bicolor* GN=SORBI_3005G126100														01
157	6.35	23.78	C5Y5U9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177500	5	1.00	1.56	1.03	1.19	1.19	0.96	1.15	0.90	0.43	0.86	0.26	0.72	1.50E-01
158	6.21	32.35	A0A1Z5RHM3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G098700	10	1.00	1.41	0.96	1.11	1.12	1.15	0.90	0.60	0.83	0.87	0.20	0.78	1.56E-01
159	6.12	12.46	A0A1B6QN00	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G360500	3	1.00	0.98	1.04	1.55	1.14	1.12	0.85	0.67	0.86	0.88	0.16	0.77	1.57E-01
160	6.04	19.84	C5WZU7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G516000	4	1.00	1.47	0.70	0.86	1.01	0.69	0.89	0.66	0.66	0.73	0.11	0.72	1.61E-01
161	6.02	17.17	C5WX01	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G050100	4	1.00	0.88	0.57	0.94	0.85	0.98	1.13	1.04	0.91	1.01	0.11	1.20	1.65E-01
162	6.02	10.18	C5YZI6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G032800	5	1.00	1.03	0.89	0.99	0.98	0.78	0.98	0.86	0.94	0.89	0.09	0.91	1.69E-01
163	6.01	5.76	A0A1B6PNV2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G255600	3	1.00	0.66	0.27	0.39	0.58	0.35	0.26	0.41	0.26	0.32	0.12	0.55	1.69E-01
164	6	7.674	C5YLE3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G002800	4	1.00	0.88	1.11	1.20	1.05	1.34	0.93	1.36	2.01	1.41	0.43	1.34	1.73E-01
165	6	11.92	A0A1W0W1X3	Carboxypeptidase OS=*Sorghum bicolor* GN=SORBI_3002G024400	3	1.00	0.95	0.87	2.05	1.22	0.69	0.71	1.06	0.60	0.76	0.16	0.63	1.77E-01
166	6	16.82	C5YD83	Uncharacterized protein OS=*Sorghum bicolor*	3	1.00	0.71	1.07	0.58	0.84	1.29	0.63	1.87	1.26	1.26	0.60	1.50	1.81E-01

				GN=SORBI_3006G031200														
167	6	12.58	C5XYB7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G229500	3	1.00	0.91	1.14	1.45	1.12	1.47	1.41	1.29	1.16	1.33	0.12	1.18	1.81E-01
168	5.98	5.873	C5XPK9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205600	5	1.00	1.11	1.26	1.17	1.13	0.95	1.12	1.11	0.77	0.99	0.15	0.87	1.86E-01
169	5.93	11.97	A0A1B6QJR7	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G189000	4	1.00	1.93	0.44	0.57	0.98	0.44	0.50	0.58	0.40	0.48	0.08	0.49	1.87E-01
170	5.83	31.84	C5WWH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G333400	4	1.00	0.96	0.99	1.14	1.02	1.07	1.13	1.02	1.33	1.14	0.13	1.11	1.91E-01
171	5.77	8.914	C5YV55	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G084300	3	1.00	1.34	1.22	1.00	1.14	1.08	1.01	1.05	0.67	0.95	0.17	0.84	1.92E-01
172	5.7	6.658	A0A1B6QC86	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G189100	3	1.00	0.64	0.99	0.80	0.86	1.06	0.79	1.50	1.08	1.11	0.34	1.29	1.94E-01
173	5.69	9.153	P13647	SWISS-PROT:P13647 Tax_Id=9606 Gene_Symbol=KRT5 Keratin, type II cytoskeletal 5	6	1.00	1.47	0.76	0.82	1.01	0.91	0.76	0.78	0.59	0.76	0.13	0.75	1.94E-01
174	5.66	12.61	C5YCI1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G154300	3	1.00	0.57	0.72	1.00	0.83	0.57	0.67	0.67	0.73	0.66	0.08	0.80	1.94E-01
175	5.61	11.38	A0A1Z5S8J9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G300800	6	1.00	2.54	1.36	1.24	1.54	1.17	0.73	0.85	1.25	1.00	0.17	0.65	1.95E-01
176	5.57	15.53	C5XQS6	Uncharacterized protein OS=*Sorghum bicolor*	3	1.00	0.90	1.78	1.95	1.41	2.32	1.21	2.42	1.89	1.96	0.39	1.39	1.98E-01

				GN=SORBI_3003G082600														
177	5.51	8.602	C5YNA1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G172100	3	1.00	1.09	0.65	1.17	0.98	1.11	0.18	0.70	0.65	0.66	0.39	0.67	2.01E-01
178	5.45	4.791	A0A1B6QN59	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G366800	3	1.00	1.54	0.59	0.58	0.93	0.65	0.68	0.45	0.60	0.60	0.11	0.64	2.04E-01
179	5.45	36.14	A0A1B6QG28	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3002G407900	4	1.00	1.00	0.67	0.82	0.87	0.59	0.91	0.74	0.65	0.72	0.16	0.83	2.05E-01
180	5.39	6.771	C5XIT6	Pectinesterase OS=*Sorghum bicolor* GN=SORBI_3003G148400	3	1.00	0.65	0.91	0.57	0.78	0.67	0.71	0.53	0.60	0.63	0.10	0.80	2.05E-01
181	5.34	22.09	C5X6P6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G140300	4	1.00	0.66	1.04	1.07	0.94	1.14	0.90	1.58	1.11	1.18	0.30	1.26	2.08E-01
182	5.09	13.42	C5X5T3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G130700	3	1.00	1.73	1.32	1.47	1.38	1.14	1.12	0.94	1.35	1.14	0.12	0.82	2.11E-01
183	4.7	9.763	C5Z6Y0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G088700	3	1.00	1.04	0.96	1.77	1.19	1.42	1.19	2.08	1.56	1.56	0.31	1.31	2.18E-01
184	4.65	9.091	A0A1B6Q0N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G005800	3	1.00	0.93	1.01	1.10	1.01	1.41	1.11	0.97	1.10	1.14	0.19	1.13	2.27E-01
185	4.49	13.81	C5XCL8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G070200	3	1.00	1.16	0.78	0.98	0.98	1.09	1.16	1.23	0.96	1.11	0.12	1.13	2.32E-01
186	4.43	7.107	A0A1Z5RAM1	Uncharacterized protein OS=*Sorghum bicolor*	3	1.00	0.82	1.02	0.45	0.82	0.61	0.40	0.87	0.54	0.61	0.23	0.74	2.38E-01

				GN=SORBI_3007G188100														
187	4.41	11.63	A0A1W0VX32	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G127100	5	1.00	1.25	0.88	0.96	1.02	1.11	1.09	1.11	1.24	1.14	0.07	1.11	2.39E-01
188	4.29	10.48	C5YC92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G018100	2	1.00	1.25	0.99	0.97	1.05	0.87	0.88	0.97	1.08	0.95	0.09	0.90	2.42E-01
189	4.25	14.19	C5XG67	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G400400	2	1.00	1.64	1.61	1.24	1.37	2.03	2.50	0.71	2.59	1.96	0.63	1.43	2.49E-01
190	4.19	6.714	C5XCI9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G068000	3	1.00	1.67	0.78	1.35	1.20	1.05	0.80	1.00	0.93	0.95	0.09	0.79	2.60E-01
191	4.18	5.882	C5X8J1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G301700	2	1.00	1.69	2.07	2.76	1.88	1.79	1.58	1.17	0.95	1.37	0.20	0.73	2.66E-01
192	4.17	6.746	C5XIY9	Dihydrolipoyl dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3003G152900	2	1.00	0.84	0.72	0.85	0.85	0.91	0.73	0.75	0.64	0.76	0.13	0.89	2.71E-01
193	4.15	5.109	C5X9Y2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G314800	3	1.00	1.86	0.78	0.95	1.14	0.95	0.92	0.72	0.79	0.85	0.09	0.74	2.75E-01
194	4.11	19.4	A0A1Z5RDM9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132100	4	1.00	1.74	0.45	1.39	1.14	0.51	0.70	1.14	0.77	0.78	0.23	0.68	2.77E-01
195	4.08	12.54	C5YYA0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G145500	3	1.00	0.76	0.92	0.86	0.88	1.20	0.85	1.04	0.89	1.00	0.18	1.13	2.84E-01
196	4.05	16.85	C5XN41	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G33	3	1.00	1.43	0.90	0.95	1.07	0.80	0.84	1.20	0.64	0.87	0.22	0.81	2.85E-01

				0300														
197	4.05	7.806	C5WY51	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G063500	2	1.00	1.22	1.18	1.28	1.17	1.14	1.14	1.00	1.09	1.09	0.06	0.93	2.99E-01
198	4.04	12.26	C5YH35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G177500	3	1.00	1.49	0.82	1.08	1.10	0.83	0.93	1.05	0.89	0.93	0.09	0.84	3.00E-01
199	4.03	10.25	A0A1B6QGB6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416800	3	1.00	1.46	0.97	1.01	1.11	0.97	1.09	0.88	0.93	0.97	0.08	0.87	3.00E-01
200	4.03	6.044	C5WT90	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G173300	2	1.00	0.59	0.71	1.62	0.98	0.52	1.00	0.47	0.76	0.69	0.25	0.70	3.01E-01
201	4.03	7.513	C5Z1Z7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G270800	2	1.00	1.10	0.48	0.60	0.79	0.73	0.61	0.51	0.62	0.62	0.11	0.78	3.02E-01
202	4.02	9.579	C5Y9W4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G108400	2	1.00	1.75	1.20	2.28	1.56	2.05	1.68	1.94	1.89	1.89	0.10	1.21	3.09E-01
203	4.01	12.32	C5Z0N8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055100	4	1.00	0.64	0.80	1.11	0.89	0.94	1.14	0.84	1.19	1.03	0.19	1.16	3.31E-01
204	4.01	7.539	A0A1B6PTZ2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G217900	4	1.00	0.90	1.09	0.83	0.96	0.86	0.96	0.88	0.88	0.89	0.05	0.93	3.33E-01
205	4.01	10.55	C5YM54	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G151300	2	1.00	1.77	1.61	1.42	1.45	1.54	1.04	1.40	0.84	1.21	0.22	0.83	3.36E-01
206	4	6.173	C5WNS8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G123100	4	1.00	1.32	0.65	0.92	0.97	0.87	0.88	0.78	0.78	0.83	0.06	0.85	3.37E-01

207	4	3.474	A0A194YN27	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G064900	3	1.00	1.03	0.75	0.81	0.90	0.87	1.01	0.55	0.64	0.77	0.23	0.86	3.44E-01
208	4	13.24	C5YBF1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132700	3	1.00	0.76	0.84	1.04	0.91	0.91	0.86	1.02	1.67	1.11	0.41	1.22	3.48E-01
209	4	3.115	C5WN09	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G113800	2	1.00	0.89	0.62	0.69	0.80	0.88	1.00	0.81	0.87	0.89	0.10	1.12	3.68E-01
211	4	8.993	C5Z861	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G231900	2	1.00	1.76	1.12	1.24	1.28	0.94	1.13	0.93	1.37	1.09	0.16	0.85	3.80E-01
212	4	8.169	C5XFH6	Fructose-bisphosphate aldolase OS=*Sorghum bicolor* GN=SORBI_3003G393900	2	1.00	1.29	0.78	1.06	1.03	0.92	0.98	0.92	0.92	0.93	0.03	0.90	3.87E-01
213	4	9.687	C5Z8S3	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G245400	2	1.00	0.83	1.09	1.07	1.00	1.23	0.91	0.75	0.53	0.85	0.30	0.85	3.96E-01
214	4	19.08	C5YU74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G068000	3	1.00	0.98	0.66	0.65	0.82	0.90	0.54	0.89	0.38	0.68	0.31	0.83	4.06E-01
215	4	8.989	C5XAQ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G326100	2	1.00	1.12	2.14	2.41	1.67	1.16	1.22	2.03	0.65	1.27	0.34	0.76	4.11E-01
216	4	15.33	C5X972	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3002G306900	2	1.00	1.00	1.14	1.00	1.03	0.99	0.81	1.14	0.94	0.97	0.13	0.94	4.16E-01
217	4	8.108	A0A1Z5R4X8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G048100	2	1.00	1.11	0.72	0.92	0.94	1.02	0.87	0.98	1.33	1.05	0.21	1.12	4.16E-01

218	4	11.46	A0A1W0VZJ3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G294800	2	1.00	1.20	1.03	1.14	1.09	1.02	1.12	1.03	1.01	1.05	0.05	0.96	4.16E-01
219	4	10.1	A0A1B6Q242	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085300	4	1.00	0.62	0.66	0.80	0.77	0.92	0.79	1.00	0.74	0.86	0.16	1.12	4.18E-01
220	4	7.595	A0A1B6PHX9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G150800	3	1.00	1.11	0.61	1.22	0.98	0.97	1.28	1.05	1.15	1.11	0.13	1.13	4.21E-01
221	3.93	15.57	A0A1B6QD45	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G237000	10	1.00	0.65	0.42	0.52	0.65	0.58	0.54	0.48	0.55	0.54	0.07	0.83	4.23E-01
222	3.93	6.571	tr|A0A1B6QQP5|A0A1B6QQP5_SORBI	Uncharacterized protein OS=Sorghum bicolor OX=4558 GN=SORBI_3001G515500 PE=4 SV=1	5	1.00	1.35	0.82	0.87	1.01	0.91	0.91	0.88	0.93	0.91	0.02	0.90	4.23E-01
223	3.9	17.12	C5Z8E7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G110800	2	1.00	0.66	0.67	0.89	0.80	0.77	0.66	0.65	0.82	0.72	0.10	0.90	4.31E-01
224	3.89	13.21	C5XIY2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152200	7	1.00	2.22	1.03	1.24	1.37	1.24	1.22	1.05	0.99	1.12	0.09	0.82	4.34E-01
225	3.85	11.67	C5XGM0	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G126800	2	1.00	3.17	4.74	2.99	2.98	5.45	6.29	2.17	2.34	4.06	0.71	1.37	4.37E-01
226	3.82	3.618	C5XL53	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3003G024000	2	1.00	1.23	0.82	0.64	0.92	0.74	0.80	0.78	0.95	0.82	0.10	0.88	4.55E-01
227	3.69	4.624	C5YBP8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G009000	2	1.00	1.55	1.08	0.81	1.11	1.10	1.02	0.91	0.90	0.98	0.09	0.88	4.65E-01

229	3.62	5	A0A1W0W0W5	Pectin acetylesterase OS=*Sorghum bicolor* GN=SORBI_3003G384700	2	1.00	0.75	0.84	1.70	1.07	1.14	0.98	1.52	1.39	1.26	0.23	1.17	4.79E-01
230	3.58	8.197	C5XQ07	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G072300	2	1.00	0.93	0.62	0.44	0.75	0.56	0.83	0.65	0.50	0.64	0.19	0.85	4.85E-01
231	3.57	14.47	A0A194YL19	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G236500	3	1.00	1.62	0.33	0.85	0.95	0.40	0.83	0.79	0.93	0.74	0.24	0.78	4.91E-01
232	3.53	5.23	A0A1W0W1H5	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3003G440900	3	1.00	1.45	0.85	0.98	1.07	1.11	0.85	1.45	1.44	1.21	0.27	1.13	4.92E-01
233	3.53	12.15	C5YBA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G004300	2	1.00	0.46	0.74	0.28	0.62	0.79	0.31	0.34	0.48	0.48	0.36	0.77	4.94E-01
234	3.52	8.055	C5XQP2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G078400	3	1.00	1.57	0.74	0.88	1.05	1.15	1.21	0.97	1.46	1.20	0.19	1.14	4.97E-01
235	3.51	7.66	C5XLV5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G030700	2	1.00	0.42	1.55	1.11	1.02	0.01	1.35	0.94	0.73	0.76	0.55	0.74	4.99E-01
236	3.44	16.78	C5WRH5	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3001G295200	2	1.00	0.83	0.79	0.89	0.88	0.66	0.73	1.02	0.85	0.82	0.18	0.93	5.24E-01
237	3.42	9.701	C5Z4N3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G168200	2	1.00	1.45	0.94	0.78	1.04	0.75	0.92	0.83	1.20	0.93	0.19	0.89	5.29E-01
238	3.39	9.278	C5X750	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G003800	2	1.00	0.76	1.05	1.20	1.00	1.28	1.31	0.70	1.16	1.11	0.28	1.11	5.34E-01

239	3.38	3.938	C5WWQ2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G342600	2	1.00	0.98	0.65	0.88	0.88	0.71	0.86	1.15	1.15	0.97	0.25	1.10	5.45E-01
240	3.32	14.18	C5WVM2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G330500	2	1.00	1.13	1.03	1.42	1.15	1.24	1.17	1.13	1.31	1.21	0.07	1.06	5.46E-01
241	3.29	19.45	C5Z471	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G161800	5	1.00	0.87	1.09	1.25	1.05	0.98	1.18	0.93	0.85	0.98	0.13	0.94	5.52E-01
242	3.29	3.838	C5X733	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G002100	2	1.00	1.00	1.38	1.37	1.19	1.22	1.31	1.21	1.29	1.26	0.04	1.06	5.54E-01
243	3.27	16.07	C5Y7Z1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G215900	11	1.00	0.73	0.70	0.66	0.77	0.78	0.96	0.74	0.83	0.83	0.13	1.07	5.78E-01
244	3.24	2.712	A0A1Z5RC44	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G213300	2	1.00	1.14	0.91	0.81	0.97	0.99	1.13	1.08	0.87	1.02	0.12	1.06	5.78E-01
245	3.21	5.244	A0A1W0W1V5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G016400	2	1.00	0.49	0.51	0.45	0.61	0.10	0.45	1.40	1.30	0.81	1.05	1.33	5.84E-01
246	3.09	12.2	A0A1Z5S8P9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324700	2	1.00	0.89	1.09	1.20	1.04	1.04	0.95	1.10	0.91	1.00	0.08	0.96	5.86E-01
247	3.01	9.231	A0A1Z5RDF6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G119700	2	1.00	1.22	1.08	1.01	1.08	0.96	1.05	1.22	0.89	1.03	0.13	0.95	5.86E-01
248	2.97	13.35	C5YY92	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G144600	3	1.00	0.96	1.49	1.41	1.21	1.13	1.37	1.46	1.25	1.30	0.12	1.07	5.90E-01
249	2.95	13.83	C5WSG0	Uncharacterized	4	1.00	0.67	0.79	0.87	0.83	0.89	0.49	0.90	0.79	0.77	0.23	0.92	6.03E-

				protein OS=*Sorghum bicolor* GN=SORBI_3001G301600														01
250	2.95	8.178	C5X578	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G128000	2	1.00	0.98	2.40	2.32	1.67	1.62	0.93	1.85	1.34	1.43	0.24	0.86	6.04E-01
252	2.84	4.783	C5XQD5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G214900	2	1.00	1.07	0.92	0.83	0.96	0.82	1.15	0.91	0.71	0.90	0.20	0.94	6.08E-01
253	2.82	19.33	A0A1Z5R5E6	Non-specific lipid-transfer protein OS=*Sorghum bicolor* GN=SORBI_3008G030900	8	1.00	0.66	0.70	0.75	0.78	1.04	1.02	0.69	0.64	0.85	0.27	1.09	6.08E-01
254	2.79	9.774	C5YBE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132300	3	1.00	1.96	1.18	2.36	1.62	0.54	1.67	3.15	2.52	1.97	0.70	1.21	6.14E-01
255	2.79	8.225	C5Y4W3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G160700	2	1.00	0.75	0.74	1.01	0.88	1.15	0.81	0.80	0.98	0.93	0.19	1.07	6.20E-01
256	2.77	27.22	A0A1B6PJ93	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G227300	9	1.00	0.77	1.09	1.13	1.00	1.02	1.00	0.77	1.00	0.95	0.12	0.95	6.42E-01
257	2.76	6.371	C5XKY2	Uncharacterized protein OS=S*orghum bicolor* GN=SORBI_3003G308300	2	1.00	0.99	0.75	0.85	0.90	0.94	0.79	0.82	0.91	0.86	0.08	0.96	6.52E-01
258	2.68	12.66	C5YBF3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132900	7	1.00	1.15	0.75	0.82	0.93	0.89	0.74	0.79	1.07	0.87	0.16	0.94	6.53E-01
259	2.64	6.126	A0A1B6Q6G6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G314800	3	1.00	0.71	0.25	0.53	0.62	0.65	0.54	0.99	0.67	0.71	0.31	1.14	6.60E-01

260	2.61	3.525	A0A1B6PEC9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G178800	2	1.00	0.20	3.56	0.95	1.43	1.01	0.80	1.54	1.00	1.09	0.22	0.76	6.67E-01
261	2.58	5.725	A0A1W0W287	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050100	2	1.00	1.14	1.16	1.22	1.13	1.30	1.07	1.14	1.13	1.16	0.09	1.03	6.67E-01
262	2.54	39.73	C5YYF4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G009800	2	1.00	1.15	1.30	0.68	1.03	1.02	1.12	1.00	0.71	0.96	0.17	0.93	6.69E-01
263	2.52	5.655	C5YHR8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3007G192300	2	1.00	1.03	0.92	1.23	1.04	0.99	1.16	1.03	1.12	1.08	0.08	1.03	6.70E-01
264	2.51	16.95	A0A1W0W2G8	Thioredoxin OS=*Sorghum bicolor* GN=SORBI_3002G057900	2	1.00	0.75	0.92	1.00	0.92	0.88	1.08	0.90	0.94	0.95	0.10	1.03	6.82E-01
266	2.37	3.093	C5WP98	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G414000	3	1.00	1.73	1.50	1.38	1.40	1.41	1.50	1.27	1.13	1.33	0.12	0.95	6.89E-01
268	2.37	7.547	C5X4M5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255000	2	1.00	0.57	1.08	0.37	0.75	1.12	0.77	0.45	0.26	0.65	0.50	0.86	6.92E-01
269	2.36	3.968	C5XKG0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G016800	2	1.00	2.14	3.02	2.16	2.08	2.77	2.46	1.53	0.48	1.81	0.50	0.87	6.99E-01
272	2.31	7.101	C5YZJ2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G033400	6	1.00	0.92	0.98	0.83	0.93	1.05	1.30	0.53	0.54	0.85	0.41	0.92	7.11E-01
273	2.28	14.2	C5WPH2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G130400	4	1.00	0.33	0.57	0.73	0.66	0.85	0.64	0.86	0.52	0.72	0.26	1.09	7.28E-01
275	2.26	7.066	C5XP10	Carboxypeptidase OS=*Sorghum bicolor*	2	1.00	1.44	1.20	0.57	1.05	0.95	0.89	1.48	0.50	0.95	0.38	0.91	7.32E-01

				GN=SORBI_3003G345700														
276	2.25	3.286	A0A1Z5S3X9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G020500	2	1.00	1.84	0.65	0.83	1.08	1.07	1.11	0.93	0.85	0.99	0.11	0.91	7.45E-01
279	2.17	7.524	A0A1B6QEM0	Xyloglucan endotransglucosylase/ hydrolase OS=*Sorghum bicolor* GN=SORBI_3002G324100	3	1.00	0.94	1.07	1.12	1.03	0.98	0.97	1.20	1.06	1.05	0.10	1.02	7.49E-01
281	2.16	24.11	C5WQD8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G145600	3	1.00	1.20	1.04	0.94	1.04	0.78	0.99	1.17	1.11	1.01	0.16	0.97	7.55E-01
284	2.11	15.43	A0A194YMM6	Glyceraldehyde-3-phosphate dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3010G262500	5	1.00	1.10	0.72	0.33	0.79	0.83	1.10	0.73	0.73	0.85	0.22	1.08	7.63E-01
286	2.09	10.4	C5Y1M2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G097400	2	1.00	0.77	0.52	0.77	0.76	0.93	0.87	0.77	0.62	0.80	0.18	1.04	7.89E-01
287	2.09	6.436	C5XEB7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G226000	2	1.00	1.39	0.99	1.01	1.10	1.27	0.80	1.16	1.01	1.06	0.19	0.97	7.98E-01
288	2.09	8.017	C5Y981	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G094000	2	1.00	0.98	0.29	1.21	0.87	1.39	0.88	0.29	1.27	0.95	0.57	1.10	8.03E-01
299	2.02	6.789	A0A1Z5S810	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3001G267600	3	1.00	1.49	1.05	1.19	1.18	1.07	1.35	0.97	1.21	1.15	0.14	0.97	8.03E-01
305	2.01	16	A0A1Z5S985	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G353501	5	1.00	1.13	1.36	1.23	1.18	1.19	1.16	1.28	1.17	1.20	0.05	1.02	8.19E-01

318	2	26.46	A0A194YGY2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G027000	7	1.00	0.96	0.51	0.81	0.82	0.90	0.50	1.18	0.52	0.77	0.40	0.94	8.27E-01
321	2	27.91	C5WN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G119100	4	1.00	0.72	1.01	1.29	1.00	1.12	1.02	0.99	0.99	1.03	0.06	1.03	8.34E-01
322	2	32.21	C5X1U2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G390300	4	1.00	1.35	1.02	1.04	1.10	0.95	0.88	1.47	0.98	1.07	0.25	0.97	8.46E-01
323	2	9.398	A0A1Z5RIM0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G145201	2	1.00	1.20	2.40	3.35	1.99	1.78	2.54	1.83	1.31	1.87	0.26	0.94	8.47E-01
324	2	16.45	A0A1B6QN96	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3001G371900	2	1.00	0.63	0.93	0.75	0.83	0.94	0.96	0.89	0.61	0.85	0.20	1.03	8.53E-01
329	2	7.477	A0A1Z5RGB0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3006G243600	2	1.00	0.97	0.74	1.24	0.99	1.11	0.96	1.12	0.84	1.01	0.13	1.02	8.63E-01
330	2	6.195	C5YI12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G065900	2	1.00	1.22	0.77	0.96	0.99	1.01	1.08	0.86	1.08	1.01	0.10	1.02	8.79E-01
364	2	6.897	C5XL87	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G027200	3	1.00	1.47	1.94	0.83	1.31	0.99	1.32	1.15	1.61	1.27	0.20	0.97	8.86E-01
365	2	8.738	C5X385	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G387100	2	1.00	1.40	0.83	0.73	0.99	0.99	0.73	1.15	1.00	0.97	0.18	0.98	8.97E-01
373	1.85	58.24	A0A194YKY7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G225300	11	1.00	1.66	1.52	1.49	1.42	1.17	1.26	1.50	1.85	1.44	0.21	1.02	9.10E-01

374	1.83	5.112	A0A194YMI7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G036800	3	1.00	2.51	1.61	3.21	2.08	2.71	1.01	1.07	3.20	2.00	0.54	0.96	9.11E-01
384	1.3	3.627	C5XMN8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G042200	2	1.00	0.65	0.64	0.77	0.76	0.66	0.75	0.87	0.74	0.75	0.11	0.99	9.11E-01
386	1.27	9.534	P02533	SWISS-PROT:P02533 Tax_Id=9606 Gene_Symbol=KRT14 Keratin, type I cytoskeletal 14	5	1.00	0.36	0.34	0.61	0.58	0.73	0.60	0.63	0.42	0.59	0.23	1.03	9.16E-01
391	1.1	12.88	C5X3C2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391400	7	1.00	1.12	1.01	1.01	1.04	1.05	0.97	0.95	1.15	1.03	0.09	0.99	9.20E-01
395	0.99	4.819	C5WRN4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G444400	2	1.00	1.08	0.87	0.63	0.90	1.23	0.76	0.74	0.91	0.91	0.25	1.02	9.21E-01
396	0.98	5.017	Q7Z794	SWISS-PROT:Q7Z794 Tax_Id=9606 Gene_Symbol=KRT77 Keratin 77	6	1.00	1.37	1.04	0.76	1.04	1.12	1.22	0.92	0.95	1.05	0.14	1.01	9.25E-01
398	0.91	9.619	C5X216	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G090400	4	1.00	1.33	1.07	1.16	1.14	0.99	1.23	1.05	1.25	1.13	0.11	0.99	9.29E-01
400	0.86	8.136	tr|A0A1W0W5X4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G258300	4	1.00	0.43	0.62	1.73	0.95	0.80	0.81	1.35	0.73	0.92	0.30	0.97	9.43E-01
403	0.75	43.82	C5Y359	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011200	97	1.00	1.00	0.52	0.92	0.86	0.98	0.91	0.82	0.70	0.85	0.14	0.99	9.58E-01
423	0.44	4.076	C5YT19	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G191400	2	1.00	0.96	0.39	1.32	0.92	0.89	0.68	0.86	1.19	0.91	0.23	0.99	9.61E-01
429	0.35	7.788	C5XYY9	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3004G10	3	1.00	0.37	0.20	0.55	0.53	0.47	0.71	0.41	0.52	0.52	0.25	0.99	9.72E-01

				5800														
433	0.31	10.12	A0A194YNU6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3004G106100	6	1.00	0.15	0.36	0.54	0.51	0.48	0.51	0.84	0.26	0.52	0.47	1.02	9.72E-01
442	0.24	11.35	C5WSF9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301500	3	1.00	0.73	0.63	1.17	0.88	1.01	0.82	1.01	0.68	0.88	0.18	1.00	9.80E-01
456	0.16	5.363	C5Z8S2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G245300	2	1.00	0.98	2.05	1.11	1.28	1.33	1.16	1.36	1.30	1.29	0.07	1.00	9.81E-01
478	0.07	3.858	C5XHS1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422000	2	1.00	1.14	1.12	2.43	1.42	1.10	1.16	1.69	1.71	1.42	0.23	1.00	9.86E-01

[a] Protein number assigned in ProteinPilot software.

[b] Protein score generated by ProteinPilot software relating to the confidence of protein identification. Proteins were identified with a 95% confidence.

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

[d] Proteins accession obtained from the TrEMBL database [within the UniProt database (http://www.uniprot.org)] searches against sequences of *Sorghum bicolor* only.

[e] Sequenced peptide refers to the number of peptide that were sequenced and gave rise to protein identity. All proteins that were identified by means of a single peptide were deleted from the dataset.

[f] Values indicate the abundance of each protein from the four replicate control samples presented as a ratio to the 113-tagged sample

[g] Mean of ratios of each protein from the control samples ($n = 4$).

[h] Values indicate the abundance of each protein from the four replicate sorbitol-treated samples presented as a ratio to the 113-tagged sample.

[i] Mean of ratios of each protein from the heat-treated samples ($n = 4$).

[j] Standard deviation of the ratios of samples ($n = 4$).

[k] Fold-change ($n = 4$) induced by heat stress treatment relative to control. Positive values indicate an up-regulation.

[l] Probability value of the quantitative difference between the treatment and control protein abundance being due to chance alone.