MOLECULAR CHANGES OF SORGHUM CELL SUSPENSION CULTURES IN RESPONSE TO EXOGENOUS ABSCISIC ACID

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May 2022

DECLARATION

I, Dakalo Muthego, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification in Botany at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

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DEDICATION

I dedicate this dissertation to my mom T.G Muthego and my late dad A.J Muthego. Thank you for the unconditional love. Mom, I truly appreciate your endless support, and your prayers keep me going and do better in life. Ndo livhuwa!

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

1D	One-dimensional
ABA	Abscisic acid
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CF	Culture filtrate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHLH	H subunit of Mg-chelatase
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
GO	Gene ontology
HILIC	Hydrophilic interaction chromatography
iTRAQ	Isobaric tags for relative and absolute quantitation
kDa	kilo Dalton
LC	Liquid chromatography
LEA	Late-embryogenesis abundant
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
PAGE	Polyacrylamide gel electrophoresis
ROS	Reactive oxygen species
PP2C	Type 2 Protein phosphatase
PYR/PYL/RCAR	Pyrabactin resistance/Pyrabactin resistance1-like/Regulatory components of ABA receptors
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SnRK2	Sucrose non-fermenting-1-related protein kinase 2
TCA	Trichloroacetic acid
TFs	Transcription factors
TSP	Total soluble protein
v/v	Volume to volume
w/v	Weight to volume

ABSTRACT

Abiotic stresses reduce the growth and productivity of crops, thus threatening food security. It is therefore, important to develop crops that can withstand harsh environmental conditions in order to ensure availability of food. In general, plants have developed a wide range of mechanisms in response to these abiotic stresses. For example, under stress conditions, plants undergo molecular changes which include alterations in gene, protein and metabolite expression patterns that are mostly regulated by the plant hormone abscisic acid (ABA). ABA-regulated stress responsive pathways are well studied in the model plant Arabidopsis (*Arabidopsis thaliana)*, yet similar processes in sorghum (*Sorghum bicolor*), a drought tolerant crop, are not yet fully understood. The aim of the study was to investigate the biochemical properties and protein expression patterns of sorghum cell suspension cultures in response to exogenous ABA. White sorghum cell suspension cultures were used, and at eight days post-subculture, the cultures were treated with 100 µM ABA prepared in 70% (v/v) methanol. For control cells, an equal volume of 70% (v/v) methanol was added, and both treatment groups were incubated with shaking at 27℃ for 72 hours. Analysis of cell viability using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that ABA does not affect the viability of the cells at 0, 24, 48 and 72 hours. However, exogenous application of ABA for 72 hours resulted in increased accumulation of proline in the sorghum cells relative to the controls. Furthermore, proteins were extracted from the cells, as total soluble proteins (TSP), and from the culture medium, as culture filtrate proteins (CF) 72 hours after the exogenous ABA treatment. The protein profiles of the two proteomes were visually analysed on Coomassie brilliant blue-stained one-dimensional sodium dodecyl sulfate polyacrylamide gels. The gels showed that the two proteomes were of good quality even under control conditions. Furthermore, following the 72-hour ABA treatment, proteins were differentially expressed in both the TSP and CF proteomes. Moreover, isobaric tags for relative and absolute

quantitation (iTRAQ) method and mass spectrometry were used to identify and quantify the differentially expressed proteins. A total of 725 and 256 proteins were identified in the TSP and CF proteomes, respectively. Of all these, 46 and 82 were ABA-responsive in the TSP and CF, respectively, and 8 proteins were common to both proteomes. Signal peptide analysis revealed that the majority of TSP found in the intracellular matrix did not have a predicted signal peptide (72%), while the majority of CF proteins found in the extracellular matrix contained signal-peptides (82%). Amongst these differentially expressed proteins in both the TSP and CF proteomes, the majority of them proteins were involved in metabolism with 37% and 35%, followed by defence with 24% and 24%, respectively. However, the metabolic processes in the CF were mainly related to carbohydrate metabolism. The signal transduction functional group was only unique to the TSP fraction, while transporters, and cell structure functional groups were unique to the CF protein fraction. The differentially expressed proteins are well-known stress proteins such as peroxidases and superoxide dismutases whose levels change under abiotic stresses. Together with causing an increase in proline content, a known osmoprotectant, exogenous ABA does indeed act as a stress phytohormone. Furthermore, these results showed that ABA influences the differential expression of both intracellular and extracellular matrix proteins, possibly suggesting the importance of both cell compartments in stress response. Furthermore, these two compartments have different roles in stress responses as suggested by the results. Therefore, the application of exogenous ABA could be the way forward to further understand plant stress response networks, and possibly to develop crops that can survive under any abiotic stress.

Keywords: Sorghum, cell suspension cultures, exogenous abscisic acid, total soluble proteins, culture filtrate proteins, proline, signal peptides, intracellular proteins, extracellular proteins, iTRAQ

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Abiotic stresses and their general effects on plants

An abiotic stress is an environmental factor that negatively affects the growth of a living organism in a particular environment (Ben-Ari and Lavi, 2012). Such stresses include drought, waterlogged soil, low and high temperatures and high soil salinity (Patanè *et al*., 2013). In agriculture, abiotic stresses reduce the growth, development and productivity of crops to variable degrees depending on the time of onset, duration and intensity (Witcombe *et al.*, 2007), as well as the plant species and genotype (Des Marais *et al*., 2013). As a result of the abiotic stresses, there is a reduction in production yields and food supply (Knight and Knight, 2001).

Currently at 7.94 billion (Worldometer, 2022), the world population is expected to reach 9.9 billion by the year 2050 (Population Reference Bureau, 2018). Furthermore, Africa's population size is also expected to double by the year 2050 to about 2.5 billion (Sitaula *et al.*, 2020). A rise in the world population by such a large margin will also increase the demand for cereal production (Rosegrant and Cline, 2003). Apart from the negative effects of population growth on food demand, climate change is also projected to increase the frequency of abiotic stresses such as drought and extreme temperatures (IPCC, 2007, Jat *et al.*, 2016). Drought, salinity and high temperatures are amongst the major threats to agricultural productivity, reducing crop yield by up to 50% (Boko *et al.*, 2007). Bray *et al.* (2000) also stated that by 2050, about 50% of arable lands will be affected by soil salinity. With the projections in global climatic change, sustaining food production in future will require the use of stress tolerant crops that yield under extreme climatic conditions (Jat *et al.,* 2016).

Such crops include sorghum (*Sorghum bicolor*), a naturally drought tolerant cereal (Tesso *et al*., 2005). Cousin *et al.* (2003), added that sorghum plants are also efficient in C_4 photosynthesis and have evolved a range of mechanisms that increase net carbon assimilation at high temperatures. In order to sustain cereal production itself, a combination of adaptive agricultural strategies will be required (Taylor, 2003). These include the application of new management and agronomic practices, and further improvement in the genetic potential of abiotic stress resistant crops (Dalal *et al*., 2012). However, plant breeding initiatives also require an understanding of how plants respond to abiotic stresses (Dalal *et al.,* 2012).

1.2 Plant responses to abiotic stresses

Plants encounter a range of abiotic stresses during their growth and development (Tari *et al.*, 2013). However, in order to complete their life cycles, plants have developed a range of adaptive mechanisms to these stressful environmental conditions (Zhu, 2016). Generally, plant responses to abiotic stresses include changes in morphological and developmental patterns as well as physiological, biochemical and molecular processes (Anjum *et al* 2011). These response mechanisms are very complex (Rizhsky *et al.*, 2004) for a number of reasons. For example, one or more type of stresses may affect the plant at different stages of development. Response can be specific to a particular stress or crosstalk between stresses (Knight and Knight, 2001).

Plant responses to stress may include the activation of molecular cascades in the perception of stress, signal transduction pathways and expression of specific stress-related genes, proteins and metabolites (Chinnusamy *et al.*, 2004; Vinocur and Altman, 2005). Figure 1.1 illustrates some of the general plant response mechanisms to drought, cold and salt stresses showing protein expression changes to control the damage caused by the stress (Tuteja *et al.,* 2011). For

example, plants accumulate scavengers of reactive oxygen species (ROS), chaperones, proteinases and phytohormones which trigger complex changes in physiology and metabolic activities, leading to defence responses (Rejeb *et al.*, 2014) and adaptation to stress (Zhang *et al*., 2006).

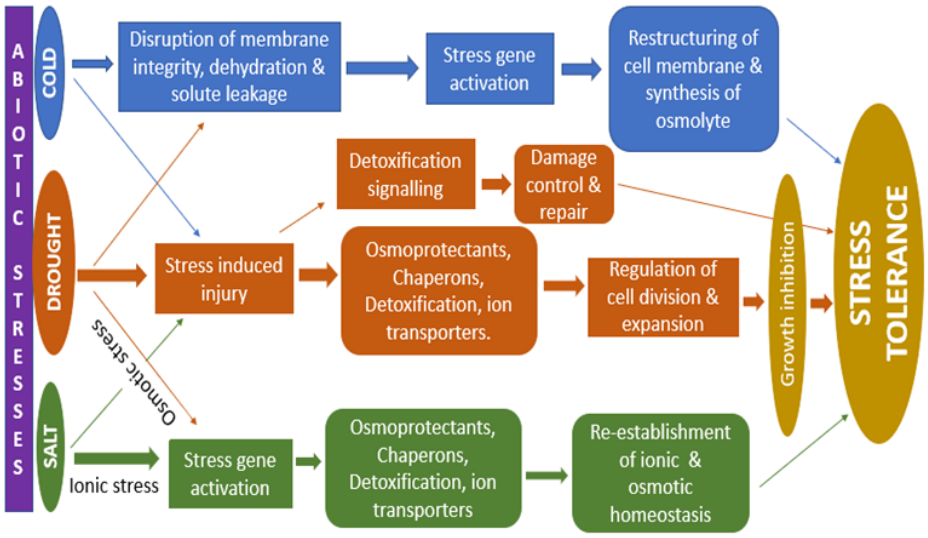


Figure 1.1: Complex nature of plant response mechanisms to abiotic stresses (Tuteja *et al*., 2011).

Molecular chaperones, such as heat shock proteins (HSP) are stress proteins that function in protein folding, assembly, translocation and stabilisation (Bukau *et al.*, 2006). Therefore, these chaperones play a major role in maintaining cellular homeostasis by restoring normal protein conformation, and ultimately their function (Wang *et al*., 2004). Reactive oxygen species are produced under both normal and stress conditions in different cellular compartments such as peroxisomes, chloroplasts and mitochondria (Gill and Tuteja, 2010). High levels of ROS are harmful to plants as they cause cellular damage due to oxidative stress. On the other hand, low ROS levels are responsible for regulating plant stress responses, serving as signalling molecules (Foyer and Noctor, 2005). In addition, after sensing the stress factor, a rapid accumulation of ROS is observed (Choudhury *et al.*, 2013). High levels of ROS are detoxified

by enzymatic antioxidants such as superoxide dismutase and glutathione reductase (Ahmad *et al*., 2008) and non-enzymatic antioxidant such as ascorbic acid, glutathione, α-tocopherol, carotenoids, phenolics, flavonoids, and proline (Gill and Tuteja, 2010). This detoxification reduces possible damage of cellular macromolecules such as membranes, lipids and proteins (Ahmad *et al*., 2010).

1.3 Abscisic acid, the plant hormone

Phytohormones are plant growth regulators that can be used at sites of synthesis or transported within the plant for growth and developmental processes under both normal and stressful conditions (Taiz and Zeiger, 2010; Peleg and Blumwald, 2011). A range of phytohormones such as auxins, gibberellins, ethylene and abscisic acid (ABA) are also involved in plant responses to abiotic stresses (Wani *et al.*, 2016; Dar *et al*., 2017). This study focusses on the phytohormone ABA. Therefore, a review of literature on ABA as a plant growth hormone and its role as a stress hormone is discussed below.

1.3.1 Occurrence of ABA

Abscisic acid is a plant hormone that occurs in all dicotyledonous and monocotyledonous plants (Milborrow, 1974). This hormone has also been detected in gymnosperms, a horsetail, as well as in the ferns (Varner, and Mense, 1972; Milborrow, 1974). However, ABA does not occur in liverworts since its role is played by lunularic acid (Tarakhovskaya *et al*., 2007). ABA is produced in many species of both higher and lower plants. Furthermore, the levels of ABA differ according to plant parts (Charlwood and Banthorpe, 1991).

1.3.2 Biosynthesis of ABA

The biosynthesis of ABA occurs in chloroplasts and other plastids via the terpenoid pathway as shown in Figure 1.2 (Seo and Koshiba, 2002). In higher plants, ABA is derived from the cleavage of C_{40} caretonoid precursors. In this pathway, the major reaction step involves the conversion of epoxycarotenoid 9-cis-neoxanthin by the 9-cis-epoxycarotenoid dioxygenase (NCED) yielding a C_{15} intermediate, xanthoxin. Xanthoxin is formed in the cytosol, and it contains ABA-like physiological properties. It is then converted into ABA-aldehyde by the enzyme short-chain alcohol dehydrogenase/reductase (SDR). The final step in the biosynthetic pathway involves abscisic aldehyde oxidase (AAO) that requires molybdenum cofactors (MCSU) to synthesize ABA (Xiong and Zhu, 2003; Assmann, 2004). ABA produced in roots is transported through the xylem vessels to leaves where it regulates stomatal closure during drought stress conditions (Jiang and Hartung, 2008).

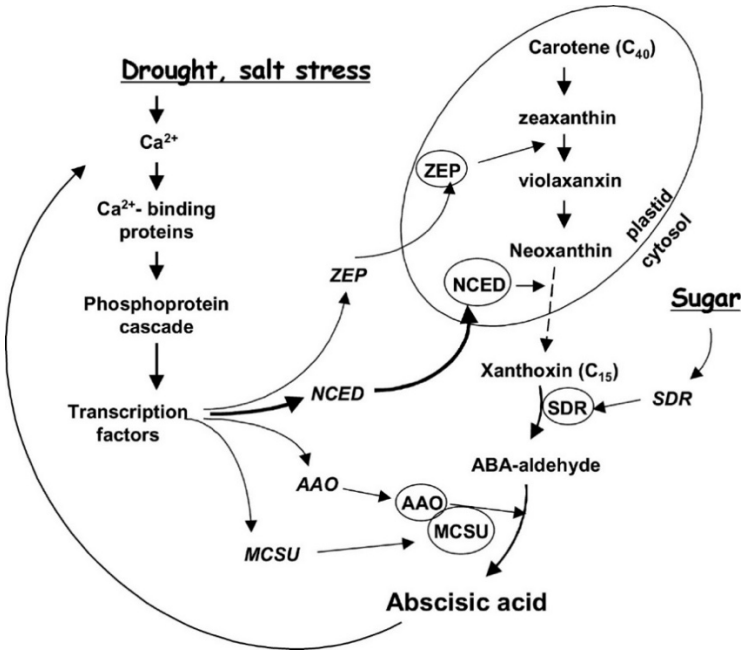


Figure 1.2: The ABA biosynthetic pathway (Xiong and Zhu, 2003).

Key: Zeaxanthin epoxidase (ZEP), 9-*cis*-epoxycarotenoid dioxygenase (NCED), ABA-aldehyde oxidase (AAO), MoCo sulfurase (MCSU), short-chain alcohol dehydrogenase/reductase (SDR).

1.3.1 The role of ABA in plants

In plants, ABA functions in plant developmental processes well as in response to different environmental conditions (Mauch-Mani and Mauch, 2005), such as drought, salt stress, high, and low temperatures (Zhang *et al*., 2006; Tuteja, 2007). Its roles in embryo development, seed dormancy, and as a stress hormone are discussed below.

1.3.3.1 Role in embryo development

ABA regulates embryo development and seed germination (Quatrano, 1986). Embryo development is categorized into three main stages, namely (i) mitosis and cell differentiation (ii) cell expansion and accumulation of food reserves, (iii) maturation, where the seed dries and goes through the dormant stage (Goldberg *et al*., 1994). Several studies have investigated the effects of ABA on embryo development under tissue culture conditions (Finkelstein *et al.,* 1985; Quatrano, 1987; Skriver and Mundy, 1990). ABA maintains embryogenic development during the storage reserve accumulation phase. When embryos of *Brassica napus* were removed from the mother plant halfway through development and cultured *in vitro,* they were able to germinate and develop into seedlings due to the ABA that had been accumulated (Finkelstein *et al.,* 1985). However, ABA inhibits premature germination of seeds whilst they are still on the parent plant (Quatrano, 1987).

1.3.3.2 Role in seed dormancy

Seed germination is the growth of embryo in the mature seed, and this depends on the availability of water, oxygen and favourable temperatures (Bewley, 1997). However, in some cases, the seed fails to germinate even if environmental conditions are favourable, a process known as dormancy (Hilhorst, and Karssen, 1992; Bewley *et al.*, 2012). Seed dormancy provides a temporal delay in the germination process, which allow additional time for seed

dispersal and also increases the chances of seed survival if it lands on unfavourable conditions (Finch- Savage and Leubner- Metzger, 2006). Abscisic acid also activates the synthesis of late embryogenesis abundant (LEA) proteins, that prepare the embryo for desiccation in the resting phase and induction of dormancy (Grappin *et al.*, 2000).

1.3.3.3 Abscisic acid as a stress hormone

Abscisic acid also plays an important role in sending stress signals when plant tissues experience unfavourable environmental conditions (Campalans *et al.,* 1999; Tuteja, 2007). When a plant is subjected to drought stress, the levels of ABA in leaves and roots increase (Griffiths *et al*., 1996). For example, upon sensing water shortages in the soil, roots will produce ABA, which is transported to the leaves via the xylem. Abscisic acid synthesized either in the roots or leaves is further transported into guard cells, resulting in stomatal closure and a reduction in transpiration water loss (Zeevaart and Creelman, 1988; Sauter *et al.*, 2001; Boursiac *et al.*, 2013). Apart from causing stomatal closure during periods of drought stress, ABA is involved in various other cellular processes such as stress signalling and the subsequent changes in molecular events which lead to stress response and adaptation (Yoshida *et al*., 2002). This study will be focused on the role of ABA as a signalling molecule and its regulatory effects on gene, protein and metabolite expression patterns in plant systems.

1.3.4 Abscisic acid signal transduction pathways

While ABA is a well-known signalling molecule (Zeevaart and Creelman, 1988), the ABA recognition processes, accumulation patterns and general signalling pathways are yet to be fully understood (Peirats-Llobet *et al*., 2016). However, ABA is thought to be recognised by a range of protein receptors (McCourt and Creelman, 2008). These ABA receptors are found both on cell surfaces and intracellularly (Ma *et al*., 2009), and directly bind to the hormone, triggering

a cascade of signalling molecules to initiate physiological responses (Klingler *et al.*, 2010). Examples of three candidate ABA receptors include (i) G protein coupled receptors (GPCR)-type G-proteins (GTG proteins), (ii) Pyrabactin Resistance/Like/ Regulatory Components of ABA Receptors (PYR/PYL/RCAR) proteins and (iii) the subunit of Mg-chelatase (CHLH) proteins (Pandey *et al*., 2006; Park *et al*., 2009; Ma *et al*., 2009). Figure 1.3 below shows a model of how these three candidate ABA receptors interact in plant cells (Taiz and Zeiger, 2010).

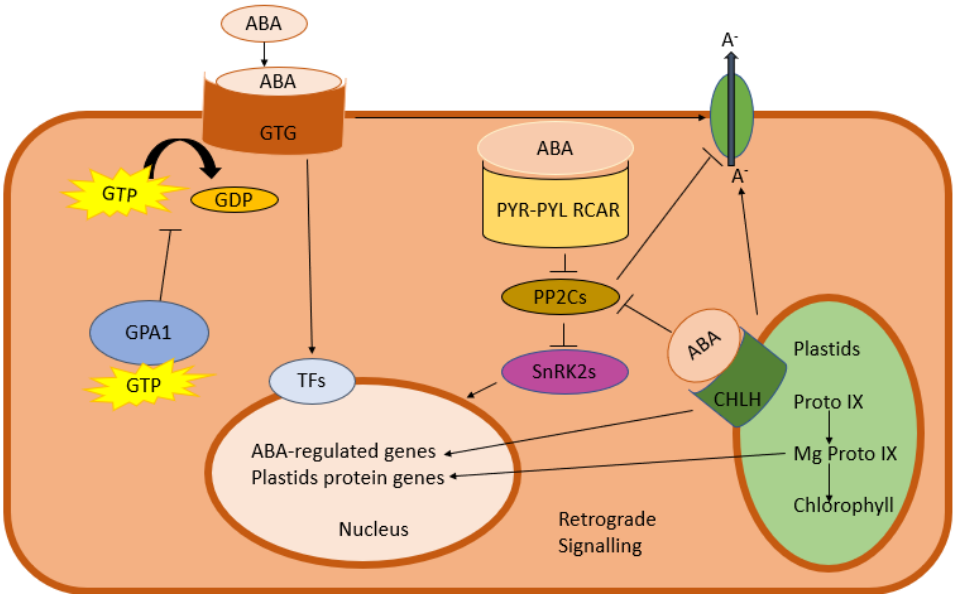


Figure 1.3: A model on how ABA interacts with its receptors in plant cells (adapted from Taiz and Zeiger, 2010).

Key: ABA (Abscisic acid), TFs (Transcription factors), PP2C (Type 2 Protein phosphatases), SnRK2 (Sucrose non-fermenting-1-related protein kinase 2), PYR-PYL/RCAR (Pyrabactin resistanc/Pyrabactin resistance1-like/ Regulatory components of ABA receptors), CHLH (H subunit of Mg-chelatase), GTG (GPCR-type G proteins), GTP (Guanosine triphosphate), GDP (Guanosine diphosphate) and GPA (guanine nucleotide-binding protein alpha-1 subunit).

The GTG receptors are located in the plasma membrane (Taiz and Zeiger, 2010) and are thought to modulate almost all aspects of ABA signalling in plants (Pandey *et al.,* 2006). On the other hand, CHLH receptors are located in the chloroplast/plastids (Taiz and Zeiger, 2010; Du *et al*., 2012) and function in ABA signalling (Wu *et al.*, 2009). However, their precise mechanism of action is still relatively unknown. Nevertheless, their localization in plastids possibly implies that some perception of ABA occurs in these organelles (Taiz and Zeiger, 2010).

Lastly, PYR/PLY/RCAR protein receptors are located in the cytosol and nucleus, and about 14 PYR/PYL/RCAR genes have been found in Arabidopsis and encode highly conserved small proteins with 159-211 amino acid residues (Ma *et al*., 2009; Park *et al*., 2009). PYR1, PYL1 and PYL2 have been shown to directly bind to ABA (Nishimura *et al*., 2009). Figure 1.4 below shows that in the absence of ABA, PYR/PYL proteins do not bind to PP2Cs. Therefore, PP2C activity is high, which prevents phosphorylation and activation of SnRK2s and downstream factors (DFs). However, in the presence of ABA, PYR/PYLs bind to and inhibit PP2Cs, thus allowing the accumulation of phosphorylated downstream factors and ABA transcriptional responses.

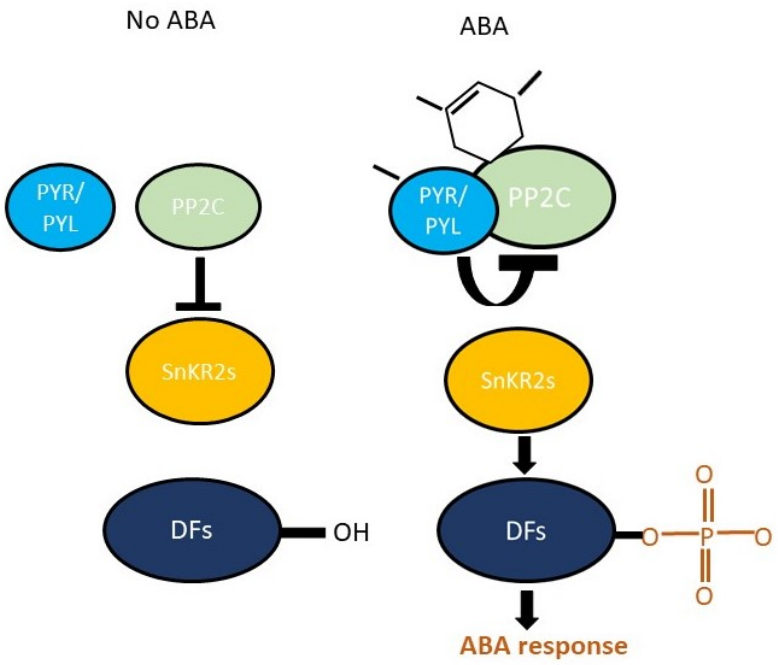


Figure 1.4: A model for PYR/PYL control of ABA signalling (Park *et al.*, 2009).

Key: ABA (Abscisic acid), PYR/PYL (Pyrabactin resistance/Pyrabactin resistance1-like), PP2C (Type 2C protein phosphatase), SnRK2 (Sucrose non-fermenting1-related protein kinase 2), and DFs (Downstream factors).

1.3.5 Regulation of gene expression by ABA

During seed germination and plant responses to abiotic stresses, ABA regulates the expression of numerous genes (Hoth *et al*., 2002). Regulatory classes of proteins known as transcription factors (TFs) coordinate signal transduction and the expression of genes during stress response (Tuteja, 2007; Wani *et al*., 2013). Some of the main classes of TFs include the basic leucine zipper (bZIP), B3 domain (B3), myeloblastosis (MYB), and myelocytomatosis (MYC) families (Alves *et al.*, 2014).

The expression of ABA responsive genes is specifically regulated by TFs that identify and bind to a *cis*–element in the promoter regions up-stream of their target gene (Zhang *et al*., 2005). Furthermore, TFs use receptors, secondary messengers, protein kinase/phosphatase cascades and chromatin-re-modeling factors to control the expression of the ABA responsive genes (Fujita *et al*., 2011). In addition, microRNA (miRNA) targeting, mRNA maturation and stability, and protein degradation all seem to regulate ABA-responsive gene expression (Fujita *et al*., 2011). Under stress conditions, the expression of TFs may be ABA-dependent or ABA-independent (Agarwal and Jha, 2010). Furthermore, some adaptive mechanisms of plants in response to a range of abiotic stresses are induced by the ABA and stress-responsive genes (Shinozaki and Yamaguchi-Shinozak, 1997), proteins and metabolites (Seki *et al.,* 2007).

1.4 Plant response to exogenous ABA

There are several studies that have investigated the effect of exogenous ABA on plants with or without environmental stresses. Wang *et al*. (2013) conducted a study on tomato (*Solanum lycopersicum*) plants, where 60 pots with four seedlings each were grown in a greenhouse. Seedlings were watered every other day. After 45 days of growth, the pots were divided into two groups of 30 pots each for the control and ABA treatment, respectively. The ABA treatment was imposed by spraying plants with 400 mL of 7.58 μmol L^{-1} ABA solution, while for the control, the same volume of purified water was used. After 24 hours of treatment, young leaves were randomly selected for transcriptome analysis using RNA-sequencing technology. Of the 50 616 transcripts that were generated, 21 712 (54.73%) responded to exogenous ABA treatment and 2 787 were differentially expressed. Since ABA mediates the signalling pathway with the help of PYR/PYL/RCARs-PP2Cs-SnRK2s family of proteins, a number of these respective genes were identified in the study. For example, 18 PYL genes, 23 PPC2s and 12 SnRK2s were detected. Of these, 10 PYL transcripts, 13 PPC2s and 5 SnRK2s were

differentially expressed in response to the ABA treatment. Other up-regulated transcripts in response to exogenous ABA included those of heat shock proteins, protein pathogen resistance and those related to salicyclic acid, jasmonic acid, and ethylene signalling pathways. The authors concluded that exogenous ABA is a potential hormone to improve pathogen-resistance and abiotic stress tolerance in tomato (Wang *et al*., 2013).

In another study, Zhou *et al.* (2014) conducted a leaf proteomic analysis of 2-year old tea plants (*Camellia sinensis*) that were grown in a greenhouse in response to exogenous ABA and drought stress imposed by withholding water to the plant. For the treatments, 250 mL of a 50 mg/L ABA solution was sprayed on the plants, while control plants were sprayed with distilled water. After 3 days, drought stress was induced on both ABA treated plants using 10% polyethylene glycol (PEG) and leaves were sampled at 0, 12 and 72 hours post-treatment. Leaf proteins were extracted and separated by two-dimensional gel electrophoresis (2-DE) and identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The results indicated that 21 protein spots were responsive to drought stress in the ABA pre-treated plants. Examples of the up-regulated proteins in response to ABA plus drought stress included lipoxygenase and chloroplastic glutamine synthetase. The authors concluded that during drought stress, ABA played an important role by increasing protein transport and expression of resistance proteins (Zhou *et al*., 2014).

1.5 Aim, objectives and significance of the study

The **aim** of the study was to investigate the biochemical properties and protein expression patterns of sorghum cell suspension cultures in response to exogenous ABA.

The **objectives** of the study were to:

(i) analyse the metabolic activity, and proline content of the sorghum cell suspension cultures in response to exogenous ABA treatment,

(ii) identify differentially expressed intracellular and extracellular matrix proteins of sorghum cell cultures in response to exogenous ABA, and

(iii) bioinformatically analyse the differentially expressed intracellular and extracellular matrix proteins.

Abscisic acid is an important plant hormone that functions as an endogenous messenger and is involved in plant adaptation to biotic and abiotic stresses by mediating a wide range of responses. This study sought to improve our understanding of the proteomic changes of sorghum cell suspension cultures in response to exogenous ABA, and ultimately the identification of ABA-responsive genes for potential use in breeding for abiotic stress-tolerant crops in the future.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant material

White sorghum cell suspension cultures were used in this study. The cell suspension cultures were initiated from friable callus previously established in our research group (Ramulifho, 2017; Ramulifho *et al*., 2019). However, the phenotypic trait of the white sorghum cell cultures is not yet known (Ngara *et al*., 2008; Ramulifho *et al*., 2019).

2.2 Plant tissue culture methods

2.2.1 Maintenance of white sorghum callus

White sorghum callus was maintained on sorghum callus medium [4.4 g/L Murashige and Skoog basal salt with minimal organics (MSMO) medium (Murashige and Skoog, 1962); 3% (w/v) sucrose; 3 mg/L 2,4-dichorophenoxyacetic acid (2,4D); 2.5 mg/L 1-naphaleneacetic acid (NAA); pH adjusted to 5.8 using 1 M NaOH; 0.8% (w/v) bacteriological agar] as previously described (Ngara *et al*., 2008; Ramulifho *et al*., 2019). Briefly, six pea-sized easily breakable callus masses were transferred from 5-week-old callus into a petri dish containing fresh callus medium. Seven petri dishes were prepared, sealed with parafilm and incubated under dark conditions in a growth chamber (Labcon, Mariasburg, South Africa) at 27ºC. Callus growth was visually monitored over a 5-week period.

2.2.2 Initiation and maintenance of white sorghum cell suspension cultures

White sorghum cell suspension cultures were initiated from 5-week-old friable callus as previously described (Ngara *et al*., 2008; Ramulifho *et al*., 2019). About six callus masses were placed in a 250 mL Erlenmeyer flask containing 50 mL of sorghum cell suspension culture

medium. The cell suspension culture medium had the same composition as the callus medium except for bacteriological agar. Four biological replicate cell cultures were initiated, and the flasks were incubated in a shaking incubator at 130 rpm, under dark conditions at 27ºC. After 4 days, 50 mL of fresh medium was added into the flasks and the cultures were further incubated for about two weeks. The cell suspension cultures were maintained by transferring 30 mL of 10-12 days old cells into 250 mL Erlenmeyer flask followed by 70 mL of fresh medium. Cells suspension cultures were sub-cultured four times before treating with abscisic acid (ABA).

2.3 Abscisic acid treatment of white sorghum cell suspension cultures

Eight-day old cultures, growing at the mid-log phase (Ramulifho *et al*., 2019) were aliquoted into two, 30 mL sub-cultures each for the control and ABA treatments. Four biological replicate cultures were prepared for each treatment group. For the ABA treatment, the cell suspension cultures were treated with a final concentration of 100 µM ABA using a 0.1 M ABA (Catalog number: A1049, SIGMA ALDRICH, Saint Louis, USA) stock solution. The 0.1 M ABA stock solution was prepared in 70% (v/v) methanol and filter sterilized using a sterile Millex-GP syringe filter with a Polyethersulfone (PES) membrane of 0.22 µm pore size (Merck, Kenilworth, New Jersey, USA) and stored at -80℃. Control cell cultures were spiked with the same volume of 70% (v/v) methanol as that of the ABA stock solution used in the treatments. All control and ABA-treated cells were incubated at 27℃ in a shaking incubator under dark conditions for 72 hours. During the incubation period, cells were sampled for the determination of cell viability, cell growth and proline content analysis, and protein extraction and expression analyses.

2.4 Cell viability testing using the MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the viability of white sorghum cell suspension cultures following ABA treatment as previously described by Ngara (2009). Four biological replicates of 8 days old white sorghum cell suspension cultures were prepared for both the control and ABA treatments. From both treatment groups, 150 µL of cells were sampled into 1.5 mL Eppendorf tubes at 0, 24, 48 and 72 hours after treatment. Two technical replicates were also prepared for each biological replicate. Into each sample, 50 µL of a 5 mg/mL MTT stock solution was added and tubes were incubated for 30 minutes with gently shaking at room temperature. Thereafter, cells were left to settle at room temperature and then the supernatant was discarded without disrupting the cells. In all tubes, 1 mL of 100% dimethyl sulfoxide (DMSO) was added and incubated for another 10 minutes with gently shaking. Thereafter, the MTT treated cells were left to settle for 5 min at room temperature. Lastly, the supernatant was collected and absorbance was read using a spectrophotometer at 490 nm, starting with the DMSO blank solution.

2.5 Cell growth measurements

The effect of ABA on the growth of white sorghum cell cultures was estimated using the fresh and dry weight measurements at 0 and 72 hours following treatment. Four biological replicates for both the control and ABA treated samples were used. Immediately after treating the cell cultures at 0 hours, 1 mL of the cells was transferred into pre-weighed 1.5 mL Eppendorf tubes for each of the control and ABA treated biological replicate samples. Four technical replicates of each control and ABA treated cell suspension culture were centrifuged at 21 200 × *g* for 5 minutes and the medium was discarded. The fresh weight of the cells was then determined prior to oven-drying the cells for 72 hours at 60℃ for dry weight measurements. At 72 hours

of treatment, the procedure for estimating the fresh and dry weight of the cells was also repeated as described above.

2.6 Proline content analysis

Proline content analysis was estimated for the control and ABA treated cell samples at 72 hours after treatment. Each treatment group of cells had four biological replicates. Samples were prepared following the experimental procedure previously described by Bates *et al.* (1973) with minor modifications. Approximately 100 mg of the ground cell material was homogenized in 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 6000 rpm for 15 minutes to remove cell residue. One millilitre of the extract was mixed with 1 mL of acid nihydrin and 1 mL of glacial acetic acid in a 15 mL Falcon tube before incubation in a water bath at 100℃for 1 hour. The reaction mixture was cooled to room temperature and was extracted with 2 mL toluene by mixing vigorously for 15-20 seconds. The chromophore containing toluene was aspirated from the aqueous phase and warmed at room temperature. Proline standards were prepared in duplicates as indicated in Appendix A, Table A1. Then absorbance readings were taken at 520 nm using a spectrophotometer. The proline concentration was determined using the following equation by Carrillo and Gibon (2011):

Proline in $nmol.mg^{-1}$ FW or in $\mu mol.g^{-1}$ FW = ($Abs_{extract}$ − blank)/slope*$Vol_{extract}$/$Vol_{aliquot}$*1/FW

Key: Absextract is the absorbance determined with the extract, blank (expressed as absorbance) and slope (expressed as absorbance·$nmol^{-1}$) are determined by linear regression, Vol_{extrac}t is the total volume of the extract, $Vol_{aliquot}$ is the volume used in the assay, FW (expressed in mg) is the amount of plant material extracted. It is assumed that $Abs_{extract}$ is within the linear range.

2.7 Protein extraction from white sorghum cell suspension cultures

Eight-day old white sorghum cell suspension cultures were treated with a final concentration of 100 µM ABA. The control samples were spike with 70% (v/v) methanol. Both treatment groups were incubated at 27℃ for 72 hours. After 72 hours, the medium was separated from the cells by filtration over four layers of Miracloth. The cells were briefly washed with sterile distilled water and stored at -80ºC for later use, while the medium was clarified at 2 500 × *g* for 10 minutes. Culture filtrate (CF) proteins were subsequently extracted from the medium for both ABA treated and control cell cultures. On the other hand, the total soluble protein (TSP) was extracted from the cells, for both treatment groups. Both CF and TSP extraction processes are briefly explained below.

2.7.1 Culture filtrate protein extraction

The filtered culture medium, also known as the CF, was transferred into 15 mL Falcon tubes and centrifuged at 2 500 × *g* for 10 minutes. The clarified supernatant was collected and mixed with four volumes of absolute acetone to precipitate secreted proteins, overnight at -20ºC. Thereafter, the samples were centrifuged at 2 500 × *g* for 10 minutes and the supernatant was discarded. The protein pellets were washed three times with ice-cold 80% (v/v) acetone by centrifuging at 21 200 × *g* for 10 minutes per wash, briefly air-dried and resolubilised in extraction buffer [7 M urea, 2 M thiourea and 4% 3-(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)] according to the pellet size. Culture filtrate protein extraction was carried out with vigorous vortexing overnight at room temperature. The solubilized CF proteins were collected in the supernatant fraction after centrifugation at 21 200 × *g* for 10 minutes and stored at -20ºC for use in protein quantification, one-dimensional (1D) gel electrophoresis and isobaric tags for relative and absolute quantitation (iTRAQ) experiments.

2.7.2 Total soluble protein extraction

White sorghum cells previously stored at -80℃ were ground into a fine powder using chilled mortar and pestles. About 0.5 g of the fine powder was transferred into 2 mL Eppendorf tubes, mixed with 1 mL of 10% trichloroacetic acid (TCA) before centrifuging at 9 400 × *g* for 10 minutes. The pellets were washed three times with 1.5 mL of ice-cold 80% (v/v) acetone, briefly air-dried before extracting the TSP with 1.2 mL of extraction buffer by vigorous vortexing overnight at room temperature. The TSP samples were collected from the supernatant fraction after centrifugation at 21 200 × *g* for 10 minutes and stored at -20℃ for use in protein quantification, 1D gel electrophoresis and iTRAQ experiments.

2.8 Protein quantification

The concentration of the protein extracts was estimated using the Bradford assay (Bradford, 1976), with modifications as previously described (Ngara, 2009). Bovine serum albumin (BSA) was used as a protein standard and prepared in duplicates from a 5 mg/mL stock solution as indicated in Appendix A, Table A2. Protein samples were also prepared in duplicate in 2 ml plastic cuvette by adding 10 µL of protein sample, 10 µL 0.1 M HCl and 80 µL distilled water. A ratio of 1:4 was used to dilute the protein assay dye reagent concentrate (Bradford reagent) (BIO-RAD, Hercules, USA) with distilled water. Thereafter, 900 µL of the diluted Bradford reagent was added to all BSA standard solutions and protein samples, mixed well and incubated at room temperature for 5 minutes. The absorbance of all standards and protein samples was measured at 595 nm using the 0 mg/ml BSA standard as a blank solution. The BSA standard solutions were used to plot a standard curve to estimate the concentration of all unknown protein samples.

2.9 One-dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

White sorghum CF and TSP extracts were electrophoresed by 1D SDS PAGE as previously described (Laemmli, 1970). Resolving and stacking gels of 12% (v/v) and 5% (v/v), respectively were prepared as indicated in Appendix A, Table A3 and cast on 1 mm thick plates using a Mini – PROTEAN® Tetra cell (BIO-RAD) gel casting system, according to the manufacturer's instructions. Protein samples were mixed with equal volumes of the protein sample buffer [100 mM Tris HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 200 mM 1,4-dithiothreitol (DTT), and a tint of bromophenol blue], pulse vortexed, centrifuged and incubated on a heat block at 100ºC for 5 minutes. A 5 µL protein ladder (Unstained Protein Standard, Broad Range, catalogue number: P7704S, New England BioLabs, Massachusetts, USA) and 10 µg of each protein extract were loaded on the gels. Gel electrophoresis was performed in running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS], starting at 100 V for the first 30 minutes then at 150 V until the bromophenol blue reached the bottom of the gel.

2.10 Coomassie brilliant blue (CBB) Staining

Coomassie brilliant blue (CBB) R-250 was used to stain the gels to visually analyse protein quality. The gels were stained using three staining solutions: CBB I [0,025% (w/v) CBB R-250, 10% (v/v) glacial acid, 25% (v/v) propan-2-ol], CBB II [10% (v/v) glacial acetic acid, 0.003% (w/v) CBB R-250, 10% (v/v) propan-2-ol] and CBB III [0,003% (w/v) CBB R-250, 10% (v/v) glacial acetic acid]. The gels were incubated in stain I and II for 30 minutes each with gentle shaking, and overnight in stain III. Thereafter, gels were immersed in a de-staining solution (10% (v/v) acetic acid and 1% (v/v) glycerol) until the proteins were clearly

visible. The gels were imaged and documented using a Molecular Imager Gel Doc™ XR+ with Image Lab™ Software version 5.2.1 (BIO-RAD).

2.11 Acetone precipitation of protein samples

The CF and TSP protein samples were acetone precipitated for iTRAQ analysis at Durham University, UK. Both CF and TSP protein extracts were precipitated with 80% (v/v) acetone and incubated at -20℃ overnight. After incubation, samples were centrifuged at 9 400 x *g* for 10 minutes. The CF protein pellets were transferred into 2 mL microcentrifuge tubes and washed three times, whereas the TSP pellets were only washed once with ice-cold 80% (v/v) acetone. Both CF and TSP pellets were stored in 50 µL of ice-cold 80% (v/v) acetone before posting to Durham University, UK for iTRAQ labelling and mass spectrometry. However, the cleaning up of iTRAQ data, statistical analyses for differentially accumulated proteins, and the subsequent bioinformatics analyses of the proteins was done at the University of the Free State, QwaQwa campus.

2.12 The iTRAQ analysis

White sorghum TSP and CF proteins were analysed using an iTRAQ method as previously described by Smith *et al*. (2015) with minor modifications (Goche *et al*., 2020). Prior to iTRAQ analysis, the acetone precipitated TSP and CF samples (Section 2.11) were pelleted by centrifugation at 15 000 × *g* for 5 minutes, before discarding the supernatant, followed by brief pellet air-drying and re-suspension in 100 µL of extraction buffer and vortexing. Thereafter, the protein samples were centrifuged at 15 000 × *g* for 10 minutes and the supernatants containing either TSP or CF proteins was collected for each proteome. The protein samples were quantified using the Bradford assay (Section 2.8) and electrophoresed on a 12% (w/v) 1D

SDS polyacrylamide gel (Section 2.9) to visually analyse the protein quality. For each proteome, four biological replicates of ABA treated and controls were used for analysis.

2.12.1 iTRAQ sample labelling

Sample labelling was done for both the CF and TSP proteomes on 12.5 µg of protein per sample. Four biological replicates of control and ABA treated protein samples were used for both the CF and TSP. Both proteome samples were acetone precipitated overnight at -20℃, left on a bench for an hour at room temperature, and centrifuged at 10 000 x *g* for 10 minutes. The collected protein pellets were air-dried and resolubilized using an iTRAQ Reagent-Multiplex Buffer Kit (AB Sciex, Redwood city, USA) according to the manufacturer's instructions. A volume of 2.5 µL of the denaturant was added to each protein pellet and incubated at 60℃ for 1 hour. Thereafter, 47.5 µL of dissolution buffer was added and vortexed for 20 minutes, before centrifuging for 10 minutes at 15 000 × *g*. The supernatant was collected and mixed with 1 µL of the reducing agent. The samples were incubated for an hour at 60°C and alkylated with 0.5 µL of cysteine blocking agent, vortexed and incubated for 10 minutes at room temperature.

The CF and TSP protein samples were separately digested with trypsin (Promega, Madison, USA), overnight at 37°C. Thereafter, samples were vacuum-dried and resuspended in MilliQ water before adjusting pH for all samples to 7.5 using dissolution buffer. The labelling was conducted for all samples using the 8-plex iTRAQ reagent kit from ABI Sciex according to the manufacturer's instructions. The four control samples of the CF and TSP samples were separately labelled with isobaric tags 113, 114, 115 and 116, whereas the ABA treated samples of the two proteomes were separately labelled with tags 117, 118, 119 and 121. In summary, this study consisted of two separate iTRAQ experiments, one for the control and ABA-treated CF proteins, and the other for the control and ABA-treated TSP samples.

2.12.2 iTRAQ sample clean-up process

Hydrophilic interaction chromatography (HILIC) solid phase extraction (SPE) cartridges (PolyLC Inc.) were used to clean-up samples. The cartridges contained 300 mg of 12 µm polyhydroxyethyl-A, to remove unincorporated label and buffer salts. The cartridges were equilibrated by the sequential addition of 4 x 3 mL releasing solution (5% acetonitrile (ACN), 30 mM ammonium formate, pH 3.0) followed by 4 x 3 mL binding solution (85% ACN, 30 mM ammonium formate, pH 3.0). The freeze-dried iTRAQ-labelled peptide residues were dissolved in 75 µL of 3% 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 using trifluoroacetic acid (TFA). Samples were clarified by centrifugation at 10 000 x *g* for 10 minutes and 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- tandem mass spectrometry (LC-MS/MS).

2.12.3 LC-MS/MS analysis

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

minutes and 35 to 80% B over 2 minutes were followed by a 3-minute column wash in 80% B. Return to 3% B was over 1 minute before column re-equilibration for 8 minutes. Data-dependent top-30 MS-MS acquisition, with collision energy adjusted for iTRAQ-labelled peptides, was started immediately upon gradient initiation and was for 85 minutes. Throughout this period, precursor-ion scans (400 to 1600 m/z) of 250 ms enabled selection of up to 30 multiply charged ions (>500 cps) for CID fragmentation and MS/MS spectrum acquisition (m/z 100-1500) for 50 ms. The cycle time was 1.8 sec and a rolling precursor exclusion of 15 seconds was applied to limit multiple fragmentation of the same peptide. Analyst TF 1.7.1 instrument control and data processing software (AB Sciex) was used to acquire spectrometer data.

2.12.4 Mass spectra data analysis

Protein identification and relative quantification was performed by processing the raw data-files against UniProt protein sequences of *Sorghum bicolor* (downloaded 25 May 2018) using ProteinPilot™ 5.0.1 version 4895 software, incorporating the Paragon™ Algorithm 5.0.1.0.4874, (AB Sciex).

Peptide and protein tables were exported from ProteinPilot for subsequent manual data-handling and filtering. All proteins identified with duplicate proteins and zero unused scores were removed from the data list. Furthermore, all proteins identified with less than two sequenced peptides were removed from the protein list. The abundance of each protein in all samples was calculated as a ratio to the 113-tagged sample in each of the two proteome experiments, CF and TSP. Averages of the ratios for each protein across the 4 replicates in control and ABA- treated of each of the two experiments CF and TSP were calculated. The fold change in

protein expression was denoted by the ratio of control average to ABA treated average samples. Student *t* test was used for statistical analysis at p value ≤ 0.05.

2.12.5 Bioinformatics analysis

The identified proteins from both CF and TSP iTRAQ experiments were functionally annotated using the UniProt database (https://www.uniprot.org). This database was also used to collate the Gene Ontology (GO) information using three terms of Biological Process, Molecular Function and Cellular Component and also to predict the presence or the absence of signal peptide. The Interpro database (https://www.ebi.ac.uk/interpro) was used to determine the conserved domains and family names of the identified proteins.

CHAPTER 3

WHITE SORGHUM CELL SUSPENSION CULTURES: A VALUABLE EXPERIMENATAL SYSTEM FOR SUB-CELLULAR PROTEOMICS STUDIES

3.1 Introduction

Cell suspension cultures are individual plant cells that multiply at a high rate in liquid medium (Cia *et al*., 1987). Their growth and viability can be assessed using a range of methods. For example, cell growth can be determined by measuring the fresh and dry weight of cells at specific time-points during the growth cycle (Evans *et al*., 2003). On the other hand, cell viability assays are important in determining the overall metabolic state of cells before and after specific treatments (Aslantürk, 2018). For example, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a widely used and reliable technique for cell viability measurements (Fotakis and Timbrell, 2006). When cells are incubated with a yellow tetrazolium reagent, viable cell will convert it into a purple formazan product (Yang *et al.*, 2015), which is then quantified as absorbance at 490 nm (Berridge *et al*., 2005).

Plant cell suspension cultures are frequently employed in various plant biology studies (Mustafa *et al*., 2011). They are used in the investigation of physiological processes at the cellular and molecular levels due to the homogeneity of cell populations, the large availability of experimental material, rapid cell growth, and well-controlled growth conditions (Hall, 2000). Cell cultures are also excellent experimental tools in studies of the composition of proteins in both the intracellular [total soluble proteins, (TSP)] and extracellular [culture filtrate (CF)/secreted proteins] compartments of cells, as well as for gene expression analysis

(Ramulifho *et al*., 2019) in response to biotic and abiotic stress factors, including exogenous abscisic acid (ABA).

Both the TSP and secreted proteins can be separated and initially analysed using gel-based techniques, such as one dimensional (1D) or two-dimensional (2D) gel electrophoresis, prior to the more expensive gel-free methods (Baggerman *et al*., 2005). The 1D gel electrophoretic method is a widely used, and separates proteins based on their molecular weight (Shi and Jackowski, 1998). There are studies reported on the application of exogenous abscisic acid (ABA) under different abiotic stresses such as cold (Rajashekar and Lafta, 1996), drought stress (Pattanagul, 2011), and under normal conditions in transcriptomics analysis of tomato (*Solanum lycopersicum*) (Wang *et al*., 2013). The advantage of using 1D gels is that they are not complex to run and they are relatively cheap (Galeva and Altermann, 2002). Therefore, this chapter aimed to explore the potential application of white sorghum cell cultures in studying the TSP and secreted protein fractions of sorghum in response to exogenous ABA.

3.2 Results

3.2.1 Maintenance of white sorghum callus

White sorghum callus was maintained on sorghum callus medium at 27℃ and sub-cultured every five weeks as described in Section 2.2.1. Figure 3.1 below shows 5-week old callus masses ready for sub-culturing.

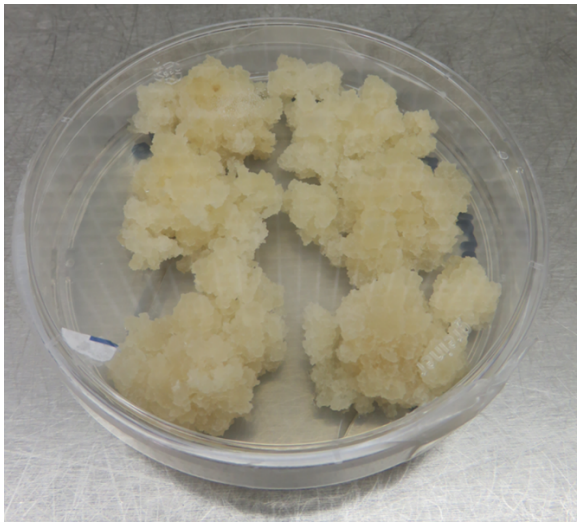


Figure 3.1: Five weeks old white sorghum callus masses.

3.2.2 Initiation, maintenance, and ABA treatment of cell suspension cultures

Easily breakable callus was used to initiate cell suspension cultures in liquid medium at 27℃ as described in Section 2.2.2. Figure 3.2A shows a fine white sorghum cell suspension culture ready for sub-culturing at day 12. During the growth cycle, such cell cultures were treated with 100 µM ABA for 72 hours and filtered to generate cells (Figure 3.2B) and spent medium (Figure 3.4C) fractions for use in sub-cellular proteomic analyses in response to the exogenous ABA treatment (Chapter 4). Proteins obtained from the cells (Figure 3.2B) are known as the TSP, while those obtained from the medium (Figure 3.2C) are known as the CF or secreted proteins.

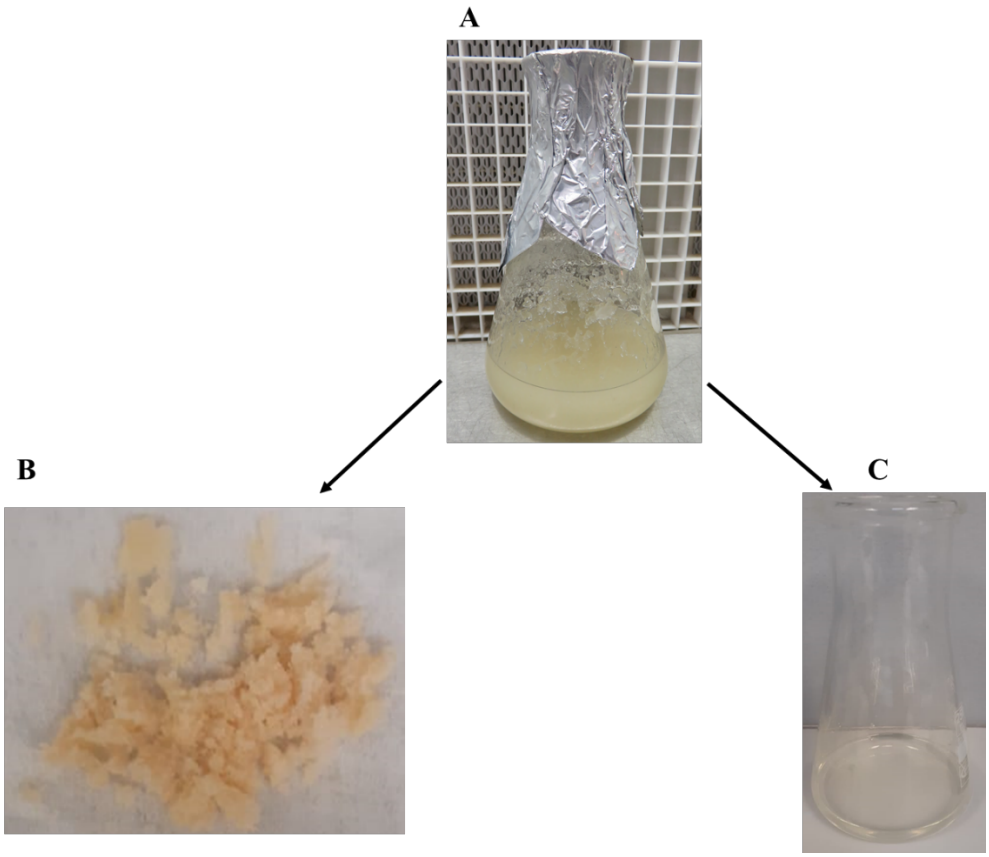


Figure 3.2: A white sorghum cell suspension culture and its compartments for sub-cellular proteomics. (A) shows fine white sorghum cell suspension culture, (B) shows filtered cells and (C) shows medium.

3.2.3 The viability of the sorghum cell cultures

White sorghum cell suspension cultures were treated with 100 µM ABA for 72 hours and the MTT assay was used to determine cell viability. The viability was determined in four biological replicates of the cell cultures of both the control and ABA treatment groups at 0, 24, 48, and 72 hours (Section 2.4). The Student's *t*-test $p \leq 0.05$, was used for statistical analysis, and results showed that there was no significant difference in cell viability between the controls and ABA treated cells throughout the 72-hour treatment period (Figure 3.3).

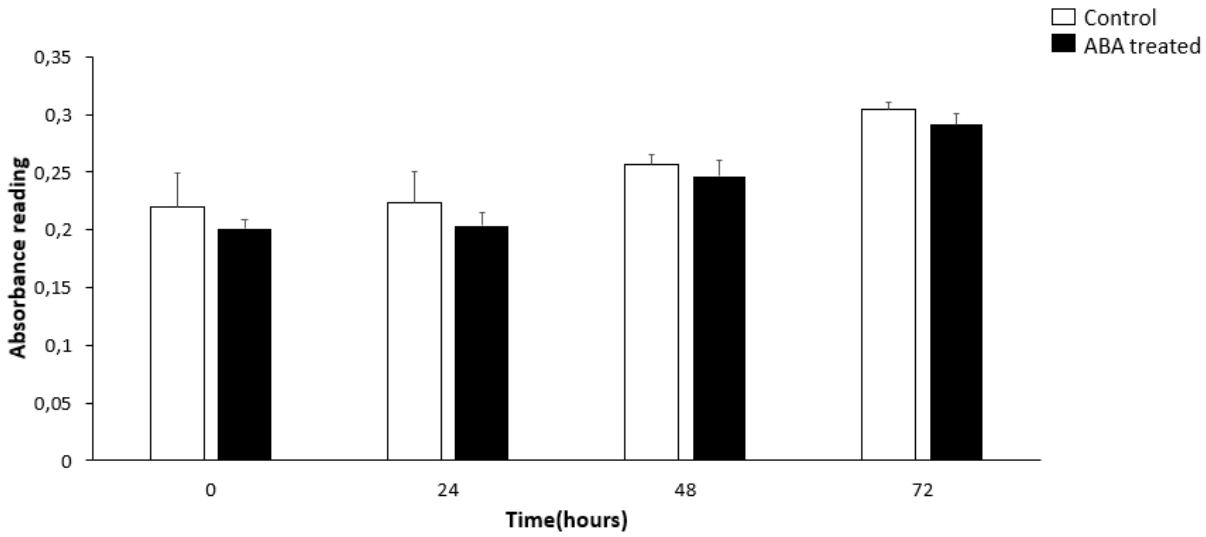


Figure 3.3: Cell viability of white sorghum cell suspension cultures following exogenous ABA treatment. White sorghum cell suspension cultures were treated with 100 µM ABA for 72 hours and viability was estimated using the MTT assay at 0, 24, 48 and 72 hours.

3.2.4 Cell growth measurements

The growth of control and ABA treated white sorghum cell cultures was assessed by measuring fresh and dry weight of the cells at 0 and 72 hours following treatment. Four biological replicates for both control and ABA treated samples were used. Results shown in Figure 3.4 illustrate that there was no significant different between the control and ABA treated cells for both fresh (Figure 3.4A) and dry (Figure 3.4B) weight readings at both time points.

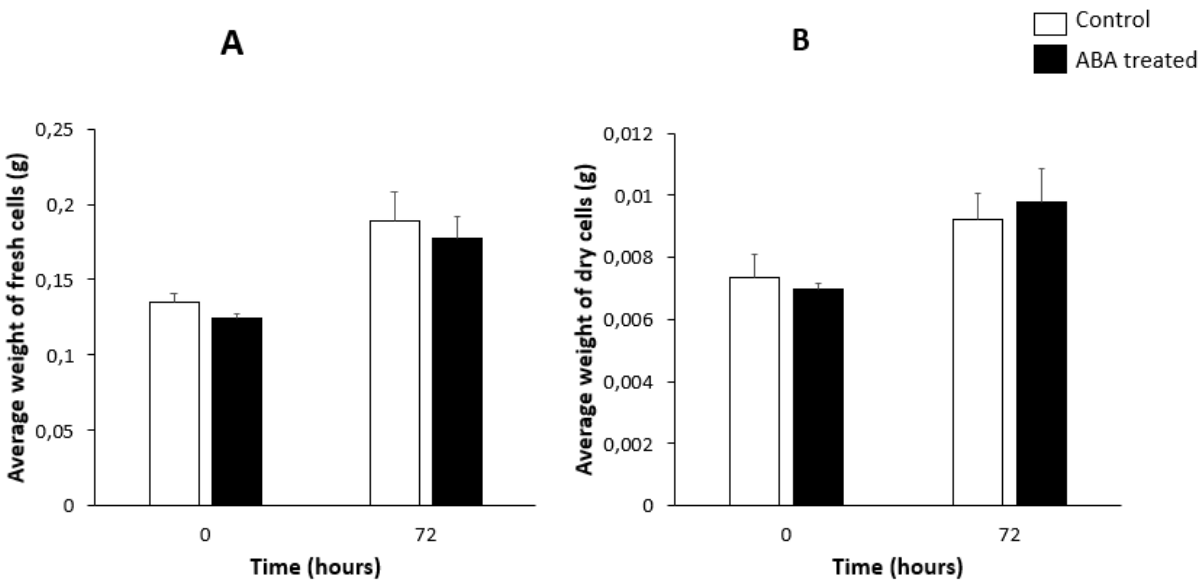


Figure 3.4: Growth estimation of cells at 0 and 72 hours after ABA treatment. (A) is the average weight of fresh cells in grams, and (B) is the average weight of oven-dried cells in grams.

3.2.5 Proline content analysis

Proline is one of the osmolytes that plays an important role when plants are experiencing abiotic stresses (Rejeb *et al*., 2014). Proline was determined in the control and ABA treated cell samples and results are shown in Figure 3.5. The results showed that the exogenous 100 µM ABA application increased proline content by two-fold compared to the control (Figure 3.5).

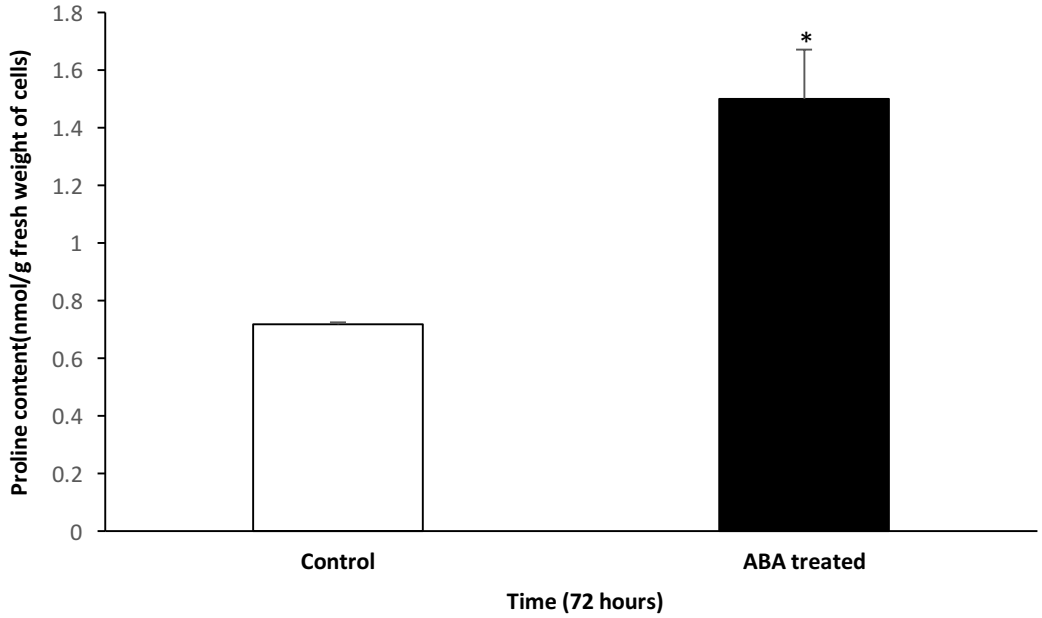


Figure 3.5: Proline content of white sorghum cell suspension cultures after exogenous ABA treatment. The proline content analysis was conducted at 72 hours following treatment. Data presented as mean ± SE (*n=4*). * Represents the statistical significant difference at $p \leq 0.05$ using Student's *t*-test.

3.2.6 1D gel analysis of the white sorghum cell suspension total soluble proteins and secreted proteins

Eight-day old white sorghum cell cultures were treated with 100 μM ABA for 72 hours. Following the ABA treatment, cells were separated from the cell culture medium using filtration and centrifugation techniques to obtain two cell culture compartments (Figure 3.2) for subcellular proteome analyses. The extracted proteins from the two compartments are the TSP from the cell component (Figure 3.6A), and the CF proteins also knows as secreted proteins from the spent medium (Figure 3.6B), and are illustrated in the 1D gels pictures.

It was observed that the protein profiles in both proteomes showed up- and down-regulation of specific proteins (Figure 3.6). For example, at around 15 kDa region, both proteomes showed an up-regulation of proteins (yellow arrows) (Figures 3.6 A and B) and at around 25 kDa region, the TSP showed a down-regulation (pink arrow) (Figure 3.6A). On the other hand, the

secreted proteins showed a down-regulation at around 80 kDa (Figure 3.6B). Overall, the 1D SDS-PAGE yielded good results by showing good, well-separated protein profiles with up- and down-regulations of proteins in response to the ABA treatment. Therefore, a gel free proteomic method using isobaric tags for relative and absolute quantitation (iTRAQ) analysis was conducted to investigate the differential expression of these two proteomes in response to 100 µM ABA (Chapter 4).

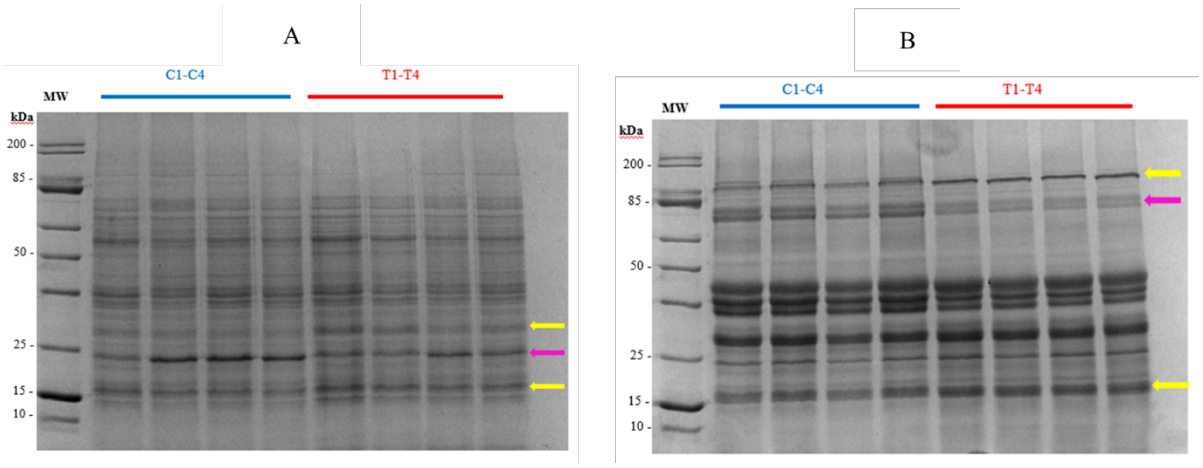


Figure 3.6: 1D SDS-PAGE profile of the two proteomes of white sorghum cell suspension cultures. (A) show the TSP and (B) shows the secreted protein samples. Lane 1 represents the molecular weight markers in kDa. C1-C4 represents the control samples, while T1-T4 represents the 100 µM ABA treated samples for each proteome. Gels were stained using Coomassie Brilliant Blue (CBB) R-250, imaged and documented using a Molecular Imager Gel DocTM XR+ with Image LabTM Software version 5.2.1 (BIO-RAD).

3.3 Discussion

In this study, white sorghum cell suspension cultures were used as an experimental system to study two distinct proteomes of plant cells in response to an exogenous application of 100 µM ABA. ABA is a plant hormone that plays important roles in many aspects of a plant's life such as growth and development, seed dormancy, stimulation of stomatal closure during drought, and also the induction of genes, proteins and metabolites expression in response to stresses (Shu *et al*., 2016; Chen *et al*., 2020).

Following the ABA treatment, cell growth parameters were assessed by measuring fresh and dry weight of the control and ABA treated cells. The results showed that cells were growing well for both treatment groups (Figure 3.4) with no obvious signs of cell growth retardation after the exogenous ABA application. Only a few studies have reported on the effects of exogenous application of ABA in plants under normal conditions (Chen *et al*., 2022). However, Li and co-workers observed that exogenous ABA promotes adventitious root development in cucumber (*Cucumis sativus*) (Li *et al*., 2022). In other studies, exogenous ABA was observed to induce the expression of genes that promote growth of plants under different types of stresses such as drought (Gai *et al*., 2020), and salt (Li *et al*., 2020).

The MTT assay was used to determine the viability of the cells, in particular their metabolic activity following the ABA treatment relative to the control. The results showed that exogenous ABA did not affect the viability of sorghum cell cultures over 72 hours of treatment (Figure 3.3). Therefore, it was of interest to further investigate if the ABA treatment used in the current study had some effect on a known metabolic process – such as the accumulation of proline. Proline is an osmolyte that is known to accumulate in plants that are subjected to adverse

environmental conditions (Hare *et al*., 1999). It protects protein structures against denaturation and stabilises cell membranes (Bohnert and Jensen, 1996).

The levels of proline have been reported to increase in many different plant species under different stress conditions (Delauney and Verma, 1993; Mansour, 2000; Claussen, 2005). Exogenous ABA has also been reported to increase the proline content under drought (Latif, 2014) and salinity (Kaur and Asthir, 2020) stresses. In the current study, similar results were observed, with a two-fold increased accumulation of proline in the 100 µM ABA treated samples relative to the control (Figure 3.5). These results are also supported by Aroca *et al.* (2008), who reported increased proline content in tomato plants under drought stress.

One dimensional gel electrophoresis was used to visually analyse the quality of the two proteomes, namely the TSP from the cells and the secreted proteins from the culture medium. The results showed that both extracts were of good quality without any vertical or horizontal streaking (Figure 3.6). Furthermore, while there could be common proteins between the two profiles (Figure 3.6), the overall protein banding patterns of the TSP (Figure 3.6A) and that of the secreted proteins (Figure 3.6B) were different both in the control samples and those after the application of exogenous ABA. This shows that sorghum cell suspension cultures can be used to study subcellular proteomics of plants (Ngara *et al*., 2008). It was also observed from the gels that exogenous ABA treatment induces the up- and down-regulation of proteins in both the TSP (Figure 3.6A) and secreted proteins (Figure 3.6B). Therefore, it would be of great interest to study these differentially expressed proteins of the two cell compartments in response to the stress phytohormone ABA.

3.4 Conclusion

In conclusion, the application of exogenous 100 μM ABA neither affects the growth of sorghum cell cultures nor its viability. However, 100 μM ABA enhances the accumulation of proline in the sorghum cells, possibly highlighting the importance of ABA during stress response. The 1D gel-based protein separation method showed that some proteins were differentially expressed in the TSP and secreted protein fractions in response to exogenous ABA, and further analysis of these proteins would be conducted by iTRAQ and mass spectrometry in Chapter 4.

CHAPTER 4

COMPARATIVE PROTEOMIC ANALYSIS OF ABA-RESPONSIVE INTRACELLULAR AND EXTRACELLULAR MATRIX PROTEINS OF SORGHUM CELL CULTURES

4.1 Introduction

Global warming negatively affects plant growth by increasing environmental temperatures (IPCC, 2014) and reducing the amount of rainfall (Gibson-Forty *et al*., 2016). In turn, plants respond to these stresses through a range of morphological, physiological, biochemical and molecular mechanisms (Howarth & Ougham, 1993). For example, when exposed to cellular dehydration, plants accumulate the endogenous phytohormone abscisic acid (ABA), which controls osmotic adjustment, stomatal closure and gene expression (Finkelstein *et al.,* 2002; Hirayama and Shinozaki 2007; Cutler *et al.,* 2010). To date, numerous dehydration-responsive genes have been identified, and many of them can also be induced by exogenous ABA treatment (Yamaguchi-Shinozaki and Shinozaki, 2006). Genes are important because they contain the genetic information required to synthesise proteins and metabolites.

Proteins are involved in almost all biological processes such as structural support, cellular transport, and enzymatic activity (Whitford, 2013). So, protein identification and analysis of their expression profiles provides an understanding of how these molecules interact to produce and sustain a well-functioning biological system (Vercauteren *et al.,* 2007). The cell also regulates the level and activity of its proteins in response to internal and external changes. As a result, proteome expression patterns change, whether qualitative or quantitative in nature (Wu *et al.,* 2014).

On the basis of their cellular location, proteins can be classified into two major groups. Proteins found inside a cell are known as intracellular or total soluble proteins (TSPs), whereas the ones that are transported out of the cell into the extracellular space are known as extracellular, culture filtrate (CF) or secreted proteins (Denecke *et al.,* 1990). The current chapter is a comparative analysis of TSP and CF protein expression changes of sorghum cell cultures in response to an exogenous application of ABA.

Plant proteomics technologies are diverse and range from gel-based and non-gel-based methods as well as label-free and label-based methods to separate and analyse both TSP and secreted proteins (Abdallah *et al.,* 2012). In the current study, the gel-free and label-based isobaric tags for relative and absolute quantification (iTRAQ) method was used for the identification of proteins that are responsive to exogenous ABA. Previous proteomic studies have shown that the amounts of TSP in plant tissues are altered in response to drought stress (Mohammadkhani and Heidari, 2008) as well as exogenous ABA (Hirayama and Shinozaki, 2007; Zhou *et al.,* 2014). Mohammadkhani and Heidari (2008) conducted a polyethylene glycol (PEG)-6000-induced drought experiment on two maize (*Zea mays*) genotypes and analysed its effects on root and leaf TSP accumulated by on one dimensional (1D) gel electrophoresis. After 24 hours of treatment, the two maize varieties initially showed an increase in TSP content followed by a decrease as the stress progressed (Mohammadkhani and Heidari, 2008).

In another study by Zhou *et al.* (2014), the leaf TSP of tea plants (*Camellia sinensis*) was up-regulated after exogenous ABA application on drought stressed plants. Briefly, two years old tea plants were sprayed with 250 mL of a 50 mg.-L^{-1} ABA solution, while controls were sprayed with distilled water. After 3 days, 10% PEG-6000 was used to induce drought stress.

Treated leaves were sampled and total soluble proteins were extracted. Two-dimensional (2-D) gel electrophoresis was used to separate differentially expressed proteins. Over 700 protein spots were detected, and 18 of these were differentially expressed under ABA treatment. These proteins include lipoxygenase, glutamine synthetase, glutathione transferase lambda 2, heat shock proteins (HSP) 90, and ascorbate peroxidase.

Currently, there is no proteomic information on the TSP and CF proteins of cell cultures after the application of exogenous ABA. Therefore, the objective of this chapter is to identify and comparatively analyse the differentially expressed TSP and CF proteins of white sorghum cell suspension cultures in response to exogenous ABA using the iTRAQ method.

4.2 Results

4.2.1 iTRAQ analysis of ABA-responsive TSP and CF proteins of white sorghum cell cultures

In this study, a total of 725 total soluble proteins were positively identified and 46 of these were differentially expressed in response to the exogenous ABA treatment. Out of the 46 differentially expressed TSP proteins, 30 were up-regulated, while 16 were down-regulated. In contrast, 256 secreted proteins were positively identified in the iTRAQ experiment of which 82 were differentially expressed in response to the exogenous ABA treatment. Forty-eight of these secreted proteins were up-regulated while 34 was down-regulated. A summary of the total number of positively identified proteins, those that are ABA-responsive, as well as up and down-regulated ABA-responsive proteins of TSP and CF proteomes is shown in Table 4.1.

Table 4.1: Summary of TSP and CF proteins from iTRAQ data.

Proteome	Total number of proteins	Total number of ABA responsive proteins	Up regulated proteins	Down regulated proteins
TSP	725	46 (6.3%)[a]	30 (65.2%)[b]	16 (34.8%)[c]
CF	256	82 (32%)[a]	48 (58.5%)[b]	34 (41.5%)[c]

[a] % of ABA responsive proteins as a proportion of the total number of proteins
[b]total number of up-regulated proteins as a proportion of the ABA responsive proteins
[c]total number of down-regulated proteins as a proportion of the ABA responsive proteins

A total of 128 ABA-responsive proteins were identified in this study in the combined proteomes. Of these 128 proteins, 8 proteins were common to both the TSP and CF proteomes as shown in Figure 4.1, while 38 proteins were unique to the TSP and the remaining 74 were unique to the CF (Figure 4.1).

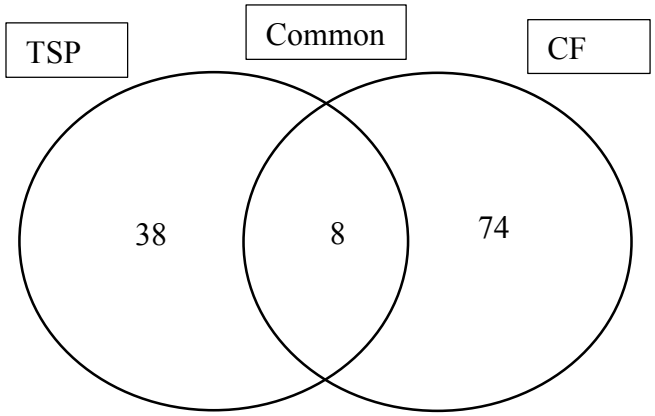


Figure 4.1: The distribution of ABA responsive proteins of the sorghum intracellular and extracellular proteomes.

Bioinformatics analyses were also conducted on the ABA-responsive proteins of the two proteomes to get information on the presence or absence of signal peptides, Gene Ontology (GO) and conserved domain and protein family names. Separate lists of the ABA-responsive

white sorghum cell culture TSP and CF proteomes are provided in Tables 4.2 and 4.3, respectively.

Table 4.2: List of ABA-responsive total soluble proteins of white sorghum cell cultures.

Pro.#[a]	Access..#[b]	Protein Name	Score[c]	%Cov[d]	Npd[e]	Fold change	StDEV[f]	p-Value[g]	Sp[h]	GO analysis[i]			Conserved domain and family name[j]
										P	F	C	
METABOLISM													
44	A0A1B6PEZ5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G014700	28.7	52.99	16	1.15	0.11	4.45E-02	No	Fructose metabolic process	Fructokinase activity	Cytosol	Carbohydrate kinase PfkB Family not predicted
51	C5YU02	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G083400	27.74	31.20	15	1.15	0.04	2.00E-02	No	Argininosuccinate metabolic process	Argininosuccinate synthase activity	Cytoplasm	Domain not predicted; Argininosuccinate synthase
76	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	22.32	21.25	14	1.55	0.14	8.60E-04	No	Cellulose catabolic process	Cellulase activity	Extracellular region	Carbohydrate binding domain CBM49; Glycoside hydrolase family 9
81	A0A194YT53	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G345800	21.79	33.69	14	1.41	0.15	2.70E-03	No	Phenylacetate catabolic process	Acetyl-CoA C-acyltransferase activity	Peroxisome	Thiolase, N-terminal, Thiolase, C-terminal; Thiolase
113	A0A1W0VUE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G227400	18.01	13.83	9	1.26	0.12	1.74E-02	No	Carbohydrate metabolic process	Carbohydrate binding	None	Glycoside hydrolase family 31, N-terminal domain; Glycoside hydrolase family 31
181	C5WXC7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	13.48	23.71	10	0.73	0.09	2.10E-02	Yes	Carbohydrate metabolic process	Raffinose alpha-galactosidase activity	Cell Wall	Alpha galactosidase, C-terminal beta sandwich domain; Glycoside hydrolase, family 27
308	C5WY32	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G061900	8.12	8.48	4	1.32	0.16	3.05E-02	Yes	Carbohydrate metabolic process	Glucan endo-1,3-beta-D-glucosidase activity	Plasma Membrane	X8 domain; Glycoside hydrolase family 17
323	A0A194YRE9	Glutamine synthetase OS=*Sorghum bicolor* GN=SORBI_3004G247000	8.37	18.82	5	1.33	0.23	4.76E-02	No	Glutamine biosynthetic process	Glutamate-ammonia ligase activity	Cytoplasm	Glutamine synthetase, catalytic domain; Glutamine synthetase.
333	A0A1Z5RB28	Patatin OS=*Sorghum bicolor* GN=SORBI_3007G158800	7.45	11.97	5	1.35	0.21	1.67E-02	No	Lipid catabolic process	Phospholipase activity	None	Patatin-like phospholipase domain; Patatin Famaily
340	C5XI18	S-adenosylmethionine synthase OS=*Sorghum bicolor* GN=SORBI_3003G140000	6.95	8.59	3	0.73	0.05	1.07E-03	No	S-adenosylmethionine biosynthetic process	Methionine adenosyltransferase activity	Cytoplasm	S-adenosylmethionine synthetase, N-terminal, S-adenosylmethionine synthetase, C-terminal; S-adenosylmethionine synthetase
424	C5YE18	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G181300	5.5	6.18	4	1.82	0.28	4.99E-02	No	Pigment biosynthetic process	Catechol oxidase activity	None	Polyphenol oxidase, central domain, Tyrosinase copper-binding domain; Polyphenol oxidase
510	C5X4M5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255000	4.02	8.68	2	0.69	0.11	3.61E-02	Yes	None	None	None	DOMON domain; Family not predicted
552	C5YGY0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G043400	3.78	3.81	2	1.65	0.29	3.99E-02	No	Carbohydrate metabolic process	Sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate	Chloroplast	Doman not predicted; Transaldolase/Fructose-6-phosphate aldolase

											glyceronetransferase activity		
577	C5YIY2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G079600	3.56	6.25	2	1.27	0.16	3.35E-02	No	Fatty acid biosynthetic process	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase activity	Cytoplasm	Domain not predicted; Beta-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ
594	Q94IP1	Cinnamic acid 4-hydroxylase OS=*Sorghum bicolor* GN=C4H	3.42	4.39	2	0.83	0.06	3.79E-02	No	Lignin metabolic process	Trans-cinnamate 4-monooxygenase activity	Membrane	Domain not predicted; Cytochrome P450
596	C5XRZ8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G296800	3.28	8.80	2	1.28	0.13	8.89E-03	No	None	Lyase activity	None	Aconitase A/isopropylmalate dehydratase small subunit, swivel domain, 3-isopropylmalate dehydratase, swivel domain, Family not predicted
855	A0A1W0W560	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G195700	2.09	5.98	2	1.31	0.18	4.55E-02	No	Lignin biosynthetic process	Cinnamyl-alcohol dehydrogenase activity	None	Alcohol dehydrogenase, C-terminal,Alcohol dehydrogenase, N-terminal; Family not predicted
PROTEIN DESTINATION AND STORAGE													
378	C5WNX2	Proteasome subunit beta type OS=*Sorghum bicolor* GN=SORBI_3001G128400	6.17	21.43	4	0.80	0.07	4.58E-02	No	Proteasomal protein catabolic process	Endopeptidase activity	Nucleus	Domain not predicted; Proteasome, subunit alpha/beta
525	C5Z6F3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G080300	4	4.33	2	0.65	0.10	4.28E-02	Yes	Cellular amino acid metabolic process	Aminoacylase activity	Cytoplasm	Peptidase M20, dimerisation domain; Peptidase M20
561	C5Z3R9	Proteasome subunit beta type OS=*Sorghum bicolor* GN=SORBI_3010G029400	3.71	7.76	2	1.30	0.04	6.25E-04	No	Proteasomal protein catabolic process	Endopeptidase activity	Nucleus	Domain not predicted; Proteasome, subunit alpha/beta.
DEFENCE													
35	A0A194YU12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G341200	31.7	39.39	19	1.41	0.26	2.76E-02	No	Cell redox homeostasis	Thioredoxin-disulfide reductase activity	Cytoplasm	Pyridine nucleotide-disulphide oxidoreductase, dimerization; Glutathione-disulphide reductase family
70	C5XIY1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152100	23.45	37.82	19	2.98	1.22	1.74E-02	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
63	A0A1B6PFE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G038600	24.37	28.57	12	1.15	0.06	4.73E-02	No	Response to cold	Oxidoreductase activity	Chloroplast	FAD/NAD(P)-binding domain; FAD/NAD(P)-binding domain superfamily
117	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600	18.63	36.94	13	2.10	0.14	5.53E-05	Yes	Negative regulation of catalytic activity	Enzyme inhibitor activity	None	Leucine-rich repeat-containing N-terminal, plant-type; Family not predicted
132	C5WWQ2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G342600	16.6	14.73	7	0.63	0.07	9.51E-03	No	None	None	None	Thioredoxin domain; Thioredoxin-like superfamily
235	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	10.58	21.83	5	1.39	0.21	2.17E-02	Yes	Defense response	None	Extracellular region or secreted	Domain not predicted;Thaumatin family

243	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G129700	10.16	30.22	5	1.33	0.20	2.54E-02	Yes	None	Manganese ion binding	Extracellular region or secreted	Cupin 1; Germin
346	C5XG44	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G254300	6.78	25.93	3	1.31	0.21	4.88E-02	No	Cell redox homeostasis	Thioredoxin peroxidase activity	Cytoplasm	Redoxin, Thioredoxin domain; Peroxiredoxin-5-like
401	C5Z4V3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G051100	6.06	10.28	4	0.85	0.11	4.16E-02	Yes	Response to endoplasmic reticulum stress	Protein disulfide isomerase activity	Endoplasmic reticulum	Thioredoxin domain; Thioredoxin-like superfamily
456	C5Z513	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G171800	4.74	4.84	2	0.76	0.09	6.98E-03	Yes	None	Oxidoreductase activity	Extracellular region	Multicopper oxidase, type 1, Multicopper oxidase, type 2; L-ascorbate oxidase, plants
600	A0A1B6QFT1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G392000	10.7	11.87	5	0.72	0.15	2.01E-02	No	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase, Plant peroxidase
SIGNAL TRANSDUCTION													
47	C5WMM0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G400900	28.41	92.50	23	1.47	0.23	8.53E-03	No	Abscisic acid-activated signaling pathway	Abscisic acid binding	Nucleus	Bet v I/Major latex protein; Bet v I type allergen
167	Q4VQB4	Pathogenesis-related protein 10c OS=*Sorghum bicolor* GN=PR10	24.5	84.91	23	1.55	0.34	2.21E-02	No	Abscisic acid-activated signaling pathway	Abscisic acid binding	Nucleus	Bet v I/Major latex protein; Bet v I type allergen
CELL GROWTH/DIVISION													
160	C5XV51	Proliferating cell nuclear antigen OS=*Sorghum bicolor* GN=SORBI_3004G336600	14.58	36.50	10	1.27	0.12	2.61E-02	No	Mismatch repair	DNA polymerase processivity factor activity	Nucleus	Proliferating cell nuclear antigen, PCNA, N-terminal, Proliferating cell nuclear antigen, PCNA, C-terminal; Proliferating cell nuclear antigen, PCNA
268	C5WV02	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G033300	9.32	17.94	6	1.32	0.16	4.58E-02	Yes	Plant-type cell wall organization	None	Cell wall	Expansin/pollen allergen, DPBB domain; Expansin
423	C5WSF9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301500 P	6.55	10.28	5	1.32	0.17	1.70E-02	Yes	Sexual reproduction	None	Extracellular region	Expansin/pollen allergen, DPBB domain; Expansin/Lol pI
ENERGY													
31	C5XFH6	Fructose-bisphosphate aldolase OS=*Sorghum bicolor* GN=SORBI_3003G393900	33.14	56.9	21	0.79	0.13	2.83E-02	No	Glycolytic process	Fructose-bisphosphate aldolase activity	Cytosol	Domain not predicted; Fructose-bisphosphate aldolase, class-I family
41	C5XW45	Glyceraldehyde-3-phosphate dehydrogenase OS=*Sorghum bicolor* N=SORBI_3004G056400	36.1	47.94	22	1.28	0.18	2.12E-02	No	Glycolytic process	Glyceraldehyde-3-phosphate dehydrogenase (NAD+)(phosphorylating) activity	Cytosol	Glyceraldehyde 3-phosphate dehydrogenase, NAD(P) binding domain; Glyceraldehyde-3-phosphate dehydrogenase, type I
205	C5YAI8	Pyruvate kinase OS=*Sorghum bicolor* GN=SORBI_3006G267200	12.06	14.04	6	0.74	0.09	2.36E-02	No	Glycolytic process	Pyruvate kinase activity	Cytoplasm	Pyruvate kinase, barrel, Pyruvate kinase, C-terminal; Pyruvate kinase

496	C5X951	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G167000	12.71	9.10	9	0.77	0.05	3.61E-02	No	Photosynthesis	Phosphoenolpyruvate carboxylase activity	Chloroplast	Domain not predicted; Phosphoenolpyruvate carboxylase
36	A0A194YGV9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G023700	31.25	23.24	16	1.23	0.18	4.74E-02	No	Pentose-phosphate shunt	Transketolase activity	Cytosol	Transketolase, N-terminal; Transketolase family
573	C5YK12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G100600	3.54	13.50	2	1.65	0.38	4.08E-02	Yes	None	Electron transfer activity	Plasma Membrane	Phytocyanin domain; Phytocyanin
UNCLEAR CLASSIFICATION													
168	C5XNL6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G189000	14.19	33.99	7	0.83	0.08	1.79E-02	No	None	GTPase activity	None	Small GTP-binding protein domain; Small GTPase
288	C5WQD6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G145400	9.43	8.39	6	0.72	0.12	3.46E-02	No	Threonyl-tRNA aminoacylation	Aminoacyl-tRNA ligase activity	Chloroplast	Aminoacyl-tRNA synthetase, class II (G/ P/ S/T), Aminoacyl-tRNA synthetase, class II; Threonine-tRNA ligase, class IIa
342	C5X487	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G111200	6.86	15.42	4	0.75	0.06	4.47E-02	No	None	Oxidoreductase activity	None	Domain not predicted; Short-chain dehydrogenase/reductase SDR
571	C5XVQ6	Glycosyltransferase OS=*Sorghum bicolor* GN=SORBI_3004G191000	3.73	6.86	3	1.33	0.12	4.93E-03	No	None	Quercetin 3-O-glucosyltransferase activity	None	UDP-glycosyltransferase family, conserved site; UDP-glucuronosyl/UDP-glucosyltransferase

[a] Protein number assigned in ProteinPilot software.

[b] Protein accession numbers obtained from the UniProt (https://www.uniprot.org/uniprot/) database against sequences of *Sorghum bicolor* only.

[c] Protein score generated by ProteinPilot software relating to the confidence of protein identification. Only proteins with 95% confidence interval were retained

[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] Number of peptide that were sequenced and contributed towards the protein identity. Proteins with 0 or 1 peptide were filtered out.

[f]Standard deviation of the protein samples (n=4).

[g]Probability value of the quantitative difference between the proteins from the control and the ABA-treated samples.

[h] Signal peptide predicted using the UniProt database (http://www.uniprot.org). Yes, means the presence of the signal peptide and the No means no signal peptide was predicted.

[i] 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.

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

Table 4.3: List of ABA-responsive culture filtrate proteins of white sorghum cell cultures.

Pro.#[a]	Access #[b]	Protein name	Score[c]	% Cov[d]	Npd[e]	Fold Change	StDEV[f]	p-Value[g]	Sp[h]	GO analysis[i]			Conserved domain and family name[j]
										P	F	C	
DEFENCE													
1	C5Z475	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G162000	931	84	199	1.42	0.24	1.49E-02	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase Family
5	C5X5K6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416700	55.45	56.55	76	0.72	0.03	3.53E-03	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
6	C5WYQ4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G360400	53.31	65.96	70	0.61	0.09	3.82E-04	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
12	C5Y360	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011300	41.26	58.84	62	0.66	0.09	2.73E-02	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
15	C5Z240	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G003100	33.57	27.63	33	0.83	0.07	1.94E-02	Yes	None	Oxidoreductase activity	Anchored component of plasma Membrane	Multicopper oxidase, type 1, Ascorbate oxidase homologue, first cupredoxin domain; Cupredoxins
26	C5XCE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G351400	23.2	42.92	19	1.63	0.27	6.06E-03	Yes	Defence response	None	Extracellular region	Domain not predicted; Thaumatin family
27	C5X3C1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391300	22.05	29.27	23	0.44	0.03	2.00E-02	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
29	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	21.49	62.88	26	2.21	0.22	4.79E-05	Yes	Defence response	None	Extracellular region	Domain not predicted; Thaumatin family
33	A0A1W0W7I8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391900	22.96	21.26	18	0.60	0.13	3.00E-02	No	Response to oxidative stress	Peroxidase activity	Cell Wall	Haem peroxidase; Plant peroxidase
42	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600	17.29	30.03	23	1.82	0.35	4.37E-03	Yes	Negative regulation of	Enzyme inhibitor activity	None	Leucine-rich repeat-containing N-terminal, plant-type; Family not predicted

										catalytic activity			
54	C5YM54	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G151300	14.38	42.2	12	0.52	0.02	7.23E-03	Yes	None	Manganese ion binding	Extracellular region	Cupin 1; Germin
62	A0A1W0VX32	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G127100	13.74	32.85	10	0.49	0.08	3.45E-02	No	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
142	C5YC92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G018100	6.21	25.76	8	1.43	0.08	5.84E-05	Yes	None	Manganese ion binding	Extracellular region	Cupin 1; Germin
161	C5YQ75	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3008G010500	17.62	31.71	20	0.54	0.11	2.75E-04	Yes	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
180	C5Y5D5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G169300	4.48	19.21	4	2.68	0.58	1.40E-03	No	Defence response	Ribonuclease activity	None	Barwin domain; Pathogenesis-related protein-4
183	A0A1B6QJR7	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G189000	4.38	10.22	3	1.80	0.35	3.98E-03	No	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
216	A0A1B6QN96	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3001G371900	3.35	21.05	2	0.56	0.16	2.39E-02	No	Removal of superoxide radicals	Superoxide dismutase activity	None	Superoxide dismutase, copper/zinc binding domain; Superoxide dismutase (Cu/Zn) / superoxide dismutase copper chaperone
265	C5XIY0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152000	4.02	6.044	3	0.67	0.05	7.51E-03	No	Response to oxidative stress	Peroxidase activity	Extracellular region	Haem peroxidase; Plant peroxidase
269	A0A1B6QG28	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3002G407900	3.35	18.67	2	0.63	0.26	3.49E-02	No	Removal of superoxide radicals	Superoxide dismutase activity	None	Superoxide dismutase, copper/zinc binding domain; Superoxide dismutase (Cu/Zn) / superoxide dismutase copper chaperone
281	C5Y2R8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G126200	2	7.692	2	0.30	0.10	9.82E-03	Yes	None	None	None	Leucine-rich repeat-containing N-terminal, plant-type; Family not predicted

TRANSPORTERS

14	A0A1Z5R5E6	Non-specific lipid-transfer protein OS=*Sorghum bicolor* GN=SORBI_3008G030900	36.43	78.15	49	4.83	2.07	1.02E-02	Yes	Lipid transport	Lipid binding	Membrane	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain; Plant non-specific lipid-transfer protein/Par allergen
105	C5YRL0	Non-specific lipid-transfer protein OS=*Sorghum bicolor* GN=SORBI_3008G030700	9.66	56.1	13	2.41	0.54	2.19E-03	Yes	Lipid transport	Lipid binding	Membrane	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain; Plant non-specific lipid-transfer protein/Par allergen
213	C5XAF8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050400	3.55	6.111	2	0.70	0.19	2.85E-02	Yes	Lipid transport	Lipid binding	None	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain; Plant non-specific lipid-transfer protein/Par allergen
PROTEIN DESTINATION AND STORAGE													
17	C5XQ74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G208800	32.44	37.64	22	1.17	0.10	2.95E-02	No	Proteolysis	Aspartic-type endopeptidase activity	None	Peptidase family A1 domain; Aspartic peptidase A1 family
49	C5Y675	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G064200	15.19	21.69	10	0.77	0.09	8.37E-03	Yes	Proteolysis	Aspartic-type endopeptidase activity	None	Peptidase family A1 domain, Xylanase inhibitor, C-terminal; Aspartic peptidase A1 family
82	C5WVG9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324800	11.27	30.37	10	0.63	0.09	3.44E-03	Yes	Negative regulation of endopeptidase activity	Cysteine-type endopeptidase inhibitor activity	None	Cystatin domain; Cystatin
86	C5XHP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419300	10.52	12.06	5	1.57	0.40	4.95E-02	No	Proteolysis	Aspartic-type endopeptidase activity	None	Xylanase inhibitor, C-terminal, Xylanase inhibitor, N-terminal; Aspartic peptidase A1 family
117	A0A1B6Q6M7	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G327700	8.64	29.32	7	1.91	0.26	7.40E-04	Yes	Negative regulation of endopeptidase activity	Cysteine-type endopeptidase inhibitor activity	None	Cystatin domain; Cystatin
131	C5XQP2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G078400	7.1	11.79	5	1.41	0.13	2.64E-02	Yes	Response to abscisic acid	Aspartic-type endopeptidase activity	Endoplasmic reticulum	Xylanase inhibitor, C-terminal, Xylanase inhibitor, N-terminal; Aspartic peptidase A1 family
132	A0A1B6P5R2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G009600	7.04	24.68	4	2.02	0.34	2.32E-03	No	Negative regulation of endopeptidase activity	Serine-type endopeptidase inhibitor activityU	None	Domain not predicted; Proteinase inhibitor I13, potato inhibitor I

140	C5YA35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G260300	6.33	10.71	5	0.78	0.06	1.53E-02	Yes	Proteolysis	Cysteine-type endopeptidase activity	Extracellular region	Granulin, Peptidase C1A, papain C-terminal; Cysteine proteinases
164	C5YPF6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G119900	5.35	5.882	4	1.71	0.45	2.53E-02	Yes	Proteolysis	Serine-type endopeptidase activity	None	Peptidase S8/S53 domain, Peptidase S8 propeptide/proteinase inhibitor I9; Peptidase S8, subtilisin-related
203	A0A1B6Q242	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085300	4	20.2	2	1.64	0.48	4.85E-02	Yes	Negative regulation of endopeptidase activity	Serine-type endopeptidase inhibitor activity	Extracellular region	Proteinase inhibitor I12, Bowman-Birk; Bowman-Birk type wound-induced proteinase inhibitor WIP1
226	C5YNA1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G172100	2.85	5.108	3	1.47	0.10	1.33E-03	Yes	Proteolysis	Cysteine-type endopeptidase activity	Extracellular space	Peptidase C1A, papain C-terminal, Cathepsin propeptide inhibitor domain (I29); Cysteine proteinases
235	C5X0Y6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G529700	2.6	3.046	2	1.70	0.14	3.77E-04	Yes	Proteolysis	Serine-type endopeptidase activity	None	Peptidase S8/S53 domain, Cucumisin-like catalytic domain; Peptidase S8, subtilisin-related
CELL STRUCTURE													
22	C5Z8T4	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246600	25.8	51.39	30	0.68	0.06	6.01E-05	Yes	Cell wall biogenesis	Carbohydrate binding	Cell wall	Glycoside hydrolase family 16, Xyloglucan endo-transglycosylase, C-terminal; Xyloglucan endotransglucosylase/hydrolase
44	C5YVJ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G232100	15.81	35.1	12	0.52	0.04	3.01E-02	Yes	Plant-type secondary cell wall biogenesis	None	Plasma Membrane	FAS1 domain; Family not predicted
71	C5Z8T5	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246700	13.07	29.57	12	1.44	0.13	1.22E-02	Yes	Cell wall biogenesis	Carbohydrate binding	Cell wall	Glycoside hydrolase family 16, Xyloglucan endo-transglycosylase, C-terminal; Xyloglucan endotransglucosylase/hydrolase
13	C5Z8N0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G118900	37.19	41.01	52	0.64	0.06	2.95E-03	Yes	None	None	Anchored component of plasma membrane	FAS1 domain; Fasciclin-like arabinogalactan protein
155	A0A1B6Q7A6	Pectinesterase OS=*Sorghum bicolor* GN=SORBI_3003G376900	6	7.792	3	1.31	0.22	4.36E-02	Yes	Pectin catabolic process	Pectinesterase activity	Cell wall	Pectinesterase, catalytic; Family not predicted

225	C5XIT5	Pectinesterase OS=*Sorghum bicolor* GN=SORBI_3003G148300	2.85	4.956	4	0.65	0.06	5.68E-03	Yes	Pectin catabolic process	Pectinesterase activity	Cell wall	Pectinesterase, catalytic; Family not predicted
CELL GROWTH/DIVISION													
57	C5XRX3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G294500	14.33	24.24	12	1.29	0.14	4.28E-02	Yes	Sexual reproduction	None	Extracellular region	Expansin/pollen allergen, DPBB domain, Expansin, cellulose-binding-like domain; Expansin/Lol pI
90	C5WSE5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G300400	10.3	23.79	9	1.66	0.27	4.82E-03	Yes	Sexual reproduction	None	Extracellular region	Expansin/pollen allergen, DPBB domain, Expansin, cellulose-binding-like domain; Expansin/Lol pI
127	C5WSF9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301500	7.52	17.38	8	1.62	0.41	4.54E-02	Yes	Sexual reproduction	None	Extracellular region	Expansin/pollen allergen, DPBB domain, Expansin, cellulose-binding-like domain; Expansin/Lol pI
236	C5WV02	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G033300	2.51	7.634	2	1.52	0.16	3.64E-02	Yes	Plant-type cell wall organization	None	Cell wall	Expansin/pollen allergen, DPBB domain, Expansin, cellulose-binding-like domain; Expansin/Lol pI
ENERGY													
41	C5YK12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G100600	16.76	42.5	15	0.76	0.10	3.91E-02	Yes	Electron transport chain	Electron transfer activity	Plasma Membrane	Phytocyanin domain, Early nodulin-like protein domain; Phytocyanin
METABOLISM													
3	C5XYP5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G233700	73.35	40.56	57	0.78	0.03	6.49E-04	Yes	Carbohydrate metabolic process	Alpha-L-arabinofuranosidase activity	Cell Wall	Glycoside hydrolase, family 3, N-terminal, Glycoside hydrolase family 3 C-terminal domain; Glycoside hydrolase superfamily
9	C5WXC7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	43.28	45.07	43	0.58	0.03	1.62E-02	Yes	Carbohydrate metabolic process	Raffinose alpha-galactosidase activity	Cell Wall	Alpha galactosidase, C-terminal beta sandwich domain; Glycoside hydrolase, family 27
11	C5Y1P4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G099000	40.5	51.92	43	1.60	0.32	1.07E-02	Yes	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; Glycoside hydrolase superfamily
16	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	32.39	29.97	27	3.30	0.88	2.22E-03	No	Cellulose catabolic process	Cellulase activity	Extracellular region	Carbohydrate binding domain CBM49; Glycoside hydrolase family 9

19	C5YBE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132400	28.55	56.78	39	3.93	0.31	1.73E-06	Yes	Carbohydrate metabolic process	Chitinase activity	None	Glycoside hydrolase, family 19, catalytic; Glycoside hydrolase, family 19
25	C5XB38	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055600	23.31	30.94	30	2.05	0.19	8.37E-05	Yes	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; Glycoside hydrolase superfamily
34	C5XB39	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055700	21.68	40.39	25	1.40	0.29	4.93E-02	Yes	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; Glycoside hydrolase family 18
35	A0A1Z5RDM9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132100	20.05	40.52	17	1.34	0.10	2.39E-02	Yes	Carbohydrate metabolic process.	Chitinase activity	None	Glycoside hydrolase, family 19, catalytic; Glycoside hydrolase, family 19
43	C5XWE5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G197600	16.27	12.22	10	0.77	0.04	1.62E-02	Yes	Glycerol metabolic process	Glycerophosphodiester phosphodiesterase activity	None	Glycerophosphodiester phosphodiesterase domain; Family not predicted
50	C5X022	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G525000	14.82	16.63	12	0.63	0.01	3.90E-04	Yes	Carbohydrate metabolic process	Polygalacturonase activity	Cell wall	Domain not predicted; Glycoside hydrolase, family 28
51	C5YBE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132300	17.37	49.25	29	4.81	0.89	1,62E-04	Yes	Carbohydrate metabolic process.	Chitinase activity	None	Glycoside hydrolase, family 19, catalytic, Chitin-binding, type 1; Glycoside hydrolase, family 19
64	A0A1B6Q838	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422200	13.28	16.37	7	1.84	0.23	1.68E-03	Yes	Carbohydrate metabolic process	Carbohydrate binding	Plasma Membrane	Domain not predicted; Glycoside hydrolase family 17
66	A0A1B6QI05	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089100	25.26	19	17	0.79	0.12	2.45E-02	Yes	Carbohydrate metabolic process	Beta-glucosidase activity	Extracellular region	Glycoside hydrolase, family 3, N-terminal, Glycoside hydrolase family 3 C-terminal domain; Glycoside hydrolase superfamily
74	C5Y5V0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177600	12.66	24.17	9	1.40	0.07	8.58E-03	Yes	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; Glycoside hydrolase, family
84	A0A109NDM1	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3010G273600	10.86	28.44	10	1.76	0.55	3.50E-02	Yes	Carbohydrate metabolic process	Chitinase activity	None	Glycoside hydrolase, family 19, catalytic, Chitin-binding, type 1; Glycoside hydrolase, family 19
85	A0A1W0VUE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G227400	10.71	8.939	6	1.28	0.17	4.70E-02	No	Carbohydrate metabolic process	Carbohydrate binding	Extracellular region	Glycoside hydrolase family 31, N-terminal domain, Galactose mutarotase,

													N-terminal barrel; Glycoside hydrolase family 31
106	C5Y9T3	Aldose 1-epimerase OS=*Sorghum bicolor* GN=SORBI_3006G105200	9.49	17.62	6	1.42	0.25	2.07E-02	Yes	Carbohydrate metabolic process	Carbohydrate binding	None	Domain not predicted; Aldose 1-/Glucose-6-phosphate 1-epimerase
107	A0A1W0VY92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205900	9.49	19.42	6	0.82	0.14	4.52E-02	Yes	None	Hydrolase activity, acting on ester bonds	None	Domain not predicted; GDSL lipase/esterase
118	A0A1B6Q4S6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G226300	8.42	8.564	7	1.30	0.06	1.71E-02	Yes	Ceramide catabolic process	Ceramidase activity	Extracellular region	Neutral/alkaline non-lysosomal ceramidase, N-terminal, Neutral/alkaline non-lysosomal ceramidase, C-terminal; Neutral/alkaline nonlysosomal ceramidase
134	A0A1B6QE21	UTP--glucose-1-phosphate uridylyltransferase OS=*Sorghum bicolor* GN=SORBI_3002G291200	6.79	10.08	4	1.38	0.27	3.32E-02	No	UDP-glucose metabolic process	UTP:glucose-1-phosphate uridylyltransferase activity	Cytoplasm	Domain not predicted; UDPGP family
171	C5Y5U9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177500	5.46	10.75	9	1.24	0.07	6.06E-03	Yes	Carbohydrate metabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain; Glycoside hydrolase superfamily
173	C5YI64	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G198000	4.78	6.202	2	0.64	0.14	7.04E-03	Yes	None	None	Membrane	DOMON domain; Family not predicted
178	C5WSY5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G014700	4.56	5.797	3	0.58	0.16	4.95E-02	Yes	Carbohydrate metabolic process	Carbohydrate binding	Plasma Membrane	X8 domain; Glycoside hydrolase family 17
186	C5Y1P6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G099500	4.22	6.291	2	0.62	0.16	1.04E-02	Yes	Nucleoside diphosphate catabolic process	Nucleoside-diphosphatase activity	Membrane	Domain not predicted; Nucleoside phosphatase GDA1/CD39
188	C5XHS1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422000	4.1	10.39	2	2.58	0.43	5.39E-03	Yes	Carbohydrate metabolic process	Carbohydrate binding	Plasma Membrane	Domain not predicted; Glycoside hydrolase family 17
191	C5Z4E5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044900	4.04	7.357	5	0.60	0.09	6.64E-04	Yes	None	Hydrolase activity, acting on ester bonds	None	Domain not predicted; GDSL lipase/esterase

208	A0A194YIA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044500	3.75	9.392	3	2.92	0.44	2.22E-04	Yes	None	Hydrolase activity, acting on ester bonds	None	Domain not predicted; GDSL lipase/esterase
221	C5XHR8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G421700	3.11	7.53	2	1.55	0.29	1.04E-02	Yes	Carbohydrate metabolic process	Carbohydrate binding	Plasma Membrane	Domain not predicted; Glycoside hydrolase family 17
229	C5XCD4	Beta-hexosaminidase OS=*Sorghum bicolor* GN=SORBI_3002G350700	2.67	3.253	2	1.44	0.25	3.79E-02	Yes	Carbohydrate metabolic process	N-acetyl-beta-D-galactosaminidase activity	None	Glycoside hydrolase family 20, catalytic domain; Beta-hexosaminidase
UNCLEAR CLASSIFICATION													
55	C5YBH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G135500	14.43	20.07	18	0.70	0.05	1.14E-02	Yes	None	None	None	Glyoxal oxidase, N-terminal, Galactose oxidase-like, Early set domain; Family not predicted
61	C5Z6D9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G079100	14.25	19.16	11	0.76	0.03	4.06E-02	Yes	None	None	None	LysM domain; Family not predicted
81	C5XYB4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G229300	11.37	11.52	8	2.36	0.28	3.93E-03	No	None	None	Extracellular region	Domain not predicted; Protein EXORDIUM-like
103	C5XL56	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G023800	9.68	9.075	5	1.48	0.33	4.50E-02	Yes	None	None	Vacuole	Domain not predicted; Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase A
116	C5WPH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G131100	8.5	6.657	4	0.79	0.05	1.84E-02	Yes	None	Catalytic activity	None	Glucose/Sorbosone dehydrogenase; Family not predicted
137	C5Z6Y0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G088700	7.37	18.05	5	2.73	0.22	1.35E-05	Yes	None	None	Extracellular region	Domain not predicted; Protein EXORDIUM-like
150	A0A1Z5RBA4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G225400	6.02	7.595	3	2.59	0.84	1.04E-02	Yes	None	None	Extracellular region	Domain not predicted; Protein EXORDIUM-like

[a] Protein number assigned in ProteinPilot software.

[b] Protein accession numbers obtained from the UniProt (https://www.uniprot.org/uniprot/) database search engine against sequences of *Sorghum bicolor* only.

[c] Protein score generated by ProteinPilot software relating to the confidence of protein identification. Only proteins with 95% confidence interval were retained.

[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] Number of peptide that were sequenced and yielded protein identity. Proteins with 0 or 1 peptide were filtered out.

[f]Standard deviation of the protein samples (n=4).

[g]Probability value of the quantitative difference between the ABA treatment and control proteins.

[h] Signal peptide predicted from UniProt database (http://www.uniprot.org). Yes, means the presence of the signal peptide and No, means no signal peptide.

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

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

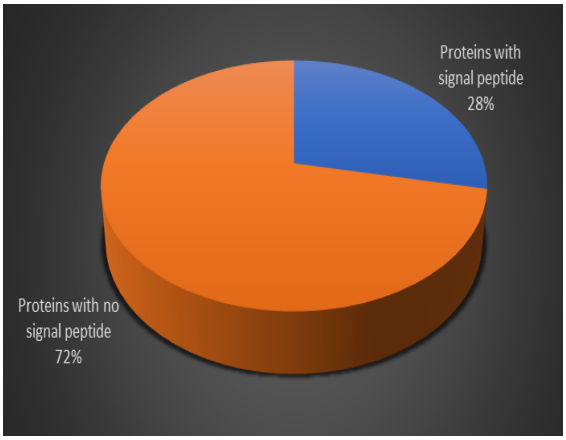
4.2.2 Bioinformatic analyses on the ABA-responsive TSP and CF proteins of white sorghum cell cultures

4.2.2.1 Signal peptide prediction

Signal peptides (SPs) are short amino acid sequences situated on the N-terminal end of proteins (Owji *et al*., 2018). They carry information for the translocation of newly synthesized proteins towards the secretory pathway in both prokaryotic and eukaryotic cells (Peng *et al*., 2019). Information available on the UniProt database was used to identify the presence/absence of signal peptides in the ABA-responsive TSP and secreted proteins. In the TSP fraction, out of 46 differential expressed proteins in response to exogenous ABA, only 13 (28%) were predicted to have signal peptides, whereas 33 (72%) did not (Table 4.2; Figure 4.2A). The majority (11) of the total soluble proteins containing signal peptides were uncharacterized and the other two signal peptide-containing proteins were on alpha-galactosidase and a peroxidase (Table 4.2). Plant alpha-galactosidases and peroxidases are well-known signal containing proteins and have been identified in white sorghum cell suspension cultures (Ramulifho, 2017).

However, of the 82 ABA-responsive secreted proteins, a large proportion of 68 (82%) contained predicted signal peptides, while 14 (18%) did not (Table 4.3; Figure 4.2B). Most of the differentially expressed secreted proteins (8) that did not contain signal peptides were characterized (Table 4.3), and include xyloglucan endotransglucosylase/hydrolase, alpha-galactosidases and peroxidases. Similar protein families containing signal peptides have been previously identified in other sorghum cell culture secretome studies in response to osmotic (Ngara *et al*., 2018), and heat (Ngcala *et al*., 2020) stresses.

A



B

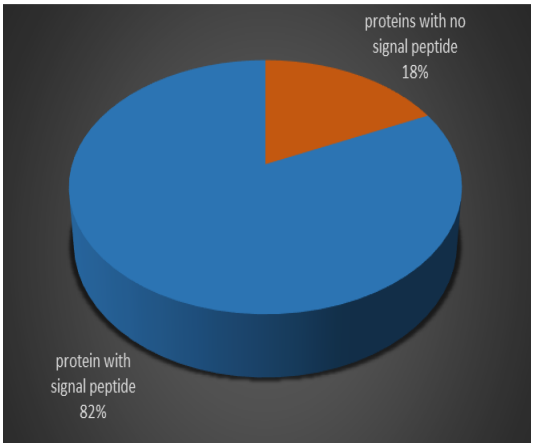


Figure 4.2: Signal peptide prediction of ABA-responsive proteins of white sorghum cell cultures. (A) shows the TSP and (B) shows the CF proteomes signal peptide results.

4.2.2.2 Gene Ontology analyses

The UniProt and InterPro databases were used to determine the GO terms for the ABA-responsive TSP and secreted proteins of white sorghum cell cultures. Gene ontology is a bioinformatic tool that is used to provide information about a gene from several domains of molecular and cellular biology (Gene Ontology Consortium, 2004). Gene ontology terms include the biological process (P), molecular function (F), and cellular components (C). Tables 4.2 and 4.3 also include results of these three GO terms for the ABA-responsive proteins of both proteomes. These results are further illustrated in a simplified manner in Figures 4.3 - 4.5 below.

4.2.2.2.1 Cellular components

Cellular components denotes the specific location, where proteins are transported to and/or maintained to perform their function (Botstein *et al*., 2000). For example, proteins in the cell wall are there to maintain cell shape, stability, growth and/or to protect against environmental stresses (Wu *et al*., 2018). Thirty-five (76%) of the differentially expressed total soluble proteins were predicted to have cellular components, while the rest did not (Table 4.2). The majority of these total soluble proteins were predicted to be located in the cytoplasm/cytosol (29%), followed by the extracellular region (15%) and nucleus (11%) (Table 4.2; Figure 4.3). Other intracellular locations of the total soluble proteins included the chloroplast (9%), endoplasmic reticulum (2%), and peroxisome (2%). About 4% of the ABA-responsive TSP proteins were predicted to be located in the membrane. Lastly, 15% of the TSP proteins were predicted to be localised in the extracellular region, and most of these proteins were predicted to have signal peptides (Table 4.2). However, the cellular location of 24% of the ABA-responsive TSP is unknown (Table 4.2; Figure 4.3). Interestingly, 9 of these ABA-responsive total soluble proteins without a predicted cellular location are uncharacterised (Table 4.2).

Fifty-five (72%) of the ABA-responsive secreted proteins were predicted to have known cellular components (Table 4.3). The majority of these proteins were located in the extracellular region (28%), followed by the cell wall (9%), plasma membrane (6%), membrane (5%) and anchored component of the plasma membrane (4%) as shown in Table 4.3 and Figure 4.3. All these cellular components are associated with the extracellular location of plant cells.

A more detailed comparison of the cellular components data of the two proteomes showed that some protein locations were unique to each proteome, while others were shared by both (Figure 4.3). For example, the cellular locations found in the TSP fraction only included the nucleus, chloroplast, and peroxisome, while the vacuole, and anchored component of plasma membrane were exclusively identified in the secreted protein sample (Figure 4.3). Furthermore, the TSP dominated the main intracellular-related region, namely the cytoplasm/cytosol when compared to the secretome (Figure 4.3). On the other hand, the extracellular-related locations such as the extracellular region, cell wall, and membrane were dominated by CF proteins (Figure 4.3).

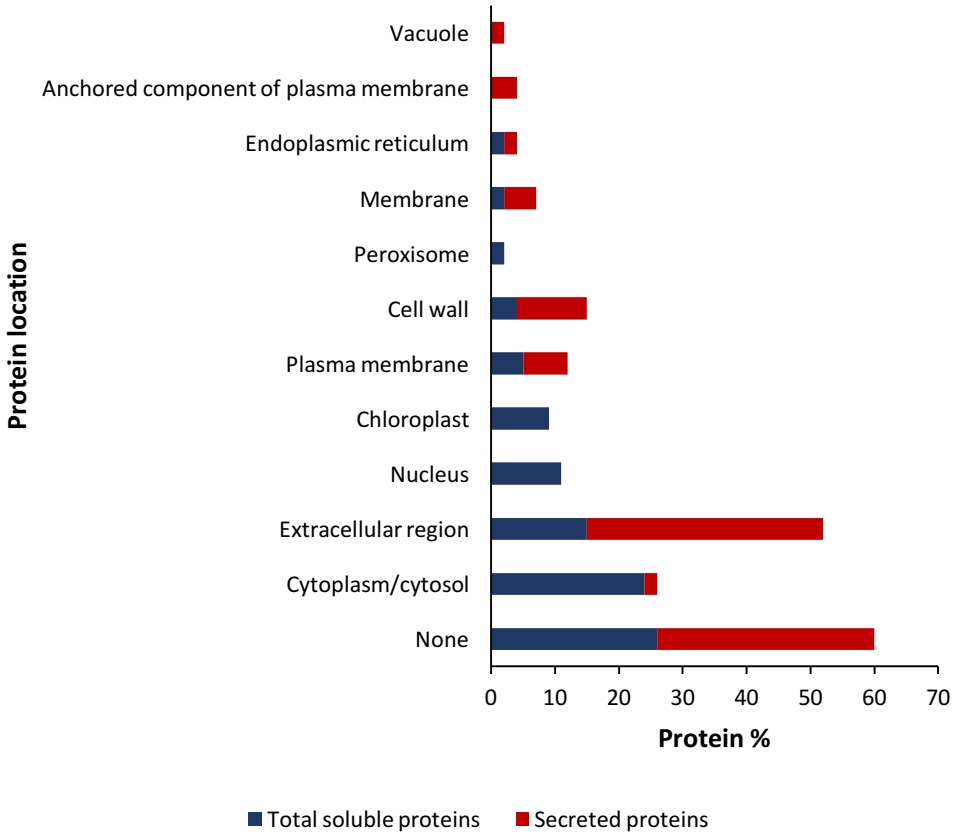


Figure 4.3: Predicted cellular components of ABA-responsive total soluble proteins and secreted proteins of white sorghum cell cultures.

4.2.2.2.2 Biological processes

Biological processes (B) are described as one or more organized molecular functional activities, for example photosynthesis and proteolysis. About 80% of the ABA-responsive proteins of both proteomes had predicted biological processes (Tables 4.2 and 4.3; Figure 4.4) but largely dominated by the carbohydrate metabolic process in both the TSP proteome (10%) and the secretome (25%) (Figure 4.4). Other biological processes that were shared by both proteomes include response to oxidative stress, cellulose catabolic process, negative regulation of catalytic activity, and defence response (Figure 4.4).

Nevertheless, these two proteomes did not share all the biological processes. For example, some of the biological processes that were unique to the TSP fraction included the glycolytic process with 7% of the proteins and was the second most dominant biological process, followed by cell redox homeostasis (4%), abscisic acid-activated signalling pathway (4%), and proteasomal protein catabolic process (4%) (Figure 4.4). Other biological process that were unique to the TSP included mismatch repair, plant-type cell wall organization, glutamine biosynthetic process and lipid catabolic process to name a few (Figure 4.4).

In comparison to the TSP, the second most represented biological process in the secreted protein fraction after carbohydrate metabolic processes (25%) was response to oxidative stress with 12% of the ABA-responsive proteins (Figure 4.4), followed by proteolysis (9%) and then negative regulation of endopeptidase activity (5%). Examples of biological processes that were unique to the secreted protein fraction included proteolysis (9%), negative regulation of endopeptidase activity (5%), lipid transport (4%), cell wall biogenesis (2%), pectin catabolic process (2%), and removal of superoxide radicals to mention a few (Figure 4.4). However, 20% each of the proteomes did not have predicted biological processes (Figure 4.4).

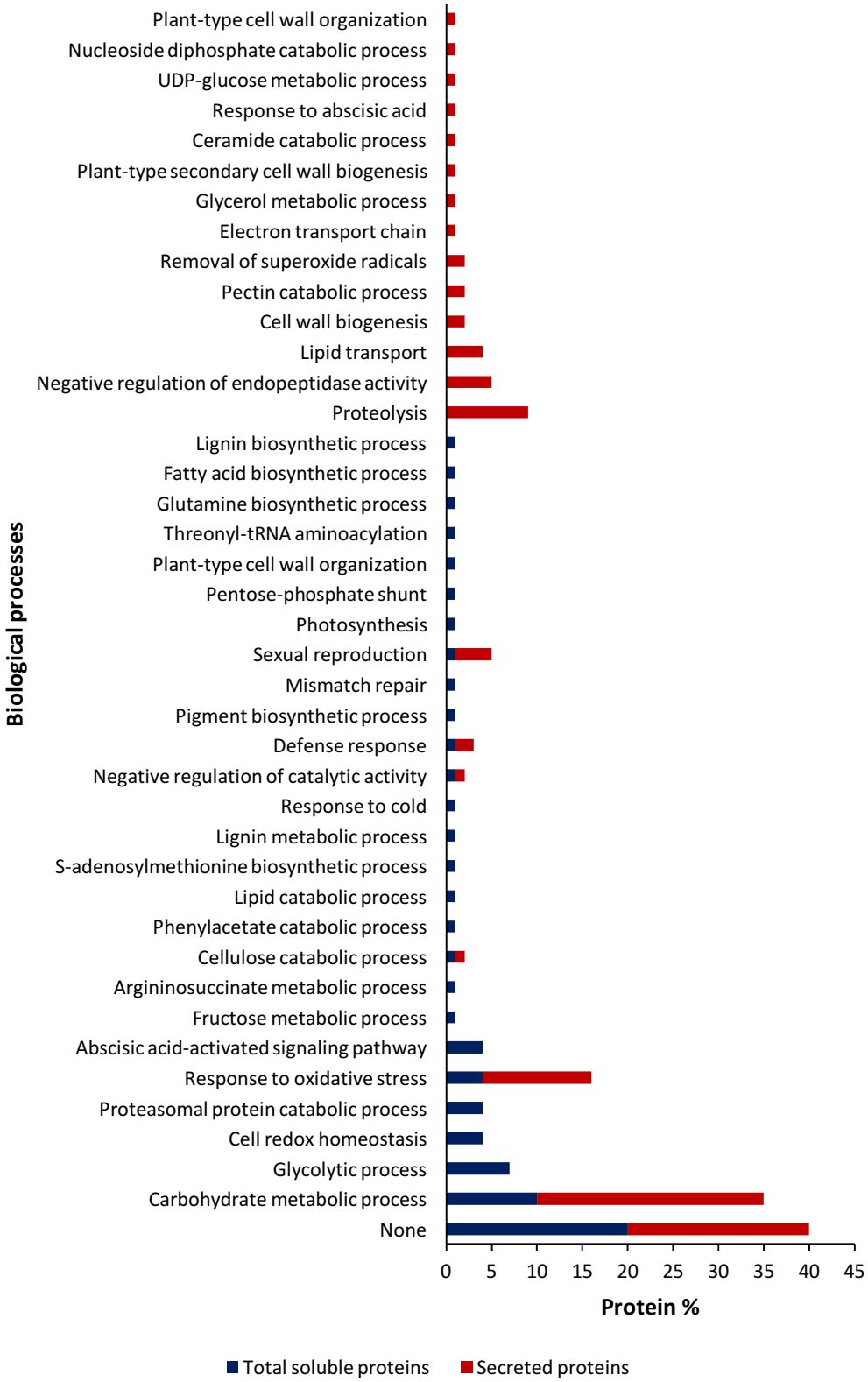


Figure 4.4: Predicted biological processes of ABA-responsive total soluble proteins and secreted proteins of white sorghum cell culture.

4.2.2.2.3 Molecular functions

The molecular function GO terms describe the activities of a gene product at the molecular level such as catalytic and binding activities (Smith *et al*., 2003). The molecular functions of 5 (11%) of the 46 ABA-responsive total soluble proteins and 16 (20%) of the 82 secreted protein were not predicted (Tables 4.2 and 4.3; Figure 4.5). Nevertheless, the two proteomes shared eight common molecular functional groups namely; carbohydrate binding, oxidoreductase activity, cellulase activity, raffinose alpha-galactosidase activity, peroxidase activity, enzyme inhibitor activity, manganese ion binding, and electron transfer activity (Figure 4.5). The dominant molecular functional groups in the TSP proteome included oxidoreductase activity and peroxidase activity both with 7% of the ABA-responsive proteins. On the other hand, the secreted protein fraction was dominated by the peroxidase activity (12%), chitinase activity (11%) and carbohydrate binding (11%).

The molecular functions that were both unique to and dominant in the total soluble proteome included abscisic acid binding (4%) and endopeptidase activity (4%). Other molecular functions that were unique to the TSP include fructokinase activity (2%), argininosuccinate synthase activity (2%), acetyl-CoA C-acyltransferase activity (2%), (Figure 4.5).

For the secretome, the highly represented molecular functional groups included peroxidase activity (12%) followed by chitinase and carbohydrate binding both with 11% (Figure 4.5). The secretome also contained unique molecular functional groups such as, chitinase activity 11%), aspartic-type endopeptidase activity (5%), lipid binding (4%), hydrolase activity (4%), superoxide dismutase activity (2%) (Figure 4.5).

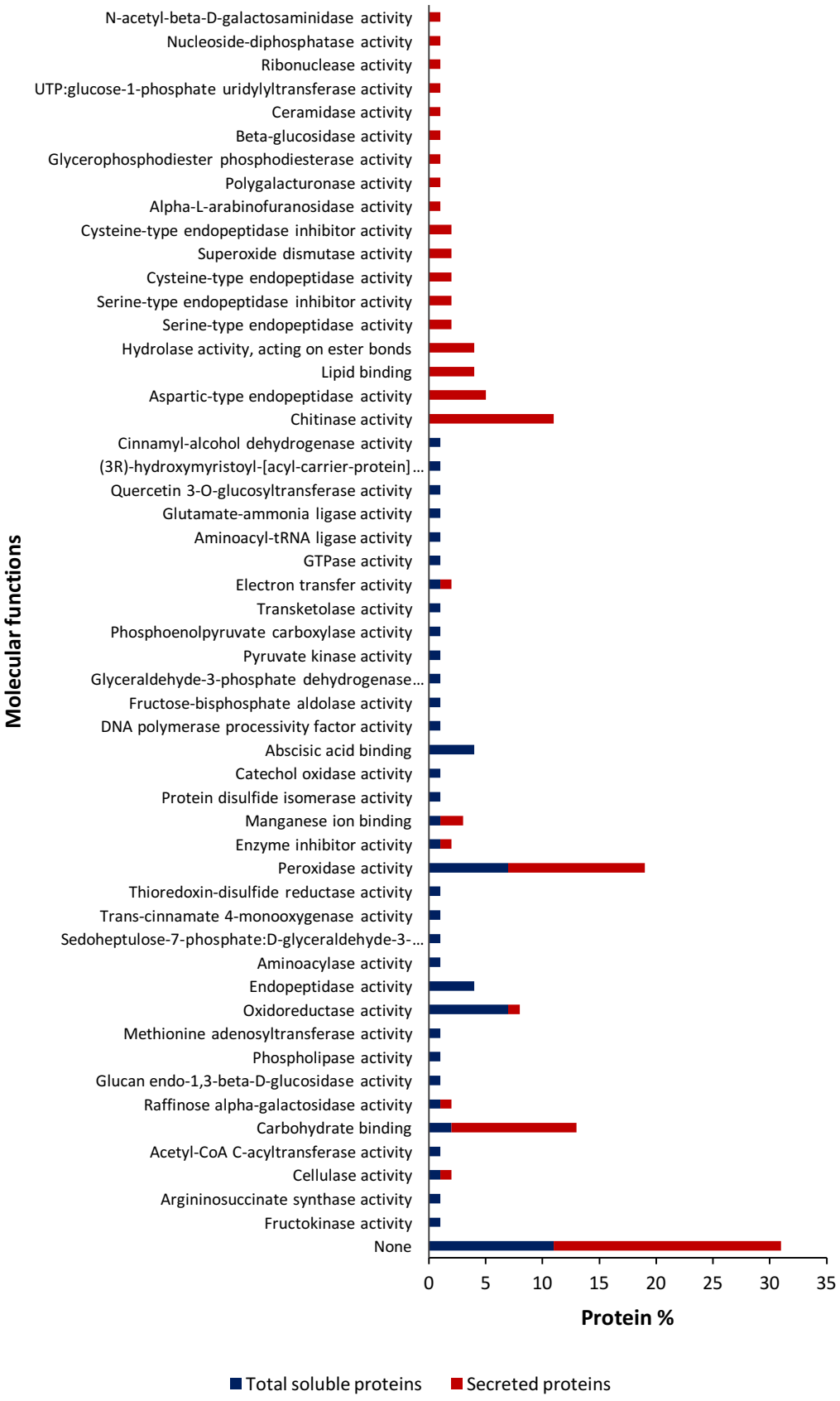


Figure 4.5: Predicted molecular functions of ABA-responsive total soluble proteins and secreted proteins of white sorghum cell culture.

4.2.3 Classification of conserved domain and protein family names

In this study, both the conserved domain and protein family names were identified for the 46 and 82 ABA-responsive total soluble and secreted proteins, respectively, using information available on the InterPro database. This was done because most of the identified ABA-responsive proteins were uncharacterised for both the TSP (65%) and the CF (69%) proteomes (Tables 4.2 and 4.3). The information on conserved domain and protein family names was subsequently used to functionally group the uncharacterized proteins (Tables 4.2 and 4.3). Protein domains are defined as distinct functional or structural units of a protein, while a protein family is a group of proteins that share a common evolutionary origin and similar sequence, structure and function. Proteins are grouped together based on their sequence, structural and/or functional similarities. When a new protein is discovered, its functional features can be anticipated based on its similarity with a specific protein group.

Only 5 of the 46 ABA-responsive total soluble proteins did not have predicted family names and all were uncharacterized (Table 4.2). In the secretome dataset, 10 of the 82 ABA-responsive proteins did not have predicted protein families (Table 4.3). Some proteins such as protein number, 1, 5, 6, 12, 14, 15, 17, 27, 33, 49, 54, 62, 82, 86, 105, 117, 131, 142, 161, 180, 183, 213, 216, 265 and 269 had both domain and family names, whereas others only had either their protein domains or protein family name predicted (Table 4.2). The TSP protein entries that had predicted family names only include protein numbers 31, 51, 243, 342, 378, 561, 577 and 594 (Table 4.2), the majority of which were uncharacterized (Table 4.2). In the secretome, protein numbers 26, 29, 50, 64, 81, 103, 106, 107, 132, 134, 137, 150, 186, 188,191, 208 and 221 had predicted family name only (Table 4.3). The ABA-responsive total soluble proteins with only a predicted domain included protein number 44, 117, 510, 596 and 85, and all were uncharacterized (Table 4.2). For the secretome proteins, protein entries 42, 43, 44, 55, 61, 116,

155, 173, 225 and 281 only had predicted domains. Some of these domain and family names were shared by the two proteomes. For example, the alpha-galactosidase proteins and glycoside hydrolase, family 27 were present in both the TSP (protein 181) and the CF (protein 9) samples (Tables 4.2 and 4.3).

4.2.4 Functional categories of differentially expressed ABA-responsive total soluble proteins and secreted proteins

The 46 differentially expressed ABA-responsive total soluble proteins were grouped into seven functional groups (Figure 4.6A). In contrast, the 82 differentially expressed secreted proteins were grouped into eight functional groups (Figure 4.6B). In both proteomes, biological process and molecular functions GO terms as well as the conserved domain and/or protein family names were used to functionally categorise each protein (Tables 4.2 and 4.3).

The majority of proteins in the TSP fraction were involved in metabolism (37%), followed by defence (24%) and energy (13%), protein destination and storage (6%), signal transduction (4%), and cell growth/division (7%) (Figure 4.6A). On the other hand, most of the ABA-responsive secreted proteins were involved in metabolism (35%) and defence (24%) followed by protein destination (15%) (Figure 4.6B). Two functional groups of transporters (4%) and cell structure (7%) were only identified in the ABA-responsive secretome but not in the intracellular proteome (Figure 4.6B), whereas the signal transduction functional group was unique to the TSP proteome (Figure 4.6A). Proteins were assigned to functional group using Bevan *et al*. (1998) and other literature sources (Figure 4.6A and Figure 4.6B).

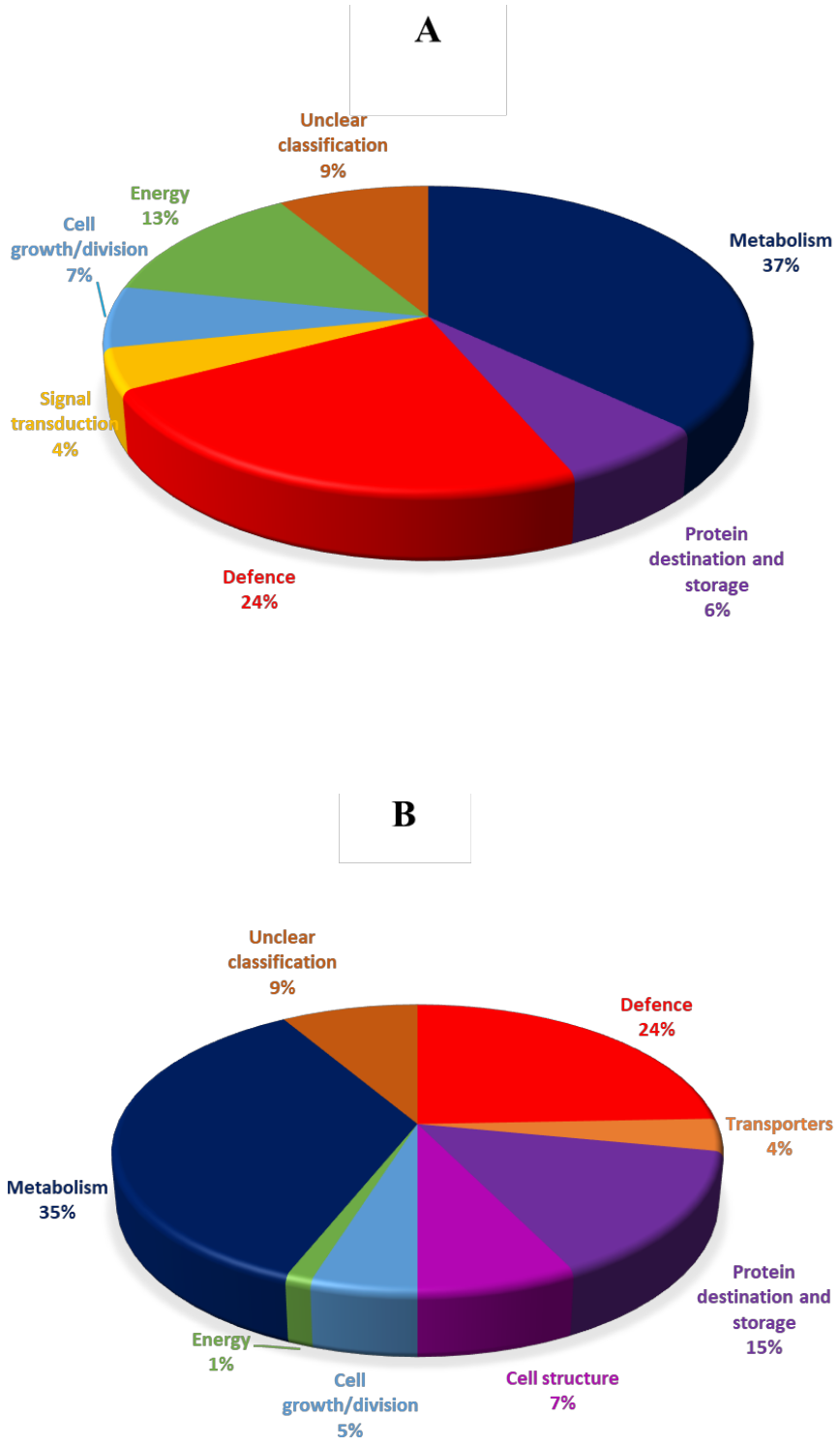


Figure 4.6: Functional groups of ABA-responsive total soluble proteins and secreted proteins of white sorghum cell cultures. (A) shows functional groups identified in the TSP, while (B) shows the functional groups identified in CF.

4.3 Discussion

Abscisic acid is a phytohormone that plays important roles in regulating plant growth, development, and response to biotic/abiotic stresses (Zehra *et al*., 2020). As a result of exposure to environmental challenges, a rapid increase in endogenous ABA levels has been documented and reported to trigger specific signalling pathways and regulate gene expression in plants (O'Brien and Benková, 2013). Some studies have shown that exogenous ABA induces specific protein and gene expression changes under drought stress (Zhou *et al*., 2014), and increases the accumulation of various sugars in buds, including oligosaccharides from the raffinose family (RFOs) under cold stress (Wang *et al*., 2020). Furthermore, when *Artemisia annua* plants were exposed to copper toxicity, exogenous ABA increased the activity of antioxidant enzymes (Zehra *et al*., 2020). Yet in another study of Wang and co-workers, exogenous ABA application improved pathogen-resistance and abiotic stress tolerance (Wang *et al*., 2013). Without doubt, ABA appears to be central to a range of plant responses towards different abiotic and biotic stresses.

In the current study, the iTRAQ method and mass spectrometry were used to identify proteins from both the TSP and CF samples of white sorghum cell cultures in response to exogenous ABA. These two protein fractions represent two different subcellular proteomes of a plant cell; the TSP being proteins located inside the cell's intracellular matrix, while the CF proteome represents proteins that are found outside the cell in the extracellular matrix (Ngara *et al*., 2008). While all proteins are synthesised in the cytoplasm (Silver, 1991), they are eventually translocated to various cellular compartments including the extracellular space (Peng *et al*., 2019). For example, signal peptides tag proteins for secretion (Madzak and Beckerich, 2013). Likewise, in this study more signal peptide containing proteins were identified in the CF fraction (82%) (Figure 4.2B) compared to the TSP fraction (28%) (Figure 4.2A). This

observation shows the importance of signal peptides in protein secretion. Since all the proteins are synthesized in the cytoplasm it is not surprising that some of the proteins from the TSP fraction contain signal peptides. It is possible that proteins were harvested before they could reach their final cellular compartments.

Of the 82 ABA-responsive secreted proteins, 68 (82%) were predicted to have signal peptides, while 14 (18%) did not (Table 4.3; Figure 4.2B). Eight of the differentially expressed secreted proteins that did not contain signal peptides were characterized, including one UTP--glucose-1-phosphate uridylyltransferase, one endoglucanase, four peroxidases, and two superoxide dismutase (Table 4.3). In some cases, proteins were secreted into the extracellular regions without the aid of signal peptides (Owji *et al*., 2018). Such proteins are referred to as leaderless proteins and are thought to be secreted via the unconventional protein secretion pathway (Ding *et al*., 2012). However, it is possible that some proteins could have ended up in the extracellular matrix through leakage of proteins according to their low molecular weight sizes or because of contamination from the intracellular compartment during protein extraction, or due to cell death and lysed cells (Krause *et al.*, 2013; Miernyk *et al*., 2016). The majority of proteins that did not have predicted signal peptides in the CF proteome included peroxidases and superoxide dismutases. These signal peptide-less proteins were also observed in a heat stress secretome experiment by Ngcala *et al*. (2020).

Most of differentially expressed proteins had predicted GO terms of cellular component, biological process and molecular function (Tables 4.2. and 4.3). Furthermore, most of the characterized proteins in the CF were peroxidases with 12 protein entries. These proteins were located in the extracellular regions and most of them predicted to contain signal peptides and

possibly secreted into the extracellular region via the conventional pathway (Table 4.3). In other sorghum proteomic studies, secretory peroxidases dominated the secreted protein fraction of sorghum cell suspension cultures without stress (Ngara and Ndimba, 2011) and in response to osmotic (Ngara *et al*., 2018) and heat (Ngcala *et al*., 2020) stresses.

Peroxidases are involved in the detoxification of the reactive oxygen species (ROS) produced during oxidative stress (Omari and Nhiri, 2015). In this study, 10 peroxidases (protein numbers: 1, 5, 6, 12, 27, 33, 62, 161, 183 and 265) were identified in the CF fraction and classified under the defence functional group (Table 4.3; Figure 4.6), compared to only two peroxidase proteins (number 70 and 600) identified in the TSP fraction. In plants, different types of stresses such as heat, and combined heat and drought, may results in damaging of cellular components. Therefore, the production of antioxidant proteins such as peroxidase assists in redox homeostasis (Foyer and Noctor, 2005). In the current study, in the TSP two peroxidases were identified and one of the two proteins was involved in cell redox homeostasis (Table 4.2). The up-regulation of peroxidase in response to the application of exogenous ABA was also observed under cold temperatures (Guo *et al*., 2012), water and salt stresses (Kaur and Zhawar, 2015; Fahad *et al*., 2015). Overall, proteins under defence functional group are important in protecting plants against the negative effects of stress.

The GO analysis results suggest that exogenous ABA regulates the expression of the proteins related to metabolism, defence, cell growth, transporters, energy, protein destination and storage, signal transduction and cell structure (Figure 4.6). These proteins may have a range of functions from the generation of energy compounds, the synthesis and degradation of primary and secondary metabolites, the maintenance of cell structure and function, cell signalling

processes, protection against stress factors, and the promotion of intra- and extracellular transportation and trafficking processes (Tables 4.2 and 4.3; Figure 4.6).

In both TSP and CF proteomes, metabolism dominated the functional groups followed by defence (Figure 4.6 A and B). Also, in other proteomic studies using sorghum cell suspension cultures in response osmotic (Ramulifho, 2017) and heat (Ngcala *et al*., 2020) stresses, metabolism functional group dominated stress responsive proteins followed by defence. This observation suggests that ABA is responsible for the induction of metabolic changes and defence related proteins in response to abiotic stress factors.

In the current study, the majority of proteins involved in metabolism, were uncharacterized in both proteomes. However, in the TSP, ABA regulated proteins under the metabolism functional group were involved in a broad range of biological processes such as carbohydrate metabolic process, fructose metabolic process, cellulose catabolic process, argininosuccinate metabolic process, glutamine biosynthetic process, lipid catabolic process, pigment biosynthetic process, fatty acid biosynthetic process, S-adenosylmethionine biosynthetic process and lignin metabolic process. These biological processes are involved in the metabolism of a variety of biomolecules such as sugars, lipids, fatty acids and secondary metabolites. Different metabolic pathways enable cells to maintain life with basic carbon and nitrogenous compounds (Coruzzi and Zhou, 2001). Often, intracellular metabolites have a regulatory role in organisms during normal growth and development (Rolland *et al*., 2006). For example, fructose is a signalling molecule during growth and developmental stages, and thus plays a role in germination, seedling growth, root and leaf differentiation, fruit ripening, embryogenesis and senescence (Smeekens, 2000; Rolland *et al*., 2006). On the other hand,

lipids are important components of plant cell membranes (Weber, 2002). They operate as second messengers in signal transduction pathways that regulate plant growth, development, and stress adaption, and form a physical barrier on epidermal cells that protects the plant from external challenges (Laxalt and Munnik, 2002; Wang, 2002; Shah, 2005). The current study suggests that the application of exogenous ABA promotes the expression of proteins that protect a plant from environmental stresses, for example protein alpha-galactosidase (Protein number 9) improves freezing tolerance in plants (Pennycooke *et al*., 2003).

In comparison, the metabolism functional group in the CF proteome had the carbohydrate metabolic process with the largest number of proteins (20). The other biological processes in this metabolic group included cellulose catabolic process, glycerol metabolic process, cerenide catabolic process, UDP-glucose metabolic process and nucleoside diphosphate. Almost all proteins (69%) under the metabolism functional group in the CF fraction belonged to the glycoside hydrolase family except protein 103, 106, 107, 118, 134, 137, 150, 173, 186, 191 and 208. Glycoside hydrolases are enzymes that hydrolyze the glycosidic link between two or more carbohydrates, or a carbohydrate and a non-carbohydrate component (Ahn *et al*., 2007). These enzymes function in a variety of biological activities, including glycan biosynthesis, cell wall metabolism, plant defence, signalling, and storage reserve mobilization (Minic, 2008). By regulating plant development, establishing a physical barrier, or releasing compounds that stimulate defence signalling, these activities assist plants in surviving under stressful conditions (Sharma *et al*., 2013). The current results suggest that the application of exogenous ABA influences the expression of glycoside hydrolases-related proteins in high numbers.

To maintain growth, cell cycle-related processes require the activation of major cell energy-generating mechanisms. In plants, energy is essential for progressing through the many phases of the cell cycle (Kaplon *et al*., 2015). In the current study, the energy functional group was identified in both proteomes. However, in the TSP proteome there were more energy-related proteins compared to the ones in the CF proteome. The reason could be that such proteins are mainly required in the intracellular matrix to maintain the cell by building and repairing cell components and production of energy. For example, in the TSP, a photosynthesis-related protein was identified (protein number 496). Within energy functional group, proteins related to glycolytic process were also identified.

In the secretome, transporters were non-specific lipid-transfer proteins (protein numbers 14, 105 and 213). These are known as cationic proteins that play a role in intracellular lipid shuttling during growth and reproduction, as well as in defence against harmful pathogens. They also promote lipid transport (Shenkarev *et al*., 2017). This could possibly mean that ABA is responsible for lipid transport and defence against harmful pathogens.

In the total soluble proteins, signal transduction proteins namely pathogenesis-related protein 10c (protein number 167) and uncharacterized (protein number 47) were identified. The pathogenesis-related protein (PR-10c) is a member of the intracellular pathogenesis-related (IPR) protein and are thought to function in plant defence since their genes are generally activated in response to pathogen attacks and environmental disturbances (Jain and Kumar, 2015). These proteins are also activated by ABA (Wang *et al*., 1999; Jain and Kumar, 2015) which correlates well with their up-regulation in the current study in response to application of exogenous ABA (Table 4.2). The signal transduction functional group was unique to TSP.

4.4 Conclusion

In conclusion, ABA plays an important role in regulating the expression of proteins in both intracellular and extracellular matrix of plant cells. The results also showed that a huge number of proteins with predicted signal peptide were located in the extracellular region and these proteins where mainly found in the metabolism functional group. As such, ABA stimulates the expression of carbohydrates metabolism in the extracellular matrix. On the other hand, most of the proteins in the intracellular fraction did not have predicted signal peptides. However, most of the proteins were dominating in the metabolism functional group, making metabolism an important part of the plant cell activities. The two proteomes are different with different proportions of proteins containing signal peptides, and in various location and also functional groups.

CHAPTER 5

OVERALL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Global warming is predicted to increase the frequency of abiotic stresses such as drought and extreme temperatures (Jat *et al*., 2016). On the other hand, the world population is increasing, thus increasing the need in cereal crop production to feed the population. It is therefore important to develop agricultural crops that can withstand these extreme abiotic stresses (Costa and Farrant, 2019). To sustain cereal crop production, a combination of adaptive agricultural strategies is required including understanding of how plants response to exogenous abscisic acid (ABA).

Abscisic acid is a phytohormone that is generally produced endogenously by plants during normal developmental, and in response to abiotic stresses such as high/low temperature, salinity and drought (Xiong *et al*., 2002; Zhang *et al*., 2006). Endogenous levels of ABA in vegetative plant tissues increases in response to abiotic stresses (Lee and Luan, 2012). For example, during drought, one of the primary adaptive responses of plants is stomatal closure that is mediated by ABA to prevent transpiration water loss (Bray, 2002; Lee and Luan, 2012; Buckley, 2019). The closure of stomata during water deficit subsequently triggers the activation of many other stress responsive genes to increase plant stress tolerant (Rock, 2000; Bray, 2002). These genes assist in protein expression, they synthesize metabolites, and osmoprotectants such as proline (Planchet *et al*., 2014). Proteins can either be found in the intracellular or extracellular matrix (Ngara *et al*., 2008). This study aimed to investigate the protein expression patterns of white sorghum cultures in response to exogenous ABA in the intracellular and extracellular matrices.

In this study, the molecular changes of sorghum in response to exogenous abscisic acid was investigated using a white sorghum culture line (Figure 3.2). Cell suspension cultures were used because they are a good material to obtain proteins from both the intra and extra-cellular matrices of cells (Ngara *et al*., 2008). Furthermore, proteins are produced in large amount, and the cell cultures can be controlled and easily handled compared to whole plants (Agrawal *et al*., 2010). The results showed that the application of exogenous ABA did not affect the cell viability at 0, 24, 48 and 72 hours of treatment (Figure 3.3). Osmolytes such as proline, glycine betaine and sugars are important for the maintenance of cellular homeostasis against environmental factors such as oxidative and osmotic stresses (Rejeb *et al*., 2014). In the current study, results also showed an increase in proline content in response to exogenous 100 µM ABA (Figure 3.5). These results suggest that the exogenous application of ABA increases the internal cell content of proline, even without exposure to abiotic stresses. In 2007, Verslues and colleagues reported that an increase in endogenous levels of ABA also increased the proline content (Verslues *et al*., 2007). As such, this study recommends other studies to focus on the proline content changes of different types abiotic stresses in response to exogenous ABA to investigate its role in osmotic adjustment and cellular protection.

Plants express genes and proteins during normal development and when the plant is exposed to both biotic and/or abiotic stresses (Crawford *et al*., 2018). In the current study, both proteomes, namely the total soluble proteins (TSP) and culture filtrate (CF) proteins were extracted to investigate the influence of ABA on protein expressional changes. One dimensional gel electrophoresis showed good quality of extracts of both TSP and CF, including some expressional changes in protein profiles (Figure 3.6). This possibly implies that ABA plays an important role in triggering the expression of both intracellular and extracellular proteins in sorghum cells. Therefore, this study recommends other studies to focus on the

effects of other phytohormones such as gibberellins, salicylic acid, ethylene and jasmonates on protein expression patterns with or without abiotic stresses.

The isobaric tags for relative and absolute quantitation (iTRAQ) method was used to identify proteins whose expression changed in response to exogenous 100 µM ABA in the TSP and CF proteomes. A total of 725 and 256 proteins were positively identified for TSP and CF fractions, respectively. The majority of these proteins were uncharacterized, meaning these proteins have not been characterized experimentally (Ngara *et al.*, 2012). In most sorghum studies, the majority of the proteins have not been verified experimentally (Swami *et al*., 2011; Jedmowski *et al*., 2014; Ngara *et al*., 2018). Therefore, I highly recommend more research focusing on the characterization of all expressed proteins with unknown function in sorghum studies to increase the functional annotation of such proteins.

Of the 725 and 256 positively identified proteins for TSP and CF fractions, 46 and 82 proteins were differentially expressed in response to 100 µM ABA, respectively (Tables 4.2 and 4.3). This suggest that exogenous ABA induced the different expression of proteins in both the intracellular and extracellular matrices of sorghum cells. Some of these ABA-responsive proteins are linked to ROS detoxification such as peroxidases, pathogenesis-related protein 10c; cell growth and division such as the proliferating cell nuclear antigen protein; transporters such as non-specific lipid transfer; and cell structure such as pectinesterase (Tables 4.2 and 4.3). Some of the above-mentioned proteins have also been identified in sorghum plants exposed to heat (Ngcala *et al*., 2020) and drought stresses (Goche, 2018) thus making them stress responsive proteins. As such, this study showed that ABA is able to induce the expression of a wide range of proteins without any additional stress factors being applied, thus validating

its role as a very important phytohormone in plant stress response. This study thus would recommend for further studies that would validate the expression of genes of these proteins using quantitative real-time polymerase chain reaction.

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APPENDICES

Table A1: The preparation of proline standard solutions.

Concentration (µg)	Proline (µl)	Nihydrin (µl)	1,2 Glacial acetic acid (µl)
0	0	500	500
5	50	475	475
10	100	450	450
20	200	400	400
40	400	300	300
50	500	250	250

Table A2: The preparation of BSA standard solutions for protein quantification.

Concentration (µg)	5 mg/mL BSA stock solution (µL)	Extraction buffer (µL)	0.1M Hydrochloric acid (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	0	10	80

Table A3: The preparation of resolving and stacking gels for 1D-SDS PAGE.

	Resolving gel 12% (v/v)) (mL)	Stacking gel (5% (v/v)) (mL)
Distilled water	4.3	3.6
40% Acryl-bisacrylamide mix	3	0.625
0.5 Tris- HCL (pH 6.8)	0	0.63
1.5 Tris- HCL (pH 6.8)	2.5	0
10% SDS	0.1	0.05
10% APS	0.1	0,05
TEMED	0.006	0.005
Total volume	**10 mL**	**5 mL**

Table B1: List of the white sorghum total soluble proteins

protein #[a]	Score [b]	%Cov [c]	Accession#[d]	Name and Species[e]	Seq Pep [e]	Ratio of control samples[f]				Mean[g]	Ratio of ABA treated samples[h]				Mean[i]	StDEV[j]	Fold change[k]	p-Value[l]
						113:113	114:113	115:113	116:113		117:113	118:113	119:113	121:113				
31	33.14	56.90	C5XFH6	Fructose-bisphosphate aldolase OS=*Sorghum bicolor* GN=SORBI_3003G393900	21	1.00	1.14	1.05	1.16	1.09	0.81	0.70	1.02	0.92	0.86	0.13	0.79	2.83 E-02
35	31.7	39.39	A0A194YU12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G341200	19	1.00	0.78	0.90	0.95	0.91	1.56	1.40	1.10	1.06	1.28	0.26	1.41	2.76 E-02
36	31.25	23.24	A0A194YGV9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G023700	16	1.00	1.03	1.03	0.96	1.01	1.51	1.16	1.12	1.16	1.24	0.18	1.23	4.74 E-02
41	36.1	47.94	C5XW45	Glyceraldehyde-3-phosphate dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3004G056400	22	1.00	0.99	0.93	0.96	0.97	1.32	1.43	1.05	1.14	1.24	0.18	1.28	2.12 E-02
44	28.7	52.99	A0A1B6PEZ5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G014700	16	1.00	1.10	0.99	1.11	1.05	1.31	1.31	1.14	1.09	1.21	0.11	1.15	4.45 E-02
47	28.41	92.50	C5WMM0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G400900	23	1.00	1.00	0.87	0.83	0.92	1.59	1.36	1.09	1.41	1.36	0.23	1.47	8.53 E-03
51	27.74	31.20	C5YU02	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G083400	15	1.00	0.96	1.01	1.16	1.03	1.21	1.13	1.16	1.24	1.18	0.04	1.15	2.00 E-02
63	24.37	28.57	A0A1B6PFE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G038600	12	1.00	1.08	1.08	1.28	1.11	1.26	1.27	1.21	1.36	1.28	0.06	1.15	4.73 E-02
70	23.45	37.82	C5XIY1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152100	19	1.00	0.97	0.90	0.87	0.94	2.86	4.37	1.85	2.08	2.79	1.22	2.98	1.74 E-02

76	22.32	21.25	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	14	1.00	1.20	1.26	1.03	1.12	1.70	1.57	1.73	1.95	1.74	0.14	1.55	8.60 E-04
81	21.79	33.69	A0A194YT53	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G345800	14	1.00	1.18	1.15	1.11	1.11	1.59	1.70	1.33	1.67	1.57	0.15	1.41	2.70 E-03
113	18.01	13.83	A0A1W0VUE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G227400	9	1.00	0.78	0.95	0.94	0.92	1.33	1.11	1.11	1.08	1.16	0.12	1.26	1.74 E-02
117	18.63	36.94	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600	13	1.00	0.93	0.67	0.91	0.88	1.94	1.91	1.66	1.85	1.84	0.14	2.10	5.53 E-05
132	16.6	14.73	C5WWQ2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G342600	7	1.00	1.52	1.13	1.30	1.24	0.70	0.71	0.87	0.85	0.78	0.07	0.63	9.51 E-03
160	14.58	36.50	C5XV51	Proliferating cell nuclear antigen OS=*Sorghum bicolor* GN=SORBI_3004G33660	10	1.00	0.95	0.74	0.78	0.87	1.24	1.10	1.08	1.00	1.11	0.12	1.27	2.61 E-02
167	24.5	84.91	Q4VQB4	Pathogenesis-related protein 10c OS=*Sorghum bicolor* GN=PR10	23	1.00	0.88	0.83	0.76	0.87	1.62	1.58	1.05	1.13	1.35	0.34	1.55	2.21 E-02
168	14.19	33.99	C5XNL6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G189000	7	1.00	0.90	0.95	0.86	0.93	0.79	0.65	0.80	0.83	0.77	0.08	0.83	1.79 E-02
181	13.48	23.71	C5WXC7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	10	1.00	0.81	1.17	1.05	1.01	0.83	0.79	0.71	0.63	0.74	0.09	0.73	2.10 E-02
205	12.06	14.04	C5YAI8	Pyruvate kinase OS=*Sorghum bicolor* GN=SORBI_3006G267200	6	1.00	1.37	1.36	1.13	1.22	0.82	0.80	1.00	0.98	0.90	0.09	0.74	2.36 E-02
235	10.58	21.83	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	5	1.00	0.73	0.92	0.79	0.86	1.24	1.44	1.07	1.04	1.20	0.21	1.39	2.17 E-02

243	10.16	30.22	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G129700	5	1.00	1.10	1.02	0.86	0.99	1.29	1.58	1.11	1.28	1.32	0.20	1.33	2.54 E-02
268	9.32	17.94	C5WV02	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G033300	6	1.00	0.72	0.74	0.65	0.78	1.10	1.12	0.85	1.04	1.03	0.16	1.32	4.58 E-02
288	9.43	8.39	C5WQD6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G145400	6	1.00	1.47	1.20	1.13	1.20	0.75	0.81	1.08	0.82	0.87	0.12	0.72	3.46 E-02
308	8.12	8.48	C5WY32	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G061900	4	1.00	1.18	0.81	0.91	0.97	1.39	1.46	1.17	1.13	1.29	0.16	1.32	3.05 E-02
323	8.37	18.82	A0A194YRE9	Glutamine synthetase OS=*Sorghum bicolor* GN=SORBI_3004G247000	5	1.00	0.79	0.78	0.76	0.83	1.28	1.22	0.84	1.10	1.11	0.23	1.33	4.76 E-02
333	7.45	11.97	A0A1Z5RB28	Patatin OS=*Sorghum bicolor* GN=SORBI_3007G158800	5	1.00	1.10	1.00	1.03	1.03	1.41	1.68	1.17	1.31	1.39	0.21	1.35	1.67 E-02
340	6.95	8.59	C5XI18	S-adenosylmethionine synthase OS=*Sorghum bicolor* GN=SORBI_3003G140000	3	1.00	0.95	1.10	0.92	0.99	0.75	0.67	0.71	0.77	0.72	0.05	0.73	1.07 E-03
342	6.86	15.42	C5X487	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G111200	4	1.00	1.59	1.33	1.35	1.32	1.01	1.04	1.05	0.87	0.99	0.06	0.75	4.47 E-02
346	6.78	25.93	C5XG44	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G254300	3	1.00	0.85	0.74	0.97	0.89	1.40	1.12	0.94	1.20	1.17	0.21	1.31	4.88 E-02
378	6.17	21.43	C5WNX2	Proteasome subunit beta type OS=*Sorghum bicolor* GN=SORBI_3001G128400	4	1.00	1.27	1.01	0.91	1.05	0.73	0.88	0.85	0.88	0.83	0.07	0.80	4.58 E-02
401	6.06	10.28	C5Z4V3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G051100	4	1.00	1.01	1.03	1.02	1.02	0.85	0.75	1.03	0.85	0.87	0.11	0.85	4.16 E-02

423	6.55	10.28	C5WSF9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301500	5	1.00	0.88	1.07	0.92	0.97	1.17	1.14	1.26	1.51	1.27	0.17	1.32	1.70 E-02
424	5.5	6.18	C5YE18	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G181300	4	1.00	2.37	0.72	0.88	1.24	2.26	2.13	1.93	2.74	2.27	0.28	1.82	4.99 E-02
456	4.74	4.84	C5Z513	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G171800	2	1.00	0.85	0.90	0.84	0.90	0.62	0.77	0.63	0.73	0.69	0.09	0.76	6.98 E-03
496	12.71	9.10	C5X951	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G167000	9	1.00	1.48	1.40	1.39	1.32	1.02	0.92	1.10	1.02	1.01	0.05	0.77	3.61 E-02
510	4.02	8.68	C5X4M5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G255000	2	1.00	0.73	0.77	0.61	0.78	0.48	0.65	0.47	0.54	0.53	0.11	0.69	3.61 E-02
525	4	4.33	C5Z6F3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G080300	2	1.00	0.55	0.84	1.00	0.85	0.48	0.64	0.62	0.48	0.55	0.10	0.65	4.28 E-02
552	3.78	3.81	C5YGY0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G043400	2	1.00	0.70	0.51	1.35	0.89	1.54	1.80	1.29	1.26	1.47	0.29	1.65	3.99 E-02
561	3.71	7.76	C5Z3R9	Proteasome subunit beta type OS=*Sorghum bicolor* GN=SORBI_3010G029400	2	1.00	0.90	0.82	0.89	0.90	1.13	1.16	1.18	1.21	1.17	0.04	1.30	6.25 E-04
571	3.73	6.86	C5XVQ6	Glycosyltransferase OS=*Sorghum bicolor* GN=SORBI_3004G191000	3	1.00	1.21	1.10	0.99	1.07	1.46	1.26	1.58	1.41	1.43	0.12	1.33	4.93 E-03
573	3.54	13.50	C5YK12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G100600	2	1.00	0.53	0.77	1.17	0.87	1.89	1.28	1.12	1.43	1.43	0.38	1.65	4.08 E-02
577	3.56	6.25	C5YIY2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G079600	2	1.00	0.85	1.00	1.13	1.00	1.45	1.27	1.07	1.26	1.26	0.16	1.27	3.35 E-02

594	3.42	4.39	Q94IP1	Cinnamic acid 4-hydroxylase OS=*Sorghum bicolor* OX=4558 GN=C4H	2	1.00	0.79	0.82	0.82	0.86	0.72	0.78	0.70	0.66	0.72	0.06	0.83	3.79 E-02
596	3.28	8.80	C5XRZ8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G296800	2	1.00	0.87	0.95	0.89	0.93	1.10	1.18	1.36	1.10	1.19	0.13	1.28	8.89 E-03
600	10.7	11.87	A0A1B6QFT1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G392000	5	1.00	1.00	0.87	0.81	0.92	0.60	0.84	0.53	0.66	0.66	0.15	0.72	2.01 E-02
855	2.09	5.98	A0A1W0W560	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G195700	2	1.00	0.75	1.13	0.98	0.96	1.00	1.32	1.33	1.39	1.26	0.18	1.31	4.55 E-02

[a] Protein number assigned in ProteinPilot software.

[b] Protein score generated by ProteinPilot software relating to the confidence of protein identification. Only proteins with 95% confidence interval were retained

[c] 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.

[d] Protein accession numbers obtained from the UniProt () database against sequences of *Sorghum bicolor* only.

[e] Number of peptide that were sequenced and contributed towards the protein identity. Proteins with 0 or 1 peptide were filtered out.

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

[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 ABA-treated samples presented as a ratio to the 113-tagged sample.

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

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

[k]Probability value of the quantitative difference between the proteins from the control and the ABA-treated samples.

Table B2: List of the white sorghum culture filtrate secreted proteins

Protein #[a]	Score[b]	%Cov[c]	Accession#[d]	Name and Species	SeqPep[e]	Ratio of control samples				Mean[g]	Ratio of ABA treated samples				Mean[i]	StDEV[j]	Fold Change[k]	p-Value[l]
						113:113	114:113	115:113	116:113		117:113	118:113	119:113	121:113				
1	93.31	84.00	C5Z475	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3010G162000	199	1.00	0.87	0.88	0.90	0.91	1.31	1.56	1.28	1.03	1.29	0.24	1.42	1.49 E-02
3	73.35	40.56	C5XYP5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G233700	57	1.00	0.99	1.07	0.92	1.00	0.74	0.82	0.76	0.78	0.78	0.03	0.78	6.49 E-04
5	55.45	56.55	C5X5K6	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G416700	76	1.00	0.88	1.04	0.81	0.93	0.71	0.69	0.64	0.66	0.67	0.03	0.72	3.53 E-03
6	53.31	65.96	C5WYQ4	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G360400	70	1.00	0.97	0.86	0.96	0.95	0.64	0.66	0.50	0.50	0.57	0.09	0.61	3.82 E-04
9	43.28	45.07	C5WXC7	Alpha-galactosidase OS=*Sorghum bicolor* GN=SORBI_3001G208100	43	1.00	0.96	1.44	0.83	1.06	0.65	0.62	0.57	0.62	0.62	0.03	0.58	1.62 E-02
11	40.5	51.92	C5Y1P4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G099000	43	1.00	1.19	1.17	1.23	1.15	1.52	1.67	1.80	2.35	1.84	0.32	1.60	1.07 E-02
12	41.26	58.84	C5Y360	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011300	62	1.00	0.78	1.13	0.71	0.91	0.69	0.64	0.56	0.50	0.60	0.09	0.66	2.73 E-02
13	37.19	41.01	C5Z8N0	Uncharacterized protein OS=*Sorghum bicolor*	52	1.00	0.83	1.07	0.82	0.93	0.67	0.57	0.63	0.53	0.60	0.06	0.64	2.95 E-03

				GN=SORBI_3010G118900														
14	36.43	78.15	A0A1Z5R5E6	Non-specific lipid-transfer protein OS=*Sorghum bicolor* GN=SORBI_3008G030900	49	1.00	1.01	1.11	1.44	1.14	4.08	3.97	5.01	8.97	5.51	2.07	4.83	1.02 E-02
15	33.57	27.63	C5Z240	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G003100	33	1.00	1.22	1.12	1.14	1.12	0.95	1.02	0.92	0.84	0.93	0.07	0.83	1.94 E-02
16	32.39	29.97	C5XKE9	Endoglucanase OS=*Sorghum bicolor* GN=SORBI_3003G015700	27	1.00	1.41	0.87	1.12	1.10	2.59	3.13	4.02	4.77	3.63	0.88	3.30	2.22 E-03
17	32.44	37.64	C5XQ74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G208800	22	1.00	0.99	1.10	0.96	1.01	1.29	1.15	1.06	1.26	1.19	0.10	1.17	2.95 E-02
19	28.55	56.78	C5YBE9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132400	39	1.00	1.10	0.89	1.02	1.00	4.36	3.64	3.98	3.79	3.94	0.31	3.93	1.73 E-06
22	25.8	51.39	C5Z8T4	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246600	30	1.00	1.05	1.00	1.03	1.02	0.75	0.70	0.73	0.61	0.70	0.06	0.68	6.01 E-05
25	23.31	30.94	C5XB38	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055600	30	1.00	1.20	0.99	1.23	1.11	1.99	2.32	2.26	2.50	2.27	0.19	2.05	8.37 E-05
26	23.2	42.92	C5XCE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G351400	19	1.00	1.00	0.86	1.17	1.01	1.48	1.39	1.68	2.01	1.64	0.27	1.63	6.06 E-03

27	22.05	29.27	C5X3C1	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391300	23	1.00	0.79	1.55	0.77	1.03	0.47	0.49	0.40	0.47	0.46	0.03	0.44	2.00 E-02
29	21.49	62.88	C5XN52	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G331700	26	1.00	0.96	1.12	1.14	1.06	2.29	2.09	2.28	2.65	2.33	0.22	2.21	4.79 E-05
33	22.96	21.26	A0A1W0W7I8	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3002G391900	18	1.00	0.63	1.09	0.73	0.86	0.55	0.52	0.64	0.37	0.52	0.13	0.60	3.00 E-02
34	21.68	40.39	C5XB39	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G055700	25	1.00	0.97	1.29	0.93	1.05	1.39	1.90	1.39	1.20	1.47	0.29	1.40	4.93 E-02
35	20.05	40.52	A0A1Z5RDM9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132100	17	1.00	0.91	1.38	0.98	1.07	1.42	1.48	1.53	1.29	1.43	0.10	1.34	2.39 E-02
41	16.76	42.50	C5YK12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G100600	15	1.00	0.87	1.15	0.83	0.96	0.71	0.79	0.82	0.61	0.73	0.10	0.76	3.91 E-02
42	17.29	30.03	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600 1	23	1.00	0.98	1.00	0.76	0.94	2.13	1.80	1.41	1.48	1.71	0.35	1.82	4.37 E-03
43	16.27	12.22	C5XWE5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G197600	10	1.00	0.91	1.12	0.83	0.97	0.71	0.72	0.78	0.79	0.75	0.04	0.77	1.62 E-02
44	15.81	35.10	C5YVJ7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G232100	12	1.00	0.71	1.28	0.62	0.90	0.44	0.52	0.49	0.44	0.47	0.04	0.52	3.01 E-02

49	15.19	21.69	C5Y675	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G064200	10	1.00	1.11	1.22	1.12	1.11	0.82	0.97	0.75	0.91	0.86	0.09	0.77	8.37 E-03
50	14.82	16.63	C5X022	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G525000	12	1.00	0.99	1.23	1.08	1.07	0.66	0.68	0.68	0.69	0.68	0.01	0.63	3.90 E-04
51	17.37	49.25	C5YBE8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G132300	29	1.00	1.32	0.99	1.47	1.20	5.98	5.07	4.80	7.17	5.75	0.89	4.81	1.62 E-04
54	14.38	42.20	C5YM54	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G151300	12	1.00	0.75	1.16	0.70	0.90	0.50	0.45	0.47	0.46	0.47	0.02	0.52	7.23 E-03
55	14.43	20.07	C5YBH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G135500	18	1.00	0.97	1.18	0.80	0.98	0.63	0.67	0.75	0.70	0.69	0.05	0.70	1.14 E-02
57	14.33	24.24	C5XRX3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G294500	12	1.00	1.32	0.87	1.16	1.09	1.44	1.20	1.41	1.56	1.40	0.14	1.29	4.28 E-02
61	14.25	19.16	C5Z6D9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G079100	11	1.00	1.13	0.75	1.14	1.00	0.77	0.74	0.76	0.81	0.77	0.03	0.76	4.06 E-02
62	13.74	32.85	A0A1W0VX32	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G127100	10	1.00	0.38	1.01	0.88	0.82	0.38	0.34	0.39	0.50	0.40	0.08	0.49	3.45 E-02
64	13.28	16.37	A0A1B6Q838	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422200	7	1.00	1.27	0.78	0.88	0.98	2.03	1.73	1.93	1.53	1.81	0.23	1.84	1.68 E-03

66	25.26	19.00	A0A1B6QI05	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G089100	17	1.00	1.18	1.08	1.02	1.07	0.87	1.02	0.75	0.75	0.85	0.12	0.79	2.45 E-02
71	13.07	29.57	C5Z8T5	Xyloglucan endotransglucosylase/hydrolase OS=*Sorghum bicolor* GN=SORBI_3010G246700	12	1.00	1.63	1.51	1.66	1.45	2.10	2.31	2.04	1.87	2.08	0.13	1.44	1.22 E-02
74	12.66	24.17	C5Y5V0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177600	9	1.00	1.00	1.35	0.87	1.06	1.38	1.47	1.52	1.56	1.48	0.07	1.40	8.58 E-03
81	11.37	11.52	C5XYB4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G229300	8	1.00	2.63	0.96	1.32	1.48	3.08	3.46	3.37	4.06	3.49	0.28	2.36	3.93 E-03
82	11.27	30.37	C5WVG9	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3001G324800	10	1.00	0.91	1.04	1.24	1.05	0.62	0.63	0.61	0.80	0.66	0.09	0.63	3.44 E-03
84	10.86	28.44	A0A109NDM1	Uncharacterized protein (Fragment) OS=*Sorghum bicolor* GN=SORBI_3010G273600	10	1.00	0.82	0.88	0.91	0.90	1.87	2.00	1.59	0.88	1.59	0.55	1.76	3.50 E-02
85	10.71	8.94	A0A1W0VUE2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G227400	6	1.00	1.12	1.18	0.82	1.03	1.55	1.17	1.35	1.20	1.32	0.17	1.28	4.70 E-02
86	10.52	12.06	C5XHP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G419300	5	1.00	1.47	0.85	1.22	1.14	1.89	2.18	1.13	1.91	1.78	0.40	1.57	4.95 E-02
90	10.3	23.79	C5WSE5	Uncharacterized protein OS=*Sorghum bicolor*	9	1.00	1.37	1.07	1.20	1.16	2.02	2.12	1.46	2.12	1.93	0.27	1.66	4.82 E-03

				GN=SORBI_3001G300400														
103	9.68	9.08	C5XL56	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G023800	5	1.00	0.95	0.93	1.38	1.07	1.54	1.23	1.50	2.06	1.58	0.33	1.48	4.50 E-02
105	9.66	56.10	C5YRL0	Non-specific lipid-transfer protein OS=*Sorghum bicolor* GN=SORBI_3008G030700	13	1.00	1.12	1.26	1.30	1.17	1.91	3.24	3.26	2.86	2.82	0.54	2.41	2.19 E-03
106	9.49	17.62	C5Y9T3	Aldose 1-epimerase OS=*Sorghum bicolor* GN=SORBI_3006G105200	6	1.00	0.99	0.97	0.83	0.95	1.10	1.19	1.63	1.44	1.34	0.25	1.42	2.07 E-02
107	9.49	19.42	A0A1W0VY92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G205900	6	1.00	1.01	1.03	0.95	1.00	0.77	0.95	0.91	0.66	0.82	0.14	0.82	4.52 E-02
116	8.5	6.66	C5WPH7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G131100	4	1.00	1.15	1.34	1.09	1.15	0.87	0.90	0.98	0.86	0.90	0.05	0.79	1.84 E-02
117	8.64	29.32	A0A1B6Q6M7	Cysteine proteinase inhibitor OS=*Sorghum bicolor* GN=SORBI_3003G327700	7	1.00	1.24	0.93	1.06	1.06	1.95	2.18	2.26	1.65	2.01	0.26	1.91	7.40 E-04
118	8.42	8.56	A0A1B6Q4S6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G226300	7	1.00	1.18	0.77	0.95	0.98	1.34	1.20	1.25	1.31	1.27	0.06	1.30	1.71 E-02
127	7.52	17.38	C5WSF9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G301500	8	1.00	1.56	0.85	1.11	1.13	2.17	2.27	1.53	1.34	1.83	0.41	1.62	4.54 E-02

131	7.1	11.79	C5XQP2	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G078400	5	1.00	0.78	0.60	0.62	0.75	0.92	1.10	1.15	1.05	1.06	0.13	1.41	2.64 E-02
132	7.04	24.68	A0A1B6P5R2	Uncharacterized protein OS=*Sorghum bicolo*r GN=SORBI_3009G009600	4	1.00	1.45	0.90	1.03	1.10	1.76	2.07	2.47	2.56	2.22	0.34	2.02	2.32 E-03
134	6.79	10.08	A0A1B6QE21	UTP--glucose-1-phosphate uridylyltransferase OS=*Sorghum bicolor* GN=SORBI_3002G291200	4	1.00	1.18	1.09	1.00	1.07	1.62	1.05	1.65	1.58	1.48	0.27	1.38	3.32 E-02
137	7.37	18.05	C5Z6Y0	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G088700	5	1.00	1.07	0.74	0.86	0.92	2.34	2.46	2.79	2.41	2.50	0.22	2.73	1.35 E-05
140	6.33	10.71	C5YA35	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G260300	5	1.00	1.11	0.83	0.98	0.98	0.69	0.83	0.79	0.74	0.76	0.06	0.78	1.53 E-02
142	6.21	25.76	C5YC92	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G018100	8	1.00	1.09	1.02	1.00	1.03	1.44	1.49	1.40	1.57	1.48	0.08	1.43	5.84 E-05
150	6.02	7.59	A0A1Z5RBA4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G225400	3	1.00	1.41	0.90	1.07	1.09	2.82	1.62	3.09	3.84	2.84	0.84	2.59	1.04 E-02
155	6	7.79	A0A1B6Q7A6	Pectinesterase OS=*Sorghum bicolor* GN=SORBI_3003G376900	3	1.00	0.99	1.01	0.81	0.95	1.08	1.05	1.41	1.46	1.25	0.22	1.31	4.36 E-02
161	17.62	31.71	C5YQ75	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3008G010500	20	1.00	0.99	1.01	0.89	0.97	0.63	0.52	0.56	0.39	0.52	0.11	0.54	2.75 E-04

164	5.35	5.88	C5YPF6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3008G119900	4	1.00	1.14	0.75	1.08	0.99	1.50	1.56	1.37	2.36	1.70	0.45	1.71	2.53 E-02
171	5.46	10.75	C5Y5U9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G177500	9	1.00	0.86	1.03	1.04	0.98	1.17	1.21	1.17	1.32	1.22	0.07	1.24	6.06 E-03
173	4.78	6.20	C5YI64	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G198000	2	1.00	1.03	1.02	1.25	1.07	0.66	0.56	0.91	0.62	0.69	0.14	0.64	7.04 E-03
178	4.56	5.80	C5WSY5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G014700	3	1.00	1.03	0.59	1.31	0.98	0.54	0.79	0.54	0.41	0.57	0.16	0.58	4.95 E-02
180	4.48	19.21	C5Y5D5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G169300	4	1.00	0.89	1.05	1.29	1.06	2.39	2.88	2.39	3.69	2.84	0.58	2.68	1.40 E-03
183	4.38	10.22	A0A1B6QJR7	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3001G189000	3	1.00	0.91	0.87	0.97	0.94	1.41	1.67	1.53	2.16	1.69	0.35	1.80	3.98 E-03
186	4.22	6.29	C5Y1P6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G099500	2	1.00	0.84	1.14	1.03	1.00	0.69	0.74	0.69	0.38	0.62	0.16	0.62	1.04 E-02
188	4.1	10.39	C5XHS1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G422000	2	1.00	0.30	0.82	0.28	0.60	1.40	1.39	1.49	1.93	1.55	0.43	2.58	5.39 E-03
191	4.04	7.36	C5Z4E5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044900	5	1.00	0.86	1.04	1.06	0.99	0.52	0.72	0.58	0.55	0.59	0.09	0.60	6.64 E-04

203	4	20.20	A0A1B6Q242	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G085300	2	1.00	1.34	1.48	1.03	1.21	2.19	2.04	2.56	1.18	1.99	0.48	1.64	4.85 E-02
208	3.75	9.39	A0A194YIA9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G044500	3	1.00	1.35	0.82	0.96	1.03	2.35	3.18	3.30	3.24	3.02	0.44	2.92	2.22 E-04
213	3.55	6.11	C5XAF8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G050400	2	1.00	1.19	0.98	0.99	1.04	0.91	0.88	0.55	0.57	0.73	0.19	0.70	2.85 E-02
216	3.35	21.05	A0A1B6QN96	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3001G371900	2	1.00	0.74	1.30	0.85	0.97	0.70	0.38	0.65	0.43	0.54	0.16	0.56	2.39 E-02
221	3.11	7.53	C5XHR8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G421700	2	1.00	0.98	1.00	0.87	0.96	1.65	1.64	1.61	1.07	1.49	0.29	1.55	1.04 E-02
225	2.85	4.96	C5XIT5	Pectinesterase OS=*Sorghum bicolor* GN=SORBI_3003G148300	4	1.00	1.06	1.38	1.05	1.12	0.75	0.82	0.67	0.68	0.73	0.06	0.65	5.68 E-03
226	2.85	5.11	C5YNA1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G172100	3	1.00	1.18	0.86	1.08	1.03	1.36	1.61	1.54	1.54	1.51	0.10	1.47	1.33 E-03
229	2.67	3.25	C5XCD4	Beta-hexosaminidase OS=*Sorghum bicolor* GN=SORBI_3002G350700	2	1.00	1.38	0.85	1.00	1.06	1.80	1.16	1.55	1.57	1.52	0.25	1.44	3.79 E-02
235	2.6	3.05	C5X0Y6	Uncharacterized protein OS=S*orghum bicolor* GN=SORBI_3001G529700	2	1.00	1.16	1.09	1.36	1.15	1.92	2.07	1.75	2.11	1.96	0.14	1.70	3.77 E-04

236	2.51	7.63	C5WV02	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G033300	2	1.00	0.94	1.95	1.62	1.38	2.28	2.17	1.79	2.15	2.10	0.16	1.52	3.64 E-02
265	4.02	6.04	C5XIY0	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152000	3	1.00	1.24	0.94	0.87	1.01	0.73	0.71	0.63	0.65	0.68	0.05	0.67	7.51 E-03
269	3.35	18.67	A0A1B6QG28	Superoxide dismutase [Cu-Zn] OS=*Sorghum bicolor* GN=SORBI_3002G407900	2	1.00	1.20	1.04	1.03	1.07	0.96	0.71	0.73	0.29	0.67	0.26	0.63	3.49 E-02
281	2	7.69	C5Y2R8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G126200	2	1.00	0.73	1.22	0.50	0.86	0.37	0.17	0.25	0.24	0.26	0.10	0.30	9.82 E-03

[a] Protein number assigned in ProteinPilot software.

[b] Protein score generated by ProteinPilot software relating to the confidence of protein identification. Only proteins with 95% confidence interval were retained

[c] 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.

[d] Protein accession numbers obtained from the UniProt () database against sequences of *Sorghum bicolor* only.

[e] Number of peptide that were sequenced and contributed towards the protein identity. Proteins with 0 or 1 peptide were filtered out.

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

[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 ABA-treated samples presented as a ratio to the 113-tagged sample.

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

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

[k]Probability value of the quantitative difference between the proteins from the control and the ABA-treated samples.