A comparative proteomic analysis of two contrasting *Salvia hispanica* L. genotypes under salinity stress

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ABSTRACT

A comparative proteomic analysis of two contrasting *Salvia hispanica* L. genotypes under salinity stress

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Salvia hispanica L. is an annual pseudocereal food crop, locally known as chia that has the ability to grow in water stress environments. The importance of chia dates back to the pre-columbian era where it was consumed as staple food by the indigenous South Americans due to its high nutritional and medicinal benefits. A single chia plant produces two seed variants: white seed genotype (denoted as WSG) and black seed genotype (denoted as BSG). Chia seeds have been proven to have a huge potential as a healthy food source and contained various medicinal properties. However, these plants are still prone to environmental stress conditions such as salinity that is one of the major abiotic stresses that influence crop production and yield worldwide. Despite the nutritional impact of the chia seeds, limited information regarding their molecular responses to abiotic stress conditions are known. This study was divided into two distinct parts. Firstly, the study comparatively analysed the leaf proteomes of two chia genotypes using gel-based proteomic analysis coupled with mass spectrometry. Total soluble proteins were extracted from chia leaves and subjected to 2-D PAGE analysis. Proteins were visualized by CBB and identified by MALDI-TOF MS/MS. A total of 284 and 209 spots were detected in WSG and BSG, respectively. Using mass spectrometry, 36 differentially expressed protein spots were successfully

identified based on their protein abundance using homology database searches. Interestingly, two defensive-related proteins (osmotin-like protein and the chalcone isomerase) were only present in WSG and absent in BSG. In light of previous information regarding the nutritional profiles (no significant difference) of these two genotypes, this study has shown that there are distinct molecular differences between these genotypes. Therefore, WSG will be used in further downstream analysis.

The second part of this study focused on the influence of salt stress (imposed by 100 mM NaCl) on the leaf proteome of WSG. Using gel-based proteomic analysis, 61 differentially expressed proteins were identified and classified into nine functional categories. Most of the proteins identified in this study were up-regulated by salt stress. Interesting to note, 12 proteins identified in this study were only present in response to salt stress but were absent in the control. These proteins include ATP-dependent zinc metalloprotease FTSH 2 (spot 48), HSP70 proteins (spots 46 and 47), superoxide dismutases (spots 10, 41 and 42) and an ascorbate peroxidase (spot 56). All these proteins are important antioxidants that play a significant role in scavenging reactive oxygen species (ROS). Previous studies have shown that these antioxidants play vital roles in stress tolerance. These proteins could serve as potential biomarkers that could be used to enhance salt stress tolerance in pseudocereals and cereal food crops.

DECLARATION

I declare that **Comparative proteomic analysis of two contrasting *Salvia hispanica* L. genotypes under salinity stress** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Achmat Williams

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Signature

November 2016

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Nelson Mandela: "*It always seems impossible until it's done*"

LIST OF ABBREVIATION

µg	Micrograms
µl	Microliters
1-D	One-dimensional gel electrophoresis
2-D	Two-dimensional gel electrophoresis
ACN	Acetonitrile
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSG	Black seed genotype
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3cholamidopropyl)dimethylammonio]-propanesulfonate
dH_2O	Distilled water
DTT	Dithiothreitol Cleland's reagent
g	Grams
HSP70	Heat shock protein 70
HEPES	4-(2- hydroxyethyl)-1-

	piperazineethanesulfonic acid
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kDa	Kilo Daltons
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight
Mascot	Matrix Science
mg	Mili grams
min	Minutes
ml	Mililiter
mM	Milli molar
MOWSE	Molecular weight search
MS	Mass spectrometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
PAGE	Polyacrylamide gel electrophoresis
PMF	Peptide mass fingerprinting

PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidenedifluoride
RuBP	Ribulose-1, 5-biphosphate
s	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis gels
SOD	Superoxide dismutase
T	Treated
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
U	Untreated
Uniprot	Universal protein resource
V	Volts
WSG	White seed genotype

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The impact of climate change will drastically affect Africa due to the long-term shift in weather conditions. With a fast increasing population that is expected to surpass 9 billion within the next three decades, there is an increasing need for sustainable food production, efficient yields and high quality crops (Komatsu *et al*., 2013). African communities are dependent on agricultural activities for sustainable food security. Rapid population growth coupled with severe environmental changes is threatening food security on the African continent. One third of African populations are amongst those that are suffering from famine and malnutrition due to the unavailability of nutrient dense food sources (Slingo *et al*., 2005; Wlokas, 2008). Abiotic stresses such as salinity, extreme temperatures, drought and toxic heavy metals are contributing factors that reduce crop production on the African continent.

Plants are the backbone of life on earth and it is an essential resource for human existence. Due to climate change and increasing global population, novel tools are required to protect crops against unfavourable conditions that may restrict plant growth and development. Important food crops such as rice, maize and wheat are major food sources for human consumption and contribute to food security. These crops form part of the staple diet for more than half of the world's population. Due to rapid population growth coupled with environmental factors associated with

climate change, more Africans are suffering from hunger and malnutrition as the economically important food crops are affected by these unfavourable conditions. In order to improve food security on the African continent, it is imperative to explore alternative food sources that are rich in proteins and other nutrients with more resilience to environmental changes.

1.2 Chia (*Salvia hispanica* L.) as an alternative food source

Salvia hispanica L. is a biannual cultivated food crop and member of the *Labiatae* family that originated from Mexico and Guatemala (Figure 1.1). This desert crop is deemed to be drought tolerant although no scientific evidence exists to prove this theory. Chia is a pseudocereal crop plant that was consumed as staple food by the indigenous South Americans including the Mayan and Aztec populations during the pre-columbian era (Sandoval and Paredes, 2013). To date, chia is commercially cultivated in various regions across the globe including Mexico, Guatemala, Argentina, Bolivia, Ecuador and Australia. Over the past decade, Australia has become the leading producers of chia seeds in the world (Crawford *et al*., 2012). Chia plants are primarily cultivated for its seeds although the entire plant can be utilised. The seeds are oval in shape and ranges from 1-2 mm in size (Mohd *et al*., 2013). A single plant produces two colour variants commonly known as black and white (Figure 1.2). Ayerza (2009) have shown that no significant difference exists in the nutritional profile of the two seed variants. Recent studies have shown that chia seeds have huge potential for food consumption and medicinal uses (Ayerza, 2009).

Figure 1.1: Mature *Salvia hispanica* cultivars. Adapted from: https://treasurecoastnatives.wordpress.com/

Figure 1.2: The colour variation between black and white chia seeds genotypes. Adapted from: http://jeanetteshealthyliving.com

1.2.1 Nutritional composition of chia seeds

Chia seeds have been suggested by economical historians as an important food source due to its numerous desirable characteristics (Cahill, 2003; Ixtaina *et al*., 2008). The main characteristics which makes it so desirable is the botanical α-linolenic acid and protein contents, which have been shown to be higher compared to other major crops such as oats, maize, wheat and rice (Ixtaina *et al*., 2008). The nutritional profile of chia seeds is illustrated in Table 1.1 (Mohd *et al*., 2013). Furthermore, chia seeds have been shown to contain at least three times more antioxidants than blueberries which allows for the inhibition of free radicals and defence against reactive oxygen species (Ayerza, 2009). Chia seeds are highly recommended as an alternative food source for both human and animal consumption due to it being hypoallergenic because it is mycotoxin and gluten free (Ayerza, 2010). Research have shown that chia seeds can be used successfully to increase the ω-3 fatty acid composition in animal products such as milk, eggs and various meats (Mohd *et al*., 2013).

Table 1.1: Chia seeds constituents and distribution of each constituent (Mohd *et al*., 2013).

Constituents	Percentage composition (%)
Carbohydrates	26–41
Fats	30– 33
dietary fibre	18–30
Protein	15–25
Ash	4-5
Minerals, Dry matter, and vitamins	90–93

1.2.2 Medicinal value of chia seeds

Life style related diseases are a major concern globally due to a lack of a well-balanced diet and exercise regime. In both developed and developing regions, death and disability remains a problem due to chronic diseases such as cardiovascular heart disease (CVD), high blood pressure, obesity, diabetes, and other related diseases (Ayerza, 2009). Studies have shown that increased intake of saturated lipids, trans-fatty acids and polyunsaturated ω-6 fatty acids have resulted in chronic diseases (Ayerza, 2009; Mohd *et al*., 2013). However, consuming lipids rich in ω-3 fatty acids have shown to reduce the risk of CVD (Mohd *et al*., 2013). Chia seeds contain high levels of ω-3 fatty acids, which have been shown to normalize blood sugar levels. Chia seeds also contain high levels of dietary fibre, which is ideal for weight management and preventing constipation. Despite the vast array of nutritional and medicinal characteristics associated with the consumption of chia seeds to promote a healthy lifestyle, most consumers are unaware of these benefits due to the lack of sufficient and relevant information and knowledge in the public domain.

1.3 Influence of abiotic stresses on plants

Plants are sessile organisms and remain in their habitat throughout their entire life cycle. During their life cycle, plants are exposed to diverse environmental factors. These factors negatively influence plant growth and development. Abiotic stress conditions such as salinity, drought, high temperatures and mineral deficiency has major limitations on plant growth and development (Cushman and Bohnert, 2000; Nakashima *et al*., 2009; Wanga and Freib, 2011). These stresses negatively influence plant growth, crop productivity and various metabolic processes (Debnath

et al., 2011). Therefore, there is an increasing need for stress tolerant food crops due to the negative impacts caused by the effects of climate change (Cushman and Bohnert, 2000). In order to mitigate the negative effects caused by abiotic stress conditions, plants have evolved their defensive strategies at molecular and cellular levels in order to survive (Nakashima *et al*., 2009). When the plant is exposed to various stresses, it triggers a specific response or it alters gene expression. The genes that are expressed are regarded as potential biomarkers that assist the plant in enduring these stress conditions. In most cases, plants may experience multiple stresses at any given time during its growing season. Osmotic stress caused by water deficit affects up to 23 % of all arid regions (Grebosz *et al*., 2014). Due to the increasing demand for food production and security, researchers have focused on the mechanisms of plant adaptation to water stress. Osmotic stress has been shown to limit productivity and distribution of cereal crops that is found to be a consequence of diverse abiotic stressors such as drought, salinity and extreme temperatures (Grebosz *et al*., 2014; Valentovič *et al*., 2006). Abiotic stresses are similar as all stresses affects or causes a disruption in the plants water status. This is mainly caused by decreasing the water availability (drought) and decreasing the ion content and water uptake (salinity), which may lead to cell death (Verslues *et al*., 2006). Furthermore, other negative effects are the formation of the reactive oxygen species (ROS). These species will result in oxidation of proteins, amino and nucleic acids, lipid peroxidation, oxidative stress and ultimately cell death.

1.3.1 Drought stress

Drought or water deficiency is known as a period without substantial rainfall (Jaleel *et al*., 2009). Water plays an important role in the functioning of all forms of life.

Generally, due to transpiration and evaporation, water is continuously loss, which may result in drought stress. Drought stress occurs when soil water/moister content is significantly reduced (Jaleel *et al*., 2009). In various cropping systems around the world, drought is considered the main abiotic stressor, and is estimated to drastically reduce crop yields. This poses various challenges to food productivity, which negatively impacts food security (Hsiao and Acevedo, 1974; Jogaiah *et al*., 2013). The degree of damage to plants is based on the severity of the stress as well as the species, genotype, developmental stage and duration of exposure to the stress (Obidiegwu *et al*., 2015).

The effects of drought stress on plants are visible and may affect the plant's morphology, physiology and biochemical responses. Drought is a major limiting factor in the plant's initial growth phase. According to research, drought stress causes major morphological changes (Jaleel *et al*., 2009). Morphological changes were observed in the stem, plant length; the reduction in photosynthetic pigments such as chlorophylls and carotenoids, leaf morphology, decrease in CO_2 influx and it affects the electron transport system. Furthermore, drought-induced stress affects the plants metabolic pathways and mineral uptake. Drought induced stress causes a loss in water availability and cellular dehydration, which results in a cellular metabolic changes. This in turn cause changes in the proteome, which was intensively studied by Bogeat-Triboulot *et al.* (2007) and Wang *et al*. (2015) where drought stress has an effect at a molecular level.

1.3.2 Salinity stress

Salinity stress refers to the amounts of salt content found within soil or water that negatively affect the normal functions of plants. Similarly, to drought stress, salinity

is regarded as one of the most important factors that limit crop production and yield. Based on all water sources on the Earth's crust, 97.5 % are salt water therefore, salinity is a major problem globally (Shannon *et al*., 2008). There are mainly two sources causing salinity namely primary and secondary salinity. Primary salinity known as "natural salinity", refers to salt ion accumulation as a consequence of long term exposure to saline soils or ground water. This is caused by weathering perennial materials such as rocks and/or oceanic salt carried inland by wind or rainfall. Whereas, secondary salinity known as "human-induced salinity" which is caused by human activities. Some of the major human activities are land clearing, replacement of perennial vegetation with annual crops and irrigation activities using salt-rich water (Le Gall *et al*., 2015; Munns, 2002).

Exposure to salinity-induced stress has been shown to influence the growth, survival and biomass production of plants, which will negatively affect the food crop industry (Hasanuzzaman, *et al*., 2013). Salinity stress is a major problem in many regions, and may cause serious damage in the next 25 years (Rajendran *et al*., 2009; Wang *et al*., 2003). High saline soils are not only found in deserts and semi-deserts regions, there are a considerable amount of land in the world that is affected by salinity stress. To date, over 20 % of total land has been affected by salinity stress (Hasanuzzaman, *et al*., 2013; Le Gall *et al*., 2015). However, with increasing saline levels, the destructive damage is expected to increase causing a staggering 50 % loss of cultivated lands in the next 15 years (Le Gall *et al*., 2015).

The physiological effects of salinity stress on plant growth resemble comparable effects as observed for other abiotic stresses such as drought and high temperatures. According to Munns and Tester (2008), high salt concentration prevents roots from

extracting water from the soil, thus increasing toxicity. Salts on the outside of roots have a direct effect on the metabolism and the development of the cell, whereas salts inside the plant take time before influencing plants' functions (Munns and Tester, 2008). These negative effects caused by salinity stress are a consequence of Na^+ and Cl^- ion accumulation. However, both ions cause significant physiological changes in plants, where Cl^- ions are more destructive than Na^+ ions. The uptake of these ions is highly depended on the plant (growth stage and genetic characteristics) and its environment (temperature, humidity and light intensity). This uptake of ions is the main cause of plant damage and ultimately may cause plant death. Plants require Cl^- ions as it plays a role in regulating enzymes activities within the cytoplasm. It is essential for photosynthesis, turgor and pH regulation (Hasanuzzaman, *et al*., 2013). However, in high concentrations it causes more harm than good as it reduces growth and water use efficiency in plant cells.

1.4 Heat shock proteins

The heat shock proteins were initially identified as proteins that were found in plants when exposed to a rapid increase in temperature (Sung *et al*., 2001). Most heat shock proteins are recognised as chaperones and are mainly located in cytoplasm under basal conditions, but rapidly transferred to the nucleus when exposed to stress. Chaperones are proteins that help proteins fold under abnormal conditions. When a living cell experiences changes in temperature, salinity, and heavy metals (Xu *et al*., 2012); the molecular chaperones will react against the stress by preventing aggregation and refold stress-mediated unfolded polypeptides, thus, making molecular chaperones a key component in maintaining homeostasis (Wang *et al*., 2004). There are five major families of HSPs/chaperones namely the

HSP70 (DnaK) family; the small HSP (sHSP) family; the HSP90 family; the HSP100 (Clp) family; and the chaperonins (GroEL and HSP60) (Wang *et al*., 2004; Xu *et al*., 2012). The most studied chaperone is the HSP70 family. In plants, scientists have identified 18 HSP70 genes within *Arabidopsis thaliana* alone (Wang *et al*., 2004). These HSP70 genes have shown to be highly expressed as a result of environmental stress conditions. HSP70 is a known stress responsive protein and has been used to in various studies as a protein to determine whether the applied stress is physiologically relevant by HSP70 immunoblotting.

1.5 Proteomics

Proteomics is the study of protein structure and their function within a biological system (Graves and Haystead, 2002). The term proteome is a combination of two words "protein" and "genome". For more than 20 years, major advances have been developed and discovered within the field of proteomics. Proteomic analysis has become an integral part within crop plant studies for more than 10 years (Komatsu *et al*., 2013). Techniques in proteomics have been use in various disciplines (Kushalappa and Gunnaiah, 2013; McGarry *et al*., 2015; Ngara *et al*., 2012). Proteomics is highly important as the cell state can be determined by analysing the protein content. Therefore it is important to have rapid and efficient tools for characterisation of proteins (Chmelik *et al*., 2002).

The use of proteomics in plant science has progressed tremendously, where crucial proteins have been identified that are directly linked to plant growth and development (Komatsu *et al*., 2013). As previously mentioned in this review, climate changes are evident which can be a major limiting factor to agricultural important crops such as maize, wheat, soybean and barley. Proteomic techniques

have been used to research model systems in plant science and have been applied to several agriculturally important crops under abiotic and biotic stresses in order to determine protein-protein interaction, protein function and localization (Chan, 2013; Wang *et al*., 2013). Proteomic methods are divided mainly into gel-based and gel-free proteomics.

1.5.1 Gel-based proteomics

The traditional gel-based proteomic approach is one of the widely used techniques to study proteins and was first introduced in 1975 (O'Farrell, 1975). Gel-based proteomics has evolved and became one of the main methods of choice for studying differential expression (Abdallah *et al*., 2012). The standard method for studying proteins is by separating soluble proteins by 2 dimensional polyacrylamide gel electrophoresis (2-D PAGE), followed by identification by using a mass spectrometer. Today, this workhorse method has the ability to visualize over 10000 spots, which corresponds to over 1000 proteins on a single 2-D gel. This is all due to the high resolving power of 2-D PAGE.

Various techniques have been used in proteomic studies such as separation techniques, mass spectrometry, immunoblotting and bioinformatics (Chmelik *et al*., 2002). The mentioned techniques have been applied successfully in identification of proteins in countless biological systems such as soybean (Koo *et al*., 2011), pea (Bourgeois *et al*., 2009), peanut (Kottapalli *et al*., 2008), lupin (Islam *et al*., 2012) , rapeseed (Hajduch *et al*., 2006), medicago (Gallargo *et al*., 2003), *Arabidopsis* (Gallargo *et al*., 2002), wheat (Islam *et al*., 2002), sorghum (Ngara *et al*., 2012; Roy *et al*., 2014), barley (Finnie *et al*., 2004) and more recently pseudocereal crops (Huerta-Ocampo *et al*., 2015). In general proteomic studies, proteins are separated

at a one dimension (1-D) based on the molecular weight (range of 10 and 300 kDa) of the proteins or in a two dimension (2-D) based on the proteins' isoelectric point and molecular weight (Graves and Haystead, 2002; Vadivel, 2015). In 2-D PAGE, these parameters are not related so one would expect a uniform distribution of protein spots on the gel, which is considered as a protein fingerprint of a specific sample.

In both 1-D and 2-D, polyacrylamide is used which has the same UV absorbance as proteins. Therefore, in order to visualise proteins, proteins needs to be stained on the gel. One of the most commonly used stains is Coomassie blue staining method because it is inexpensive, easy to use and safer (Baggerman *et al*., 2005). However, it is less sensitive, which leaves a large amount of proteins undetected compared to other staining methods such as silver staining method. The silver staining method is 20 to 50 times more sensitive however; one of the major disadvantages is that it is not compatible with the mass spectrometer. Therefore, newer methods have been developed to overcome these limitations such as fluorescent staining methods (Spyro Ruby, Lava and Deep Purple) (Abdallah *et al*., 2012; Baggerman *et al*., 2005). The fluorescent staining method makes use of radioactive or fluorescent labels. This allows one to separate more than one protein sample on a single gel. This method was coined by Ünlü and co-workers in 1997 and was termed Two Dimensional Difference Gel Electrophoresis (2-D DIGE) (Ünlü *et al*., 1997).

One of the major advantages of using 2-D allows proteins to be resolved that have gone through post-translational modification. The 2-D technique has been enhanced and improved over the past years and have introduced various pH gradients which assist in the reproducibility of 2-D (Görg *et al*., 2004).The implementation of 2-D

DIGE allows for the comparison of more than one protein sample (control vs experiment) on a single gel. With larger experiments with three samples, a third fluorescent dye was introduced (Alban *et al*., 2003). Although 2-D is well-established and meaningful progress and evolution was made, it has its limitation when studying certain classes of proteins like those with extreme p*I*'s or molecular weights, lower abundance proteins, and hydrophobic membrane proteins (Gygi *et al*., 2000). In practice, 2-D can only visualised approximately 30-50 % of the proteome, which is highly depended on sample and tissue type. Even though, gel-based approaches are under pressure due to these limitations, it remains one of the widely used approaches in plant sciences. However, new developments of alternative MS-based approaches have been developed.

1.5.2 Gel-free proteomics

Due to the limitations identified in gel-based proteomics in recent years, researchers have been highly focused on exploring alternative approaches (Vadivel, 2015). Gel-free proteomics analysis has been explored although these techniques complement each other. This approach is MS-based but with an entirely new toolbox for quantitative analysis (Abdallah *et al*., 2012). The gel-free approach has been more routinely used and has great potential to give information about subsets of proteins that were not found by the traditional 2-D approaches (gel-based). The most commonly used gel-free method among researchers is the multi-dimensional protein identification (MudPIT) which include a strong cation-exchange (SCX) fractionation, reversed-phase (RP) chromatography and tandem mass spectrometer (MS/MS) (Vadivel, 2015). This method consists of an in-solution digestion instead of in-gel digestion when compared to gel-based proteomics. The digested peptide

solution is subjected to liquid chromatography columns which are in line with the MS/MS system. At least 2000 proteins can be identified in a sample using the MudPIT approach. Over 12,000 proteins have been identified in different organs of *Arabidopsis* and in maize leaf using gel-free techniques (Hernandez *et al*., 2012; Vadivel, 2015). However, both platforms (gel-based and gel-free) are high through-put methods therefore the choice between them is determined solely based on the biological questions being asked. Eventually, both approaches (gel-based and gel-free) have great impacts within plant science and often add corresponding information for a holistic analysis (Gevaert *et al*., 2007).

1.5.3 Applications of proteomics in plants studies

The applications of proteomic analysis in various plant species have been shown to be a very effective tool. The expression of the proteome between plant cultivars of varying tolerance compared to stress treated plants has a potential of investigating stress responsive mechanisms in plants that can be link to specific phenotypic traits (Salekdeh *et al*., 2002). The stress responsive proteins may either show qualitative or quantitative changes between the control and treated groups (Thiellement *et al*., 2002). By using mass spectrometry, positive identification of proteins has led to the discovery of proteins that play a vital role in stress tolerance (Salekdeh *et al*., 2002). Therefore, plant proteomics can identify candidate genes that can be used for the genetic improvement of plants against stresses (Cushman and Bohnert, 2000). However, not all proteins that are expressed may play a role in tolerance (Zhu, 2000). The overexpression of other proteins may be due to the stress treatment that caused cell damage. For this reason, after the proteomic analysis the proteins of interest are identified using MS and subjected to bioinformatics analysis for further

characterisation. It would also be of great importance to perform further functional studies to obtain a true reflection of the proteins identified.

1.6 Aims of this study

The aim of this study is to comparative analyse the leaf proteomes of two contrasting chia genotypes and their responses to exogenously applied salinity stress using 2-D gel electrophoresis coupled with MALDI-TOF MS/MS analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant growth and treatment

Chia (*Salvia hispanica* L.) seeds (purchased from Faithful to nature, Sea Point, Cape Town) code-named WSG (white seed genotype) and BSG (black seed genotype) were germinated on wet filter paper in a dark environment for 2-3 days. Germinated seedlings were transplanted (1 per pot) in a moist (distilled water) promix growth medium (Stodels Nurseries, Brackenfell, South Africa) and were allowed to grow in a growth room on a 16 hours light/8 hours dark cycle at 25°C until the first leaves were fully expanded. At this stage germinated seedlings were irrigated with 50 ml of nutrient solution [1 mM K_2SO_4, 1 μM $ZnSO_4$, 5 mM $CaCl_2$, 5 μM $MnSO_4$, 10 mM NH_4NO_3, 5 mM KNO_3, 5 μM H_3BO_3, 1 mM K_2HPO_4 buffer at pH 6.4, 10 mM 4-(2- hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) at pH 6.4, 100 μM Fe-NaEDTA, 2 μM Na_2MO_4, 1 μM $CoSO_4$, 1 μM $CuSO_4$ and 2 mM $MgSO_4$] at 2 day intervals for a period of 21 days.

For treatment with NaCl to impose salinity stress, 50 ml of nutrient solution containing NaCl at a final concentration of 100 mM was applied (at intervals of 2 days between each treatment) to each plant by adding the solution directly to the base of the stem of the plant for a total period of 21 days. Control plants were treated in a similar manner except that nutrient solution without NaCl was used for the control plants.

2.2 Protein Extraction and quantification

Total leaf soluble protein for each genotype was extracted using the phenol/SDS method as previously described by Wang *et al*. (2006) with various modifications. Protein extracts were obtained by pulverizing 0.25 g of leaf tissue into a fine powder in liquid nitrogen and homogenizing leaf tissue with 1 ml of 10 % (v/v) acetone. The resulting homogenates were centrifuged at 16,000 x g for 10 min at 4°C and the supernatant decanted. The pellet was washed once with methanolic ammonium acetate (0.1 M ammonium acetate dissolved in 80 % (v/v) methanol) and 80 % (v/v) acetone and the supernatant decanted after each centrifugation (16,000 x g for 5 min) step. The pellet was dried at room temperature and briefly re-suspended in 0.8 ml dense sodium dodecyl sulfate (SDS) buffer (2 % (w/v) SDS, 0.1 M Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 % (v/v) 2-mercaptoethanol, 30 % (w/v) sucrose,) and 0.8 ml phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) in a 2 ml eppendorf tube. The mixture was vortexed thoroughly for 3 min and the phenol phase was partitioned by centrifugation at 16,000 x g for 10 min at 4°C. The upper phase (phenol) was transferred to fresh eppendorf tubes (0.5 ml for 2 ml tube). The extraction process was repeated and phenol fractions were mixed. Proteins were precipitated overnight with 3 volumes of pre-cooled methanolic ammonium acetate (0.1 M ammonium acetate dissolved in 80 % (v/v) menthanol). Precipitated proteins were recovered at 16,000 x g for 10 min (4°C), and washed with cold methanolic ammonium acetate and cold 80 % (v/v) acetone. The final pellet was dried at room temperature and dissolved in 100 µl isoelectric focusing (IEF) buffer containing 7 M urea, 2 M thiourea, 4 % (w/v) 3-[(3cholamidopropyl)

dimethylammonio]-1 propanesulfonate (CHAPS) and 20 mM dithiothreitol (DTT). Each sample was sonicated twice for 30s in a water bath at 25°C.

Total protein concentration for each sample was determined according to the method of Bradford (1976). The protein concentration was calculated using bovine serum albumin (BSA) as standard.

2.3 1-D and 2-D analysis

Approximately 10 µg of leaf protein extract were separated on a 1-D SDS gel to evaluate for quality and loading quantities prior to 2-D analysis. For the 2-D, protein samples (100 µg) in a final volume of 125 µl Destreak rehydration solution (GE Healthcare) containing 0.2 % (v/v) carrier ampholytes (pH 3–10; Bio-Lyte, Bio-Rad, Hercules, CA, USA) were loaded into a focusing tray. Immobilized pH gradient (IPG) strips (4-7 NL, 7 cm, Bio-Rad) were passively rehydrated overnight. Isoelectric focusing (IEF) was carried out using a Protean IEF Cell system (Bio-Rad) under the following conditions: 250 V for 15 min with a linear ramp, 8000 V for 1 h with a linear ramp, and finally 8000 V for 12,000 V-h with a rapid ramp. After IEF, the strips were incubated for 15 min in equilibration buffer I consisting of 130 mM DTT, 6 M urea, 20 % (v/v) glycerol, 0.375 M Tris-HCl (pH 8.8) and 2 % (w/v) SDS. The strips were incubated for 15 min in equilibration buffer II, consisting of 135 mM iodoacetamide (IOA), 6 M urea, 20 % (v/v) glycerol, 0.375 M Tris-HCl (pH 8.8) and 2 % (w/v) SDS. The strips were then placed onto a 12 % SDS-PAGE and sealed using 1 % (w/v) low-melting temperature agarose. Second dimensional electrophoresis was performed at a constant current of 30 mA. After electrophoresis, the gels were stained for 1 h in a solution of Coomassie brilliant blue (CBB) containing 0.02 % (w/v) CBB,

0.25 % (v/v) Propan-2-ol and 10 % (v/v) acetic acid, and then destained for 12 h in a solution of 1 % (v/v) glycerol and 10 % (v/v) acetic acid.

2.4 2-D gel image analysis

Gel image analysis was performed using PDQuest software (version 8.0.1, Bio-Rad). Spot intensities were subjected to statistical analysis to obtain the differentially abundant protein spots. A protein spot was considered differentially abundant between samples when it had both a *p*-value of less than 0.05 and a fold change of more or less than 1.5. Three biological replicates were used for the analysis.

2.5 In-gel digest and peptide extraction

Briefly, the differential spots were manually excised from 2-D gels and washed twice in distilled water for 10 min. The gel pieces were then subjected to destaining solution (50 % (v/v) acetonitrile and 25 mM ammonium bicarbonate) which was followed by sonication for 3-5 min. The gel pieces were dehydrated by washing twice in 50 % (v/v) acetonitrile (ACN) for 10 min. After dehydration the gel pieces were digested overnight in 50 ng of sequencing grade trypsin (Promega Madison, Wisconsin, United States) at 37°C according to the manufacturer's guide. Peptides were then extracted with a 10 µl solution of 30 % (v/v) ACN and 0.1 % (v/v) trifluoroacetic acid (TFA) (Sigma, St. Louis, Missouri, United States) for 30 min at room temperature and stored at 4°C until analysis.

2.6 Protein identification by MALDI-TOF MS/MS

Differential expressed proteins were identified using the ultrafleXtreme MALDI-TOF system (Bruker Daltonics, Germany) with instrument control through Flex

control 3.4. A small fraction (1 µl) of peptide extract produced by the in-gel digestion was placed on the MALDI anchor chip and allowed to air-dry at room temperature. Each sample on the anchor chip was covered with 1 µl solution of 0.4 mg/ml α-cyano-4-hydroxycinnamic acid in a mixture of acetonitrile and 0.1 % (v/v) trifluoroacetic acid (TFA) (70:30) and then air-dried. The mass spectra were acquired on an ultrafleXtreme TOF mass spectrometer (Bruker Daltonics). Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 Da to 4000 Da. Data captured by MALDI-TOF MS/MS were a result coupled with Mascot version 2.2 (http://www.matrixscience.com) against NCBI [Taxonomy: *Viridiplantae* (Green Plants)] and SwissProt using the following parameters: 0.2 Da mass tolerance, one missed cleavage, carbamidomethylation of cysteines as fixed modifications and oxidation of methionine as variable modifications.

2.7 Bioinformatics analysis

Theoretical Mr and pI of MS identified proteins were estimated using the Compute pI/MW tool available on ExPASy (http://expasy.org). Proteins were grouped into functional categories using data available on the UniProt database (www.uniprot.org) as well as literature sources.

2.8 Western blot analysis

Western Blot analysis for HSP70 on chia leaf protein extracts separated on a 1-D gels were performed as described in section 2.3. The 1-D gel were not stained with CBB, instead it was pre-equilibrated overnight in cold transfer buffer at 4°C.

A 7 cm x 9 cm polyvinylidenedifluoride (PVDF) membrane was flooded with 100 % isopropanol for 30s to activate the membrane. The PVDF membrane was then placed in cold transfer buffer with six 7 cm x 9 cm pieces of filter paper. It was then incubated at room temperature for 15 min. Three pieces of the filter paper was placed on the electrode cassette of the Transblot® Electrophoresis Transfer Cell (BIO-RAD). The PVDF membrane was then placed on top of the three filter papers followed by the 1-D SDS gel. Another three layers of filter paper was placed on top of the gel to form a gel-membrane sandwich. Bubbles were eliminated by rolling the sandwich with a 2 cm stripette. The transfer was performed at 24 V for 20 min.

After protein transfer, the membrane was placed in 1 % (w/v) blocking buffer [casein dissolved in 1 X PBS containing 0.1 % (v/v) Tween 20 (PBST)] for 1 hour. Thereafter, it was washed three times with PBST and the membrane was then incubated in primary antibody (Human HeLa cells anti-HSP 70 monoclonal antibody raised in mouse; Biomol International LP) diluted 1:1000 in 1 % (w/v) PBST solution for an hour. The membrane was washed three times with PBST. After the wash steps, the membrane was incubated for one hour with the secondary antibody (goat anti-mouse IgG (H and L) Horseradish peroxidase conjugated (Invitrogen corp., Carlsbad, CA, USA) diluted 1:1000 in 1 % (w/v) PBST solution for an hour. After incubation, the membrane was washed three times in PBST for 10 min per wash. The heat shock proteins were detected with ClarityTM Western ECL Substrate Lumino/enhancer and ClarityTM Western ECL peroxide solution (BIO-RAD). The two solutions were prepared in a 1:1 ratio and 2 ml of the mixture was transferred onto the membrane. The membrane

was imaged with the UVP BioSpectrum® Imaging System (Ultra Violet Productions, Cambridge, UK).

2.9 Statistical analysis

All experiments described were performed three times independently, with five different plants from each genotype for each treatment in each of the three independent experiments. For statistical analysis, One-way analysis of variance (ANOVA) test was used for all data and means (for three independent experiments) were compared according to the Tukey-Kramer test at 5 % level of significance, using GraphPad Prism 5.03 software.

CHAPTER 3

A COMPARATIVE ANALYSIS OF THE LEAF PROTEOMES OF TWO SALVIA *HISPANICA* L. GENOTYPES

3.1 Introduction

Salvia hispanica L. commonly known as chia is a biannual cultivated food crop and member of the *Labiatae* family. Chia is predominantly grown in arid regions and was first identified in Mexico and Guatemala (Cahill, 2003). It was mainly a primary food source by the native South Americans (Mayan and Aztec populations) because of its nutritional benefits (Sandoval and Paredes, 2013). Recent research undertakings into this ancient food crop have revitalised interest in chia as a potential crop. Due to its nutritional benefits and potential as an alternative food source, chia is commercially grown in Mexico, Peru, Columbia, Guatemala, Argentina, Bolivia, Ecuador and Australia (Patel, 2015).

The chia plant produces both small white seeds (WSG) and black seed (BSG). The WSG is produced in low quantities, which is a result of a single recessive gene and may have more commercial preference compared to the BSG (Ixtaina *et al*., 2008). Chia seeds have been proven to have a huge potential as a food source and contained various medicinal properties (Ayerza, 2009). The seed has about 25–38 % oil by weight, and it comprises the highest percentage of α-linolenic acid (~60 %) compared to other natural sources known to date (Palma *et al*., 1947; Ayerza, 1995), and also higher levels of protein (19–23 %) compared to the usual cereals such as wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), rice (*Oryza*

sativa L.), oats (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) as presented by Ayerza and Coates (1996). Research has shown that there are no nutritional differences between these two seeds genotypes. Despite the nutritional impact of chia seeds, limited molecular information and knowledge of chia plants exist in the public domain.

The use of proteomics as a tool to identify protein biomarkers have extensively been used in plant science, but limited information about its use in pseudocereals have been published to date. Recent data have been published on the use of gel-based proteomics to identify differentially expressed proteins in the roots of *Amaranthus* in response to salinity stress (Huerta-Ocampo *et al*., 2014) and leaves under drought stress (Huerta-Ocampo *et al*., 2009). To our knowledge these are some of a few documented cases that describe the use of proteomics in pseudocereals, although limited information exists on chia plants. Proteomic analysis has been successfully used to link genotypes and phenotypes during growth and development (Thomas *et al*., 2010; Graves and Haystead, 2002) and has become an integral part within crop science for the past decade (Komatsu *et al*., 2013). In view of its considerable economic potential in foods and chemical industries we analysed the leaf proteomes of two chia genotypes using gel-based proteomic analysis.

3.2 Results

3.2.1 One-dimensional protein profiles of chia leaf tissue

Chia seeds (WSG and BSG) were grown as described in section 2.1. Total leaf protein extracts for each genotype (10 µg) was separated by 1-D SDS-PAGE

(Figure 3.1) to assess the loading quantities and quality of the protein extracts prior to 2-D SDS-PAGE analysis. The result shows the CBB stained 1-D leaf protein profiles (in triplicate) of the two chia genotypes. Lanes M represents the molecular weight markers on both gels. Lanes 1-3 shows protein profiles from three independent biological replicate extractions for the leaf tissues for each genotype. Each lane was loaded with approximately 10 µg of total protein of leaf tissue extract (Figure 3.1 A-B). It was observed that the quality of the leaf protein extracts was of good quality, showing no visible signs of streaking and protein degradations.

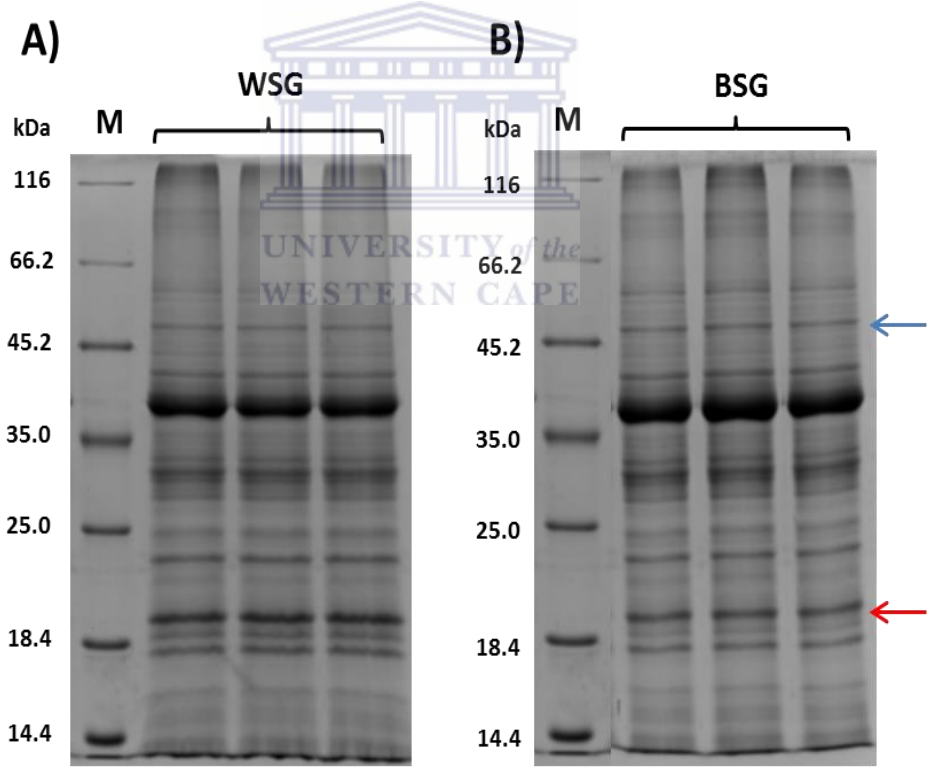

Figure 3.1: One-dimensional leaf profiles of WSG (A) and BSG (B). Total soluble protein (10 µg) of leaf tissue for each genotype was loaded onto 12 % SDS-PAGE gels. Lane M is the molecular weight marker. Lanes 1-3 represent the protein extracts for the leaf samples of WSG (A) and BSG (B) from three independent biological replicates.

The biological replicates (Lane 1-3) within an experiment (Figure 3.1 A-B) also showed high similarity in terms of protein expression, abundance and banding patterns (example indicated by the blue arrow). This suggests that protein preparation was reproducible between independent extractions. The results further presented noteworthy differences in band intensities between the 1-D profile of these genotypes where various bands were either up-regulated or down-regulated (example indicated by the red arrow). Protein extracts from leaf tissue for each genotype covered the MW range of between 10 and 116 kDa.

3.2.2 Two dimensional leaf protein profiles of chia genotypes

For 2-D PAGE, 100 µg of leaf protein extracts of both genotypes was focused and resolved using IPG strips in the 4-7 pH range (Section 2.3). Detected protein spots showed good resolution in this pH range (4-7) and protein abundance between three biological replicate gels for each sample was uniform. This indicates that 2-D PAGE analysis was reproducible between different samples within an experiment.

Figure 3.2 illustrates representative 2-D gels of leaf samples for WSG and BSG respectively. The 2-D gels from both genotypes were subjected to PDQuest analysis and on average a total of 284 and 209 spots were identified in the WSG and the BSG, respectively. Although there are similarities between the two genotypes there are also clear differences as seen by the 2-D profiles.

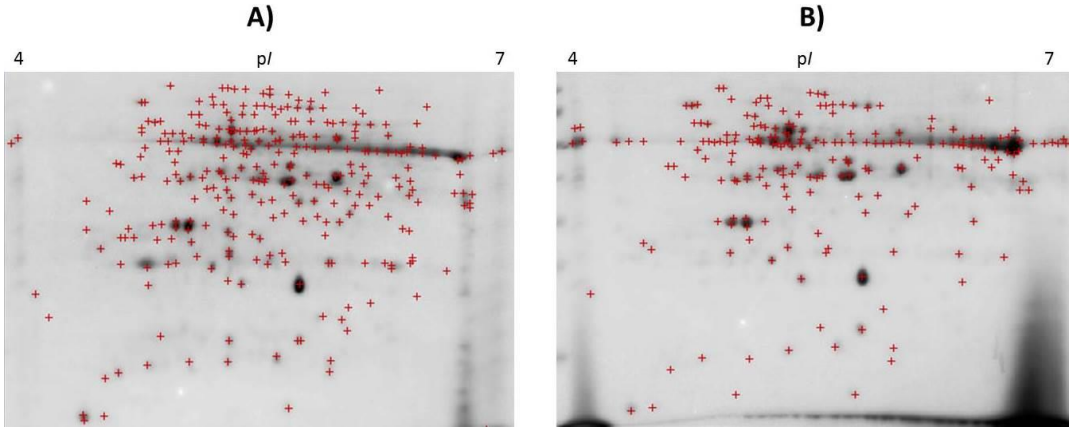

Figure 3.2: Representative 2-D gel illustrating the leaf proteome profiles of WSG (A) and BSG (B). Total soluble protein (100 µg) was separated on the 2-D SDS polyacrylamide gel and stained with CBB.

3.2.3 Protein identification using the MALDI-TOF MS/MS system

This part of the work focusses on identifying differential expressed proteins in the leaves of the two genotypes. A total of 50 well resolved protein spots of varying degrees of abundance and MW were selected for identification using MALDI-TOF MS/MS analysis and database searches (Figure 3.3). The red arrows indicate the proteins that are present in both genotypes whereas the blue arrows represent proteins that are only present in WSG. On the other-hand the orange coloured arrows shows protein spots that were selected for identification although no mass spectrometry data were obtained.

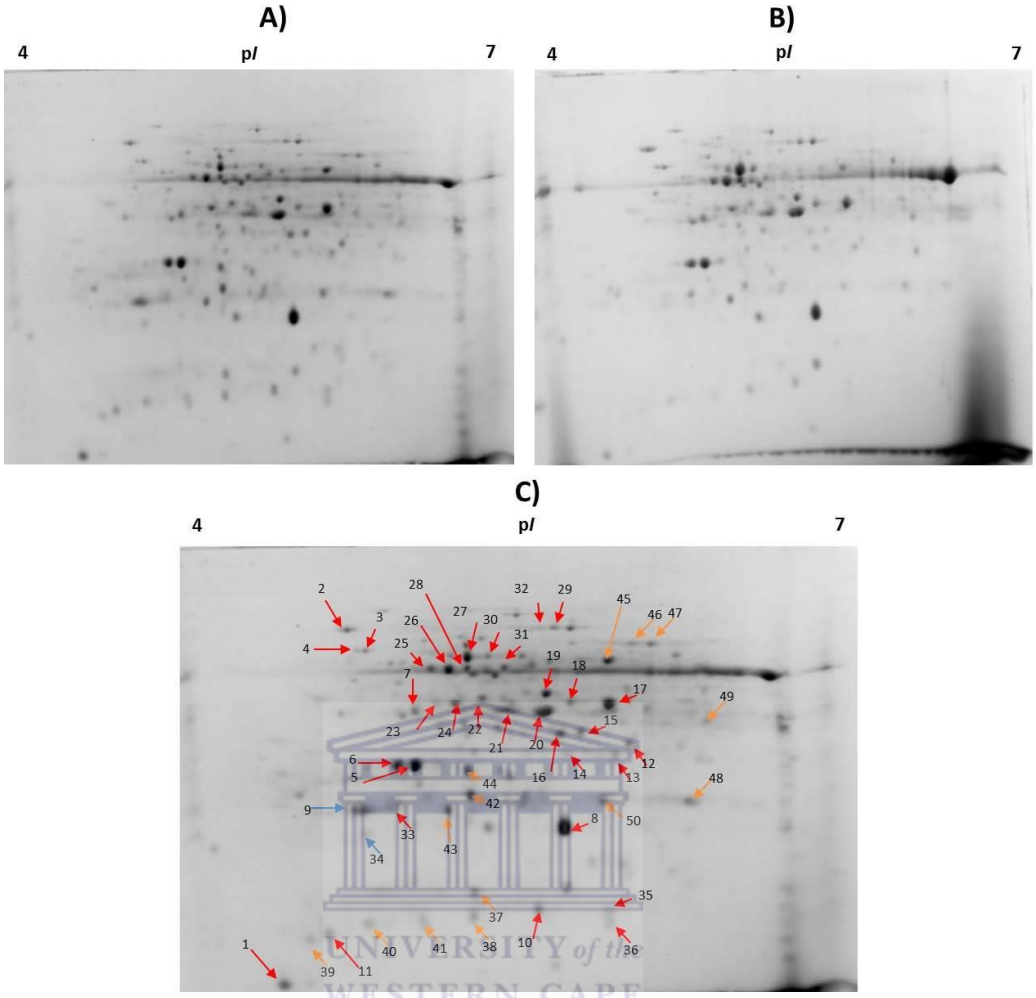

Figure 3.3: Two dimensional leaf proteome profile of WSG (A) and BSG (B) chia genotypes including the master gel (C) indicating selected proteins spots for mass spectrometry analysis. Leaf protein extract (100 µg) was separated in the first dimension by IEF using 7 cm NL IPG strips, pH range 4-7; and size fractionated on a 12 % SDS PAGE gels in the second dimension. Protein spots (1-50) were selected for identification using a combination of MALDI-TOF MS/MS coupled with database searches.

The selected protein spots (spots 1-50; Figure 3.3 C) were manually excised (using sterile blades) from the CBB stained SDS gels. Excised gel plugs were trypsinised and digested peptides were analysed using the ultrafleXtreme MALDI-TOF MS/MS system (Section 2.6). The resultant peptide mass fingerprints (PMF) for each protein spot were searched against various sequence datasets to retrieve protein identities. Since no genome data exist for chia,

database searches were aligned to all entries in the NCBI database using Mascot version 2.2. Protein identities for each spot with the highest MOWSE score equal to or greater than 42 (p<0.05) were regarded as significant protein matches. Mass spectrometry using a combination of MALDI-TOF MS/MS and genomic database searches of the trypsinised protein spots (50) resulted in the positive identification of 36 protein spots. A visual presentation was constructed to show the uniqueness between the two contrasting chia genotypes (Figure 3.4) (Oliveros, 2007-2015). This result shows that there was 2 (5.6 %) unique protein spots (spots 9 and 34) that were only identified in WSG but were absent in BSG.

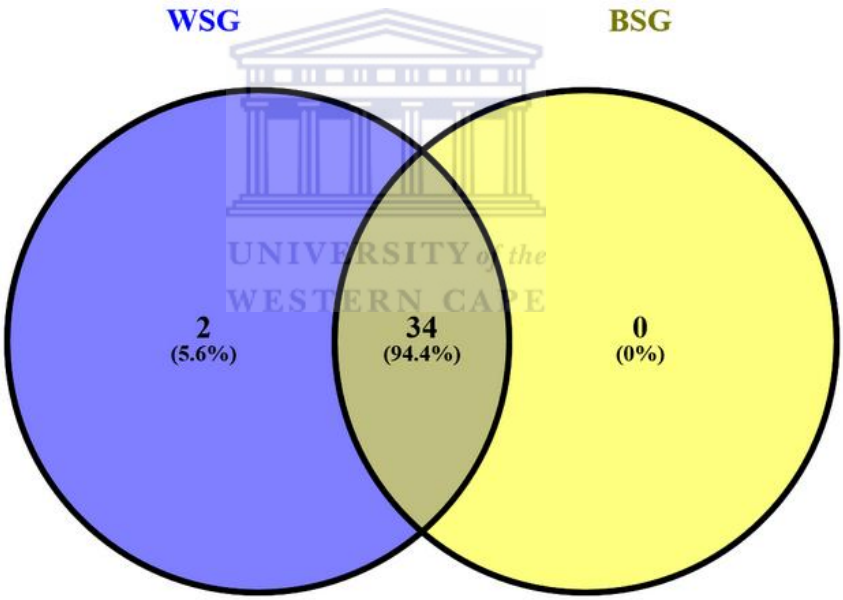

Figure 3.4: Venn diagram comparing the 36 identified protein spots between the two contrasting chia genotypes. The diagram illustrates the proteins that are unique to each genotype.

A total of 36 of 50 spots were positively identified thus giving a success rate of 72 %. The identities of the positive identified protein spots are listed in Table 3.1. The functions of the identified chia leaf proteins (Table 1) were assessed by a

combination of similarity searches against the Universal Protein Sequence database (http://www.uniprot.org) and other literature sources. Identified protein spots (36) (Table 3.1) were successfully classified into nine broad functional categories including photosynthesis (33 %), ATP production (16 %), protein folding (8 %), defence (14 %), transport (3 %), metabolism (17 %), protein synthesis (3 %), structural proteins (3 %) and other functional proteins (3 %). The functional categories and proteins in each respective class are listed in Table 3.1 while a graphical representation of the distribution of proteins in each class is illustrated in Figure 3.5.

Table 3.1: List of the 36 positively identified chia leaf proteins by a combination of MALDI-TOF MS/MS and database searches.

Spot	Best Match Protein	gi|[a]	NCBI Accession	Species	MOWSE score[b]	Exp. MW/pI[c]	Matching peptides[d]	WSG Expression relative to BSG
	Photosynthesis							
1	Plastocyanin	gi|130284	P00296	*Solanum tuberosum*	177.85	10.30/4.10	1	Down
5	Oxygen-evolving enhancer protein 1	gi |11134054	Q40459	*Nicotiana tabacum*	121	35.2/5.46	19	Down
6	Oxygen-evolving enhancer protein 1	gi|11134054	Q40459	*Nicotiana tabacum*	754.79	33.2/5.75	6	Down
7	Phosphoribulokinase	gi|125578	P27774	*Mesembryanthemum crystallinum*	565.81	44.1/6.02	8	Up
8	Oxygen-evolving enhancer protein 2	gi|131392	P12302	*Spinacia oleracea*	213.89	21.5/5.87	3	Down
11	Glycine decarboxylase subunit H	gi|1169884	P46485	*Flaveria trinervia*	52.88	3.8/6.02	1	Down
13	Ferredoxin--NADP reductase	gi|119905	P10933	*Pisum sativum*	642.73	34.80/6.58	10	Down
20	Ribulose bisphosphate carboxylase/oxygena activase	gi|132167	P23489	*Chlamydomonas reinhardtii*	64.60	45.50/5.78	1	Up
21	Ribulose bisphosphate carboxylase/oxygena activase	gi|12643998	P10871	*Spinacia oleracea*	90.82	47.80/6.67	2	Up
29	Transketolase, putative	gi|460425430	F4IW47	*Arabidopsis thaliana*	289.55	81.20/6.55	5	Up

Spot	Best Match Protein	gi|[a]	NCBI Accession	Species	MOWSE score[b]	Exp. MW/pI[c]	Matching peptides[d]	WSG Expression relative to BSG
32	Transketolase, putative	gi| 460425430	F4IW47	*Arabidopsis thaliana*	334.91	81.20/6.55	5	Down
33	Chlorophyll a-b binding protein of LHCII type I	gi|115768	P08221	*Cucumis sativus*	199.68	27.20/5.00	3	Up
	ATP production							
25	ATP synthase beta chain	gi| 114552	P06284	*Marchantia polymorpha*	125.80	40.20/4.79	3	Up
26	ATP synthase beta chain	gi| 75336630	Q9MU41	*Magnolia tripetala*	177.00	51.70/4.88	30	Up
27	ATPase alpha subunit (chloroplast)	gi|118573497	Q0ZJ35	*Vitis vinifera*	171.00	55.30/5.05	29	Up
28	ATP synthase beta subunit	gi|34582342	Q9MU80	*Chamaedorea seifrizii*	156.00	53.30/4.94	36	Up
30	ATP synthase CF1 alpha subunit	gi|118573497	Q0ZJ35	*Vitis vinifera*	536.96	55.30/5.05	10	Up
31	ATP synthase beta subunit	gi|114421	P17614	*Nicotiana plumbaginifolia*	815.36	59.80/5.92	9	Down
	Protein folding							
2	Chloroplast heat shock protein 70-1	gi|399942	Q02028	*Pisum sativum*	121.00	74.3/5.00	18	Up
3	RuBisCO large subunit-binding protein subunit alpha	gi|134101	P08824	*Ricinus communis*	122.6	52.3/4.62	2	Up

Spot	Best Match Protein	gi|[a]	NCBI Accession	Species	MOWSE score[b]	Exp. MW/pI[c]	Matching peptides[d]	WSG Expression relative to BSG
4	Stromal 70 kDa heat shock-related	gi|1708311	Q08080	*Spinacia oleracea*	252.95	64.9/4.72	3	Up
	Defence							
9	Osmotin-like protein	gi|21542444	P50700	*Arabidopsis thaliana*	75.22	13.82/4.25	1	*
10	Superoxide dismutase	gi|12230570	O65199	*Vitis vinifera*	324.73	23/6.27	3	Down
34	Chalcone isomerase	gi|75156641	Q8LKP9	*Saussurea medusa*	52.97	23.70/5.44	1	*
35	CuZn-superoxide dismutase 3	gi| 134616	P27082	*Nicotiana plumbaginifolia*	108.46	15.30/6.03	2	Down
36	CuZn-superoxide dismutase 3	gi|134616	P27082	*Nicotiana plumbaginifolia*	136.11	15.30/6.03	2	Down
	Transport							
12	Importin alpha-1b subunit	gi|3915737	O22478	*Solanum lycopersicum*	44.56	59.9/5.14	1	Down
	Metabolism							
15	Fructose-bisphosphate aldolase	gi|78099750	Q40677	*Oryza sativa subsp. japonica*	552.02	38.10/6.44	8	Down
16	Fructose-bisphosphate aldolase 2	gi|341940207	Q944G9	*Arabidopsis thaliana*	76.20	42.80/6.44	19	Down
17	Porphobilinogen deaminase	gi|129915	P12782	*Triticum aestivum*	358.93	49.80/6.69	4	Up

Spot	Best Match Protein	gi|[(a)]	NCBI Accession	Species	MOWSE score[(b)]	Exp. MW/pI[(c)]	Matching peptides[(d)]	WSG Expression relative to BSG
18	Phosphoglycerate kinase	gi|1172455	P41758	*Chlamydomonas smithii*	51.75	38.20/5.12	1	Up
23	Glutamine synthetase leaf isozyme precurso	gi|121353	P15102	*Phaseolus vulgaris*	392.27	47.40/6.88	5	Up
24	Glutamine synthetase leaf isozyme precurso	gi|121353	P15102	*Mesembryanthemum crystallinum*	410.60	47.40/6.88	6	Up
	Protein synthesis							
19	Chloroplast elongation factor TuA	gi|68566313	Q40450	*Nicotiana sylvestris*	714.79	49.70/6.09	8	Up
	Structural							
22	Putative actin protein	gi| 54035683	O81221	*Gossypium hirsutum*	1145.97	41.70/5.28	14	Down
	Other functional							
14	1,8-cineole synthase synthase	gi|62900763	O81191	*Salvia officinalis*	47.67	68.20/5.03	1	Up

(a) Accession number

(b) Probability- based molecular weight search (Mowse) score

(c) Exp. MW/p*I*- Experimental molecular weights and isoelectric point obtained from their 2-D gels Fig 3.3.

(d) Number of matching peptides

3.2.4 Proteins identified in multiple spots

Based on protein identification from several protein spots, seven classes of proteins were represented in multiple spots on the 2-D gels (Figure 3.5; Table 3.1). These include oxygen-evolving enhancer proteins (spots 5, 6 and 8); RuBisCo (spots 3, 20 and 21); transketolases (spots 29 and 32); ATP synthase proteins (spots 25, 26, 28, 30 and 31); superoxide dismutases (spot 10, 35 and 36); fructose-bisphosphate aldolases (spots 15 and 16) and glutamine synthatases (spots 23 and 24).

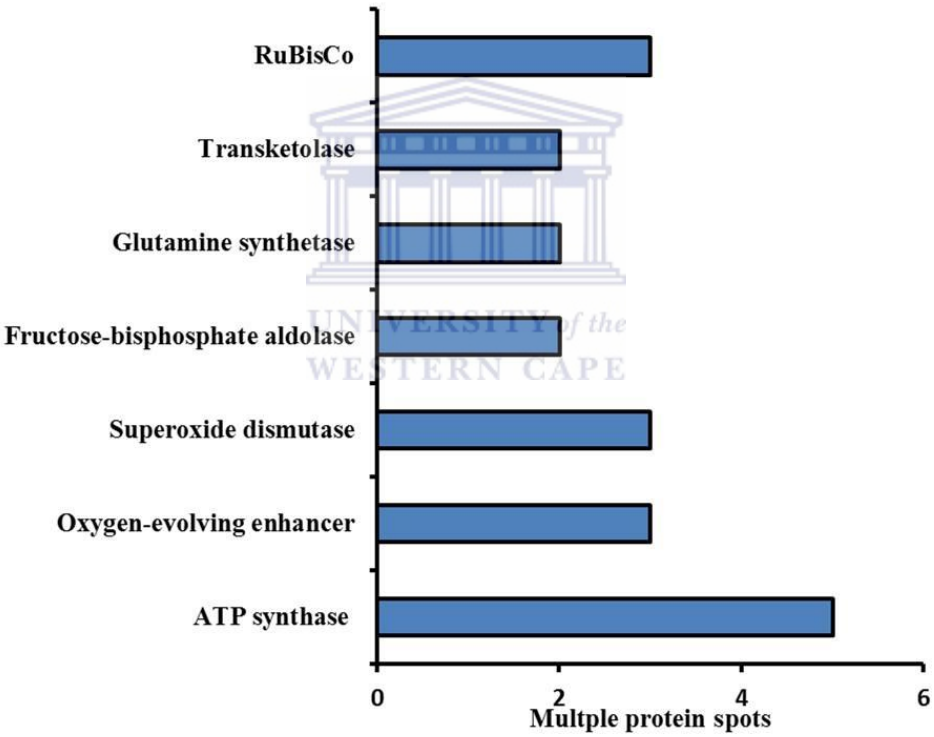

Figure 3.5: Different protein classes represented by multiple spots. The graph illustrates multiple spots associated with each protein class.

The proteins identified in multiple spots can be classified into three groups. Group one consist of protein with the same NCBI accession number and MW but different p*I*s (spots 5 and 6). Group two consist of protein with the different

accession numbers, MW and p*I*s (spots 3, 20 and 21). Group 3 consist of proteins with the same accession numbers, MW and p*I*s (spots 23 and 24; 27 and 30; 29 and 32; 35 and 36). The multiple protein-spotting patterns observed in this chia leaf proteome are associated with photosynthesis (33 %), ATP production (16 %), defence (14 %) and metabolism (17 %) (Figure 3.5; Table 3.1).

3.2.5 Functional classification of positively identified leaf proteins

After the identification of expressed proteins in chia leaf tissue their putative functions were also established. Knowledge of protein function would lead to the identification of cellular processes at work. As such, the main metabolic pathways and biological functions of the tissue under study can be elucidated. The putative functions of the identified chia leaf proteins (Table 3.1) were assessed by a combination of similarity searches on the Universal Protein Sequence database (http://www.uniprot.org) and other literature sources. Using the bioinformatics tools stated above and literature sources, all the 36 positively identified protein spots (Table 3.1) were successfully classified into nine broad functional categories These include photosynthesis, ATP production, protein folding, defence, transport, metabolism, protein synthesis, structural proteins and other functional proteins. The functional categories and proteins in each respective class are listed in Table 3.1 while a graphical representation of the distribution of proteins in each class is illustrated in Figure 3.6.

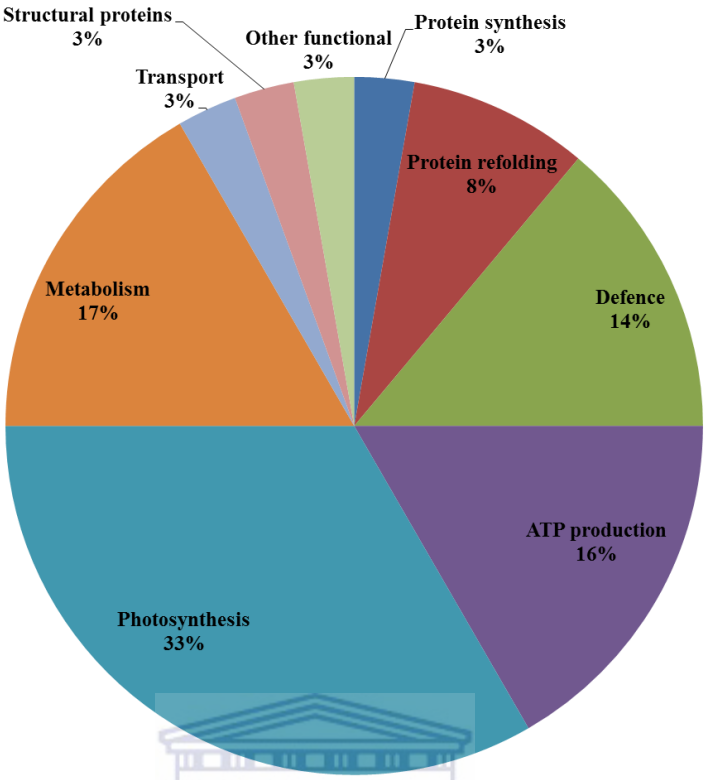

Figure 3.6: Functional classification of MALDI-TOF MS/MS identified proteins. Numbers indicated in brackets represent the proportion of proteins within each functional category expressed as a percentage of the 36 MALDI-TOF MS/MS positively identified protein spots.

3.2.6 Subcellular localization of the chia leaf proteins

Subcellular localizations of the identified chia leaf proteins were predicted using a combination of TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP); Emanuelsson *et al.*, 2007), Predotar version 1.0 (http://urgi.versailles.inra.fr/predotar/predotar.html; Small *et al.*, 2004) and other literature sources. The localisation of each positively identified protein is represented as a pie chart showing the total number of proteins in each subcellular location as shown in Figure 3.7. Chia leaf proteins identified in this study were predicted to be localised in the chloroplast (29 spots; 81 %), cytoplasm (4 spots; 11 %), mitochondrion (1 spots; 3 %), and other location (2 spot; 5 %).

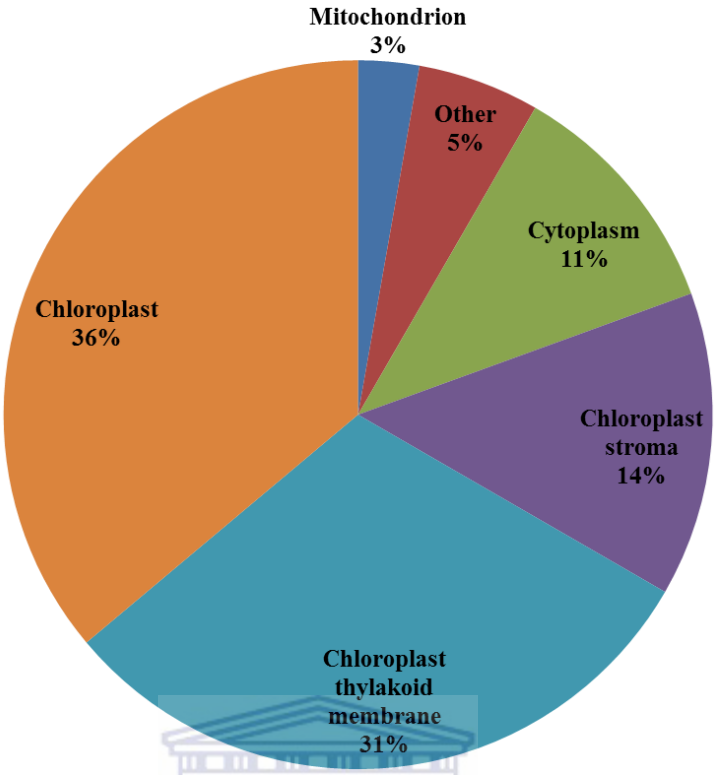

Figure 3.7: Subcellular localisation of chia leaf proteins. Subcellular localizations for chia leaf proteins were predicted using a combination of predictive software packages and literature sources. The proportion of chia leaf proteins identified within each subcellular compartment expressed as a percentage is shown.

3.3 Discussion

In this study, we comparatively analysed the leaf proteomes of two chia genotypes (WSG and BSG) using gel-based proteomic analysis. The identification of differentially expressed proteins in two chia genotypes (WSG and BSG) was achieved using 2-D PAGE coupled with MALDI-TOF MS/MS analysis.

Prior to separation on 2-D PAGE, total protein samples were extracted using the phenol-SDS method and was size fractionated on a 1-D polyacrylamide gel. Each sample was separated in triplicate and the results suggested sample uniformity base on similar intensities observed in the banding patterns. The results observed on the 1-D gels (Figure 3.1), showed differential expression between the two

samples as indicated by the arrows in figure 3.1. To confirm that observation made on the 1-D PAGE, protein samples were separated in the second dimension across a 4-7 pH range. The separation of total soluble proteins from plant samples in this pH range have been well documented (Ndimba *et al*., 2005; Wu *et al*., 2014). Although 2-D PAGE has known limitation such as excluding extreme p*I*s and MW which have been well documented (Abdallah *et al*., 2012; Baggerman *et al*., 2005), it remains one of the highly traditional used methods for protein identification (Chevalier, 2010). For comparing, the leave proteomes of two chia genotypes, 50 spots were selected for further identification (Figure 3.3). Protein identification relied on homology searches against various databases, with specific reference to green plants, as the genome for chia has not yet been sequenced. From the 50 spots that were selected for identification only 36 were positively identified (Table 3.1). Although the 14 unidentified protein spots are is clearly visible in Figure 3.3 C (denoted by the orange arrows) with varying degrees of protein abundance their identities could not be ascertained using MALDI-TOF MS analysis. A possible explanation could be the lack of sequence information from the chia plant.

From the 36 proteins that were identified using MALDI-TOF MS analysis, seven proteins were present in multiple spots detected on the 2-D gels (Figure 3.3; Figure 3.5; Table 3.1). The detection of proteins in multiple spots in various plants species have been described (Watson *et al.,* 2003; Albertin *et al.,* 2009; Ngara 2009; Gharechahi *et al*., 2014). According to Albertin *et al*. (2009) the detection of proteins in multiple spots could be ascribed to post-transcriptional modification or the presence of dimeric and monomeric forms of proteins on the same gel.

These sentiments were shared by Ngara *et al* (2012) and (Budak *et al*., 2013), All positively identified proteins were categorised into nine functional groups (Figure 3.6; Table 3.1). These groups include photosynthesis (33 %), metabolism (17 %), proton transport (16 %) and defence (14 %). A brief description of each protein and their respective functions in each of the functional categories is described below.

Proteins associated with photosynthesis

In total, eight proteins were directly involved in photosynthetic metabolism (Figure 3.4; Table 3.1). These proteins include plastocyanin (spot 1), oxygen enhancer proteins (spots 5, 6 and 8), phosphoribulokinase (spot 7), glycine decarboxylase (spot 11), ferredoxin (spot 13) and chlorophyll a/b binding protein (spot 33). The abundance of these proteins was differentially regulated between the two chia genotypes. Plastocyanin (spot 1) is a 10 kD copper protein that functions in the electron transport chain of chloroplasts where it functions as a mobile electron carrier shuttling electrons from cytochrome to P700 in Photosystem I (Gross, 1993).

Oxygen-evolving enhancer proteins (spots 5, 6 and 8) were previously identified in wheat (Faghani *et al*., 2015) and sorghum (Ngara *et al*., 2012). It consists of four manganese ions, calcium and possibly chloride ions, which are bound to extrinsic proteins (McEvoy and Brudvig, 2006). The oxygen evolving enhancer protein is believed to have a dual function; (i) optimising the manganese cluster during photolysis and (ii) protecting the reaction centre proteins from damage by oxygen radicals formed in light (van der Heide *et al*., 2004).

Phophoribulokinase (spot 7) is involved in carboxylation, reduction and regeneration phases of the Calvin cycle (Ngara *et al*., 2012). Glycine decarboxylase is an important mitochondrial multi-enzyme that plays a vital role in photorespiratory metabolism of plants (Engelmann *et al*., 2008). It consists of four subunits (P, H, T and L). In this study, subunit H (spot 11) was identified which have also been identified in pea plants (Taylor *et al*., 2005).

Transketolases (spots 29 and 32) are enzymes that are associated with the pentose phosphate pathway and the Calvin cycle in plants (Wang *et al*., 2015). Ferredoxin-NADP reductase (spot 13) catalyses the production of NADPH during photosynthesis (Deng *et al*., 1999), was previously identified in maize (Agapito-Tenfen *et al*., 2013).

Ribulose-bisphosphate carboxylase (RuBisCO) activase (spots 20 and 21) that allow for the reactivation of RuBisCO only once ribulose 1,5-bis phosphate (RuBP) or other inhibitory sugar phosphates are present (Wang and Portis, 1992). RuBisCO activase is highly important because it takes part in photorespiration and CO_2 fixation (Badger and Price, 1994) which was identified in the chloroplast stroma. This enzyme has been shown to organize a large pool of stored leaf nitrogen (20-30 %) that can be rapidly remobilized under senescence and various stresses (Demirevska *et al*., 2008).

Chlorophyll a-b binding protein of LHCII type I (spot 33) are involved in harvesting light and regulating photosynthesis (Keown *et al*., 2013). In addition, chlorophyll a/b binding proteins are believed to have a function in pigment storage (Krol *et al*., 1995). The light energy absorbed by chlorophyll a/b binding

proteins (Spot 33; Table 3.1) is used to drive the light dependent oxidation of water, releasing molecular oxygen.

Proton Transport

A total of six proteins (Figure 3, spots 25, 26, 27, 28, 30 and 31) representing various subunits of the chloroplastic, ATP synthase complex were identified. Various subunit components of this complex have also been identified in the proteomes of maize (Porubleva *et al*., 2001), rapeseed (Albertin *et al*., 2009), grapes (Giribaldi *et al*., 2007), soybean (Hoa *et al*., 2004) and peanut (Katam *et al*., 2010). These proteins convert ADP to ATP in the presence of a proton gradient through a thylakoid membrane (von Ballmoos and Dimroth, 2007).

Proteins associated with metabolism

Plants have various unique respiratory metabolic features consisting of three pathways namely glycolysis, the mitochondrial tricarboxylic acid (TCA) cycle and mitochondrial electron transport (Fernie *et al*., 2004). In this study 17 % of the proteins identified were associated with the metabolism. Majority of these proteins played a role in the glycolytic pathway (coverts glucose to pyruvate) which includes two fructose-bisphosphate aldolase (spots 15 and 16), porphobilinogen deaminase (spot 17) and phosphoglycerate kinase (spot 18). Phosphoglycerate kinase is an ATP producing enzyme that acts in the gluconeogenic, photosynthetic pathways and glycolytic (Cheng *et al*., 2013). The enzyme fructose-1,6 bisphosphate aldolase, often simply called aldolase, catalyzes a reversible aldol condensation. Fructose-1,6-bisphosphate when cleaved results in, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate which is two

different triose phosphate, which was identified in previous studies on sorghum (Ngara *et al*., 2012) and maize (Agapito-Tenfen *et al*., 2013).

Two glutamine synthetase leaf isozyme precursor proteins (spots 22 and 23) were identified that is an important nitrogen metabolic enzyme which utilise ammonia and 2-oxoglutarate as a substrate to produce glutamine and glutamate (Lightfoot *et al*., 1988; Teixeira *et al*., 2005).

Transport

Importin alpha-1b subunit (spot 12), which facilitates nuclear membrane transporting of proteins and nucleic acids. It was concluded that importin transporting pathway is substituted by other pathway, which is unclear. It has been reported that this protein may play a role in maintaining homeostasis specifically when a plant is under salinity stress (Miyamoto *et al*., 2004; Wang *et al*., 2008).

Protein synthesis

Chloroplast elongation factor TuA (EF-TuA) (spot 19) was the only protein identified to be linked to protein synthesis. The tufA gene encodes a polypeptide of 478 amino-acid residues, consisting of a putative transit peptide of 70 residues and a mature EF-TuA of 408 residues (Sugita *et al*., 1994). It was previously identified in sorghum (Ngara *et al*. 2012), tobacco (Sugita *et al*., 1994) and *Arabidopsis* (Ndimba *et al*., 2005). This protein has shown to bind to aminoacyl tRNAs and Guanosine triphosphate, and consequently directs the elongation of polypeptides (Murayama *et al*., 1993).

Protein refolding and defence

Plants contain two strategies to cope with misfolded proteins which include either removing the proteins or refolding them to its normal state. RuBisCO large subunit-binding protein subunit alpha (spot 3), belongs to the chaperonin (HSP60) family and was first discovered from young seedlings of pea plants. This protein is required for the precise gathering of specific oligomeric proteins such as the carboxylase from their subunits (Ellis and Van Der Vies, 1988). Chloroplast heat shock protein 70 (spot 2) and stromal 70 kDa heat shock-related protein (spot 4) was identified by MALDI-TOF MS/MS. According to Wang *et al*., (2004) there have been 18 HSP70 genes identified within *Arabidopsis thaliana*. It has also been reported in pea plants (Taylor *et al*., 2005). Most heat shock proteins are commonly known as chaperones found in the cytoplasm under normal conditions but rapidly relocated to the nucleus when exposed to stress conditions. Chaperones are proteins that assist in protein folding when a plant experiences an abnormal condition such as biotic and abiotic stresses (Xu *et al*., 2012). Significantly, chalcone isomerase (spot 34) which was previously reported in *A. thaliana* (Pelletier and Shirley, 1996) and tomato (Muir *et al*., 2001) was only found within the WSG but absent in the BSG. It has been shown to catalyze the conversion of chalcones to flavanones which is an important secondary metabolite (Mehdy and Lamb, 1987; Pelletier and Shirley, 1996). It has been reported that flavonoids are important signalling molecules in plant-microbe interactions, provide pigmentation to attract pollinators, and act as phytoalexins, which is an antimicrobial (Pelletier and Shirley, 1996). It has been previously reported to play a role in plant resistance and protection (Dao *et al*., 2011).

Significantly, 14 % of the proteins identified were defence related proteins that includes three superoxide dismutases (spots 10, 35 and 36) and an osmotin-like protein (spot 9). Superoxide dismutase was previously identified in various crop species including garlic (Shemesh-Mayer *et al*., 2015) and pea plants (Taylor *et al*., 2005). These enzymes have been shown to act as antioxidants when plants are exposed to various biotic and abiotic stress conditions (Shemesh-Mayer *et al*., 2015). Interestingly, the osmotin-like protein was only identified in the WSG but absent in the BSG. Osmotin is a stress responsive multifunctional protein that has been reported to be involved in osmo-tolerance of plants (Abdin *et al*., 2011) and was isolated from tobacco (Singh *et al*., 1985). According to Abdin *et al*. (2011) osmotin may be involved in modulation of plant responses to biotic and abiotic stresses by acting as transcriptional regulator for the genes encoding key enzymes or as signaling molecules acting through intra cellular receptors.

Structural proteins

The putative actin protein (spot 22) was the only protein identified in this category and has been shown to contribute significantly to plants morphogenesis and development. Plants contain actin-binding proteins, which regulate the supramolecular organization and function of the actin cytoskeleton, including monomer-binding proteins (profilin), severing and dynamizing proteins (ADF/cofilin), and side-binding proteins (fimbrin, 135-ABP/villin, 115-ABP) (McCurdy *et al*., 2001).

Other functional

Cineole isomerase (spot 14) is the only protein in this study that could not be classified into a specific functional category. The enzyme was isolated from the secretory cells of the glandular trichomes of *Salvia officinalis* (garden sage) (Wise *et al*., 1998) and have been shown to convert geranyl pyrophosphate to 1,8-cineole and diphosphate. This compound is an important component of eucalyptus oil which has been used in pharmaceutical application and has been studied as a potential biofuel additive (Shaw *et al*., 2015).

It is interesting to note that from the 50 protein spots that was selected for MALDI-TOF MS analysis two spots (spots 9 and 34) were unique to WSG and absent from BSG (Figure 3.3; Figure 3.4). These spots were identified as osmotin protein and chalcone isomerase, respectively and formed part of the defence category. In light of the significant changes in protein abundance observed in WSG compared to BSG coupled with the two unique proteins spots being identified, WSG was selected for the salinity stress experiment (See Chapter 4).

CHAPTER 4

ANALYSIS OF PROTEIN CHANGES IN *SALVIA HISPANICA* L. UNDER SALINITY STRESS BY 2-D AND MALDI-TOF MS/MS

4.1 Introduction

Salinity is one of the major problems faced by agriculture worldwide (Yan *et al*., 2005). The excessive amounts of soluble salts found within soil effects seed germination, plant strength and crop productivity, mainly in arid and semi-arid regions (Ngara *et al*, 2012; Parida and Das, 2005). The important cations contributing to high saline environments are Na^+, Ca^{2+}, Mg^{2+}, K^+ and anions are Cl^-, SO_4^{2-}, HCO^{3-}, CO_3^{2-} and NO_3^{2-}. The detrimental effects of high saline levels are due to water deficiency that results from the relatively high solute concentrations in the soil and a specific Cl^- and Na^+ stress (Manaa *et al*., 2013). High salt concentrated soils are caused by irrigation activities and/or sea water intrusion along the coastal areas (Carillo *et al*., 2011). Although irrigation is used to supply a source of water in drought prone areas, over-irrigation has been shown to increase salt levels. According to Ngara *et al.* (2012) and Manaa *et al*. (2013); salt concentrations are estimated to increase drastically, thus affecting more than 50 % of arable regions by the year 2050.

Similar to drought stress; salinity has comparable physiological effects on plant, at tissue and cellular level due to water loss. The accumulation of extreme amounts of salts in plant tissues causes an ion imbalance and hyperosmotic stress (Zhu, 2000). This limits water uptake by cells and affects metabolic functions in plant tissue that ultimately affect plant growth. Plants have evolved survival

mechanisms to assist against environmental stresses. These complex salt-responsive signalling and metabolic processes at the cellular, organ and whole plant level, is difficult to understand. This is mainly due to the complexity of salt-induced stress responses, which has both an ionic and osmotic component (Manaa *et al*., 2013). However, with an inevitable change in environmental conditions it will affect agricultural production, prices and infrastructure, which will limit the amount and quality of crops produced (Wlokas, 2008). Therefore, understanding these complex mechanisms, at which plants respond to high saline environments, is of utmost importance.

The recently rediscovered ancient super food crop, chia (*Salvia hispanica* L.) has become one of the popular food crops not only in America but also extend to Southern Asia and Australia. Chia is mainly cultivated for its seeds because it contains high levels of (omega) ω-3 alpha-linolenic acid (ALA) content and antioxidant properties (Mohd Ali *et al*., 2012). Therefore, understanding salinity stress tolerance mechanisms in chia plants is fundamentally and economically important. Proteomics tools offer a new platform for studying complex biological functions involving large numbers and networks of protein and can serve as a key tool for identifying salt-stress responsive protein biomarkers. Proteomic analysis has also been successfully used to investigate abiotic stress responses in plants during growth and development (Thomas *et al*., 2010; Graves and Haystead, 2002; Ngara *et al*., 2012) and has become an integral part in crop science for the past decade (Komatsu *et al*., 2013). According to our knowledge this is a first attempt at analysing the leaf proteome of chia under salinity stress. In view of the considerable economic potential of chia in the food and chemical industries; we

have analysed the leaf proteome of chia under salinity stress using gel-based proteomic analysis to facilitate the identification of potential protein biomarkers to improve salinity stress tolerance in chia and other pseudocereal food crops.

4.2 Results

4.2.1 Separation and visualisation of chia leaf samples on 1-D SDS PAGE

The results in Figure 4.1 shows the 1-D SDS-PAGE leaf profile of chia (WSG) treated with 100 mM NaCl as described in section 2.1. Approximately, 10 µg of protein from each sample (untreated and treated) was separated on a 1-D SDS gel to assess the loading quantities and quality of the protein extracts prior to 2-D PAGE analysis. Lane M show the protein molecular marker whereas lanes 1-3 in A and B, represents the protein profiles from three independent biological replicate extractions for the leaf tissues for each sample. The protein profile for each sample from each treatment showed that the quality of leaf protein extracted were good with no visible signs of streaking and protein degradations. The results in figure 4.1, shows a high degree of similarity in terms of banding patterns and protein abundance (see blue arrow), which confirms that there was relatively equal loading across all samples. However, there were also clear differences observed in protein expression where certain bands were either up- or down regulated (see red arrow) relative to the untreated control sample. Although differential protein expression was observed in the 1-D gels, this could be attributed to more than one protein separating as a single band. This illustrates the limitation with 1-D SDS PAGE; therefore, separating protein samples in the second dimension would be useful in identifying salinity stress responsive proteins.

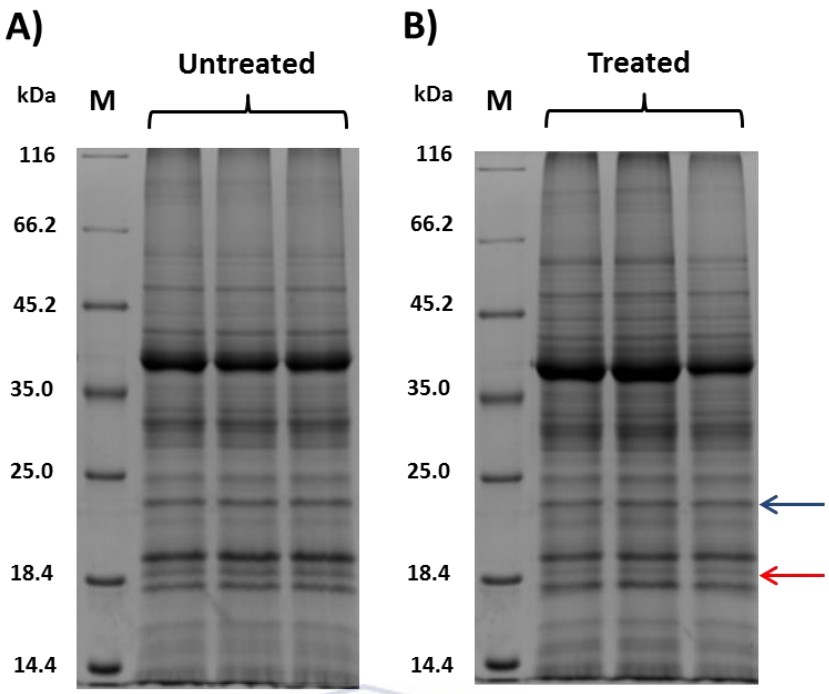

Figure 4.1: One-dimensional profiles of Chia (WSG) leaf proteome the untreated (A) and salinity treated (B) samples. The experimental plants were exposed to salinity stress (100 mM NaCl) for a period of 21 days.

4.2.2 The effect of salinity stress on HSP70 expression patterns in chia leaves

Plants adapt to environmental stress by regulating stress responsive proteins by altering gene expression (Shinozaki *et al*., 2003; 2007). In order to confirm whether the plant was placed under sufficient stress, the expression of heat shock protein 70 (HSP70) was investigated. HSP70 was first identified in *Arabidopsis* as a stress responsive protein.

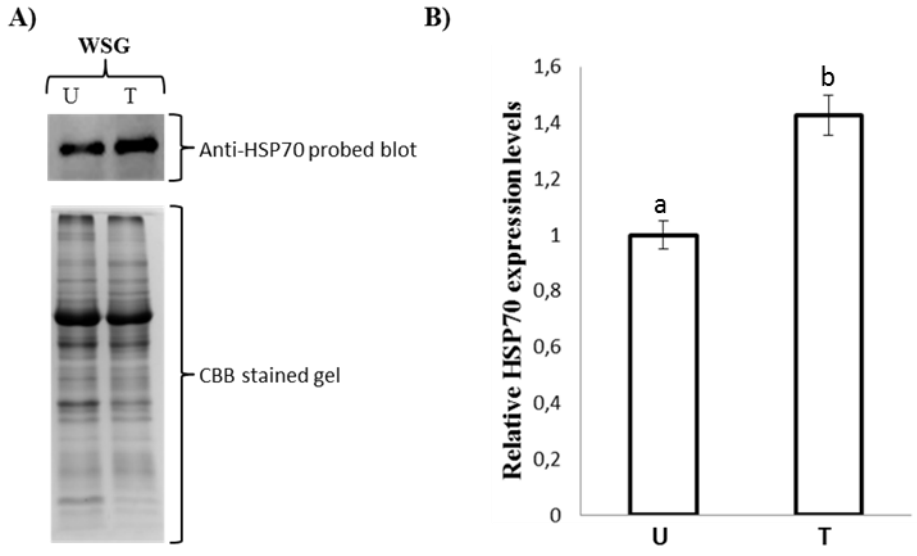

Figure 4.2: Western blot analysis of HSP70 expression in chia leaves exposed to 100 mM NaCl (U-untreated and T-treated). HSP70 was detected using goat anti-HSP70/HSC70 polyclonal antibody (A) and relatively quantified using densitometry analysis (B).

Western Blot analysis was done on all protein extracts from chia leaves using goat anti-HSP70/HSC70 polyclonal antibody as described in section 2.8. The results showed an increase in HSP70 expression in chia leaves when exposed to long term salinity stress (100 mM NaCl) compared with the untreated control plants (Figure 4.2). The increase in HSP70 observed in the salinity treatment (T) is significantly higher than the untreated (U) control (Figure 4.2 A). This result was supported by the densitometry analysis performed on the western blot gels (Figure 4.2 B). This result therefore demonstrates that the salinity stress imposed in this study was sufficient to induce stress responses in chia leaves.

4.2.3 Detection of salinity stress responsive proteins in chia leaves

This part of the work focusses on detecting differential expressed proteins in chia leaves when exposed to 100 mM NaCl using 2-D SDS gel electrophoresis coupled with PDQuest software analysis. To detect differential expressed proteins between

the untreated control and salinity treated samples, approximately 100 µg of protein extract was passively hydrated on a 7 cm IPG strip, pH range 4-7 and further separated on a 12 % SDS gel as described in section 2.3. Protein spots were comparatively analysed for differential expression amongst all treatments. Only spots with a 1.5-fold increase/decrease in intensity/abundance were selected for further analysis. A total of 61 well resolved differential expressed protein spots were selected for MALDI-TOF MS/MS analysis (Figure 4.3). Figure 4.3; illustrate the 2-D gels for both untreated (A) and treated (B) samples. The master gel (C) is a representative of both samples as it contains all selected spots (Figure 4.3). The red arrows indicate the proteins that were identified in both treated and untreated samples. More importantly the blue arrows indicate the proteins that were only identified under salinity stress (100 mM NaCl).

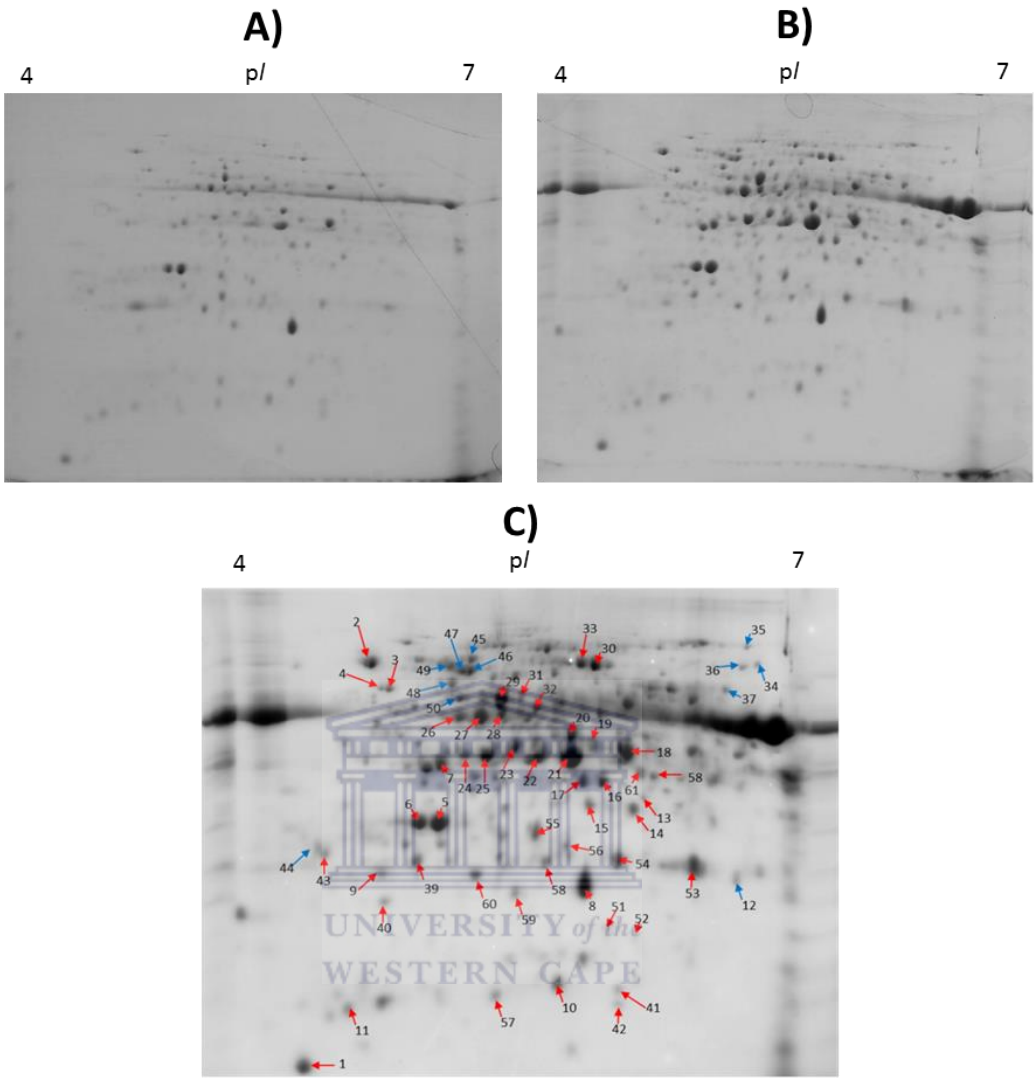

Figure 4.3: Two dimensional leaf proteome profiles of chia under salinity stress. Leaf protein extract (100 µg) was separated in the first dimension by IEF using 7 cm NL IPG strips, pH range 4-7; and size fractionated on a 12 % SDS PAGE gels in the second dimension. Protein spots (1-61) were selected for identification using a combination of MALDI-TOF MS/MS coupled with database searches.

A few protein spots were selected to demonstrate the influence of salinity stress on protein expression (Figure 4.4). Figure 4.4, shows zoomed in images of four proteins spots (spots 10, 13, 48 and 54) differential expression profiles. These proteins spots show a clear difference in expression between untreated and treated samples. Spots 10, 48 and 54, show a pronounced increase in protein expression

in the salinity treatment compared to the untreated control. These proteins are salt-induced proteins as the protein abundance exceeds the 1.5-fold threshold. Spot 13, on the other hand was inhibited by salinity (Figure 4.4 A-B). All these proteins could serve as potential protein biomarkers involved in modulating salinity stress tolerance pending their identification using mass spectrometry.

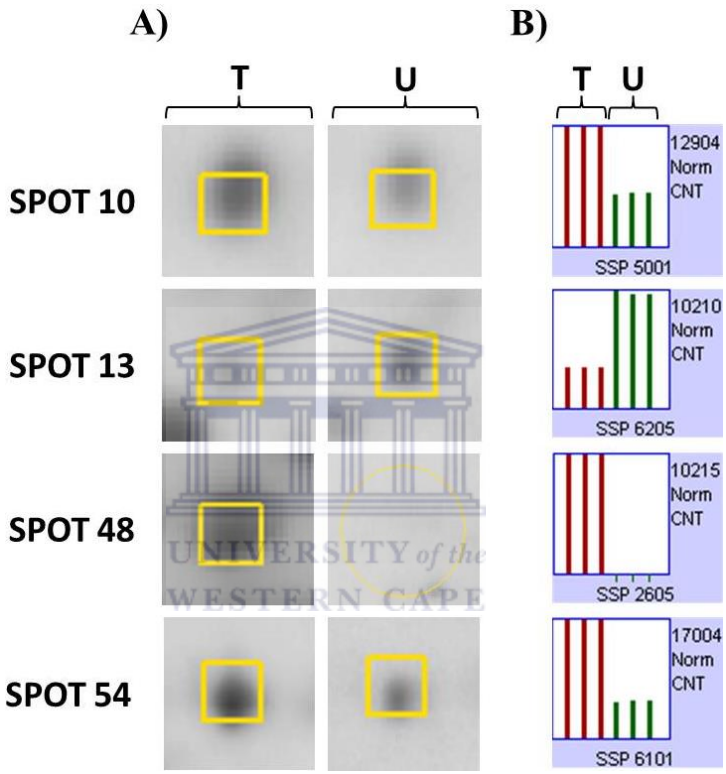

Figure 4.4: Zoomed in gel sections of representative spots showing differential expression following salinity stress from PDQuest software.

4.2.4 Identification of salinity stress responsive proteins in chia leaves

The 61 differential expressed spots of interest were manually excised (using sterile blades) from the CBB stained 2-D gels. Excised gel plugs were trypsinised and digested peptides were analysed using ultrafleXtreme MALDI-TOF MS/MS system (Section 2.6). The mass peptides generated were subjected to the SwissProt database for protein identification. These identities are shown in Tables

4.1, along with their protein identity, gi accession number, species name, MOWSE score, experimental MW and p*I* and matched peptides.

Table 4.1: A List of salinity induced responsive proteins identified by MALDI-TOF MS/MS coupled with Swissprot database searches.

Spot	Best Match Protein	gi|[a]	Species	Mowse score[b]	Exp. MW/pI[c]	Matching peptides[d]
	Photosynthesis					
1	Plastocyanin	gi|130284	*Solanum tuberosum*	177.85	10.30/4.10	1
5	Oxygen-evolving enhancer protein 1	gi |11134054	*Nicotiana tabacum*	121	35.2/5.46	19
6	Oxygen-evolving enhancer protein 1	gi|11134054	*Nicotiana tabacum*	754.79	33.2/5.75	6
7	Phosphoribulokinase	gi|125578	*Mesembryanthemum crystallinum*	565.81	44.1/6.02	8
8	Oxygen-evolving enhancer protein 2	gi|131392	*Spinacia oleracea*	213.89	21.5/5.87	3
11	glycine decarboxylase subunit H	gi|1169884	*Flaveria trinervia*	52.88	3.8/6.02	1
14	Ferredoxin--NADP reductase	gi|119905	*Pisum sativum*	642.73	34.80/6.58	10
21	Ribulose bisphosphate carboxylase/oxygenase activase	gi|132167	*Chlamydomonas reinhardtii*	64.60	45.50/5.78	1
22	Ribulose bisphosphate carboxylase/oxygen nase activase	gi|12643998	*Spinacia oleracea*	90.82	47.80/6.67	2
30	transketolase, putative	gi|460425430	*Arabidopsis thaliana*	289.55	81.20/6.55	5
33	transketolase, putative	gi| 460425430	*Arabidopsis thaliana*	334.91	81.20/6.55	5

Spot	Best Match Protein	gi|[a]	Species	Mowse score[b]	Exp. MW/pI[c]	Matching peptides[d]
39	Chlorophyll a-b binding protein of LHCII type I	gi|115768	*Cucumis sativus*	199.68	27.20/5.00	3
51	Thylakoid lumenal 19 kDa protein	gi|255571642	*Ricinus communis*	158.58	26.20/6.65	1
53	Carbonic anhydrase isoform 2	gi|4754915	*Gossypium hirsutum*	299.55	34.60/7.74	4
54	Carbonic anhydrase isoform 2	gi|4754915	*Gossypium hirsutum*	243.07	34.60/7.75	4
38	Carbonic anhydrase	gi|115473	*Nicotiana tabacum*	42.92	27.70/5.53	1
55	Carbonic anhydrase isoform 2	gi|4754915	*Gossypium hirsutum*	196.51	34.60/7.76	4
59	23 kDa OEC protein	gi|148535011	*Salicornia veneta*	117.82	21.50/5.87	1
	Proton transport					
26	ATP synthase beta chain	gi| 114552	*Marchantia polymorpha*	125.80	40.20/4.79	3
27	ATP synthase beta chain	gi| 75336630	*Magnolia tripetala*	177.00	51.70/4.88	30
28	ATPase alpha subunit (chloroplast)	gi|118573497	*Vitis vinifera*	171.00	55.30/5.05	29
32	ATP synthase beta subunit	gi|114421	*Nicotiana plumbaginifolia*	815.36	59.80/5.92	9
29	ATP synthase beta subunit	gi|34582342	*Chamaedorea seifrizii*	156.00	53.30/4.94	36
31	ATP synthase CF1 alpha subunit	gi|118573497	*Vitis vinifera*	536.96	55.30/5.05	10

Spot	Best Match Protein	gi|[a]	Species	Mowse score[b]	Exp. MW/pI[c]	Matching peptides[d]
48	ATP-dependent zinc metalloprotease FTSH 2, chloroplastic-like	gi|75318709	*Arabidopsis thaliana*	476.92	74.80/5.96	7
	Metabolism					
12	Ribulose-phosphate 3-epimerase	gi|109940150	*Oryza sativa subsp. japonica*	215.27	26.2/6.73	3
15	1,8-cineole synthase synthase	gi|62900763	*Salvia officinalis*	47.67	68.20/5.03	1
16	Fructose-bisphosphate aldolase	gi|78099750	*Oryza sativa subsp. japonica*	552.02	38.10/6.44	8
17	Fructose-bisphosphate aldolase 2	gi|341940207	*Arabidopsis thaliana*	76.20	42.80/6.44	19
18	Porphobilinogen deaminase	gi|129915	*Triticum aestivum*	358.93	49.80/6.69	4
19	Phosphoglycerate kinase	gi| 1172455	*Chlamydomonas smithii*	51.75	38.20/5.12	1
24	Glutamine synthetase leaf isozyme precursor	gi|121353	*Phaseolus vulgaris*	392.27	47.40/6.88	5
25	Glutamine synthetase leaf isozyme precursor	gi|121353	*Mesembryanthemum crystallinum*	410.60	47.40/6.88	6
37	Malic enzyme	gi|1346485	*Populus trichocarpa*	166.27	65.00/6.38	4
58	Fructose-bisphosphate aldolase	gi|224122120	*Populus trichocarpa*	197.40	38.40/8.99	2
61	NAD-dependent malate dehydrogenase	gi|307707110	*Prunus armeniaca*	172.26	34.50/5.89	5

Spot	Best Match Protein	gi|[a]	Species	Mowse score[b]	Exp. MW/pI[c]	Matching peptides[d]
	Protein synthesis					
20	Chloroplast elongation factor TuA (EF-TuA)	gi|68566313	*Nicotiana sylvestris*	714.79	49.70/6.09	8
34	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 2	gi|122203087	*Oryza sativa subsp. japonica*	428.79	84.40/5.81	5
35	Elongation factor 2	gi|6015065	*Beta vulgaris*	89.60	93.90/5.89	29
36	Vitamin-b12 independent methionine synthase-5-methyltetrahydropteroyltriglutamate-homocysteine	gi|8134570	*Catharanthus roseus*	265.60	84.50/6.27	4
45	Elongation factor G, chloroplastic-like	gi|576011128	*Glycine max*	594.73	85.30/5.42	36
	Protein folding					
2	Chloroplast heat shock protein 70-1	gi|399942	*Pisum sativum*	121.00	74.3/5.00	18
3	RuBisCO large subunit-binding protein subunit alpha	gi|134101	*Ricinus communis*	122.6	52.3/4.62	2
4	Stromal 70 kDa heat shock-related protein	gi|1708311	*Spinacia oleracea*	252.95	64.9/4.72	3

Spot	Best Match Protein	gi|[a]	Species	Mowse score[b]	Exp. MW/pI[c]	Matching peptides[d]
40	Chalcone isomerase	gi|75156641	*Saussurea medusa*	52.97	23.70/5.44	1
46	70-kDa heat shock protein	gi|123620	*Solanum lycopersicum*	858.13	71.30/4.96	17
47	70-kDa heat shock protein	gi|123620	*Solanum lycopersicum*	858.13	71.30/4.96	7
49	Luminal-binding protein 5	gi|729623	*Nicotiana tabacum*	493.31	73.50/4.96	9
50	Chaperonin 60 subunit beta 1	gi|27735252	*Arabidopsis thaliana*	421.57	61.70/5.34	7
	Defence					
9	Osmotin-like protein	gi|21542444	*Arabidopsis thaliana*	75.22	13.82/4.25	1
10	Superoxide dismutase 2	gi|12230570	*Vitis vinifera*	324.73	23/6.27	3
41	CuZn-superoxide dismutase 3	gi| 134616	*Nicotiana plumbaginifolia*	108.46	15.30/6.03	2
42	CuZn-superoxide dismutase 3	gi|134616	*Nicotiana plumbaginifolia*	136.11	15.30/6.03	2
56	Ascorbate peroxidase	gi|90811699	*Striga asiatica*	96.21	16.30/5.37	2
	Transport					
13	Importin alpha-1b subunit	gi|3915737	*Solanum lycopersicum*	44.56	59.9/5.14	1

Spot	Best Match Protein	gi|[(a)]	Species	Mowse score[(b)]	Exp. MW/pI[(c)]	Matching peptides[(d)]
44	Alpha chain of nascent polypeptide associated complex	gi|71151999	*Pinus taeda*	399.04	21.90/4.13	6
	Structural					
23	Putative actin protein	gi| 54035683	*Gossypium hirsutum*	1145.97	41.70/5.28	14
	Other					
43	28kD RNA binding protein	gi|133247	*Spinacia oleracea*	117.59	24.50/4.27	2
52	Putative uncharacterised protein Sb06g029650	gi|242074456	*Sorghum bicolor*	60.46	20.90/5.71	1
57	Uncharacterised protein	gi|194693774	*Zea mays*	132.71	13.80/5.25	2
60	Putative uncharacterised Sb06g029651	gi|242074456	*Sorghum bicolor*	120.95	26.40/8.82	1

(a) **Accession number**

(b) **Probability- based molecular weight search (Mowse) score**

(c) **Exp. MW/p*I*- Experimental molecular weights and isoelectric point from the 2-D gels in Fig 4.3**

(d) **Number of matching peptides**

The positively identified protein spots (61) as shown in Figure 4.3 and Table 4.1; were further grouped based on their uniqueness to a specific treatment. Figure 4.5 shows the number of proteins (also expressed as a percentage) that were differentially regulated by salinity stress compared to the untreated controls. Based on the data captured in the Venn diagrams no unique proteins were identified in the untreated control sample (Oliveros, 2007-2015). Interestingly a total of 12 protein spots (spots 12, 34, 35, 36, 37, 44, 45, 46, 47, 48, 49 and 50) were only detected in the salinity treatment and not present in the untreated controls (see blue arrows in Figure 4.3). The 12 proteins are associated with multiple functional groups namely protein folding (4 spots), metabolism (2 spots), protein synthesis (4 spots), proton transport (1 spot) and transport (1 spot) (Table 4.1). Some of these salt-induced proteins were highly significant contributing to salinity stress tolerance and might contribute directly or indirectly towards plant tolerance.

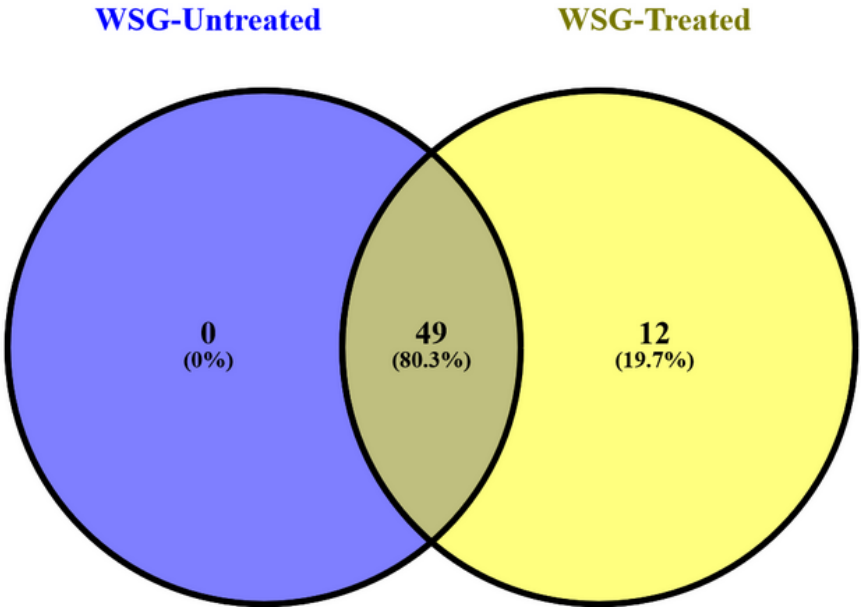

Figure 4.5: Venn diagram comparing the 61 identified protein spots in the different treated samples. The diagram illustrates the proteins that are unique to each treatment.

4.2.5 Proteins Identified in Multiple Spots

In total, 61 proteins spots were selected for mass spectrometry analysis have been positively identified (Table 4.1). From these positively identified proteins, nine classes of proteins were represented in multiple spots on the 2-D gels (Figure 4.4; Table 4.1). These proteins include fructose-bisphosphate aldolases (spots 16, 17 and 58); ATP synthases (spots 26, 27, 28, 29, 31 and 32); superoxide dismutases (spot 10, 41 and 42); glutamine synthatases (spots 24 and 25); oxygen-evolving enhancer proteins (spots 5, 6 and 8); transketolases (spots 30 and 33); heat shock proteins (spots 2, 4, 46, 47 and 50), RuBisCo (spots 3, 12, 21 and 22) and uncharacterised (spots 52, 57 and 60). The proteins identified in multiple spots observed in chia leaves are associated mainly with photosynthesis (28 %), proton transport (12 %), protein refolding (11 %) and metabolism (16 %) (Figure 4.5; Table 4.1).

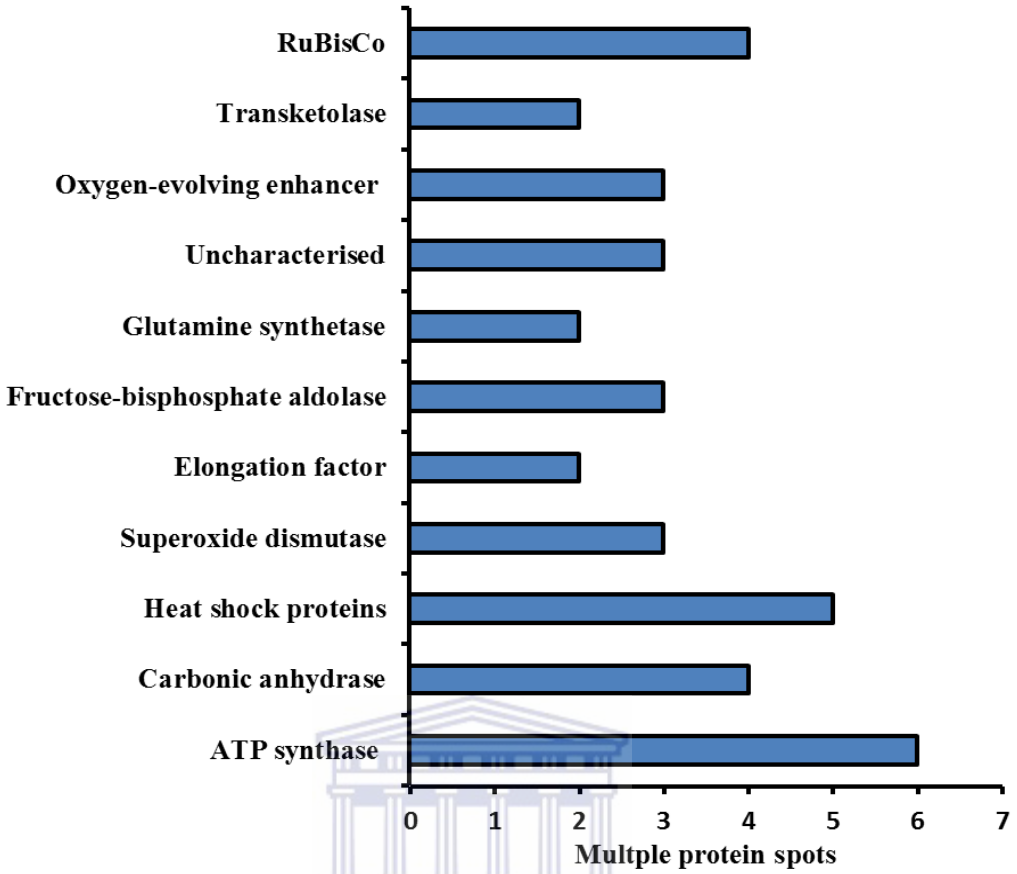

Figure 4.4: Different protein classes represented by multiple spots. The graph illustrates multiple protein spots associated with each protein class.

4.2.6 Functional classification of differential expressed protein spots

Leaf proteins spots identified in chia under salinity stress conditions were further classified into nine functional categories as described by Bevan *et al*. (1998). Knowledge of protein function would lead to the identification of cellular processes at work. These functional categories include photosynthesis (28 %, 17 spots), proton transport (12 %, 7 spots), metabolism (16 %, 10 spots), protein synthesis (7 %, 4 spots), protein folding (11 %, 7 spots), defence (8 %, 5 spots), transport (3 %, 2 spots), structural (2 %, 1 spot) and other (13 %, 8 spots) (Table 4.1; Figure 4.5). The major functional categories were photosynthesis and

metabolism which have interlinking functions. This was expected at these are major metabolic processes found in green leaves.

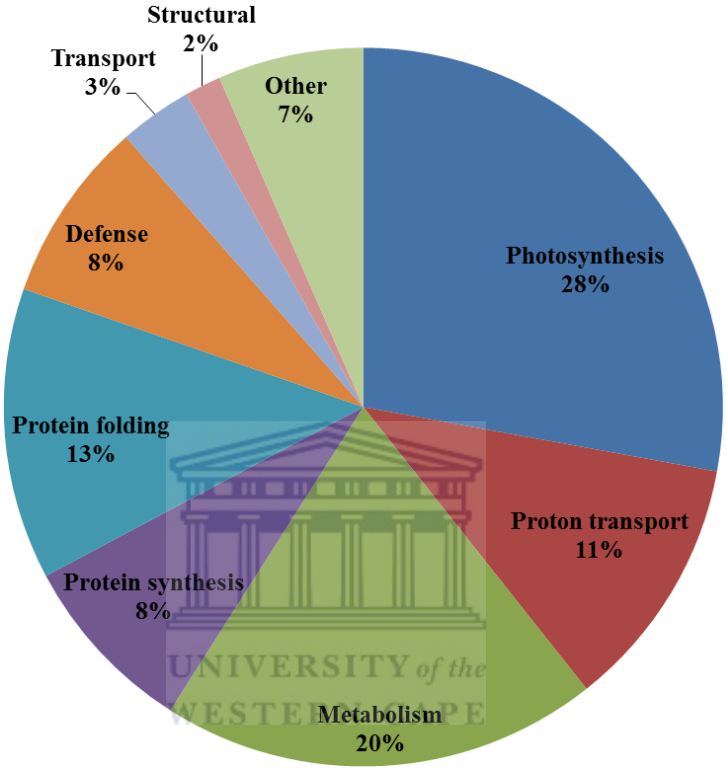

Figure 4.5: Functional characterisation of MALDI-TOF MS identified proteins. Numbers indicated in brackets represent the proportion of proteins within each functional category expressed as a percentage of the 61 MALDI-TOF MS positively identified protein spots.

4.2.7 Subcellular localization of positively identified proteins

The subcellular localisation of each positively identified protein is represented as a pie chart showing the total number of proteins in each subcellular location as shown in figure 4.6. Chia leaf proteins identified in this study were predicted to be localised in the chloroplast (44 spots; 72 %), cytoplasm (11 spots; 18 %), mitochondrion (1 spots; 2 %), and other location (4 spot; 8 %).

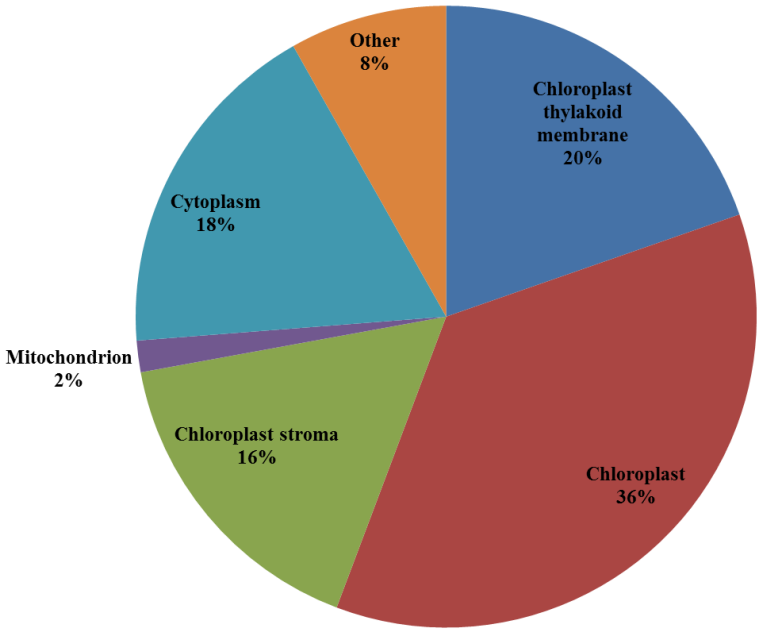

Figure 4.6: Subcellular localisation of chia leaf proteins. Subcellular localizations of chia leaf proteins were predicted using a combination of predictive software packages and literature sources. The proportion of chia leaf proteins identified within each subcellular compartment is expressed as a percentage.

4.3 Discussion

Abiotic stress conditions such as salinity stress poses serious threats to global food production. This study investigated the influence of 100 mM NaCl on the leaf proteome profile of chia plants using gel-based proteomic analysis. The aim was to identify potential protein biomarkers that could in turn enhance salinity stress tolerance in pseudocereal and other economically important food crops.

In order to determine whether the stress imposed by treatment with 100 mM NaCl we analysed the expression of HSP70 using western blot analysis. Given the outcome of the western blot analysis (HSP70 expression levels in the salinity treatment relative to the control); the level of salinity exposure imposed on chia was found to be physiologically significant (Figure 4.2). Chaperones such as

HSPs are stress responsive proteins and was extensively studied in plant science. The expression of HSPs was linked to abiotic stress conditions such as salinity, drought, heat, cold and oxidative stress conditions (Wang *et al*., 2004). Like other HSPs, HSP70 functions by preventing aggregation and support in refolding of non-native proteins under these environmental stress conditions (Scarpeci *et al*., 2008). HSP70 is found in all organisms and has been shown to be an important stress responsive protein against various environmental stress conditions (Ndimba *et al*., 2005; Sato and Yokoya, 2008). In this study we observed basal levels of HSP70 expression in the untreated sample whereas, the salinity treated sample showed a significant increase in HSP70 expression. This outcome demonstrates that 100 mM NaCl treatment of chia plants for 21 days was physiologically significant in this study.

Based on the 1-D leaf profile (Figure 4.1) of chia plants in response to salinity treatment there was a high degree of similarity in terms of loading and protein abundance. Due to the limitation of 1-D PAGE analysis it was imperative to analyse samples from each treatment in the second dimension. A comparative proteomic approach was performed using the 2-D SDS-PAGE coupled with MALDI-TOF MS/MS analysis to identify stress-induced differential expressed proteins. A total of 61 protein spots (with varying degree of expression) were identified using mass spectrometry (Figure 4.3). The positively identified proteins from the leaf tissue of chia plants were grouped into nine broad functional categories (Figure 4.5; Table 4.1). These functional groups remain putative until the functions of these proteins are determined experimentally. The main functional categories and the proportion of protein (Figure 4.5) in these classes

are; photosynthesis (28 %), metabolism (20 %), proton transport (11 %), defence (8 %), protein refolding (8 %), protein synthesis (8 %), structural proteins (2 %), transport (3 %) and other functional (7 %). The results correlate well with the functional classification of the identified proteins. A brief description of some of the salt-induced protein candidates (Table 4.1) and their respective functions in each of the functional categories is given below.

Photosynthesis

In total, 17 (28 %) of the positively identified proteins were photosynthetic related proteins and constituted the largest biological group of proteins of all the proteins identified in this study. From the 17 proteins identified in this group, the expression of six proteins (38, 51, 53, 54, 55 and 59) were upregulated in response to salinity stress These proteins include a thylakoid luminal 19 kDa protein (spot 51), carbonic anhydrase isoforms (spots 38, 53, 54 and 55) and a 23 kDa oxygen-evolving complex (OEC) protein (spot 59). Carbonic anhydrase is an important zinc-containing metalloenzyme that enables CO_2 to interact with RuBisCO (Das *et al*., 2016). These interactions play a significant role in maintaining the functional machinery of RuBisCO (Sobhanian *et al*., 2010). According to Das *et al*. (2016), by increasing the expression of carbonic anhydrases under drought stress would increase resistance to cytotoxic concentrations of H_2O_2; a reactive oxygen species (ROS). It is thus suggested that once the plant is resistant to toxic levels of H_2O_2 it would have some sort of resistance to oxidative stress.

Proton transport

In total seven proteins (spots 26, 27, 28, 32, 29, 31 and 48) were identified of which six proteins (spots 26, 27, 28, 32, 29 and 31) were identified as various subunits of ATP synthase complexes. Interestingly, the ATP-dependent zinc metalloprotease FTSH 2, chloroplastic-like protein (spot 48) was identified only under salinity stress conditions. This protein was previously identified in soybean (Das *et al*., 2016) and barley (Ashoub *et al*., 2015) under drought and heat stress. However, minimal evidence exists on the expression of ATP-dependent zinc metalloprotease FTSH 2, chloroplastic-like protein under salinity stress. ATP-dependent zinc metalloprotease in the presence of RuBisCO activase (spot 21 and 22) under normal conditions inhibits CA1P (2-carboxyarabinitol 1 phosphate, a potent inhibitor of RuBisCO). Therefore, allowing RuBisCO activase to remove the RuBP from RuBisCO and photosynthesis is not affected. However, under abiotic stress conditions if ATP-dependent zinc metalloprotease was affected this in return would affect photosynthesis and retard plant growth which demonstrates the indirect impact of these salt-induced proteins towards conferring tolerance (Das *et al*., 2016; Ashoub *et al*., 2015).

Proteins associated with metabolism

In this study 20 % (11 spots) of the proteins identified were associated with the metabolism. Proteins identified in this category include a 1,8-cineole synthase (spot 15), fructose-bisphosphate aldolases (spots 16, 17 and 58) and NAD-dependant malate dehydrogenase (spot 61) all which have been up-regulated under salinity stress. Interesting to note is that Ribulose-phosphate 3-epimerase

(RPEase) (spot 12) and malic enzyme (spot 37) was identified only under salinity stress conditions but were absent in the untreated samples. The RPEase forms part of the reductive pentose phosphate pathway (Calvin cycle) and oxidative pentose phosphate pathway thus making this enzyme an amphibolic (Guo *et al*., 2009; Kopp *et al*., 1999). It has been previously reported that RPEase were induced under salinity stress conditions in *Kosteletzkya virginica* seedlings (Guo *et al*., 2009), and similarly observed in this study.

Protein synthesis

A total of six proteins were identified and linked to protein synthesis. Significantly, the 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 2 (spot 34), vitamin-b12 independent methionine synthase-5-methyltetrahydropteroyltriglutamate-homocysteine (spot 36) and elongation factor protein (spots 35) were only identified under salinity stress conditions. These proteins play various roles in protein synthesis. It has been previously stated that under salinity stress conditions the plant tissue could be damaged and/or degraded due to oxidative stress (Omoto *et al*., 2010) which makes protein synthesis highly important for repairing damaged tissue. Increased expression of proteins linked to protein synthesis has been previously identified in *Arabidopsis* under salinity stress conditions (Ndimba *et al*., 2005).

Protein folding and defence related proteins

Plants respond to harsh environments in a complex manner. The on-going studies of molecular control mechanisms under abiotic stress conditions, with the use of molecular tools for introducing enhanced transgenic plants, is based on the

expression of specific stress responsive biomarkers. In this study, eight proteins were identified and characterised to protein folding category. These include various heat shock proteins 70 kDa (HSP70) (spots 2, 4, 46 and 47), chaperonin 60 (spot 60), RuBisCO large subunit-binding protein (spot 3), Luminal-binding protein (spot 49) and chalcone isomerase (spot 40). Interestingly, all HSP70 proteins were up-regulated under salinity stress conditions. This was expected given the expression profile of HSP70 observed in figure 3.2. These chaperones are directly linked to protecting plants against stressful environmental conditions. This phenomenon was also observed in sorghum (Ngara *et al*., 2012) and rice (Chitteti and Peng, 2007) plants exposed to salinity stress.

Under salinity stress conditions, a plant experiences oxidative stress due to ROS accumulation which is toxic to the cells. In this study we have identified four ROS scavenging proteins which have been up-regulated under salinity stress treatment. These include various superoxide dismutases (spots 10, 41 and 42) and an ascorbate peroxidase (spot 56). Interestingly, these expressions profiles have been observed in *Arabidopsis* (Ndimba *et al*., 2005; Jiang *et al*., 2007) and sorghum (Ngara *et al*., 2012) plants. An osmotin-like protein (spot 9) (see Chapter 3) linked to salinity adaptation was down regulated under salinity stress conditions. Based on the results obtained in Chapter 3 the osmotin-like protein was only present in WSG but absent in the BSG. This was a key observation and clear distinction between WSG and BSG that motivated for the use of WSG in the salinity stress experiments described in this chapter. This suggests that the osmotin-like proteins could be a potential candidate for improving salinity stress tolerance in chia plants and therefore warrants further investigation.

CHAPTER 5

CONCLUSION AND FUTURE REMARKS

In this study, we reported the first comparative proteomic analysis of two chia genotypes (WSG and BSG) to differentiate between them on a molecular level given that no significant changes were observed in their nutritional profiles. Furthermore, this study also focused on analysing the leaf protein profile of chia plants (WSG) and their responses to salinity stress. The importance of chia dates back to the pre-columbian era where it was consumed as staple food by the indigenous South Americans due to its high nutritional and medicinal benefits. Even though chia contains all these important nutritional and medicinal benefits there is limited information about chia in the public domain. With a fast growing population and ever changing environment, it is of utmost importance to counteract these challenges by instigating these highly beneficial food sources. However, before introducing chia as an alternative food source it is important to understand how these plants respond to environment stimuli (through various molecular mechanisms) that affect plant growth and development. Molecular techniques such as proteomics would contribute towards novel findings. These findings can play a vital role and might lead to further improvements such as genetic engineering of crop plants towards salinity stress tolerance.

Chapter 3 describes the comparative analysis of the leaf proteomes of two chia genotypes. In this chapter, 50 well resolved CBB stained protein spots were selected for mass spectrometry (MALDI-TOF MS/MS) analysis coupled with

homology searches against various databases. A total of 36 (72 %) protein spots were positively identified. This high protein identification success rate could be attributed to the high number of conserved genes and gene products in higher plants given the lack of genome data for the chia plants. These proteins were classified into nine broad functional categories. Functional classification and subcellular localisation of identified proteins are important parameters in clarifying the main metabolic functions that are operational in chia leaves. The work presented in Chapter 3, demonstrates the first attempt towards the analysis of the chia leaf proteome by comparing two chia genotypes (WSG and BSG). Although no significant differences were observed in their nutritional composition, this study showed that these genotypes presented significant differences at molecular level. Two proteins (osmotin and chalcone isomerase) which were only present in the WSG and absent in the BSG supports this argument. Given the limitation associated with 2-D gel based proteomics, we are certain that even more differences exist between these two genotypes and thus warrant further investigation using non-gel based proteomic analysis. In light of results presented in this chapter we have decided to used WSG (as genotype of interest) in the salinity stress experiment (Chapter 4) and omit BSG from further analysis.

Chapter 4 describes the influence of salinity stress on leaf proteome of chia plants. Chia plants were treated with 100 mM NaCl for period of 21 days to impose salinity stress. To establish whether the stress imposed was within the physiological range we used an immunoblot assay to analyse the protein expression of a chaperone protein (HSP70). The expression of HSP70 was

significantly higher in the stress treatment compare to the untreated controls. This suggested that the stress imposed in this study was within physiological range. Two-dimensional gel electrophoresis coupled with mass spectrometry (MALDI-TOF MS/MS) was used to detect and identify differential expressed proteins in the leaves of chia plants. Similar to the work presented in Chapter 3, all identified proteins were classified into nine functional categories and localised primarily to the chloroplast and the mitochondrion. In this study, 61 differentially expressed protein spots were successfully identified and categorised based on the biological and cellular functions. Some of the interesting identities were ATP-dependent zinc metalloprotease FTSH 2 (spot 48), HSP70 proteins (spots 2, 4, 46 and 47), various superoxide dismutases (spots 10, 41 and 42) and an ascorbate peroxidase (spot 56). These proteins could be potential biomarkers for enhancing salinity stress tolerance in chia plants.

According to our knowledge this is the first proteomic study analysing chia plants and their responses to exogenous applied 100 mM NaCl treatment. Due to the limited information in the public domain these protein identities remain putative and require further experimental confirmation. Proteomic profiling by 2-D SDS PAGE is a promising tool for screening for differential expression, although the number of proteins that can be analysed by 2-D SDS PAGE is still limited with respect to the predicted numbers of proteins present in the entire proteome of plants. Two-dimensional gel electrophoresis remains the most widely used tool for high-resolution protein separation and quantification. The combined development and application of validated metabolomic, proteomic, and transcriptomic approaches in plant biology will contribute to our knowledge of

biological systems, but there may be clear benefits in the area of crop safety and security because these candidates can then be used for future transgenic studies in order to analyse their role and functions in salt stress responses.

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