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.

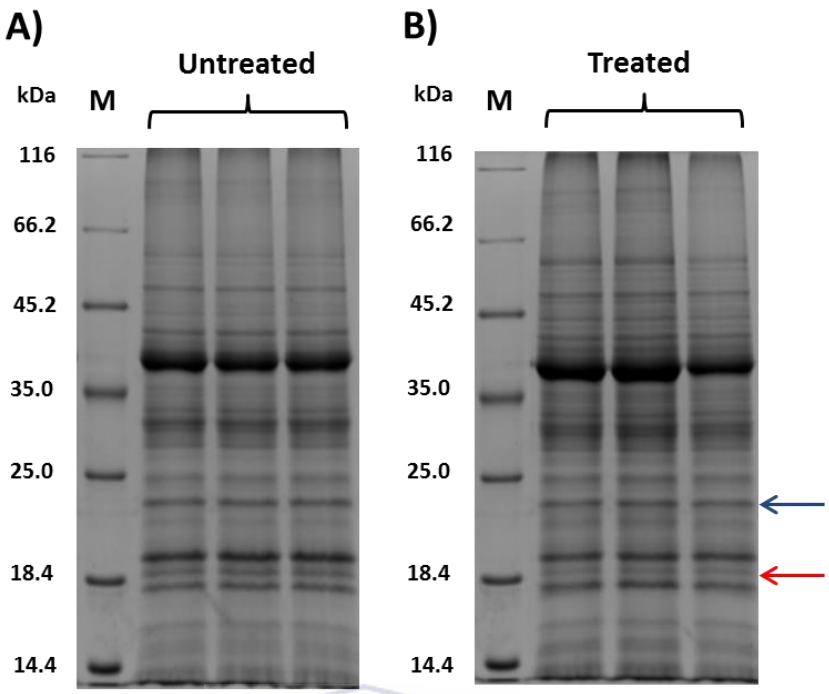

Figure 4.1: One-dimensional profiles of Chia (WSG) leaf proteome the untreated (A) and salinity treated (B) samples. The experimental plants were exposed to salinity stress (100 mM NaCl) for a period of 21 days.

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.

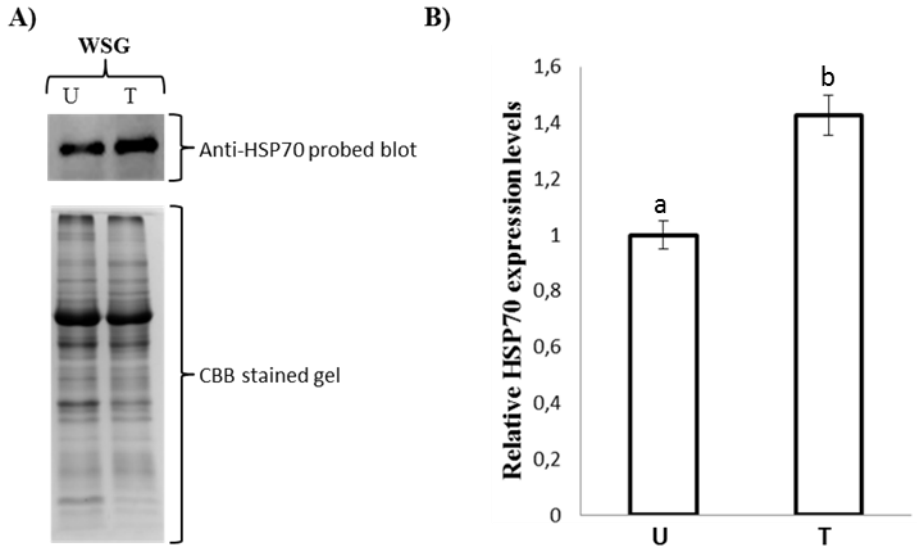

Figure 4.2: Western blot analysis of HSP70 expression in chia leaves exposed to 100 mM NaCl (U-untreated and T-treated). HSP70 was detected using goat anti-HSP70/HSC70 polyclonal antibody (A) and relatively quantified using densitometry analysis (B).

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).

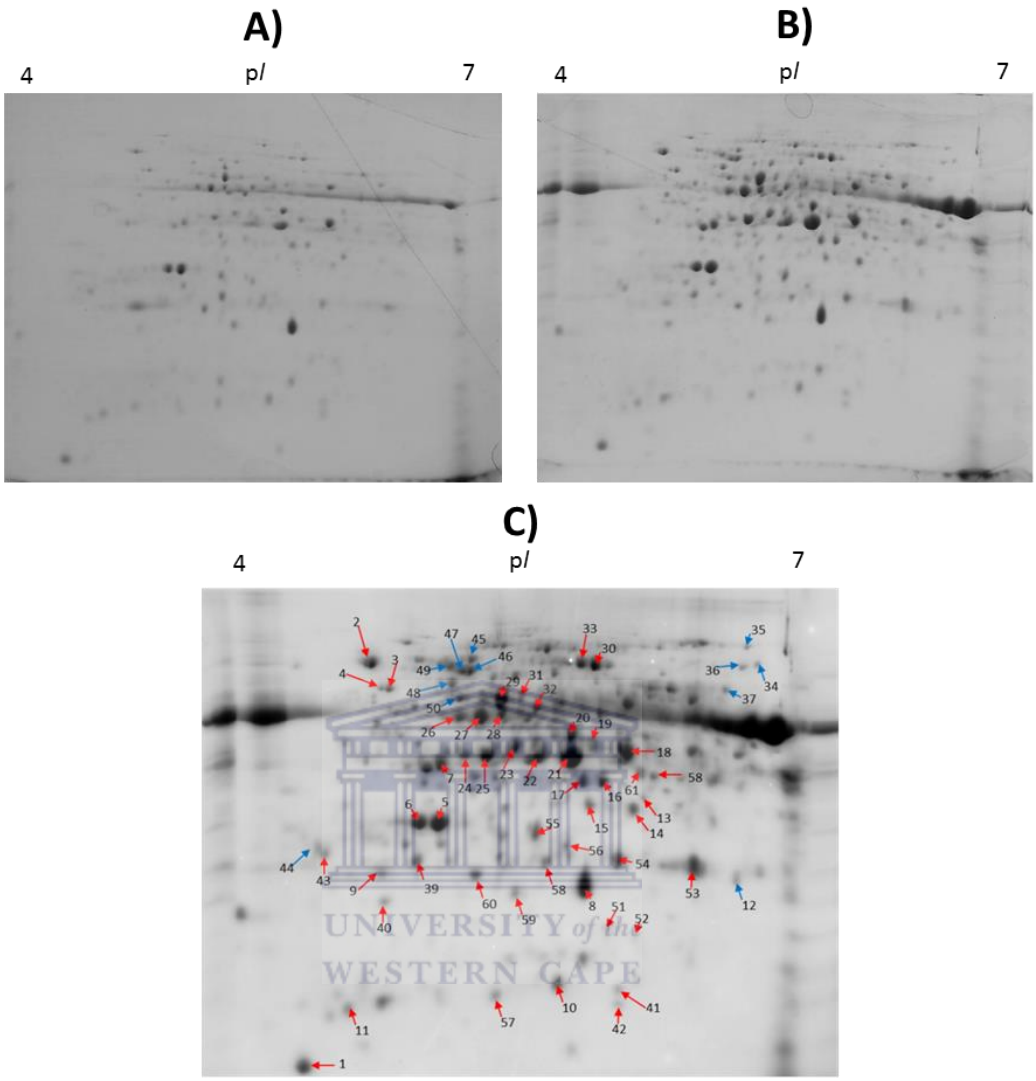

Figure 4.3: Two dimensional leaf proteome profiles of chia under salinity stress. Leaf protein extract (100 µg) was separated in the first dimension by IEF using 7 cm NL IPG strips, pH range 4-7; and size fractionated on a 12 % SDS PAGE gels in the second dimension. Protein spots (1-61) were selected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

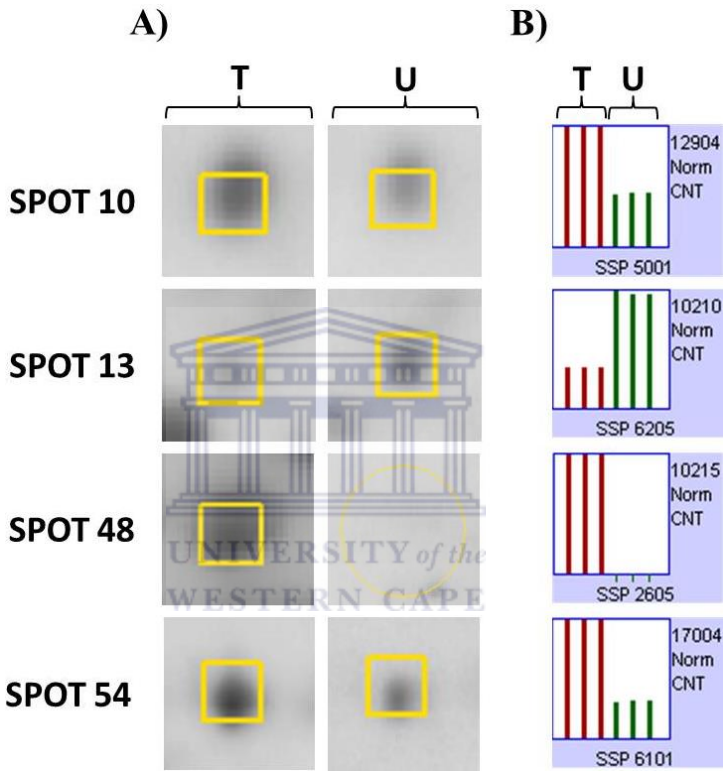

Figure 4.4: Zoomed in gel sections of representative spots showing differential expression following salinity stress from PDQuest software.

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.

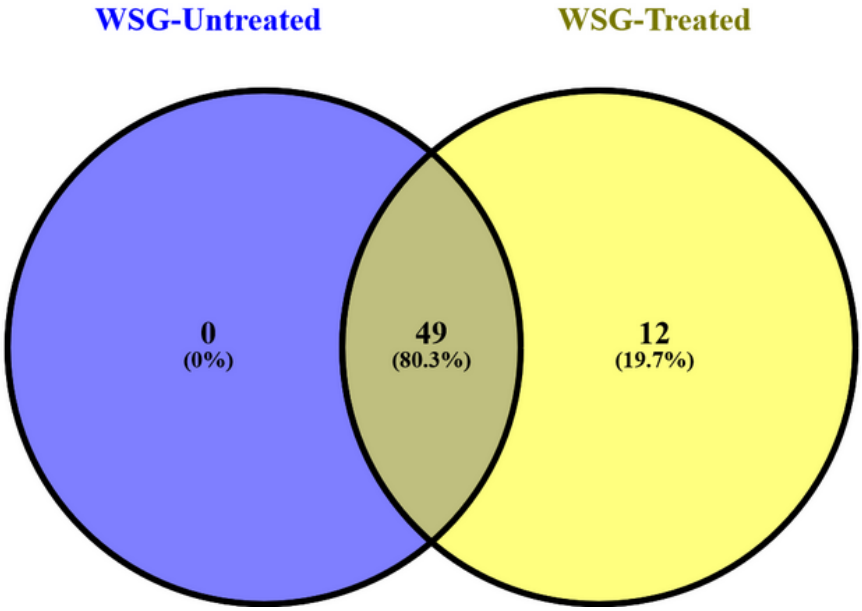

Figure 4.5: Venn diagram comparing the 61 identified protein spots in the different treated samples. The diagram illustrates the proteins that are unique to each treatment.

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).

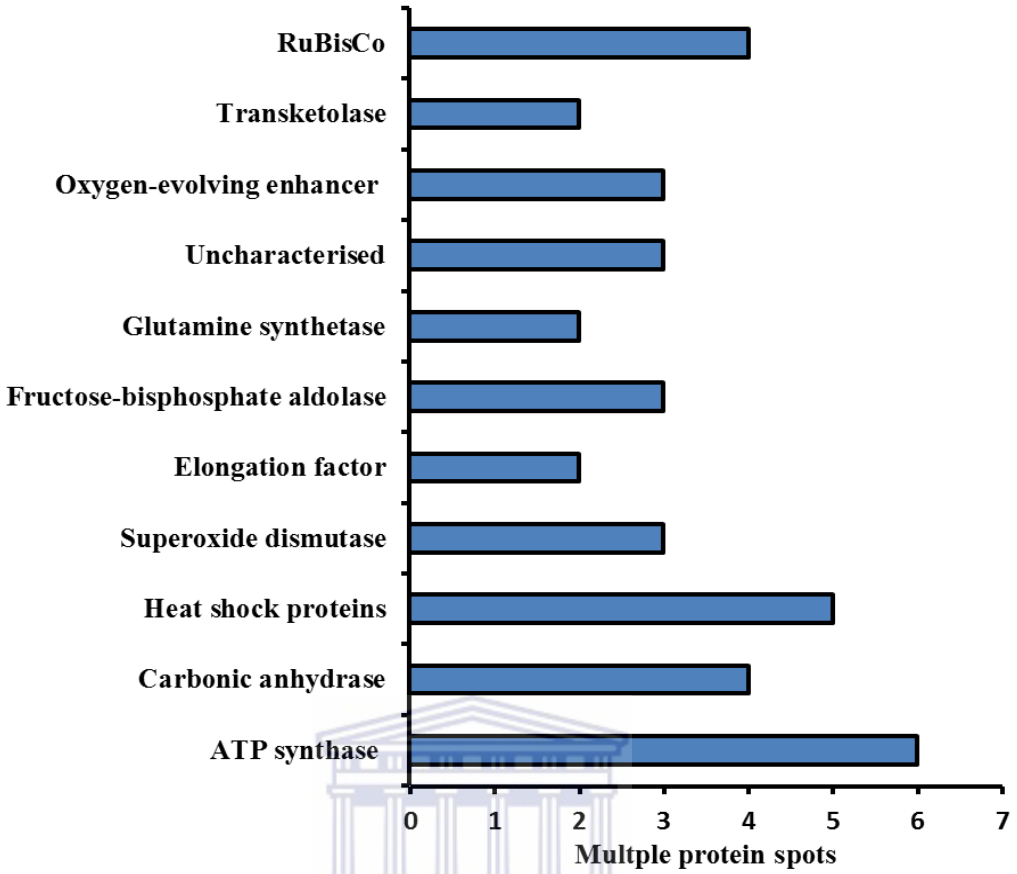

Figure 4.4: Different protein classes represented by multiple spots. The graph illustrates multiple protein spots associated with each protein class.

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.

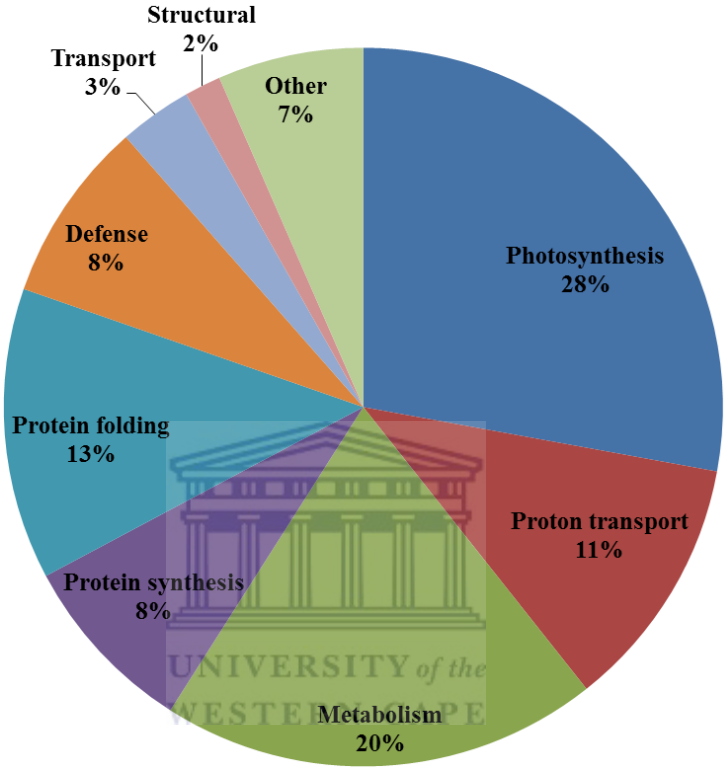

Figure 4.5: Functional characterisation of MALDI-TOF MS identified proteins. Numbers indicated in brackets represent the proportion of proteins within each functional category expressed as a percentage of the 61 MALDI-TOF MS positively identified protein spots.

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 %).

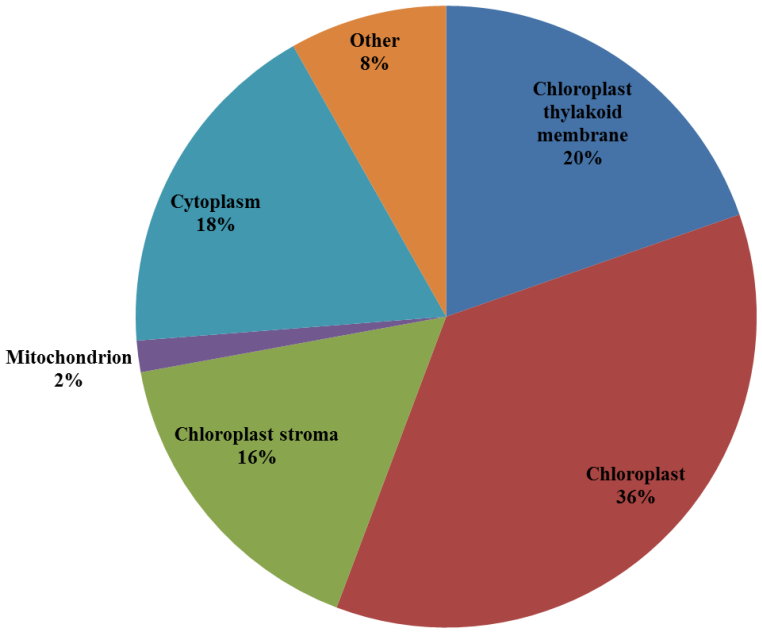

Figure 4.6: Subcellular localisation of chia leaf proteins. Subcellular localizations of chia leaf proteins were predicted using a combination of predictive software packages and literature sources. The proportion of chia leaf proteins identified within each subcellular compartment is expressed as a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