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Proteomic analysis of long term salt responsive proteins in the halophyte *Suaeda maritima*

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Abstract

A proteomic approach was carried out to identify proteins responsive to long term salt treatment in the halophyte *Suaeda maritima*. A 3-month-old *S. maritima* seedlings were hydroponically exposed to Modified Hoagland's treated with salt solution of 200mM were grown for a period of 14 days in a growth chamber maintained at 24 ± 3°C, 70-75% relative humidity with 14 h light (200 µmol m^{-2} s^{-1})/10 h dark cycle. The untreated set maintained in Modified Hoagland's solution was considered as control with similar conditions. Proteins extracted from the leaves of *S. maritima* control and salt treated seedlings were separated by Two-Dimensional gel electrophoresis (2DE). Using PDquest software analysis, we observed ~ 50 protein spots were reproducibly detected on gels, out of which 18 differentially expressed protein spots showed at least two-fold differences on 2DE maps some of them were up-regulated and few others were down-regulated in treated compared with the control. From that, we identified 6 up-regulated protein spots shows the maximum level of fold differences and these protein spots were performed trypsin digestion and Matrix Assisted Laser Desorption Ionization -Time of Flight (MALDI-TOF) analysis. The results showed that *S. maritima* could withstand up to 200mM NaCl for long term period of 14 days by up regulating proteins that are mainly involved in protein transport, vesicle trafficking, heme/iron binding, protein folding and assembly, chromosome segregation, cell maintenance. Our study has identified salt responsive proteins such as *RAB2B*, *CYP71A8*, *SCC3* that are not previously identified by expressed sequence tag analyses or transcriptome analyses in this species. This is the first report of proteome analysis and provides insights into the molecular mechanisms of long term salt tolerance in *S. maritima*.

Keywords: *Suaeda maritima*, halophyte, salt response, proteomics.
Abbreviations: 2DE_Two Dimensional gel electrophoresis, MALDI-TOF/MS_Matrix Assisted Laser Desorption Ionization Time of Flight/Mass Spectrometry, ROS_Reactive Oxygen Species, PMSF_Phenyl methyl sulfonyl fluoride, SDS_Sodium dodecyl sulfate.

Introduction

Abiotic stresses such as drought, salinity, heat, cold, flood etc are the major impediments of plant growth and metabolism that ultimately disturbs plant life (Ahmad and Prasad, 2012). Anthropogenic activities exacerbate these stress conditions to a greater extent and make a serious threat to the environment. Among these, salinity is one of the most critical abiotic stresses that adversely affect plant germination, growth and ultimately yield (Golldack et al., 2014). It has been estimated that 20% of cultivated lands are affected by soil salinity (Zhang et al., 2012). Furthermore, the salinized areas are increasing at a rate of 10% annually for various reasons, including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices. It has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Jamil et al., 2011). In this context, developing crop plants that can sustain productivity under salinity stress conditions become important.

Plants differ in their responses to salinity stress depending on species, organs and developmental stages. Halophytes are naturally evolved salt-tolerant plants that can grow in environments that inhibit the growth of most glycophytic crop plants substantially (Ramani et al., 2006). Depending on their tolerance and demands for sodium salts, the halophytes can be distinguished as obligate and facultative halophytes. Obligate halophytes need some salt for their growth and development are characterized by low morphological and taxonomical diversity with relative growth rates increasing up to 50% in seawater. Facultative halophytes are found in less saline habitats along the border between saline and non-saline upland and are characterized by broader physiological diversity which enable them to survive with saline and non-saline conditions (Parida and Jha, 2010). The survival strategies of halophytes in high salt condition include various mechanisms such as; (1) selective accumulation or exclusion of ions (Mahajan and Tuteja, 2005); (2) compartment-alization of ions at the cellular and whole plant levels (Shabala and Mackay, 2011); (3) biosynthesis of compatible solutes and osmoprotectants (Gagneul et al., 2007; Slama et al., 2015); (4) activation and synthesis of antioxidant enzyme and compounds (Wang et al., 2013); (5) change in photosynthetic pathway (Uzilday et al., 2015); (6) induction and modulation of plant hormones (Parida and Das, 2005; Gupta and Huang, 2014). Thus, halophytes are an essential genetic resource for studies to understand mechanisms of salt

stress tolerance, and to isolate candidate genes for improving salinity stress tolerance in glycophytes.

Suaeda maritima is an herbaceous, succulent, facultative annual halophyte which is native to saline soils of arid and semiarid regions. This species is a self-pollinated bisexual dicot of the family Chenopodiaceae with *2n* = 36 chromosomes and produces reddish brown seeds. It exhibits high tolerance to salt without having special salt secreting structures like salt glands or bladders on its leaves. *S. maritima* accumulates Na^+ in shoot vacuoles thus reducing the toxicity of Na^+ in the cytoplasm and limiting the osmotic potential of vacuoles to preserve cellular turgor pressure and expansion under high salt conditions.

Proteins play a crucial role in making the halophytes tolerant to salt stress and minimizing the adverse effects of Na^+. Proteomics studies are an ideal way of identifying proteins that function in salt stress tolerance (Ghosh and Xu, 2014). Two Dimensional Gel Electrophoresis (2DE) is an efficient proteomic approach have been extensively used for studying protein profiles of plants under stress to reveal the specific proteins contributing to the salt tolerance and survival of the halophytes in saline conditions. Previously, salt stress-related proteomic analyses are reported in many halophytes such as *Suaeda aegyptiaca* (Askari et al., 2006), *Bruguiera gymnorrhiza* (Tada and Kashimura, 2009), *Salicornia europaea* (Wang et al., 2009), *Aeluropus lagopoides* (Sobhanian et al., 2010), *Puccinellia tenuiflora* (Yu et al., 2011), *Cakile maritima* (Debez et al., 2012), *Thellungiella halophila* (Wang et al., 2013), *Kandelia candel* (Wang et al., 2014), *Halogeton glomeratus* (Wang et al., 2015), *Tangut Nitraria* (Cheng et al., 2015) etc. These studies have identified several salt responsive proteins which contribute to various functions such as photosynthesis, osmotic and ionic homeostasis, signal transduction, Reactive Oxygen Species (ROS) scavenging systems etc. However, there is no report on proteomic analysis of salt tolerance mechanism in *S. maritima* as yet.

In the present paper, we employed 2DE to identify proteins that are differentially expressing under salt treated conditions in *S. maritima*. Further, we analyzed few of these proteins by Matrix Assisted Laser Desorption Ionization -Time of Flight/Mass Spectrometry (MALDI-TOF/MS). Our results provide insights to the possible mechanisms of salt stress tolerance in this species at the molecular level.

Results and Discussion

Effect of salt treatment on S. maritima

Halophytes are specialized plants with well adapted morphological, anatomical and physiological characteristics that enable them to survive under high soil salt concentrations (Flowers and Colmer, 2008). We analyzed the proteomic responses of a C3 halophyte *S. maritima* under long term salt treatment to identify proteins that help the species to survive under salinity conditions.

The exposure of *S. maritima* seedlings to different NaCl concentrations resulted in various morphological changes in the leaves overtime. The plants displayed a linear decrease in leaf number and size under salt concentrations of 300 to 500mM. At 500mM NaCl, the plants showed signs of severe stress at later stages of treatment. At 100 and 200mM NaCl, the plants continued to grow comparatively similar or better than the control plants. Thus, our results show that while three month old *S. maritima* can withstand lower salt concentrations, higher concentrations above 200mM affect its growth over long periods (Fig. S1).

Two-dimensional electrophoresis analysis of total proteins in S. maritima leaves

To investigate the long-term changes of protein profiles after treatment with salt, we carried out 2DE analysis of the total proteins in *S. maritima* leaves from 3 biological replicates. For each sample, triplicate gels were performed and they showed a high level of reproducibility. Around 50 protein spots were repeatedly detected on gels for both treated and control group. Quantitative image analysis revealed that a total of 18 protein spots changed their intensities significantly ($P < 0.05$) by more than 2-fold in the treated samples compared with the control. Among the 18 differentially accumulated protein spots, 9 were more abundant and 6 less abundant in salt treated samples compared to control, and 3 spots appeared only in the control samples and were absent after salinity treatment as shown in Fig. 1 & 2.

Identification of differentially expressed proteins by MALDI-TOF analysis

Out of the 9 highly accumulating proteins spots in salt treated samples compared to control, 6 were analyzed by MALDI-TOF/MS analysis. After similarity searches, they were identified to be *Ras* related *RAB2B*, SNARE-interacting protein *KEULE*, Sister chromatid cohesion protein 3, Cytochrome P450 71A8, 16.6 KDa heat shock protein (Table 1 & 2). Localization and function distribution of identified proteins as shown in Fig. 3.

Ras related RAB2B

Ras related proteins constitute a super family of eukaryotic proteins which are thought to play a key role in the regulation of diverse cellular processes. This relatively small, monomeric protein (20-25kDa) binds GTP and has an intrinsic GTPase activity. *Ras* GTPases function as GDP/GTP regulated molecular switches (Vetter and Wittinghofer, 2001).They share a set of conserved G box GDP/GTP binding motif elements and is controlled by two main classes of regulatory proteins such as Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).These two nucleotide bound states have similar conformations but pronounced differences corresponding to the switch I (*Ras* residues 30-38) and switch II (59-67) regions. The *Ras* protein member of this family is divided into five major branches on the basis of sequence and functional similarities: *Ras*, *Rho*, *Rab*, *Ran* and *Arf*. Among these, Rab proteins are the largest branch of the super family (Pereira Leal and Seabra, 2001). Rab GTPases are regulators of intracellular vesicular transport and trafficking of proteins between different organelles of the endocytic and secretory pathways. It facilitates vesicle formation and budding from the donor compartment, transport to the acceptor compartment, and then release of the vesicle content into the acceptor compartment. Rab proteins localization is dependent on prenylation and its specificity is dictated by divergent C-terminal sequences (Zerial and McBride, 2001; Suja and Ajay, 2011). Specific Rab proteins have been found associated with the endoplasmic reticulum (*Rab1*), the post endoplasmic reticulum and pre-Golgi compartment (*Rab2*), different parts of the Golgi apparatus (*Rab6*), the plasma membrane (*Rab5*), early (*Rab5*, *Rab4*) and late (*Rab7*) endosomes (Batch, 1990). Previous studies reported that *ypt homolog 3* in *Zea mays* sharing a GTP binding and activation property with the members of *Ras* family and show 97% similarity which plays a significant role in vacuolar

trafficking and exocytosis (Palme et al., 1992). In the present study, we found *RAB2B* (spot no.1) protein shows high similarity to *Z. mays* might probably be located in the ER-Golgi membrane and could be involved in regulation of vesicular transport and delivery of proteins to the cell surface for acquiring stress tolerance.

SNARE interacting protein KEULE

SNARE (Superfamily of *N*-ethylmaleimide sensitive factor adaptor protein receptor) domain containing proteins are key players in vesicle-associated membrane fusion during transport processes between individual compartments of the endomembrane system. It plays a vital role in higher plants and is involved in various fundamental processes such as cytokinesis, gravitropism, pathogen defense, symbiosis, and abiotic stress responses (Lipka et al., 2007). SNARE proteins comprise small (200-400 amino acid) polypeptides that are characterized by the presence of a particular peptide domain called the SNARE motif (Jahn and Scheller, 2006). SNAREs can be classified either on the basis of their sub cellular localization (functional classification) or occurrence of invariant amino acid residues in the center of the SNARE motif (structural classification). Functional classification divides into vesicle-associated and target membrane-associated SNAREs (v-and t-SNAREs) and the structural classification can be grouped as Q- and R-SNAREs (Sollner et al., 1993; Fasshauer et al., 1998). Generally, t-SNAREs correspond to Q-SNAREs, and v-SNAREs correspond to R-SNAREs. The target membrane localized Q-SNAREs can be further subdivided into Qa, Qb and Qc-SNAREs (Bock et al., 2001). Due to greater diversification of SNARE isoforms in plants, it reflect the necessity for some SNAREs to be devoted to plant specific biological processes such as cytokinesis, gravitropic responses, and the transport of phytohormones etc. In Arabidopsis SNAREs genome contains six members of the *Sec1* family, of which one, *KEULE (KEU)*, has been reported to be involved in cytokinesis (Assaad et al., 2001) and this protein (spot no.3&5) were identified twice in our 2DElectophoresis experiment. This happens due to post-translational modifications such as glycosylation, phosphorylation, oxidation, etc. was also previously reported in Arabidopsis (Ndimba et al., 2005). *Sec1 KEULE* proteins are known regulators of SNARE domain and involved in promoting vesicle fusion in the cell division plane which may helps to obtain a stress tolerance.

Sister chromatid cohesion protein 3 (SCC3)

Structural maintenance of chromosome (SMC) proteins plays a critical role in chromosome folding and dynamics. Eukaryotic SMC proteins can be divided into three classes: condensins, cohesin complexes, DNA recombination and repair complexes (Michaelis et al., 1997). Of these, sister chromatid cohesion is well established along the entire length of the chromosome at the time of DNA replication and persists throughout the G2 phase (Biggins and Murray, 1998). It is mediated, in part, by a group of four highly conserved proteins, referred to as the cohesion complex (*SMC1, SMC3, SCC1 and SCC3*) which is utilized by a wide range of organisms. The cohesion complex proteins present in the nuclear matrix are involved in chromosome organization and the establishment of chromosome boundaries. The cohesion protein *SCC3* was first identified as a member of the yeast mitotic cohesin complex (Toth et al., 1999) and was subsequently identified in *Caenorhabditis elegans* and Arabidopsis (Pasierbek et al., 2003; Wang et al., 2003).This *SCC3* protein (spot no.4) was identified in our study shows 12% amino acid sequence homology with Arabidopsis. In Arabidopsis, *SCC3* protein expressed in roots, mature leaves and is mainly involved in both meiotic and mitotic divisions. The localization pattern of this protein during meiosis is very similar to that *REC8* in yeasts, and to be mainly concerned for orientation and centromere cohesion at anaphase. Hence, *SCC3* proteins are necessary not only to maintain centromere cohesion, but also for the monopolar orientation of the kinetochores during meiosis.

Cytochrome P450 71A8 (CYP 71A8)

Cytochrome P450 catalyzes the monooxygenation of a variety of hydrophobic substrates which includes steroid hormones, prostaglandins, procarcinogens, and active pharmacological compounds. P450s can be broadly classified into two classes based on sequence homology and the identity of their redox partner protein. The class I enzyme P450s (bacterial/ mitochondrial) receive their NADH-derived (bacterial) or NADPH derived (mitochondrial) electrons from a two-protein redox chain, (FAD reductase iron sulfur protein P450); the class II enzymes (membrane bound eukaryotic microsomal) receive NADPH derived electrons directly from an FAD/FMN-containing reductase (Hasemann et al., 1995).
Plant cytochromes P450s include a highly diverse array of protein sequences possessing common catalytic chemistry but different metabolic capabilities. Among these, *CYP71* is a large family of plant cytochrome P450 monooxygenases. Typically, all members of a CYP family share at least 40% amino acid sequence identity and are divided into subfamilies (A, B, C, and so forth) composed of members with greater sequence identity and the identity between members of different subfamilies can be as low as 30% (Chapple, 1998). In Arabidopsis, currently 45 members of the *CYP71* family have been identified. In the present study, *CYP71A8* protein (spot no.6) was identified and share with 33% amino acid sequence similarity with *Mentha x piperita*. This protein are mainly involved in heme binding activity which contain a conserved sequence termed as the P450 signature motif, approximately 50 to 60 amino acids from their C termini. The Cys located within this sequence serves as the ligand for the ferriprotoporphyrin IX (heme) prosthetic group (Schuler, 1996). This heme contains the B'-C turn, the central I helix, β6-1/ β1-4, the Cys-pocket, and the amino terminal L helix and is covalently bound to the invariant cysteine found in a β-bulge region called the Cys-pocket. The β-bulge appears to serve as a major role of enveloping the cysteine in a hydrophobic environment. Three residues besides the cysteine are strictly conserved and these conserved regions of the enzymes are found in the core of the protein, probably representing the conservation of regions important for proper protein folding and this perhaps supports other various processes such as lipid, nitrogen and secondary metabolism in plants against stress.

16.6 KDa heat shock protein (sHSP)

Plants are characterized by unique and diverse sHSPs that may reflect their need to rapidly adapt to ever changing environmental conditions. sHSPs are usually undetectable in vegetative tissue under normal growth conditions, but can be induced by developmental stimuli or by environmental stresses. HSPs as a major class of responsive proteins play a role via cross-talk with other mechanisms and function

Table 1. Differentially responsive proteins identified after long term salt response in *S. maritima* leaves by MALDI-TOF analysis.

Spot No.	Thr. pI/MW (KDa)	Exp. pI/MW (KDa)	Homology Proteins	Plant Species	Sequence Coverage %	Mascot Score	Peptides Matched	% Identity	Accession No.
1	6.95/ 230.46	6.96/ 230.75	*Ras* related protein *Rab2B*	*Zea mays*	54	60	112	100	NP_001106234
3	7.98/ 75.035	7.98/ 75.435	SNARE interacting protein *KEULE*	*Arabidopsis thaliana*	29	44	199	99	AAK01291
4	5.24/ 126.76	5.24/ 126.05	Sister chromatid cohesion protein 3	*Arabidopsis thaliana*	13	38	134	100	NP_566119
5	7.98/ 75.035	7.98/ 75.435	SNARE interacting protein *KEULE*	*Arabidopsis thaliana*	29	45	199	99	AAK01291
6	9.34/ 57.176	9.34/ 57.405	Cytochrome P450 71A8	*Mentha x piperita*	33	50	166	100	Q42716
12	6.31/ 16.638	6.32/ 16.696	16.6KDa heat shock protein	*Oryza sativa Japonica Group*	32	53	49	100	XP_015620983

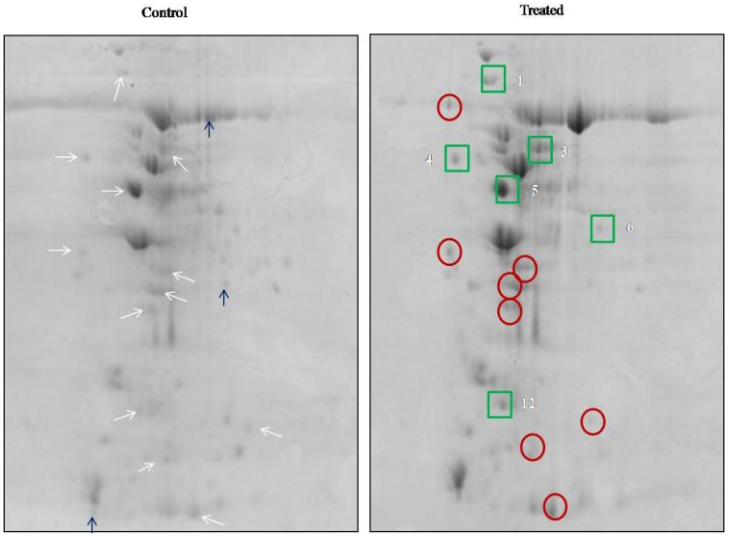

Fig 1. Representative two-dimensional gel electrophoresis (2DE) gels of proteins extracted from leaves of S. maritima. First dimension was performed using 200µg total soluble proteins on linear gradient IPG strip with pH 3-10. In the second dimension, 12 % SDS-PAGE gels were used and proteins were visualized using Coomassie Brilliant Blue (CBB) between control and 200mM NaCl treated respectively. Both red circle and green box indicates that protein changes reproducibly and significantly under salt treatments and white arrows mark the position of the same proteins from the control while green box denotes the identified protein spots by MALDI-TOF/MS analysis. Blue upward arrows indicate the proteins spot present only in the control. See Table 1 for the list of identified proteins.

Table 2. Location, role and function of identified proteins.

Spot No.	Protein	Location	Role	Function
1	*Ras* related protein *Rab2B*	ER - Golgi membranes	Protein transport	Regulates vesicle trafficking between the ER and the Golgi bodies
3&5	SNARE interacting protein *KEULE*	Plasma membrane	Protein transport	Vesicle trafficking/docking drawn in cytokinesis/exocytosis
4	Sister chromatid cohesion protein 3	Chromosome	Chromosome orientation	Centromere cohesion and kinetochore orientation
6	Cytochrome P450 71A8	Plasma membrane	Heme/iron binding	Haem binding protein involving in oxidation degradation of toxic compounds
12	16.6KDa heat shock protein	Cytoplasm	Chaperones	Molecular chaperones that suppress protein aggregation and involved in post translation modification and protein turnover

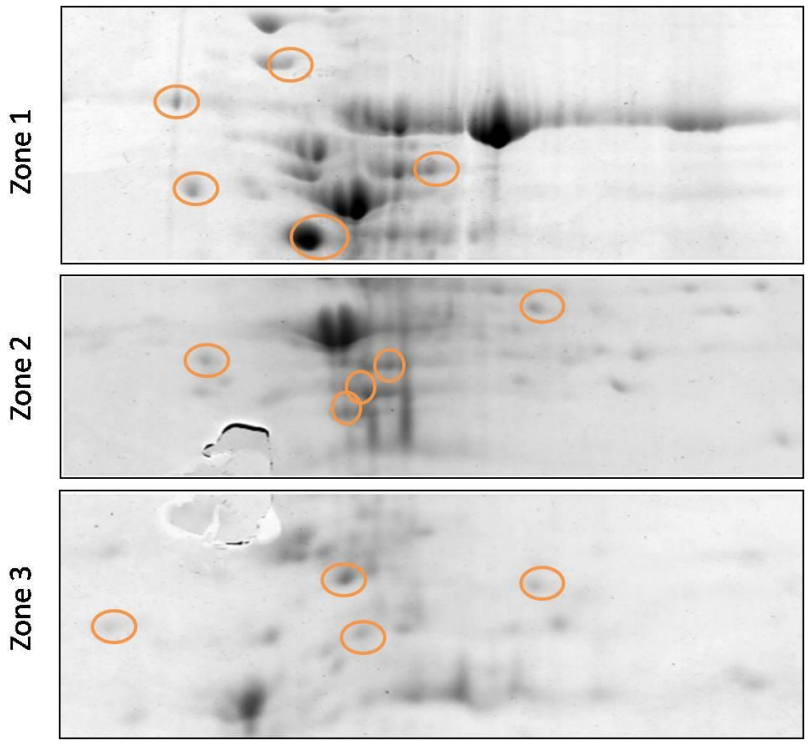

Fig 2. Enlargement of the three zones (1-3) within the 2D-PAGE gels showing the changes in the abundance of the protein spots identified upon salt-exposure of *S. maritima*. For each zone, orange circle indicate 2 fold change spot position on the gel at a given salt concentration compared with the control.

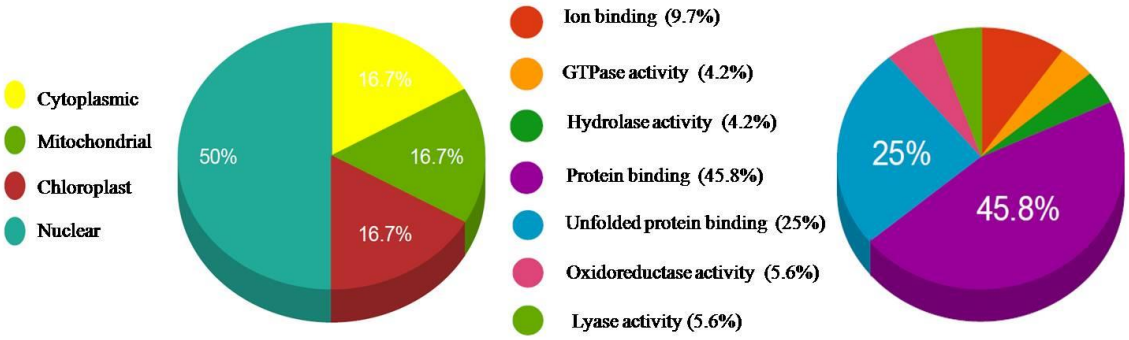

Fig 3. Localization and functional distribution of identified proteins expressed in leaf of *S. maritima* after NaCl treatment (http://cello.life.nctu.edu.tw/cello2go/).

synergistically with other components to decrease cellular damage (Wang et al., 2004). It acts as molecular chaperons in aiding the renaturation of damaged proteins to maintain protein conformation and cellular homeostasis against stress. HSPs are classified into five classes according to their approximate molecular weight: (1) Hsp100, (2) Hsp90, (3) Hsp70, (4) Hsp60 and (5) small heat-shock proteins (sHsps) (Whaibi, 2011). sHSPs represent a family of 12 to 42 kDa proteins sharing a conserved 90 amino acid C-terminal domain called α crystallin domain present in most of the organisms (waters et al., 1996). sHSPs are known to form multimers consisting of 9 to 30 monomers and to function as ATP-independent chaperones to prevent irreversible protein aggregation of denatured proteins and facilitate subsequent protein renaturation in cooperation with ATP-dependent chaperones. sHsps cannot refold non-native proteins, but they can bind to partially folded or denatured substrates proteins, preventing irreversible unfolding or wrong protein aggregation. Previous reports showed that the sHsp*18.1* and sHsp*16.9* isolated from *Pisum sativum* and wheat, as well as the sHsp*16.6* from *Synechocystis sp. PCC6803* involved in binding to unfolded proteins and allows further refolding by Hsp70/ Hsp100 complexes (Lee et al., *1998*; Mogk et al., 2003). In this study, heat shock protein *16.6* (spot no.12) were up regulated by exposure to salinity, suggesting that this protein could play a role in protein folding & assembly, signal transduction, and secondary metabolism, which are essential for cellular viability in plants to acquire salt tolerance.

Materials and Methods

Plant growth and salt treatments

S. maritima seeds were collected from an adult plant growing along the seashore of the mangrove coastal belt, Pichawaram, Chidambaram district in Tamilnadu. The seeds were germinated on vermiculite in plastic pots and allowed to grow for 3 months under natural day/night cycles in a greenhouse. The seedlings were watered with distilled water

and nutrient medium alternatively. After 3 months, the plants were transferred to Modified Hoagland's solution (Sahu and Shaw, 2009) and grew hydroponically for a week. For finding the optimal salt concentration for treatment, different concentration of NaCl (100,200,300,400 and 500mM) were added to the nutrient medium and plants were grown for a period of 14 days in a growth chamber maintained at 24 ± 3°C, 70-75% relative humidity with 14 h light (200 µmol m^{-2} s^{-1})/10 h dark cycle. The untreated set maintained in Modified Hoagland's solution was considered as control. The media was changed every two days to avoid variations in nutrients and salt concentration and to avoid contamination.

Based on the results of this experiment, 200mM NaCl was selected as the concentration for salt treatment before proteomics experiments. A set of plants were then treated with 200mM NaCl for a period of 14 days in a growth chamber maintained at similar conditions. The untreated set maintained in Modified Hoagland's solution was considered as control. After 14 days of treatment, leaves from control and salt treated plants were harvested. Three independent biological replicates each comprising of tissue from 3-4 seedlings was prepared for proteomic analyses.

Protein extraction

The extraction procedure was based on Faurobert et al., 2007 with some modifications. Samples were weighed about 3 g of tissue was powdered in a mortar and pestle using liquid nitrogen. This was mixed with 15ml of the protein extraction buffer pH 8.0 (700mM sucrose, 500mM Tris-Hcl, 50mM EDTA, 100mM KCl, 2% (v/v) β-mercaptoethanol and 1mM PMSF). After 10 min. of incubation on ice, equal volume of Tris-saturated phenol was added and the mixture was vortexed well at room temperature for 10 min and centrifuged for 15 min at 5,500 ×g and 4 °C. The centrifugation step was repeated after the upper phenolic phase containing soluble proteins had been removed carefully and the initial sample volume was restored by addition of extraction buffer. The proteins extracted in the resulting phenolic phase were precipitated at -20°C overnight by adding 100mM ammonium acetate in methanol (precipitating solution). This mixture was then centrifuged for 20 min. at 5,500 ×g and 4 °C and the pellet was resuspended in 5 ml of the precipitating solution before re-centrifugation. This latter step was repeated three times. The pellet was then rinsed with 80% (v/v) ice cold acetone, re-centrifuged, and air dried at room temperature for 15 min. Finally, the pellet were lyophilized under vacuum and then dissolved in protein rehydration buffer (8 M urea, 2% (w/v) CHAPS, 0.2% (v/v) ampholytes (3-10 Biorad), 50 mM DTT, and 2-4 mg Bromophenol Blue). The protein concentration of each pellet was determined using BCA method (Smith et al., 1985) and bovine serum albumin (BSA) was used as a standard.

Two-dimensional gel electrophoresis

The extracted proteins were separated in the first dimension by isoelectric focusing (IEF). Before IEF was performed, 200µg (200µl) protein suspensions were vortexed and centrifuged for 5 min at 12,000 ×g and 4 °C. After centrifugation, protein samples were pipetted in to rehydration tray and carefully kept the 11cm IPG Strip (pH 3-10, Biorad) on the protein sample without air bubbles at room temperature. IEF was carried out for 5 h with 11 cm dry gel strips (Protean i12 IEF, Biorad) with a current limit of 50µA/strip followed by focusing in four steps 250V (20 min), 8000 V (1 hr), 8000 V (26000 v/hrs), and 750 V (hold). Gel strips were then equilibrated for 20 min. with a solution containing 0.375mM Tris-HCl (pH 8.8), 6M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 2% (w/v) DTT. A second equilibration was carried out using the same solution in which DTT was replaced by 2.5% (w/v) iodoacetamide. Equilibrated IPG strips were then horizontally placed on a 12% Tricine SDS-PAGE gel and sealed with a solution containing 0.5% agarose and Bromophenol Blue. SDS-PAGE was carried out at 200V for 1hr at 25°C using protean gel electrophoresis unit (Biorad).

Protein staining, scanning and image analysis

Two-dimensional (2D) gels were stained overnight with Coomassie Brilliant Blue (0.1% (w/v) CBB-G250). Gels were carefully destained and again washed with distilled water to remove the background due to staining. The 2DE gels were scanned using a GS 800 calibrated densitometer (Biorad) at 600 dpi resolution and images were stored as .tif files. For spot detection and volume quantification, .tif files were transformed into .mel files and analyzed using PDQuest analysis software v8.0 (Biorad).Three images of three independent biological replicates of leaves exposed to salinity were grouped to calculate the mean volume of all the individual protein spots. Following automatic spot detection, gel images were carefully edited and one of the gel images was selected as a reference gel before spot matching. The spot abundance was normalized as relative volume according to the normalization method provided by the software to obtain the individual relative spot volume (%), i.e. the spot volume of one spot in relation to the sum of all detected spots on the gel. This method eliminates eventual protein loading differences.

MALDI-TOF analysis

Gel pieces, each containing a salt responsive protein spot, were excised manually, destained two times with 20µl of 50% acetonitrile (ACN), 50mM NH_4HCO_3 at 37°C for 30 min, dehydrated by adding 10µl ACN and dried. 20µg/ml sequencing grade trypsin (promega) was added and after 30 minutes incubation on ice remaining trypsin solution was discarded. Digestion was continued at 37°C for overnight and stopped by adding 0.1% TFA and 50% ACN. Tryptic peptides were extracted with two times 20µl 50% ACN, 0.1% formic acid (FA) for 30 min at 37°C and 10µl ACN for 30 min. at room temperature. All extracts were dried in a vacuum centrifuge. MALDI-TOF analysis was started by resolubilizing dried samples in 2µl of 2 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA), 50% ACN, and 0.1% TFA. 1µl of the solution was spotted onto a stainless steel target and analyzed with a mass spectrometer.

Database analysis

Mass spectrometric data were analyzed by Mascot search engine (www.matrixscience.com) with carbamido-methylation of cysteine as static and oxidation of methionine as variable modification against the Swiss Prot and NCBInr Database. At least two peptides with a Mascot peptide ion score higher than 35 were used as a threshold for protein identification. Localization and molecular function of identified proteins were analyzed using CELLO2Go (http://cello.life.nctu.edu.tw/cello2go/).

Conclusion

In the present study, we provided a comprehensive proteome dynamics of the leaves in the halophyte *S. maritima* under salt conditions. 18 proteins were found to be differentially expressing, showing more than a 2-fold change in abundance and 6 of them were identified by MALDI-TOF/MS analysis. Our results indicated that proteins involved in protein folding, assembly, chromosome segregation, cell maintenance, iron binding and also proteins involved in trafficking could be vital in salt stress tolerance in *S. maritima.* Further analysis of salt responsive proteins from this species would unravel novel mechanisms of salt stress tolerance in plants.

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