Identification and *in-silico* characterization of differentially expressed salt-induced proteins in the leaves of mangrove grass *Myriostachya wightiana*

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**ABSTRACT**

Salt stress is the main constraint that limits agronomic output in several regions of the world. For the development of salt-tolerant crops, it is necessary to understand the salt-responsive proteins in halophytes. *Myriostachya wightiana* is a salt marsh that belongs to Poaceae. In this work, comparative proteomic studies were used to identify the proteins responsible for salt tolerance in the *M. wightiana* using two-dimensional electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Two protein spots 48 and 14 were over-expressed from salt-stressed *M. wightiana* and they were identified as chloroplast adenosine triphosphate (ATP) synthase β and glutamine-dependent nicotinamide adenine dinucleotide (NAD⁺) synthetase. The physicochemical characterization revealed that the ATP synthase β and glutamine-dependent NAD⁺ synthetase have 31714.6 and 66481.8 Da respective molecular weights. The secondary structure of ATP synthase β and Glutamine-dependent NAD⁺ synthetase has a greater percentage of random coils and alpha helix respectively. The modeled three-dimensional structures of ATP synthase β, Glutamine-dependent NAD⁺ synthetase have close similarities with 1fx0B and 3sytaA. The functions of ATP synthase β and glutamine-dependent NAD⁺ synthetase were associated with the ATPase activity and non-covalent selective interaction with ATP correspondingly. This study provides information regarding salt-induced proteins to know the salt adaptation mechanisms for developing salt-resistant varieties.

1. INTRODUCTION

Salinity is the main abiotic stress which influences all chief metabolic activities of a plant such as photosynthesis, protein synthesis, energy production, and lipid synthesis during the development [1]. Hence, salinity is regarded as the utmost severe restrictive factor for the productivity of crops and negatively influences the plant development [2]. Adaptation to salt stress is a complicated and multigenic response, which involves physiological, biochemical, and molecular pathways that can act collectively in the cell, tissue, and plant [3]. The salinity resistance of plants was not so far fully understand but explicated to some extent by stress adaptive effector mechanisms which include homeostasis of ions, biosynthesis of osmolytes, scavenging of toxic radicals, transport of water, management of long-distance response [4], and modifications in gene transcription which alters the expression patterns of proteins. There are many proteins to increase salt tolerance in plants by protecting themselves from cytotoxic ions by excluding through the plasma membrane or accumulating these ions in their storage vacuoles [5]. The improvement of salt-resistant plant varieties is the most important challenge in the present situation. The main problem in the development of salt-resistant plants is the lack of knowledge about the molecular mechanisms involved in salinity adaptations. Therefore, the studies related to the identification and characterization of the protein-based differential expression under salinity conditions are necessary. The identified proteins will provide a better way to develop salt-tolerant crop varieties by incorporating genes through genetic engineering.

Proteomics provides a platform to analyze the protein responses of plants to environmental stimuli and establish a link between the transcriptome and metabolome [6, 7]. Proteomic approaches include extraction, purification, identification, and characterization. Protein identification can be carried out by gel-based separations followed by mass spectrometry (MS) [8]. Two-dimensional (2D) gel electrophoresis is an important technique for the separation of proteins and useful for determining the proteomic differences persuade by specific treatments or environmental alterations. Matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOF MS) analysis commonly identifies the proteins based on the unique mass spectrum of peptide fragments through mass related bioinformatics tools and protein databases such as MASCOT and SEQUEST [9]. Bioinformatics gives a way to establish protein characterization...
such as protein structure, function, modifications, localization, and protein-protein interaction.

The bioinformatics tools determine and characterize the protein’s function using the amino acid sequence. ProtParam is one of the protein analyzing tools in the ExPasy server which is used for computing various physicochemical parameters of a protein. Some bioinformatics programs have been developed for the prediction of proteins secondary structure. Self-optimized prediction method (SOPMA) is a recently developed tool to enhance the accuracy of the protein secondary structure prediction. The protein function is distinct by its structure; hence, approaches for determining protein structure from its sequence are gaining importance to integrate structural information into the annotation process [10]. Three-dimensional (3D) structure prediction is the main goal in the protein modeling from its amino acid sequence with a precision that is similar to the finest outcomes achieved experimentally [11]. Iterative Threading Assembly Refinement (I-TASSER) is a stratified protein modeling tool and it builds up possible structures by congregating fragments edited from the threading templates, where the biological perceptions of the modeled proteins are assumed by identical structures from the existed proteins in the databases [12].

Mangroves are classified as halophytes and they are found in estuaries and marine shorelines of tropical and subtropical tidal areas [13]. They divide into two groups such as true mangroves and mangroves associates. True mangroves specifically grow in intertidal regions while mangrove associates can grow in littoral and terrestrial habitats [14]. Studies on morphological, physiological, and biochemical alterations as salt stress in mangroves are inadequate to clarify the mechanism of salt adaptations. Recently some progress has been attained in perceptive the salt adaptation molecular mechanisms in mangroves, which are linked to the overexpression of salt-responsive proteins. Mangrove associates are perfect plants to study the salt-resistant mechanisms due to the transitional existences between terrestrial plants and mangroves. Myriostachya wightiana is a mangrove associate which is adapted to the two ecotypes, littoral, and terrestrial habitats.

*M. wightiana* is the imperative salt marsh in the family of Poaceae which, inhabitant laterally the muddy streams and channels in wetland mangrove swamps of Ganges Delta of India, Bangladesh and spreading into Myanmar, Malaysia, and Vietnam. In India, it is generally inhabited on the east coast of the Bay of Bengal. *M. wightiana* is typically inhabited with *Acanthus ilicifolius*, *Nypa fruticans*, and *Porteresia coarctata*. Saline water favors the growth and development of *M. wightiana* than freshwater due to the structural adaptive features such as a thick epidermis, sclerenchymatous vascular tissue, salt secreting pores, abundant metaxylem, and large phloem in the stem and leaves. The roots are adopted by the presence of thick cortex and lignified exodermis [15]. The literature review directs no research have happened on the *M. wightiana*. These characteristics make *M. wightiana* an ideal plant for the proteomic study. The current study focused on the identification and characterization of the salt-induced proteins from the leaves of *M. wightiana*.

2. MATERIALS AND METHODS

2.1. Sample Collection

Young and healthy seeds of *M. wightiana* were collected from the mangrove patch of Bhavanapadu which is located (Long: 18°33’ 52” to 18°32’ 11’’; N; Lat: 84°21’ 26” E to 84°18’ 22” E) on the North East of Andhra Pradesh, adjoining the Bay of Bengal, Tekkali, India. The collected seeds were shifted to sterile polyethylene zip bags and transported to the laboratory.

2.2. Germination of Seeds and Salt Treatment

The field-collected healthy seeds of *M. wightiana* were first cleaned under tap water and then distilled water. After cleaning, the seeds were allowed to surface sterilization with 70% C2H5OH and 2% NaOCl. Then, the sterilized seeds were soaked in distilled water overnight and kept for germination on a moisturized Whatman filter paper in a sterile Petri plate for 7 days. When the germination starts, the seedlings were shifted to pots loaded with autoclaved vermiculture. The pots were allowed to grow at 25°C 16 h photoperiod in a greenhouse and irrigated every day with ½ strength Hoagland solution. After 1 week, salinity treatment was given to test plants by irrigated with 500 mM NaCl solution whereas the control plants were irrigated only with water. Healthy and young leaves were collected from 4-week-old plants [Figure 1] to analyze the specific expression of salt-induced proteins.

2.3. Extraction and Estimation of Total Protein

Healthy and young surface-sterilized leaves (0.5 g) from the control and salt-stressed *M. wightiana* were finely pulverized separately by grinding in a motor and pestle with liquid nitrogen [16] and macerated with 2 ml homogenization buffer consists 50 mM Tris-HCl (pH 8.3), 0.5 M sucrose, 50 mM EDTA, 0.1 M KCl, 2 mM PMSF, and 0.1% 2-mercaptoethanol. The homogenate was allowed to centrifuge at 14,000 rpm for 10 min at 4°C. Finally, the supernatants from both control and test were kept at −20°C for further analysis. Lowry *et al.* [17] methodology was adopted to quantify the total protein concentration.

2.4. 2D Electrophoresis (2DE)

The protein separation from respective samples was done using 2DE. Isoelectric focusing (IEF) was used as the 1st dimension and it was done at 20°C using a 1 cm immobilized pH gradient (IPG) strip which has a 3 to 10 pH range. The IPG strips were rehydrated in 125 µl rehydration buffer (8 M urea, 2% CHAPS, 50 Mm dithiothreitol, and 0.2% biolyte) for 12 h by applying 50 V electricity and then the protein samples were loaded onto the strips. The IEF was performed in a stepwise manner: 150 V for 2 h, 300 V for 30 min, 1000 V for 30 min,
5000 V for 1.20 h, and 5000 V for 25 min. The focused strips were equilibrated 2 times for 30 min in 10 ml equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% sodium dodeyl sulfate [SDS], and 100 mg diithiothreitol) with moderate shaking and additional equilibration for 15 min in equilibration buffer having 2.2% iodoacetamide instead of diithiothreitol. After equilibration, SDS-polyacrylamide gel electrophoresis was used as the second dimension and it was done using 10% separating gel. The vertical SDS gels were loaded with the equilibrated IPG strips. Finally, the gels were allowed to stain for overnight with 0.2% Coomassie Brilliant Blue G-250 and differential protein expression between control and treatment plants were measured by counting the number of protein spots on the gels.

2.5. Peptide Mass Fingerprinting (PMF)
The PMF of distinctively induced protein spots in the 2D gel of the test sample was analyzed with MALDI-TOF-MS [18]. Differentially displayed protein dots were extracted from the Coomassie-stained gel and the gel fragments were crushed and cleaned with 25 mM NH₂HCO₃ in 50% acetonitrile, then the gel fragments were subjected to dry and hydration for overnight at 37°C in 25 mM NH₂HCO₃ with 1.0 mg of trypsin. Subsequently, the gel fragments were cleaned 3 times with distilled water, 50% acetonitrile and 5% trifluoroacetic acid at room temperature to obtain the proteins. 0.5 µl of extracted protein was placed on the matrix containing α-cyano-4-hydroxycinnamic acid made up of 50% acetonitrile and 5% trifluoroacetic acid. ABI 4800 MALDI TOF/TOF and GPS Explorer software (Applied Biosystems, Foster City, CA) was used for the collection of MS data and generation of protein masses of protein spots. The obtained mass spectrum was allowed to MASCOT (http://www.matrixscience.com) software for searching using sequence databases. The generated score by the MASCOT software indicates the chance of accurate identification and the value had to be as a minimum 50.

2.6. In-silico Characterization of Identified Proteins

2.6.1. Physicochemical characterization
The identified protein sequences were recovered from National Center for Biotechnology Information (NCBI) in the FASTA format for Insilico analysis. The physicochemical characteristics of proteins such as molecular weight, isoelectric point (pI), number of positive and negative amino acids, extinction coefficient (EC) [19], instability index [20], aliphatic index [21], and grand average hydropathy (GRAVY) [22] were calculated with the ExPASy’s ProtParam server [23].

2.6.2. Secondary structure prediction
The secondary structural features of differentially expressed salt-induced protein sequences were calculated by subjecting the sequence to the SOPMA (https://npsa-prabi.ibcp.fr) server [24].

2.6.3. 3D structure prediction and model validation
3D structural models for the identified proteins were developed by the Ab initio approach. I-TASSER Algorithms an online web server used to predict the structural models and functions of identified proteins [25]. ITASSER server predicts the protein structure depending on the multiple threading alignments built by LOMETS and iterative TASSER assembly simulations [26]. PROCHECK server was applied to generate (http://mordred.bioc.cam.ac.uk/rapper/rampage.php). Ramachandran plot was used for the accuracy validation of predicted 3D protein structural models and their stereochemical properties [27]. The quality of predicted 3D structures was confirmed by ERRAT (http://nihserver.mbi.ucla.edu/ERRATv2/) server.

2.6.4. Functional prediction by I-TASSER server
The ITASSER server was used to predict the functional properties of modeled proteins basing on the global and local resemblances with the template proteins in Protein Data Bank (PDB). The template proteins were ranked depending on the global and local similarity scores and the annotations were subjected to top-scoring hits. The outputs of the server contain functional annotations of Gene Ontology (GO), Ligand binding sites, and Enzyme Commission numbers.

3. RESULTS AND DISCUSSION

3.1. Protein Estimation
The salt-treated M. wightiana plant leaf has 11 ± 0.2% protein content per g fresh weight, whereas the control plant shows 13.2% protein. All the results were reported as mean ± SD obtained from three separate experiments. Due to NaCl treatment, total protein content was decreased in stressed plant leaves. Our findings agree with the results of earlier works like Nirjar et al. [28] observed high protein concentration in freshwater-grown plants compared to salt-stressed plants. Zeynep et al. [29] stated that the protein concentrations are considerably decreased with increasing salt concentration and time intervals in all the tested cultivars of tomato. Results of Mohammad [30] have been revealed that the soluble protein content in rice is reduced with increased salinity. Joshi and Misra [31] observed that the percentage of protein in many plant tissues is decreased during drought or saline conditions, because of proteolysis and reduced protein synthesis. The present results are evident with Parida et al. [32] who found the increased activity of acid and alkaline protease during NaCl stress. The enhanced activity of proteases can act as an indication for salt stress and promote the reduction of protein levels in Bruguiera parviflora. Palma et al. [33] observed the irreversible oxidation of proteins by carboxylation. These oxidized proteins are selectively recognized and degenerate by proteases which lead to the loss of proteins.

3.2. 2DE
2-DE provides an excellent method to compare the substantial quantitative expression of proteins between samples. In plants, salt stress triggers a huge number of stress-associated genes to produce a variety of functional proteins for counterering stress. The comparative analysis of M. wightiana leaf proteome between the control and salt-stressed using 2DE with pH range 3 and 10 revealed a broad distribution of proteins from the PI range 4 to 9.5 in both control and salt-stressed plants. The maximum protein spots were found between the PI ranges of 4.5 and 7. Contrary to this, proteins which are fall in the acidic region, especially between the pH 4.5 and 5.5 showed a superior resolution. The highest percentage of protein variation was observed between the 6 KD and 34 KD markers regions in the gels of control and salt-stressed plants. M. wightiana comparative leaf proteome analysis significantly showed considerable changes in controlled and stressed conditions. A total of 81 protein spots were observed in a control plant whereas only 75 protein spots were found on the gel of salt-treated plant. The percentage of matching spots of the two gels was 90%. Fourteen spots showed quantitative and qualitative variations between the controlled and stressed plants. Among them, seven (3, 14, 17, 35, 48, 73, and 74) were up-regulated and nine were (27, 68, 69, 74, 75, 76, 77, 78, and 81) down-regulated in stressed plant. Two protein spots numbered as 14 and 48 were appeared very sharp and prominent. The molecular weight, PI values of 14, and 48 spots are 69 KD, 5.7, and 33 KD, 5.1 respectively. The prominent spots in the stressed plant were identified to understand the mechanism of...
The present results are evident with some previous reports which found that proteins involved in energy and metabolic regulation are changed under salt stress. In a cell, apart from the constitutive genes, several proteins participate in the functional execution and adaptation to environmental stimuli [34]. Peerzada et al. [35] stated that the treatment of salt stress could induce changes in protein expression. These differentially induced proteins are engaged with various cellular and physiological functions such as osmoregulation, redox homeostasis, ion balance, photosynthesis, energy requirements, and carbohydrate metabolism. Wang et al. [36] observed that Kandelia candel can sustain at 450 mM NaCl concentration possibly with the induced expression of proteins involved in light reactions that synthesize energy equivalents essential for the carbon fixation and other vital functions during salt stress. Page et al. [37] and Manna et al. [38] reported that the heat shock proteins were overexpressed with the treatments of cold and salt stress in tomato. Our findings show similarity with the results of Gomathi et al. [39] proposed that stress-induced high protein content possible by the specific expression of salt shock proteins having MW 15, 28, and 72 KDa in resistant varieties. Our results supported the findings of Majoul et al. [40] in wheat during heat stress. Production of energy depends on the adenosine triphosphate (ATP) synthase activity which is up-regulated in the present experiment as evident with the overexpression of spot 48, to make the energy needs for the encounter the ionic toxicity and other tolerance processes.

3.3. PMF

The unknown proteins are identified based on the spectrum of peptide masses which is known as PMF. For the construction of precise assignments, high-quality protein databases are immensely required to PMF [41]. MALDI-TOFMS of both the protein spots 48 and 14 from M. wightiana salt-stressed plant has a broad range of m/z values of 700–5000 Da. The mass spectrums of protein spots 48 and 14 were presented in Figures 3 and 4. For the protein identification, the obtained masses of peptide fragments were used to search in the NCBI database with MASCOT software. The up-regulated protein spot 48 was identified as chloroplast atpB gene product. PMF results of spot 48 show the highest score, that is, 89 with Oryza sativa chloroplast atpB gene product which is having Accession number gi|552857. The up-regulated protein spot 14 was identified as glutamine-dependent nicotinamide adenine dinucleotide (NAD⁺) synthetase. PMF results of spot 14 show the highest score, that is, 50 with O. sativa indica glutamine-dependent NAD⁺ synthetase gene product which is having Accession number gi|544604189. The protein identification results of spots 48 and 14 were shown in Table 1.

In the present results, it is observed that some of the up-regulated proteins having less molecular weight than the theoretical molecular weights. This might be from the degradation of proteins as a response to salt stress. Our results evident with the results of Lee et al. [42] observed the degradation of proteins which includes Rubisco large subunit, ATP synthase β subunit, FBP aldolase, and peroxiredoxin. Komatsu and Tanaka [43] reported that the rice seedlings have greater capability to synthesize ATP during oxidative stress to withstand in an anaerobic environment. Hence, the upregulation of ATP synthase may be involved in the production of additional energy requirements to prevent the damage caused by salt stress. In salt-stressed Sorghum bicolor; the salt-induced proteins belong to the different functional groups which are involved in the salt stress adaptive mechanisms.

Table 1: Proteins identified from the 2DE gel spots of salt-stressed Myriostachya wightiana leaf.

| Spot no. | Max. homology with (Protein) | Best match organism | Expt. mw/Theor. mw (KD) | Expt. PI / Theor. PI | Score (MS/MS) | Accession no. |
|----------|------------------------------|---------------------|------------------------|---------------------|----------------|---------------|
| 48       | Chloroplast ATP synthase β subunit | Oryza sativa        | 33/31.8                | 5.1/4.7             | 89             | gi|552857       |
| 14       | Glutamine dependent NAD(+) Synthetase | Oryza sativa      | 69/66.5                | 5.7/5.86            | 50             | gi|544604189    |

ATP: Adenosine triphosphate, NAD: Nicotinamide adenine dinucleotide, PI: Isoelectric point, MS: Mass spectrometry

Figure 2: Two-dimensional (2D) gel images of Myriostachya wightiana comparative leaf proteomic expression under salt stress. (a) 2D gel image of control plant (b) 2D gel image of the salt-stressed plant.
Figure 3: Matrix-assisted laser desorption ionization-time of flight mass spectrometry spectra of up-regulated protein spot (48) of salt-stressed *Myriostachya wightiana* leaf.

Figure 4: Matrix-assisted laser desorption ionization-time of flight mass spectrometry spectra of up-regulated protein spot (14) of salt-stressed *Myriostachya wightiana* leaf.
such as energy production and causes and signal transduction [44].
Junicheng et al. [45] found the upregulation ATP synthase β subunit
in salt-stressed Medicago truncatula, and halophyte Halogeton
glomeratus. Noctor et al. [46] stated that the synthesis of NAD is by
the enzyme NAD synthetase. NAD plays an important role in various
signaling and metabolic pathways which are connected with salt stress
adaptation. Our result also implies that chloroplasts are one of the
most influenced organelles inside cells by salt stress and chloroplast
membrane proteins are highly susceptible to salt stress. To conclude,
the differential proteomic studies in the Myriostachya leaf discloses
that the salt stress affects the complex cellular network.

3.4. In-silico Characterization of Identified Proteins

3.4.1. Physicochemical characterization

The primary sequences of the proteins ATP synthase β subunit
and glutamine-dependent NAD+ synthetase of M. wightiana were
retrieved from GenBank for homology, using BLAST P suite. The
physicochemical characterization of ATP synthase β protein and
glutamine-dependent NAD+ synthetase reveals that they have 294
and 593 amino acids with molecular weights of 31714.6 Da and
66481.8 Da, respectively. The results of physicochemical parameters
shown in Table 2. The computed PI values of ATP synthase β subunit
and glutamine-dependent NAD+ synthetase were 4.74 and 5.86,
respectively. If the PI of a protein is <7, it is referred to as an acidic
protein. At the PI, all the proteins show very least mobility. Hence, a
buffer system development for the purification of protein is done using
IEF. If all the Cys residues form cystines, the EC of ATP synthase β
and glutamine-dependent NAD+ synthetase were 34505 M−1 cm−1
and 94405 M−1 cm−1, respectively. While if all the cysteines are
reduced, the EC of ATP synthase β and glutamine-dependent NAD+ synthetase
were 34380 M−1 cm−1 and 93280 M−1 cm−1 correspondingly. The EC of protein
increases with the increasing concentration of amino acids Cys, Trp, and Tyr. The EC will be useful for the quantitative analysis protein
based on UV spectral methods and the study of protein interactions
in solutions with other proteins and ligands. The instability index
of ATP synthase β and glutamine-dependent NAD+ synthetase was computed as 36.93 and 47.64 correspondingly. The instability index
provides information about the stability of the protein in in-vitro
conditions. If the protein instability index is <40, it is considered
stable. Whereas, the instability index is computed as more than 40,
the protein possibly unstable [20]. Rogers et al. [47] observed the
in vitro half-life of proteins and reported that the instability index
is more than 40, the protein shows <5 h half-life. If the protein has
an instability index of <40, it shows more than 16 h half-life in in vivo
conditions. The estimated half-life of ATP synthase β protein is
20 h in mammalian reticulocytes, 20 h in yeast. Whereas the half-life
of glutamine-dependent NAD+ synthetase is estimated as 5.5 h in
mammalian reticulocytes, and 3 min in yeast. Aliphatic index for the
protein sequences of ATP synthase β and glutamine-dependent NAD+
synthetase was measured as 79.52 and 81.06, respectively. The aliphatic index
is referred to as the concentration of aliphatic side chains present
in the protein and it increases the thermal stability of globular proteins.
The GRAVY indices of ATP synthase β and glutamine-dependent
NAD+ synthetase were measured as −0.268 and −0.241. The negative
value of the grand average of hydropathicity indicates that proteins are
non-polar, hydrophilic (GRAVY typical value for hydrophilic protein
is < −1), and better interaction of the protein with water. Our results
show similarity with the findings of Kirchhoff et al. [48] who found
ATP synthase CFβ subunit gi: 11467199 (5.31 PI) and (−0.078 gravy)
located in the chloroplast thylakoid membrane.

| Parameter | ATP synthase β protein | Glutamine-dependent NAD(+) synthetase |
|-----------|------------------------|---------------------------------------|
| Total no. of amino acids | 294 | 593 |
| Molecular weight | 31714.6 Da | 66481.8 Da |
| Theoretical PI | 4.7 | 5.86 |
| Negatively charged residues | 35 | 73 |
| Positively charged residues | 24 | 66 |
| Extinction coefficient | 34380 | 93280 |
| Instability index | 36.93 | 47.64 |
| Aliphatic index | 79.52 | 81.06 |
| GRAVY | −0.268 | −0.241 |
| Formula | C_{175}H_{210}N_{20}O_{24}S_{14} | C_{292}H_{392}N_{51}O_{77}S_{37} |
| Total number of atoms | 4404 | 9245 |
| Carbon | 1379 | 2937 |
| Hydrogen | 2181 | 4582 |
| Nitrogen | 389 | 814 |
| Oxygen | 441 | 875 |
| Sulfur | 14 | 37 |

ATP: Adenosine triphosphate, NAD: Nicotinamide adenine dinucleotide, PI: Isoelectric point, MS: Mass spectrometry

3.4.2. Secondary structure prediction

SOPMA server was used to predict the secondary structure of proteins
and it predicts the state of amino acid residues with an accuracy of
69.5%. In the secondary structure of ATP synthase β, the random
cofs and alpha-helix were found to be 39.12%, and 38.44% followed by extended strand 11.56% and Beta-turn 10.88%. The secondary
structure of glutamine-dependent NAD+ synthetase has 45.53% Alpha
helix, followed by 18.21% extended strand, 8.77% Beta-turn, and
27.49% Random coils. The secondary structural elements of ATP
synthase β subunit and glutamine-dependent NAD+ synthetase were
shown in Table 3. Secondary structure gives information regarding
the amino acid existence, whether they are present in an alpha helix,
β strand, or coil. The secondary structure of ATP synthase β and
 glutamine-dependent NAD+ synthetase reveals that these two proteins have a dominant percentage of random coils and α-helices while the extended strands and beta-turns exist in less percentage among all the
secondary structural elements. The remaining secondary structural
elements such as 3α helix, PI helix, ambiguous states, bend regions,
and β bridges were not found in ATP synthase β and glutamine-dependent
NAD+ synthetase. Secondary structure prediction of protein helps in understanding the hydrogen bonds present in the protein
which further indicates the structural and functional efficiency [49].
The present observations show a strong relation to the functional
properties of proteinaceous enzymes. The findings of this study
evident with the observations of Vidhya et al. [50] reported that the
greater concentrations of amino acid residues such as flexible glycine
and hydrophobic proline enhance the percentage of random coils in
protein secondary structure. Proline can interrupt ordered secondary
structure by generating bends in the polypeptide chains. The stability and conservation levels of proteins are increase with the dominated
percentages of random coils in structure [51]. The composition of proteins with a greater amount of hydrophobic amino acid residues
especially in the transmembrane helices enhances the formation of complementary interactions with the hydrophobic lipid bilayer [52]. Bansal et al. [53] proposed that the functions of α-helical proteins are differentiated with their topologies which include signal recognition receptors, molecules, and ion transport across the membrane, translocation, and conservation of energy. The more extended structure of an α-helical protein may also help in sliding motion and their dynamics.

### 3.4.3. 3D structure prediction and model validation

3D structure of protein provides precise information regarding their interactions, stable conformation, and molecular functions. I-TASSER is an integrated computerized platform for the prediction of protein structure and function basing on the sequence. I-TASSER generates 3D atomic structural models to target protein sequence by threading for the possible folds using profile-profile alignments with the template structures which are selected from the PDB library. The target protein 3D structural models generated by comparing with the 3D models of selected template proteins. Then, the function of the protein was predicted. The proteins ATP synthase β and glutamine-dependent NAD⁺ synthetase have the best Z-score with the PDB ID: 1e79D and 3slaA among the ten algorithms. The result of the structural alignment program reveals that the ATP synthase β and glutamine-dependent NAD⁺ synthetase protein sequences have close similarities with 1fx0B and 3sytA. Among the five models of ATP synthase β predicted by I-TASSER, the model with the best C-score (0.71) was selected with an estimated accuracy of 0.81 ± 0.09 (template modeling [TM]-Score) and 4.7 ± 3.1 Å (root-mean-squared deviation [RMSD]). The predicted best model of ATP synthase β protein was shown in Figure 5. Among the five models of glutamine-dependent NAD⁺ synthetase predicted by I-TASSER, the model with the best C-score (0.48) was selected with an estimated accuracy of 0.78 ± 0.10 (TM-Score) and 6.6 ± 4.0 Å (RMSD). The predicted best model of glutamine-dependent NAD⁺ synthetase was shown in Figure 6.

The predicted models were validated basing on the geometric properties of the backbone conformations using Ramachandran plot analysis with the PROCHECK program. Ramachandran plot of the ATP synthase β shows [Figure 7], 72.3% residues are in most favored regions, 18.8% residues are in the allowed region, and 8.9% residues in the outlier region. Ramachandran plot of the glutamine-dependent NAD⁺ synthetase shows [Figure 8]. About 71.1% residues are in most favored regions, 18.6% residues are in the allowed region, and 10.3% residues in the outlier region. The present results confirm that most of the amino acids phi-psi distributions are constant with a right-handed α-helix and the models were stable and reliable. ERRAT program predicts overall quality for protein structural models by examining with the help of statistics of pairwise atomic interactions such as CC, CN, CO, NN, NO, and OO [54]. ERRAT server predicted that the overall quality factor of ATP synthase β and glutamine-dependent NAD⁺ synthetase 3D structures as 86.022 [Figure 9] and 84.906 [Figure 10], respectively. ERRAT confirms the quality of predicted 3D structures as the most reliable and within the accepted range. Consensus predictions suggested that selected proteins of *M. wightiana* contain ATP synthase and NAD⁺ synthetase family associated with various cellular activities.

### 3.4.4. Functional prediction by I-TASSER

The functions of the query protein were predicted based on the functional analogs and the confidence score of the predicted 3D model. The protein modeling by the I-TASSER also predicts the GO term explaining the supposed function of the predicted structure. The

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**Table 3: Secondary structural elements of ATP synthase β subunit and glutamine-dependent NAD⁺ synthetase.**

| Structural elements | ATP synthase β subunit | Glutamine-dependent NAD⁺ synthetase |
|---------------------|------------------------|-----------------------------------|
|                     | Number of residues     | Percentage of residues          | Number of residues | Percentage of residues |
| Alpha helix (Hh)    | 113                    | 38.44                            | 270                | 45.53                 |
| 3_1 helix (Gg)      | 0                      | 0.00                             | 0                  | 0.00                   |
| PI helix (li)       | 0                      | 0.00                             | 0                  | 0.00                   |
| Beta bridges (Bb)   | 0                      | 0.00                             | 0                  | 0.00                   |
| Extended strands (Ee)| 34                     | 11.56                            | 108                | 18.21                  |
| Beta turn (Tt)      | 32                     | 10.88                            | 52                 | 8.77                   |
| Bend region (Ss)    | 0                      | 0.00                             | 0                  | 0.00                   |
| Random coil (Cc)    | 115                    | 39.12                            | 163                | 27.49                  |
| Ambiguous states    | 0                      | 0.00                             | 0                  | 0.00                   |
| Other states        | 0                      | 0.00                             | 0                  | 0.00                   |

ATP: Adenosine triphosphate, NAD: Nicotinamide adenine dinucleotide
Figure 7: Structural validation of adenosine triphosphate synthase β chain model by Ramachandran plot using Procheck server.

Figure 8: Structural validation of glutamine-dependent nicotinamide adenine dinucleotide’ synthetase protein model by Ramachandran plot using Procheck server.
modeled ATP synthase β molecular function, biological process, and cellular location were associated with GO:0008553, GO:0015991, and GO:0005886 with respective functions of hydrogen exporting ATPase activity, ATP hydrolysis coupled proton transport, and the protein is a component of the chloroplast thylakoid membrane. The modeled glutamine-dependent NAD⁺ synthetase molecular function, biological process, and cellular location were associated with GO:0005524, GO:0009435, and GO:0005886 with respective functions of non-covalently interacting with ATP, the formation of NAD from the nicotinic acid and the protein is a cellular component of biological membrane. The predicted numbers of GO terms and associated functions of ATP synthase β chain and glutamine-dependent NAD⁺ synthetase by I-TASSER server are listed in Table 4.

Modeled ATP synthase β subunit protein representing EC 3.6.3.14 indicates the function of alpha/beta hydrolases fold type. The predicted possible ligand binding site residues of ATP synthase β were Y¹¹⁸, E¹⁴², R¹⁴³, Y²⁵⁴, and Y²⁶⁶. Results were shown in Figure 11 and the possible binding ligand ANP (Phosphoaminophosphonic Acid-Adenylate Ester). Modeled glutamine-dependent NAD⁺ synthetase protein
representing EC 6.3.5.1 deamido NAD⁺ L-glutamine amido-ligase. The predicted possible ligand binding site residues of glutamine-dependent NAD⁺ synthetase were K292, D293, D294, N296, E299, Y331, and L432. Results were shown in Figure 12 and the possible binding ligand is ATP.

4. CONCLUSION

In this study, comparative proteomic profiling in response to salinity in mangrove associate grass *M. wightiana* was performed by 2DE, MALDI-TOF, and Bioinformatics tools. Analysis of the leaf proteome revealed that there was a significant change in controlled and stressed conditions. The percentage of matching spots in the 2D gels of control and the salt-stressed plant was 90%. Fourteen spots and stressed conditions. The percentage of matching spots in the 2D proteome revealed that there was a significant change in controlled and stressed conditions. The percentage of matching spots in the 2D gels of control and the salt-stressed plant was 90%. Fourteen spots were involved in the energy requirements of *M. wightiana* as chloroplast atpB and glutamine-dependent NAD⁺ synthetase gene products. In *silico* studies of identified proteins revealed that they were involved in the high salinity tolerance methods of *M. wightiana* by the enhanced production of ATP synthase β and maintained sufficient NAD⁺ levels by glutamine dependent NAD⁺ synthase under salt stress. The present results describe the high salinity tolerance methods of *M. wightiana* and explain the functional mechanisms involved in the salt-stress adaptation. This investigation can reflect the organization of cellular activities in *M. wightiana* under salinity stress, provides new perceptions in salt stress adaptation, and used as a source for the research works aimed to enhance salt resistance in rice and other plants.

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6. CONFLICT OF INTEREST

Authors declared that they do not have any conflicts of interest.

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