Dissecting Salt Stress-Induced Proteomic Changes in Pigeonpea (*Cajanus cajan* L. Millspaugh) Leaves: A Detailed Analysis of Stress-Responsive Protein Dynamics

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Abstract

Salinity is a major abiotic stress that poses significant challenges to global agriculture by reducing crop productivity and soil fertility. In pigeonpea (Cajanus cajan L. Millspaugh), comprehensive studies investigating proteomic responses to salt stress are limited. This study aims to establish a detailed proteomic profile for two pigeonpea varieties: ICPL 87119 (salt-tolerant) and ICPL 85063 (salt-sensitive) under NaCl-induced salt stress.

The study evaluated the effects of different NaCl concentrations (0, 50 mM, 100 mM, 150 mM and 200 mM) on seedling growth including parameters such as plant height, fresh weight and leaf necrosis. Leaf protein profiles were analyzed using two-dimensional gel electrophoresis (2-DGE) to identify differentially expressed proteins under salt stress. Proteins from plants subjected to 150 mM NaCl stress were identified and characterized using MALDI-TOF-MS with a focus on proteins related to photosynthesis, ion transport and stress response mechanisms.

Protein spots that exhibited differential expression were analyzed using PDQuest software. The results showed that salt stress significantly reduced plant growth and increased leaf necrosis, particularly at higher NaCl concentrations. Key proteins involved in photosynthesis such as the RuBisCO Large Subunit-Binding Protein Subunit Beta and ATP Synthase Subunit Beta, were differentially expressed. Proteins associated with ion transport, including the Glutamine Synthetase Leaf Isozyme and stress response proteins, such as Heat Shock Protein 7, also showed significant changes in expression. These findings highlight the critical roles of these proteins in the adaptive response of pigeonpea to salt stress. This study provides essential insights into the proteomic dynamics of pigeonpea under salinity, revealing key proteins and pathways involved in salt tolerance. The identified proteins represent potential targets for future research and biotechnological interventions to improve crop performance under adverse conditions.

Keywords: Pigeonpea, Salt stress, BLASTp, 2-DGE, MALDI-TOF-MS, Mascot.

Introduction

Salinity is recognized as one of the most severe abiotic stressors affecting agriculture today, posing critical challenges to crop productivity and soil fertility. It is estimated that over 20% of irrigated land worldwide is impacted by salinity, severely limiting agricultural yield and threatening sustainability[17,34]. High salts in the soil lead to osmotic stress, ion toxicity and nutrient imbalances, which disrupt essential plant growth processes, particularly in economically important crops[2,27]. Salt stress impacts various stages of the pigeonpea life cycle, particularly germination and early seedling growth where high salt concentrations interfere with water uptake and lead to osmotic stress, subsequently inhibiting seed germination[11]. Furthermore, the accumulation of sodium and chloride ions within plant tissues disrupts cellular processes and compromises seedling development[3]. Understanding these intricate molecular mechanisms is essential for devising effective strategies to mitigate salt-induced damage and to enhance the adaptability of pigeonpea to adverse conditions, ultimately contributing to sustainable agricultural practices in saline-affected regions[31].

Significance of Pigeon pea and Challenges of Salt Stress:
In this context, pigeon pea (*Cajanus cajan* L.) emerges as a crucial player in global food and nutritional security. Boasting a rich protein content ranging from 18% to 38%, along with vital minerals and vitamins, pigeonpea holds a unique status[9,26]. As the first seed legume plant with its complete genome sequenced, pigeonpea is a beacon of potential for addressing nutritional challenges[27].

However, despite its nutritional significance, pigeon pea faces challenges in productivity, leading to an alarming increase in its market price. This escalation makes this dietary staple increasingly unaffordable for a significant portion of the population, especially for those dependent on vegetarian meals. Through this study, we aim to contribute valuable insights that will support the development of resilient pigeonpea cultivars, ensuring food security in the face of increasing salinity challenges. Numerous studies have investigated the physiological and morphological responses of pigeonpea to salt stress, there remains a

significant gap in understanding the proteomic changes that facilitate salt tolerance in this crop[27].

Proteomic Analysis Under Abiotic Stress: Pigeonpea (*Cajanus cajan* L.) is an important legume crop cultivated in semi-arid tropical and subtropical regions, valued for its high protein content and ability to thrive under harsh environmental conditions including low soil fertility, salt, heat and drought. Its capacity to maintain stable yields despite such challenges makes it a vital component of food security in vulnerable region[23]. Proteomic studies have played a crucial role in uncovering the molecular mechanisms responsible for drought tolerance in pigeonpea. MALDI-TOF-MS/MS analysis identified 373 seed proteins with increased expression during drought stress including peroxiredoxins (Prx), antioxidant enzymes that neutralize reactive oxygen species (ROS) to mitigate oxidative damage during dehydration.

Notably, 1-Cys Prx accumulates in the aleurone layer and embryo, safeguarding seeds from oxidative stress and preserving dormancy under desiccation conditions[13]. A multi-omics approach applied to a thermo-sensitive genic male sterility (TGMS) line (Envs Sel 107) further revealed that exogenous auxin application stimulates the expression of auxin transport proteins, promoting cell wall development and nutrient uptake under stress. However, temperature-induced reductions in auxin levels hinder normal cell wall formation, contributing to male sterility in pigeonpea[21]. These findings provide critical insights into the crop's reproductive regulation under adverse conditions and offer promising molecular targets for breeding programs aimed at improving pigeonpea's resilience and performance under environmental stress.

Research on the salt stress response of pigeonpea (*Cajanus cajan*) has made significant strides in recent years, particularly through comparative proteomic and transcriptomic analyses. A study involving 7-day-old seedlings exposed to 150 mM NaCl identified 118 differentially abundant proteins in both roots and shoots under salt stress. Notable increases in amino acids such as serine, aspartate and phenylalanine were observed in these tissues, indicating potential metabolic adaptations to saline conditions. Key proteins related to DNA-binding with one finger (Dof) transcription factors and glycine betaine (GB) biosynthesis were also significantly expressed, emphasizing their roles in salt tolerance mechanisms[12]. In a parallel investigation comparing contrasting pigeonpea varieties, ICP1071 (salt-sensitive) and ICP7 (salt-tolerant), integrated proteomic and transcriptomic analyses revealed 82 differentially expressed proteins (DEPs) with a fold change of $\geq \pm 1.5$ on 2D gels.

Twenty-five of these DEPs were further characterized using MALDI-TOF/TOF and classified into functional categories via Uniprot software. Pathway analyses indicated a predominance of functional genes related to carbohydrate metabolism, followed by those involved in protein folding/degradation, amino acids and lipids. Expression studies of six selected genes revealed consistent patterns in both transcript and protein levels, highlighting the complex regulatory networks activated under salt stress[1]. Overall, these findings underscore the importance of elucidating the proteomic and transcriptomic changes in pigeonpea in response to salt stress, paving the way for future research aimed at enhancing the crop's resilience to salinity and improving its overall productivity in challenging environments.

Proteomic studies have proven invaluable in unraveling the molecular mechanisms underlying plant responses to salt stress in other crops. For instance, in sorghum, proteomic analyses using two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (MS) have identified key proteins involved in signaling, defense mechanisms and cell wall metabolism, particularly in response to salt and hyperosmotic stresses[19]. Similarly, comparative proteomic approaches in woody halophyte *Kandelia candel* have revealed that proteins associated with photosynthesis, energy metabolism and stress signaling play crucial roles in the plant's ability to withstand high NaCl concentrations[32].

Notably, sorghum seed proteomics has highlighted the significance of specific proteins such as seed storage proteins and heat shock proteins, in differentiating among genotypes and understanding their nutritional aspects[18]. Collectively, these findings underscore the importance of proteomic analyses in identifying potential biomarkers and enhancing our understanding of the molecular pathways that contribute to salt stress tolerance in various crop species. Recently, the integration of proteomic techniques has become increasingly significant, driven by advancements in methodologies such as 2D gel electrophoresis, protein identification and mass spectrometry (MS). These technological strides have greatly enhanced sensitivity and expanded the scope of proteomic analyses, enabling researchers to delve deeper into the complexities of plant biology.

The comparative study of stress-induced responses in plant proteomes has emerged as a cornerstone in developing novel plant breeding strategies. Researchers can identify key molecular players involved in stress adaptation and resilience by elucidating the dynamic changes in protein expression under stress conditions. This knowledge enhances our understanding of plant stress responses and provides valuable insights for improving crop performance and productivity[19].

The present study aims to bridge the knowledge gap by exploring the proteomic dynamics of two pigeonpea genotypes: a salt-tolerant variety (ICPL 87119) and a salt-sensitive variety (ICPL 85063). By identifying key proteins involved in essential processes such as photosynthesis, ion transport and stress responses, this research endeavors to

provide a molecular framework for breeding programs focused on enhancing salt tolerance. To ensure the future of pigeonpea cultivation and sustained nutritional availability, there is an urgent need to develop abiotic stress-resistant cultivars. This can be achieved through advanced techniques such as gene transformation and genome editing. Therefore, the present study conducts a comprehensive investigation into the salt stress proteomics of pigeonpea, specifically concentrating on the two locally cultivated genotypes: ICPL 87119 (Tall) and ICPL 85063 (Dwarf).

Material and Methods

Plant Material and Growth Conditions: In this study, two varieties of *Cajanus cajan* L., specifically the semi-dwarf spring cultivar ICPL 87119 (ASHA) and the susceptible cultivar ICPL 85063 (LAKSHMI), were carefully chosen as subjects. The plants underwent a controlled growth period of 21 days, employing a hydroponic system with Hoagland media adjusted to pH 6.6. The process initiated with the surface sterilization of mature seeds followed by germination in Petri dishes for five days. Subsequently, the germinated seeds were transferred to 15-centimeter test tubes filled with autoclaved Hoagland media.

The entire cultivation period spanned 21 days, maintaining an average temperature of 21°C, 60-80% relative humidity and a photoperiod of 16 hours light and 8 hours dark. Regular weekly changes were implemented to minimize the risk of contamination. After precisely three weeks, leaves were harvested, subjected to cleaning procedures and then stored at -80°C to preserve their integrity for later protein extraction.

Preparation of Protein Samples: The experimental design involved the collection of leaves from salt-treated plants, with 1 gram of leaves from each variety finely ground to a powder using liquid nitrogen and a mortar and pestle. The powdered material was then immersed in cold acetone containing 10% (w/v) trichloroacetic acid (TCA) and 1% (w/v) dithiothreitol (DTT). Following thorough vortexing, the suspension was placed at -20°C overnight. Subsequent centrifugation steps were performed, with the resulting pellet subjected to two washes using cold acetone containing 1% DTT followed by additional centrifugation and vacuum drying. The pellet obtained was solubilized in a lysis buffer, carefully composed of 8 M urea, 4% (w/v) CHAPS, 10 mM DTT and 1% (v/v) ampholyte at a pH range of 3–10.

After another round of centrifugation, the supernatant underwent a second clarifying centrifugation, producing the final supernatant. This supernatant was then utilized for the determination of protein content and subsequently applied to one-dimensional (1-DE) and two-dimensional (2-DE) gel electrophoresis.

Protein Quantification: The quantification of protein extracts was carried out utilizing a modified Bradford assay. For this purpose, Bovine Serum Albumin (BSA) standards were prepared and protein extracts were prepared in duplicate. The Bradford reagent, diluted fivefold with distilled water, was added to all standards and protein extracts. Following a brief incubation period, absorbance was measured at 595 nm, utilizing a Milton Roy Spectronic GENESYS 5 spectrophotometer, with a 1 mg/ml BSA standard serving as the blank solution. The resultant standards were then used to construct a standard curve, facilitating the extrapolation of concentrations for unknown protein extract samples.

Two-Dimensional Gel Electrophoresis (2-DGE)

Rehydration of 18cm IPG Strips: In the pursuit of comprehensive proteomic analysis, the 2-DGE technique was employed, initiating with the rehydration of 18 cm pH 3–10 linear Immobilized pH Gradient (IPG) strips within the IPGphor system. This phase of the process is critical for achieving optimal separation of proteins based on their isoelectric points (pI). The employed protein extracts underwent a judicious dilution in a rehydration solution consisting of 7 M urea, 2 M thiourea, 18 mM Tris–HCl at pH 8.0, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer tailored to match the pH range of the IPG strip and 0.002% bromophenol blue, serving as a tracking dye.To further enhance rehydration efficiency, 1.6% (v/v) of Destreak reagent (GE Healthcare) was incorporated into the rehydration solution. The practical implementation of this solution was carried out by loading 450 µl of the rehydration buffer onto each IPG strip.

Simultaneously, 150 µl of the respective protein sample for each variety, along with Dithiothreitol (DTT), was added to facilitate protein solubilization. To ensure uniform and consistent rehydration, the IPG strips were methodically covered with mineral oil, specifically Plus One Dry Strip Cover Fluid from GE Healthcare. This precautionary measure served the dual purpose of preventing sample evaporation during the rehydration process and maintaining an optimal environment for the passive rehydration of the strips. This passive rehydration process allowed the IPG strips to reach their original gel thickness of 0.5 mm over a duration of at least 15 hours at room temperature. This meticulous rehydration step is pivotal in preparing the IPG strips for the subsequent phases of 2-DGE.

By facilitating the absorption of the protein sample into the gel matrix under controlled conditions, it ensures uniform distribution of proteins across the pH gradient. The success of the rehydration step sets the stage for the precise separation of proteins based on both their isoelectric points and molecular weights in the subsequent dimensions of the electrophoresis process. Overall, this detailed rehydration process is integral to the success of the 2-DGE analysis, enabling accurate and reproducible protein separation for downstream analyses.

Second Dimension SDS-PAGE of Mini Format Gels: Equilibrated 18cm IPG strips were gently rinsed with 1X

SDS-PAGE running buffer and placed on top of mini format 12% SDS-PAGE resolving gels with the plastic backing against the spacer plate. Three microlitres of unstained protein ladder were spotted on small pieces of filter paper, air-dried and placed at the anodic side of each IPG strip. The IPG strips were then overlaid with 1 ml of 0.5% (w/v) molten agarose prepared in 1X SDS-PAGE running buffer containing a tint of bromophenol blue, which was used as a migration tracking dye during electrophoresis

Two-dimensional gel electrophoresis of proteins: Two-dimensional gel electrophoresis method was used in this study with the Ettan IPGphor system (GE Healthcare, Little Chalfont, UK). The leaf protein (1,500 mg) was loaded onto IPG strips (18 cm, linear gradient pH 3–10; GE Healthcare) in a rehydration tray for 12 h. Isoelectric focusing (IEF) was performed under the following conditions: 200 V for 1 h, 500 V for 1 h, 1 kV for 1 h, gradient to 8 kV for 0.5 h, and, finally, 8 kV for a total of 48,000 Vhs at 20uC. After IEF, the IPG strips were equilibrated in equilibration buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS] containing 1% (w/v) dithiothreitol for 15 min and then in equilibration buffer containing 2.5% (w/v) iodoacetamide for 15 min. The strips were transferred onto vertical 12% SDS-PAGE gels and proteins separated at 10 mA/gel for 30 min, followed by 30 mA/gel overnight. After electrophoresis, gels were visualized using Coomassie brilliant blue (CBB) R-250 staining.

Gel image analyses: Stained gels were scanned with an image scanner and analyzed with Image Master Platinum software V.50 (GE-Healthcare®). The authenticity of each spot of protein was validated and edited by visual inspection. A synthetic gel, containing only spots present in all the six gels of each mature seed stage (Six biological samples with four gels each), was obtained and used to validate the presence of spots in all repetitions. The number, percentage volume criterion and distribution of spots were evaluated. Synthetic gels were used to quantify the number of spots per stage, differential expression of proteins using the percentage volume criterion and differential distribution of proteins in accordance with molecular weight and isoelectric point. After analysis gels were dehydrated in a solution containing 30% methanol and 3% glycerol and then stored at room temperature until mass spectrometry identification of differentially expressed polypeptides.

Prior to differential protein expression analysis across treatment groups of each experiment, spots were manually edited using the group consensus tool to obtain spot expression consensus in all three biological replicates per treatment group. Differentially expressed protein spots were either qualitative (present/absent spots), quantitative (showing at least a 2-fold expression change) and/or Student's *t*-test (95% significance level) significant spots.

In-gel Digestion of Protein Spots for MALDI-TOF-MS: Protein spots were excised from the stained gel and washed first with distilled water to remove ammonium sulfate and then with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain the gel plug. The gel plug was dehydrated with 100% acetonitrile, dried under vacuum and then reswollen with 20 l of 10 l/ml trypsin in 25 mM ammonium bicarbonate. Digestion was performed overnight at 37ºC. The resulting tryptic fragments were extracted with 50% acetonitrile and 5% trifluoroacetic acid with sonication. The extract was dried to completeness and dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid 20%.

Database Search and Sequence Analysis: Protein identification was performed by searching the National Center for Biotechnology Information (NCBI) non-redundant database using the MASCOT search engine (http://www.matrixscience.com) which uses a probability-based scoring system. For database searches with MALDIMS spectra, the following parameters were used: average mass; 1.5 Da peptide and MS/MS mass tolerance; peptide charge of +1, +2, or +3; trypsin as digesting enzyme with 1 missed cleavage allowed; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modification and mass tolerance = 50, 150, 200, 250, 300, 400 ppm.

Taxonomy was limited to Taxonomy: Viridiplantae (green plants) for ion searches MALDI– TOF–MS data to qualify as an identification; a protein's score had to equal or exceed the minimum significant score. Identification of proteins by MALDI-MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score. Final sequence coverage was then calculated according to the comparison between m/z values obtained by MALDI-TOF-MS and the peptide fingerprints obtained by trypsin digestion using the mass peptide program (http://www.expasy.ch) and (http://www.ncbi.nlm.nih.gov/BLAST) for conceptual proteins.

Results

Morphological and Physiological Observations: In our initial exploration of pigeonpea genotypes for salt stress response, ICPL 87119, known as Asha (salt-tolerant), exhibited remarkable resilience under the influence of 200 mM NaCl for 21 days. The salt tolerance was evident in its germination, growth, seedling vigor and overall morphology. Conversely, ICPL 85063, identified as Lakshmi (salt-sensitive), experienced severe consequences including wilting, stunted growth and yellow necrosis, highlighting its vulnerability to salt stress. This disparity in response laid the foundation for an in-depth investigation into the impact of salinity stress on pigeonpea, particularly focusing on the molecular and proteomic aspects.

The experimental setup included salinity stress induced by varying concentrations of salt (Control, 50mM, 100mM, 150mM and 200mM NaCl). The initial observations during the control, where plants were grown hydroponically for

three weeks without infection, revealed minimal variation at the 50mM concentration, which was considered as the control. Subsequent evaluations at 100mM, 150mM and 200mM NaCl concentrations unveiled distinct effects on pigeon pea morphology (Fig. 1A and B).

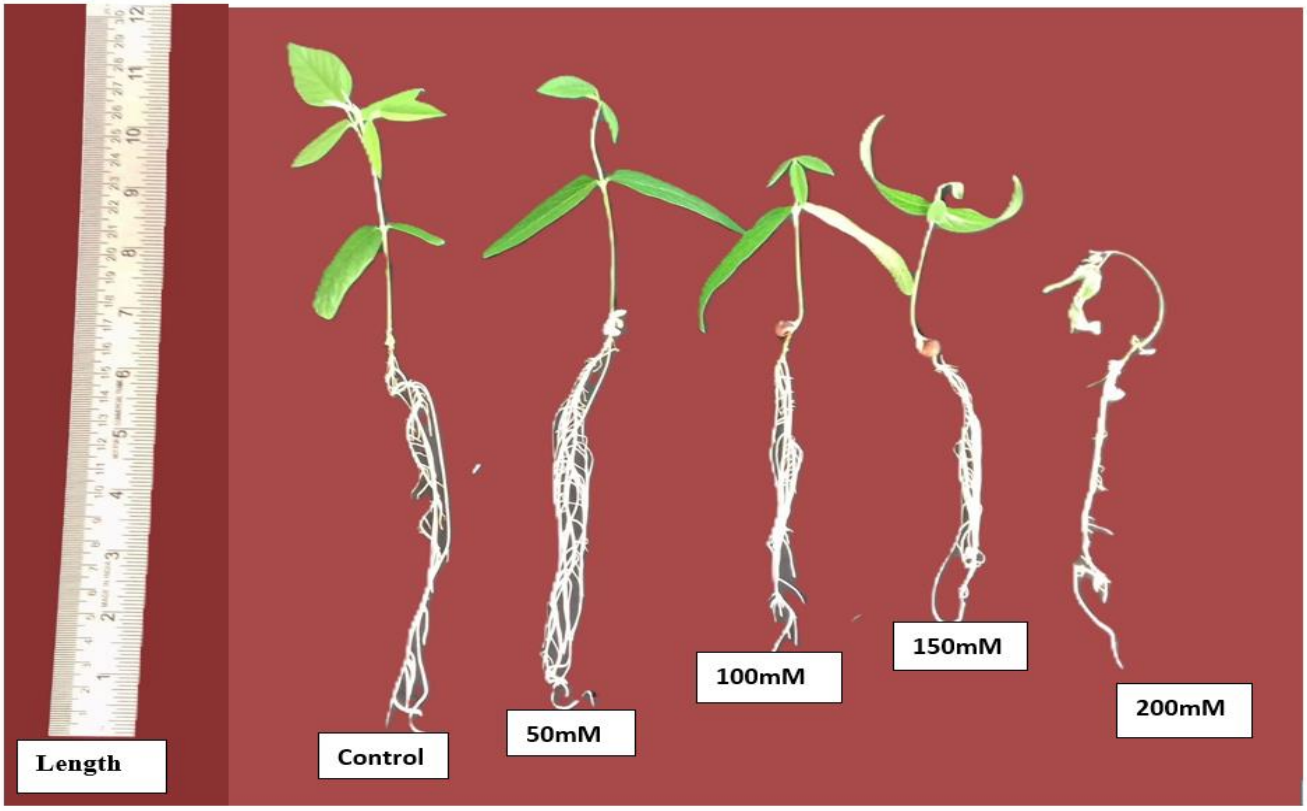

Figure 1A: Morphological Effects of Salt Stress on Pigeonpea Genotypes Leaves of ICPL 87119 at Different NaCl Concentrations

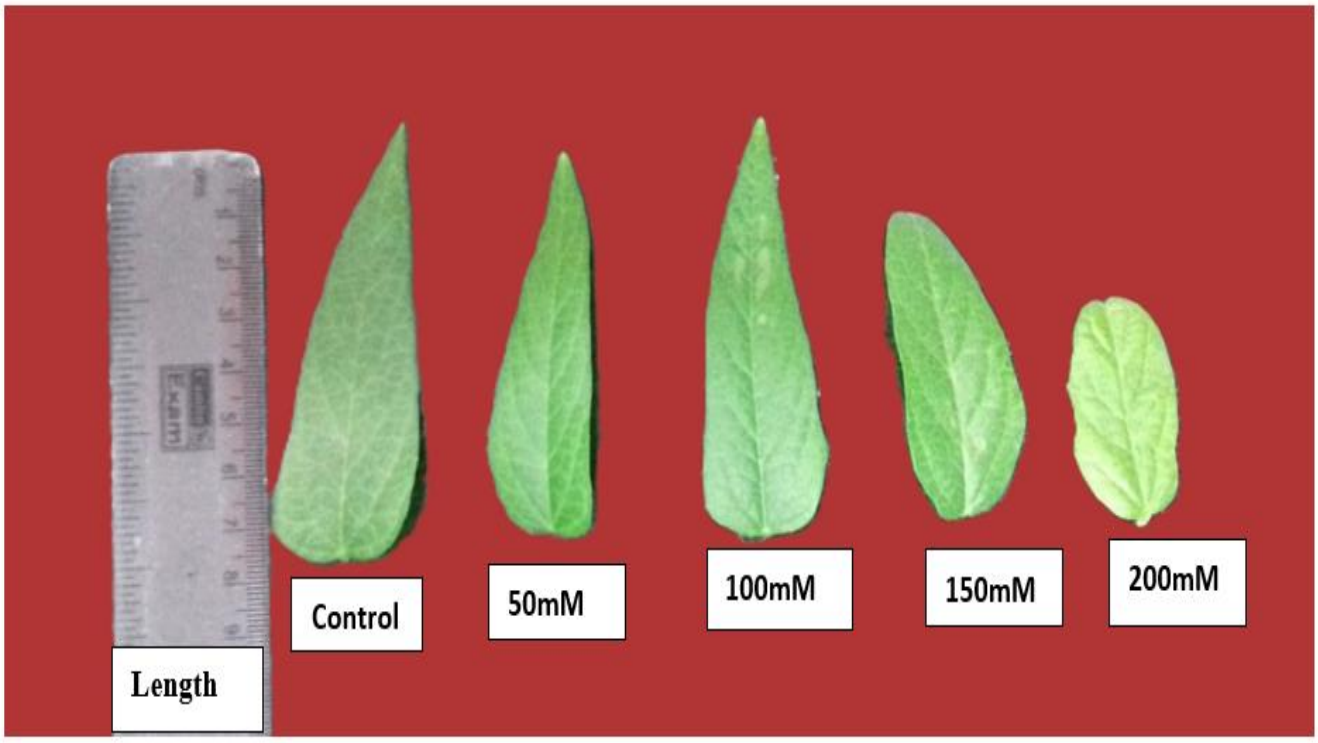

Figure 1B: Morphological Effects of Salt Stress on Pigeonpea Genotypes Leaves of ICPL 85063 at Different NaCl Concentrations

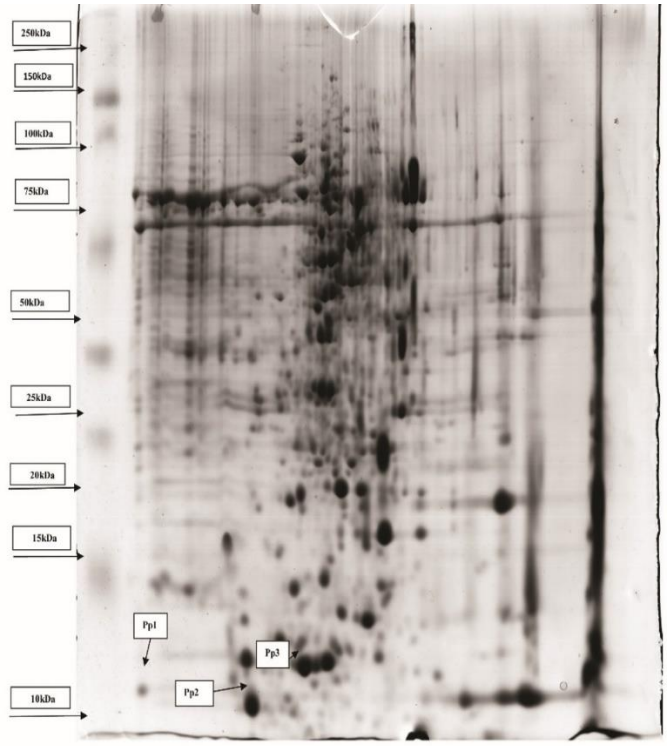

Figure 2: Control Pigeonpea Leaf of ICPL 87119

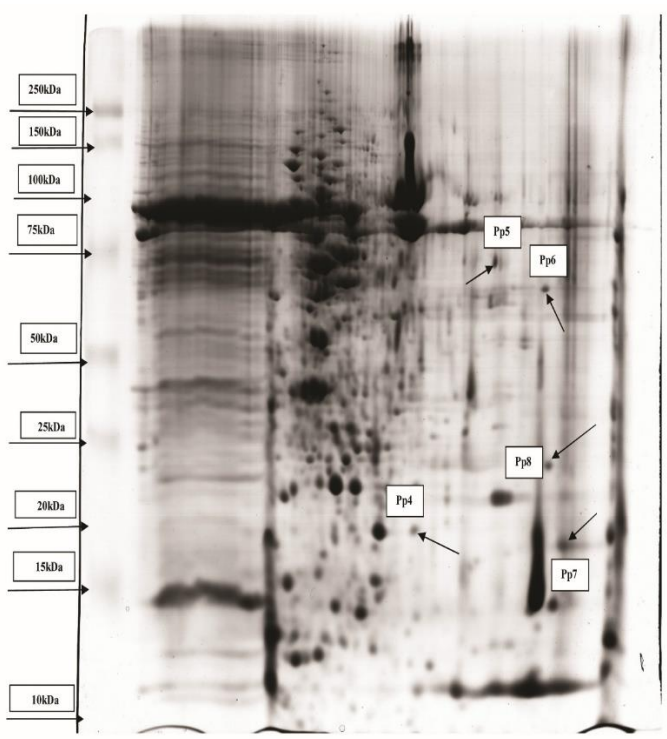

Figure 3: 150mM NaCl -Affected Pigeonpea Leaf ICPL 87119

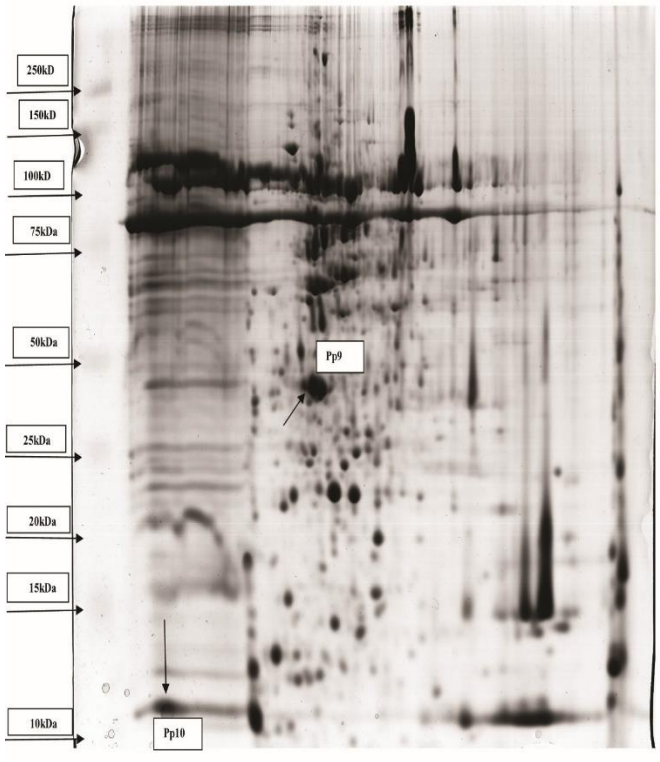

Figure 4: Control Pigeonpea Leaf of ICPL 85063

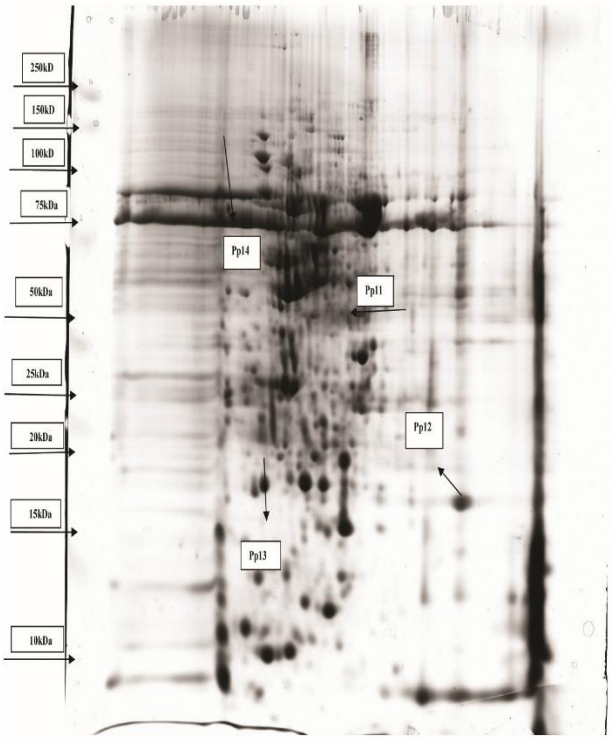

Figure 5: 150mM NaCl -Affected Pigeonpea Leaf ICPL 85063

Figures 2, 3, 4 and 5: Differential Protein Expression in Pigeonpea Leaves Under Salt Stress: A 2DGE Analysis. Figure 2: Control Leaf of Pigeonpea ICPL 87119. Figure 3: Leaf of Pigeonpea ICPL 87119 Affected by 150mM NaCl. Figure 4: Control Leaf of Pigeonpea ICPL 85063. Figure 5: Leaf of Pigeonpea ICPL 85063 Affected by 150mM NaCl

An in-depth analysis of leaf variations among the concentrations indicated that the leaf size ranged from 1-7 cm under control and 50 mM NaCl conditions. However, as salt concentrations increased, leaf sizes decreased to below 5 cm. Additionally, the weight of stress-affected leaves was notably lower than the control (Fig. 1A). The impact of 150 mM NaCl on pigeonpea seedlings was significant but not lethal. Morphological examinations including measurements

of leaf and root length and fresh weight in 21-day-old seedlings, revealed distinct responses in ICPL 87119 and ICPL 85063. Specifically, the fresh weight of leaves and roots was lowest at the 200mM concentration in ICPL 87119, whereas ICPL 85063 exhibited reduced height.

The dat not to present for leaf and root length, fresh weight and other phenotypic measurements was driven by the need to streamline the manuscript and focus on key findings. However, this omission limits the ability to fully interpret and compare the responses of ICPL 87119 and ICPL 85063 under various salt concentrations. After 7 days, control plants exhibited the opening of first and primary leaves, while those treated with 100mM and 200mM NaCl displayed closed leaves. Remarkably, plants treated with 200mM NaCl showed the absence of a first leaf (Figure 1A).

Further investigation into the length of leaves and roots revealed that NaCl treatment in ICPL 85063 resulted in a 60% and 28% reduction respectively, compared to control plants (Figure 1A). The shorter length of leaves and roots was significant at 150mM NaCl in the genotype ICPL 87119. The fresh weight of leaves and roots from plants treated with 200mM NaCl increased by 42% and 56%, respectively, compared to control plants. However, the fresh weight of leaves and roots was significantly lower at 200mM NaCl compared to the control plants in both genotypes (data not shown). While the treatment at 50mM NaCl did not severely impact plant growth even after 7 days, the effects of treatment at 150mM NaCl were significant but not lethal and the impact of treatment at 200mM NaCl was lethal.

Proteomic Analysis of Salt Stress Response: To delve into the molecular responses to the critical salt stress condition of 150 mM NaCl, a proteomics approach was employed, focusing on 21-day-old leaves. A total of 42 proteins exhibited changes in response to the salt stress. Pigeonpea seeds were sown and treated with 200 mM NaCl, followed by protein extraction from the leaves. The proteins were separated using 2-DGE and stained with CBB to assess their expression levels. PDQuest software analysis detected more than 300 reproducible protein spots on the 2-DE gels (Figures 2, 3, 4 and 5). Among these spots, 11, 12, 10 and 7 proteins were visibly up-regulated, while 8, 10, 14 and 7 proteins were down-regulated at 150 mM NaCl treatment in control and stress plants (Figures 2, 3, 4 and 5).

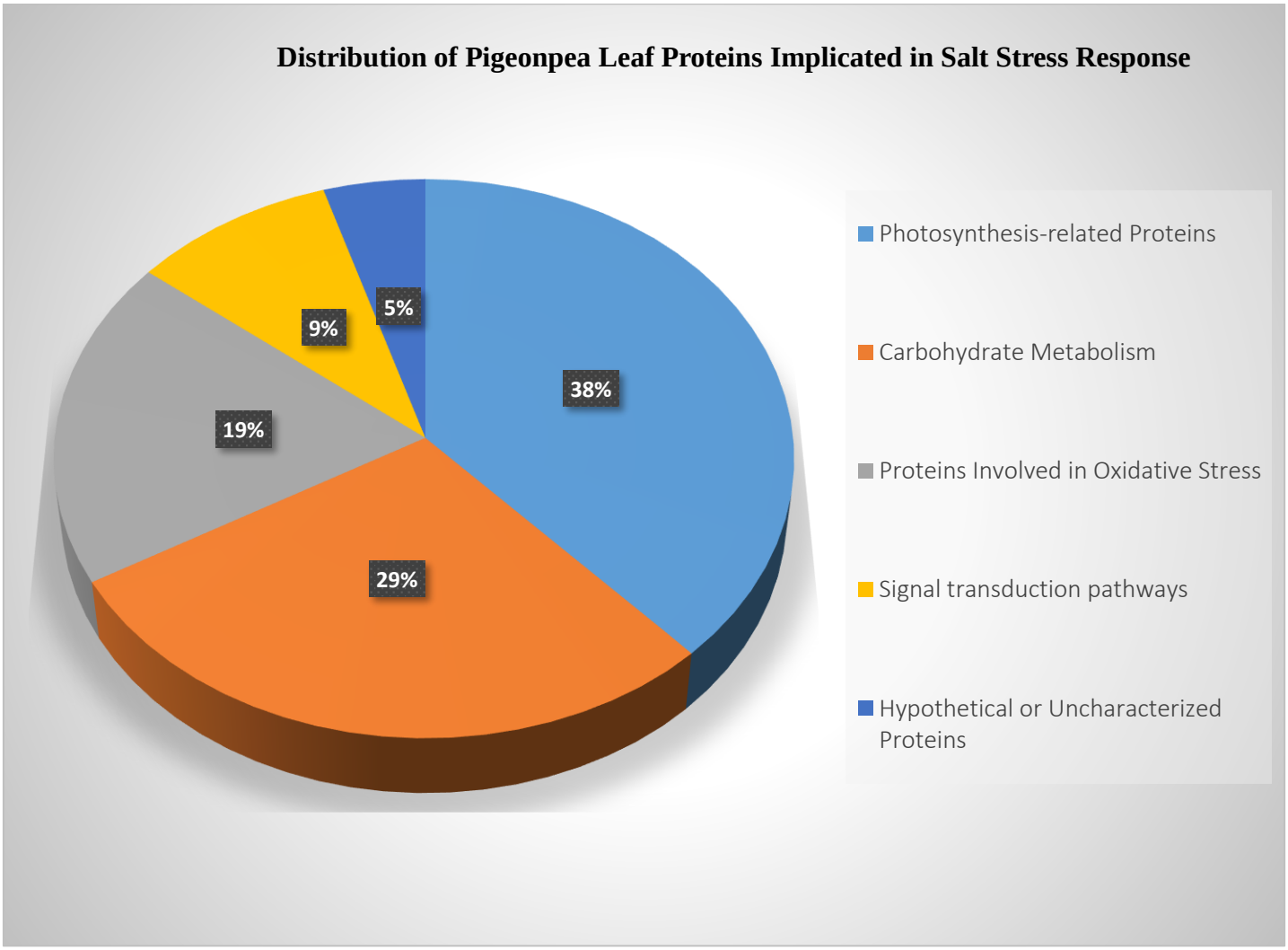

Figure 6: Functional Classification of Proteins in Germinated Pigeonpea Exposed to NaCl Stress

Table 1
Differentially Expressed Proteins Identified Through MALDI-TOF/TOF. The candidate leaf proteins were identified using MALDI-TOF/TOF in the ICPL 85063 (salt-sensitive) and ICPL 87119 (salt-tolerant) pigeon pea genotypes in response to salt stress.

Spot no.	Protein identity	Coverage (%)	MASCOT scored	TMr/EMr	TpI/EpI	Expression Level (Up/Down)	Fold Change (Up/Down-Regulation)	Accession No.
1	RuBisCO large subunit-binding protein subunit beta	44	113	55.9/58.2	4.85/4.53	Up-regulated	2.3x	XP_012567814.1
2	ATP synthase subunit beta, chloroplastic	49	105	52.9/58.7	5.16/4.99	Up-regulated	1.8x	XP_020234965
3	Glyceraldehyde-3-phosphate dehydrogenase GAPCP1, chloroplastic	55	95	24.6/23.9	4.89/5.23	Up-regulated	2.1x	XP_020206370
4	Ribulose-bisphosphate carboxylase large subunit, partial	18	55	20.5/14.5	5.61/4.94	Down-regulated	1.4x	XP_02024582
5	Glutamine synthetase leaf isozyme, chloroplastic	33	65	75.5/70.4	7.52/6.58	Up-regulated	1.6x	XP_020234965
6	Heat shock 70 kDa protein 15	15	45	73.5/71.8	9.14/8.99	Down-regulated	1.5x	XP_029130578
7	Tryptophan synthase beta chain 1, chloroplastic	20	46	18.5/20.9	10.55/9.99	Down-regulated	1.3x	XP_029128567
8	ATP-dependent Clp protease proteolytic subunit-related protein 2, chloroplastic	25	48	23.5/22.4	8.55/9.10	Up-regulated	2.0x	XP_020224873
9	Putative serine/threonine-protein kinase-like protein CCR3	24	38	50.3/53.5	4.55/5.20	Up-regulated	1.5x	XP_020220275
10	Uncharacterized protein LOC109815156, partial	30	40	12.5/14.4	3.54/3.90	Down-regulated	1.2x	XP_020235382
11	transcription initiation factor TFIID subunit 6	33	52	51.5/55.6	10.35/9.52	Down-regulated	1.7x	XP_020231936
12	ABC transporter B family member 4 isoform X2	35	55	21.2/20.5	11.55/12.05	Up-regulated	1.9x	XP_029129524
13	Uncharacterized protein LOC109807712 isoform X1	33	52	15.2/16.5	5.52/5.58	Down-regulated	1.4x	XP_029129535
14	Ferredoxin--NADP reductase, leaf isozyme, chloroplastic	32	38	75.5/74.6	4.92/5.02	Up-regulated	1.8x	XP_020216369

Mass Spectrometry and Protein Identification: The identified proteins were further analyzed through trypsinization of gel and peptide digests followed by MALDI-TOF MS. The resulting peptide mass fingerprints and fragmented ion spectra were employed in sequence database searching against the NCBI database using MASCOT version. Spot identities with the highest MOWSE score equal to or greater than 45 ($p<0.05$) were considered significant protein matches. Mass spectrometry, utilizing a combination of MALDI-TOF and MALDI-TOF-TOF MS, analyzed 42 protein spots. Matching these proteins with their homologs using the BLAST tool led to the identification of 22 protein spots, representing a 70% successful identification rate. The remaining 20 spots did not match any protein identity within acceptable MOWSE score values (Table 1).

Of the 42 spots analyzed, 14 proteins were identified, with 8

from ICPL 87119 and 6 from ICPL 85063. The identified proteins showed significant matches to the SWISSPROT and NCBI databases, with most of the proteins belonging to *Cajanus cajan* L. annotation of the protein-coding genes indicating that the majority of these proteins are involved in the biological processing of carbohydrate metabolism, followed by photosynthesis. The change in spot densities of the identified salt-related proteins was visually represented in magnified gel images (Table 1).

Functional Categorization of Salt-Responsive Proteins: Based on BLAST alignment, the pigeon pea leaf salt-responsive proteins that were identified, underwent categorization into six distinct functional groups. These groupings were established by referencing functional information obtained from the Uniprot database. The categories encompassed proteins associated with photosynthesis, carbohydrate metabolism, oxidative stress response, protein metabolism, the signaling pathway and a set of proteins that were either hypothetical or uncharacterized. This systematic classification facilitated a more structured and comprehensive understanding of the diverse roles these proteins play in the intricate molecular responses of pigeon pea leaves to salt stress.

Proteomic Responses to Salt Stress in Pigeon Peas - Key Protein Groups and Their Regulation: The study identified several key protein groups in response to salt stress in pigeon peas. Photosynthesis-related proteins including RuBisCO large subunit-binding protein subunit beta, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), oxygen-evolving enhancer protein 1 and glucose-1-phosphate adenylyl transferase large subunit 1 (chloroplastic isoform X2), showed notable up-regulation of ATP synthase subunit beta in ICPL 87119 under both control and salt-stressed conditions compared to ICPL 85063 (Figure 6). Proteins involved in carbohydrate metabolism such as triosephosphate isomerase, fructose-bisphosphate aldolase and phosphoribulokinase, were also up-regulated during salt stress [Figure 6].

Additionally, the ABC transporter B family member 4 isoform X2, part of the ATP-binding cassette (ABC) transporter superfamily, demonstrated up-regulation, indicating enhanced transport activity under stress. In the realm of signal transduction pathways, the putative serine/threonine-protein kinase-like protein CCR3, predicted to be involved in signaling, was observed **(**Figure 6). Finally, the study also identified a number of hypothetical or uncharacterized proteins, many of which were located in the chloroplasts (Figure 6).

Protein Expression and Fold Change Analysis: The RuBisCO large subunit-binding protein subunit beta is up-regulated with a 2.3-fold increase, indicating an enhanced assembly of the RuBisCO complex, critical for photosynthesis, likely aiding the plant in increasing carbon fixation under stress. The ATP synthase subunit beta, chloroplastic, also shows a 1.8-fold up-regulation, reflecting an increase in ATP production to meet the energy demands during salt stress. Glyceraldehyde-3-phosphate dehydrogenase (GAPCP1) supports increased energy metabolism with a 2.1-fold up-regulation. In contrast, the ribulose-bisphosphate carboxylase large subunit (partial) is down-regulated by 1.4x, suggesting stress-induced modulation of photosynthesis. Glutamine synthetase leaf isozyme, up-regulated by 1.6x, indicates elevated nitrogen assimilation to maintain amino acid production. The heat shock 70 kDa protein 15 is down-regulated by 1.5x, suggesting reduced protein chaperoning under prolonged stress. Tryptophan synthase beta chain 2 is down-regulated by 1.3x, indicating a shift from tryptophan biosynthesis.

ATP-dependent Clp protease is up-regulated 2.0x, highlighting the degradation of damaged proteins. The putative serine/threonine-protein kinase-like protein CCR3, up-regulated by 1.5x, suggests heightened stress signaling activity. An uncharacterized protein, LOC109815156, shows a slight down-regulation (1.2x), reflecting a diminished role in stress response. Transcription initiation factor TFIID subunit 6, down-regulated by 1.7x, implies reduced transcriptional activity. ABC transporter B family member 4 isoform X2 is up-regulated by 1.9x, enhancing molecule transport under stress. Another uncharacterized protein, LOC109807712, is down-regulated by 1.4x, suggesting reduced relevance in stress conditions. Finally, ferredoxin--NADP reductase, leaf isozyme, chloroplastic, is up-regulated by 1.8x, indicating an increase in electron transfer processes to support photosynthesis during salt stress (Table 1).

Discussion

Impact of Salt Stress on Plant Water Absorption and Growth: Salt stress poses a significant challenge to plant water absorption, primarily due to osmotic stress induced by elevated salt concentrations in the rhizosphere. This osmotic pressure disrupts the plant's ability to absorb water effectively, leading to a temporary, reversible reduction in growth. The initial symptoms of salt stress may be subtle, often going unnoticed, but as salinity levels increase, the detrimental effects become more pronounced. In particular, the developing tissues of the plant suffer damage, which subsequently impairs overall metabolism and growth. Our exploration of the salt stress responses in pigeonpea genotypes, specifically ICPL 87119 (Asha) and ICPL 85063 (Lakshmi), sheds light on the intricate relationship between morphological and proteomic responses under varying salinity conditions[29].

Dose-Dependent Morphological Responses to Salinity and Proteomic Responses in Pigeonpea Genotypes: The resilience exhibited by ICPL 87119 under high salinity conditions is indicative of effective salt tolerance mechanisms. This genotype maintained germination, growth and overall morphology, contrasting sharply with the more vulnerable ICPL 85063, which exhibited wilting, stunted

growth and yellow necrosis. These contrasting responses underscore the importance of targeted breeding programs to enhance salt tolerance in pigeonpea cultivars[26].

Our findings on the dose-dependent morphological responses to varying NaCl concentrations provide valuable insights into the salinity stress impact on plant growth and development. Notably, the significant reduction in leaf size and weight with increasing salt concentration serves as tangible evidence of the adverse effects of elevated salinity on plant morphology[8].

Key Proteins involved in Photosynthesis: Photosynthesis is a complex process reliant on specialized proteins, crucial among them being the RuBisCO Large Subunit-Binding Protein Subunit Beta. This protein is vital for the assembly and stabilization of the RuBisCO enzyme complex which plays a critical role in carbon fixation during the Calvin cycle. The importance of this protein in enhancing photosynthesis and carbon fixation efficiency has been well-documented[7,20]. Additionally, the ATP Synthase subunit beta is essential for the light-dependent reactions, converting proton flow across the thylakoid membrane into ATP, thereby providing the energy necessary for cellular functions[35]. The Glutamine Synthetase leaf isozyme, also located in chloroplasts, facilitates nitrogen assimilation and amino acid production, contributing to the overall metabolic efficiency of the plant under stress[10].

Role of Heat Shock Proteins and Stress Response: In the context of stress response, proteins like heat shock protein 7 (HSP7) play a pivotal role. Although not directly involved in photosynthesis, HSP7 acts as a molecular chaperone that ensures the stability and proper folding of proteins during stressful conditions, thereby preserving cellular integrity. The presence of HSP7 is essential for maintaining cellular homeostasis, particularly in fluctuating environmental conditions, thus enhancing the plant's resilience [25].

Chloroplastic Pathways and Amino Acid Biosynthesis: Focusing on chloroplastic pathways, the Tryptophan Synthase Beta chain 1 is integral to tryptophan biosynthesis. Tryptophan is not only essential for protein synthesis but also serves as a precursor for the plant hormone auxin, playing a vital role in developmental processes and environmental responses Additionally, the ATP-Dependent Clp Protease Proteolytic subunit-related protein 1 is crucial for quality control and protein degradation within chloroplasts. By eliminating damaged or misfolded proteins, this proteolytic subunit ensures chloroplast integrity, thereby indirectly supporting photosynthetic efficiency[22].

Signaling and Regulation Proteins: Among the diverse protein landscape, the Putative Serine/Threonine-Protein Kinase-Like Protein CCR3 suggests involvement in phosphorylation-dependent signaling pathways. While the specific functions of CCR3 require further exploration, kinases are typically key regulators of cellular processes, acting as molecular switches that modulate signaling cascades[5]. Similarly, the Transcription Initiation Factor TFIID subunit 6 plays a vital role in gene expression regulation, influencing transcription initiation processes critical for overall plant metabolism[6].

Membrane Transport and Uncharacterized Proteins: The exploration of membrane transport proteins such as ABC Transporter B Family Member 4 Isoform X2, underscores their importance in the translocation of molecules across membranes, essential for nutrient uptake and stress responses[16]. Furthermore, the existence of uncharacterized proteins presents an exciting frontier for future research, as it may hold the keys to novel biological processes and previously unknown connections to photosynthetic mechanisms[33].

Proteins involved in Starch Biosynthesis: Finally, the Glucose-1-Phosphate Adenylyl Transferase large subunit 1 is a pivotal player in chloroplasts, involved in ADP-glucose synthesis, a precursor for starch biosynthesis[14]. The proper functioning of starch biosynthesis pathways is vital for energy storage and overall plant health, particularly under salt stress conditions.

Limitations of the Study: While this study provides valuable insights into the proteomic responses of pigeonpea leaves to salt-induced stress, several limitations must be acknowledged. First, our analysis was limited to leaf tissues which may not fully capture the systemic responses of the plant to salt stress. Future studies should include proteomic analyses of other tissues such as roots which play a crucial role in water and ion uptake under salinity. Additionally, integrating metabolomic approaches alongside proteomics would offer a more comprehensive understanding of the biochemical changes involved in salt tolerance. Such multi-omics approaches could provide deeper insights into the molecular mechanisms underlying stress adaptation in pigeonpea, aiding efforts to develop more resilient crop varieties.

Conclusion

In conclusion, this comprehensive exploration of salt stress responses in pigeonpea has provided a holistic view of both morphological and proteomic aspects of the plant's adaptive mechanisms. The identified proteins, their categorization and their putative functions offer a foundation for further research aimed at developing salt-tolerant pigeonpea cultivars. The integration of morphological observations with proteomic insights enhances our understanding of the intricate interplay of molecular processes in plants subjected to salt stress. This study will serve as a roadmap for future crop improvement strategies.

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