Proteomic Investigations to Assess the Impact of Salinity on *Vigna radiata* L. Genotypes

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**Abstract:** In our previous study, six cultivars of Mungo (*Vigna radiata*) were exposed to 100-250 mM NaCl and studied for changes in growth and biomass. Among them, AEM-96 cultivar of the Mung bean (*Vigna radiata* (L.) Wilczek cv.) was found to tolerate NaCl stress at 250 mM.

**Objective:** The soluble proteome of salt-tolerant mungo cultivar (AEM-96) was compared to the proteome of control mungo to investigate the possible mechanism of salinity tolerance.

**Methods:** Gel-based two-dimensional gel electrophoresis was employed for comparative proteomics. PDQuest-based image analysis of 2D SDS-PAGE was performed. Scatter plots were prepared and total spots were analyzed for 2-fold changes in abundance of protein spot intensities in control and treated gels.

**Results:** In total 517 protein spots were detected; 36 with high significance. Among these 36 spots, 2-fold expression change was analyzed in 27 protein spots. Seven protein spots were upregulated, eight spots were down-regulated, 3 spots were newly induced and 9 spots were silenced, while 9 protein spots did not change their 2-fold abundance under salinity. Protein spots (9 in total) which were 2-fold upregulated and newly induced were excised from the respective gels. The spots were tryptically digested and run on LC-MS/MS for generating peptides and performing a comparative fingerprinting of the proteins. The peptide signal data was loaded on the Mascot (Swissprot) database to retrieve protein IDs. Proteins with the best score were selected, namely isomers of oxygen-evolving enhancer protein 1 (S1-S3), RuBisCO (S4), oxygen-evolving enhancer protein 2 (S5), Heat shock protein 70 isomers (S6-S7), RuBisCO activase (S8), rubber elongation factor (S9) and pathogen-related protein 10 (S10).

**Conclusion:** The identified proteins were found to play important roles in photosynthesis, stress response and plant growth.

**Keywords:** *Vigna mungo*, salinity, proteomics, tolerance, LC-MS/MS, plant growth.

1. **INTRODUCTION**

The mung bean (*Vigna radiata* (L.) Wilzeeck) is cultivated in several regions of the world, because of its substantial nutritional value, particularly for people facing malnutrition [1]. Being a favorite crop of arid and semi-arid regions, it faces different abiotic stress conditions, including salinity stress of varying regimes and for different times [2]. It is noted that over 800 million ha of land throughout the world is salt-affected, either by salinity (397 million ha) or the associated condition of sodicity (434 million ha) [3, 4]. Notably, the mung bean production is adversely affected by the increase in salinity prone areas worldwide [5]. However, the use of salinity resistant genotypes is one of the viable means of attaining better yield under water deficit and high salinity conditions [6, 7].

In order to develop salt-resistant varieties/lines, it is necessary to have complete knowledge of plant behaviour to salt stress [8]. Such behaviour could be in terms of modulated metabolism, physiology and molecular biology. Proteomics has developed as one of the advanced tools to analyse proteomes of desired plants and their parts. The data generated through proteomics provide information about the stress tolerance mechanism in crop plants [7]. Most of the time, it is the response of genes that proteins, which play roles in combating salinity, are over-expressed.

In our previous studies, we have already studied the impact of salinity on soybean genotypes [7]. In addition, a comprehensive study was conducted to investigate responses of the same mungo cultivars at the level of physicochemical parameters. Therefore, the prime objective of the present study will be to examine how far this stress regulates some key physio-biochemical attributes involved in the growth and development of the mung bean plant. Thus, there is a significant scope of proteomics in exploring the molecular mech-
anism of tolerance among the salinity resistant genotypes/lines for better productivity under stress environment.

2. MATERIALS AND METHODS

2.1. Procurement of Vigna Genotypes

The seeds of Mung bean [Vigna radiata (L.) Wilczek cv.] genotypes from Arid and Semi-arid regions were procured from the Pakistan Agriculture Research. In our previous study, six genotypes comprising [AEM-96 (Azri Bhakkar), NM-92 (NIAB-Faisalabad), NFM-6 (NIFA-Peshawar), NCM-1 (NARC-Islamabad), CM-6 (BARI-Chakwal)] and [NFM-12 (NIFA-Peshawar)] were analyzed to check their NaCl tolerance [8, 9]. Among the studied genotypes, AEM-96 was proven as salinity-tolerant. Therefore, in the current study, AEM-96 cultivar of the Mung bean [Vigna radiata (L.) Wilczek cv.] was selected for studying the mechanism of salinity-tolerance at the proteomic level.

2.2. Plant Growth and Treatment

As mentioned above, we have already screened six genotypes of V. radiata for comparative salinity tolerance [9]. Fifty seeds of the tolerant mung genotype (AEM-96) were washed with plain water followed by a wash with 2% detergent. 4% Sodium hypochlorite was used for 5 min and then a final rinse with distilled water was given 10 times. The surface-sterilized seeds were sown in Petri-dishes and 7 days old seedlings were transferred to the hydroponic pots containing full strength Hoagland nutrient media [10]. The hydroponic culture was maintained in a growth chamber at 28 ± 1.5 °C (daytime) and 22 ± 1.5 °C (nighttime). The plants grew at uniform irradiance of photosynthetic photon flux density of 300 μmol m⁻² S⁻¹ and relative humidity of 60-70%. The treatments were arranged in a randomized block design with three replicates and sampling was done after 20 days of NaCl treatments with sodium chloride (250 mM) for 3 days. The sodium chloride treatments prepared in the current study, AEM-96 cultivar of the Mung bean [Vigna radiata (L.) Wilczek cv.] was selected for studying the mechanism of salinity-tolerance at the proteomic level.

2.3. Statistical Analysis

Results reported as mean ± SE were subjected to one-way ANOVA using GraphPad Prism 6.0 software. The statistical significance according to Tukey’s post hoc test was P<0.05. All experiments were carried out in triplicates (n = 3).

2.4. Proteomic Analysis

2.4.1. Protein Extraction

Protein extracts were prepared by acetone-TCA method as described earlier [11]. At 3 days of treatment, V. mungo leaves (1 g) were removed and cleaned with distilled water several times followed by blot drying. The leaf samples were powdered in liquid nitrogen in chilled mortar-pestle. Samples were extracted for protein with 5 ml of extraction buffer (40 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2% PVP, 0.07% β-mercaptoethanol and 1% TritonX100). Homogenate was centrifuged at 36,000 g for 30 min at 4 °C. The supernatant was separated and incubated overnight at -20 °C with 1.5 volumes of chilled 12.5% TCA prepared in acetone containing 0.07% β-mercaptoethanol. The resultant was centrifuged at 20,000 g for 30 min. Pellet was separated and washed with chilled acetone containing 0.07% β-mercaptoethanol and 2 mM EDTA, and spun at 25000 g for 15 min. Pellet was further extracted twice with 100% chilled acetone with an incubation period of 60 min followed by centrifugation at 25,000 g for 15 min each. The pure white pellet was recovered, vacuum dried and solubilized in solubilization cocktail (9M urea, 2M thiourea, 2% Triton X 100, 4% CHAPS, 0.2% ampholine and 50 mM DTT) and vortex-mixed at room temperature for 60 min. Protein concentration was determined by standard Bradford assay using bovine serum albumin as a standard (Bio-Rad, Hercules, CA, USA). The experiments were repeated thrice (with three biological samplings) to authenticate the obtained data. All chemicals used, if not further specified in the text, were of electrophoresis grade. All electrophoresis units employed were from Bio-Rad USA.

2.5. Two-Dimensional Separation and Image Analysis

Immobile pH Gradient (IPG) strips (ReadyStrip™, Bio-Rad, USA) of 11 cm, non-linear with pH 3-10 for leaf protein, were passively rehydrated overnight with protein sample (150 μg of protein in 130 μl of solubilization cocktail). Isoelectric Focusing (IEF) of proteins was performed using the following program: 50 volts for 60 min, 150 volts for 30 min, 300 volts for 30 min, 300 volts for 120 min, 3000 volts until a total of 72000 volt-hours was achieved. After IEF, strips were equilibrated in buffer containing 7 M urea, 2% SDS, 375 mM Tris (pH 8.8), and 20% glycerol plus either 130 mM DTT for reduction or 135 mM IAA for alkylation. Equilibrated IPG strips were loaded onto a 12% T acrylamide gel, sealed with 1% agarose, and electrophoresed by applying a voltage of 100 volts at 10°C. Gels were stained with Coomassie Brilliant Blue G-250 (Blue Silver stain) and images were acquired on a gel documentation system (Bio-Rad, USA) at 300 dpi and saved as a grayscale TIFF file. Experimental molecular weight and pI values were calculated from digitized images using molecular weight marker proteins and the predicted non-linear pH gradient provided by Bio-Rad USA. Protein spots were detected and numbered with PDQuest image analysis software (Bio-Rad, USA).

Relative volume was used to quantify and compare the protein spots. The protein expression patterns were determined as up-regulated, down-regulated and unchanged compared to control. Three independent experimental replicates were used for proteomic analysis.

2.6. Peptide Mass Fingerprinting for Retrieval of Protein Ids

Selective protein spots were selected and digested with trypsin [12]. The digested peptide mixtures were separated
using a MALDI-TOF-MS/MS system. The peptide signal data was loaded on the Mascot (Swissprot) database to retrieve protein IDs. Proteins with the best score were selected and discussed.

3. RESULTS

Comparative proteomics revealed that there was a huge modulation at the level of the proteome as indicated by PDQuest image analysis of 2D gels (Fig. 1). Scatter plots were prepared and total spots were analyzed for changes in 2-fold abundance of protein spot intensities in control and treated-plant gels (Fig. 2). In total 517 protein spots were detected on the 2D gels. 36 spots changed their abundance with high significance. Among these, the 2-fold expression change was analysed. Seven spots were upregulated, 8 spots were down-regulated, 3 spots were newly induced, 9 spots were silenced and 27 spots did not change their 2-fold abundance under salinity.

Fig. (1). 2D Gels of Vigna radiata leaf proteins (A) control (B) salt-treated.
Proteomic data (Table 1) showed that some of the important primary metabolism pathways were strongly affected by salinity stress. Proteins namely Oxygen-evolving enhancer protein 1 (S1), isoform Oxygen-evolving enhancer protein 1 (S2), isoform 3 Oxygen-evolving enhancer protein 1 (S3), RuBisCO (S4), Oxygen-evolving enhancer protein 2 (S5), Heat shock protein 70 (S6), isomer heat Shock Protein 70 (S7), RuBisCO activase (S8), rubber elongation factor (S9), pathogen-related protein 10 (S10) were identified.

4. DISCUSSION

Our proteomic data suggest some important mechanisms of Mungo adaptations to salinity stress. In order to minimize the impact of salinity, regulation of the gene expression and protein composition is necessary for plants. A comparative proteomic study showed that salinity altered the expression of a large number of proteins. It is obvious that plants regulate their metabolism for curtailing oxidative stress, osmotic stress and upkeep their photosynthetic efficiency [13, 14]. This is also evident in our study while observing the expression of proteins in the salt-tolerant genotype of mung bean under salinity stress.

Up-regulation of oxygen-evolving enhancer proteins (Spots 1, 2, 3, and 5) indicates that the plant tries to improve its photosynthesis-related proteins. This is also evident from the increased abundance of RuBisCO (S5) and RubisCO activase (Spot 8) which have also been identified in Spinacia oleracea, Solanum lycopersicum, Solanum tuberosum, and

Fig. (2). Scatter plot of 2D Gels of Vigna radiata leaf proteins control and salt-treated. (A higher resolution/colour version of this figure is available in the electronic copy of the article).
Table 1. List of identified proteins in leaves of *Vigna radiata*.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spot Identity</th>
<th>Protein ID/Homologous Organism</th>
<th>Mr/pI</th>
<th>Peptides</th>
<th>Score</th>
<th>Change Over Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1</td>
<td>Oxygen-evolving enhancer protein 1, (<em>Pisum sativum</em>)</td>
<td>25100 / 6.25</td>
<td>AEGAPKRLTF DEIQSKTYLE VKGTTGANQC SYRGSSFLDP KGRGASTGYD NAVALPAGGR AKAPKDVKIQ GVWYAQLDES</td>
<td>183</td>
<td>41758 Norm INT’Area</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>Oxygen-evolving enhancer protein 1, (<em>Vitis vinifera</em>)</td>
<td>33441 / 5.87</td>
<td>YMEVKGTGTA NQCPTIDGVV DSFAFKSGKY LVPSYRGSSF LPDKGRGGST SKEPETGVEVG FESIQPSD DLGAKTPKEV</td>
<td>105</td>
<td>242790 Norm INT’Area</td>
</tr>
<tr>
<td>3</td>
<td>S3</td>
<td>Oxygen-evolving enhancer protein 1, (<em>Bruguiera gymnorhiza</em>)</td>
<td>35344 / 6.48</td>
<td>LTYDEIQSKT YLEVKGTGTA LVPSYRGSSF LPDKGRGGST SKEPETGVEVG FESIQPSD DLGAKVP-KDV KIQGIWYAGLDS</td>
<td>74</td>
<td>27360 Norm INT’Area</td>
</tr>
<tr>
<td>4</td>
<td>S4</td>
<td>RUBISCO (<em>Harpagophytum grandiflor</em>)</td>
<td>42139 / 6.81</td>
<td>TKDIDILAAF RVTPQPGVPP RVALEACVKA</td>
<td>107</td>
<td>60083 Norm INT’Area</td>
</tr>
<tr>
<td>5</td>
<td>S5</td>
<td>Oxygen evolving enhancer protein 2, (<em>Solanum lycopersicum</em>)</td>
<td>34946 / 8.28</td>
<td>FGKPKENTDF LPYNDDGFKL EVEYPGQVLRA KSITDYGSPE EFLSKVDYLL</td>
<td>115</td>
<td>16751 Norm INT’Area</td>
</tr>
<tr>
<td>6</td>
<td>S6</td>
<td>Heat shock protein 70 (<em>Cucumis sativus</em>)</td>
<td>55480 / 6.35</td>
<td>ECPAIGKQFA AEEISAQVLRA NKDVTCAVVT VPAYFNDSQR TATKDAlia GLEVLRINE IPAQELVKKMM</td>
<td>180</td>
<td>37010 Norm INT’Area</td>
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<tr>
<td>7</td>
<td>S7</td>
<td>Heat shock protein 70 (<em>Ostreococcus tauri</em>)</td>
<td>50098 / 7.21</td>
<td>IDCALGKQ FAEEISAQQLR AVVT-PAYFS QR</td>
<td>145</td>
<td>13805 Norm INT’Area</td>
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(Table 1) contd....
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<th>S. No.</th>
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<th>Mr/pI</th>
<th>Peptides</th>
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<th>Change Over Control</th>
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<tbody>
<tr>
<td>8</td>
<td>S8</td>
<td>RUBP/oxygenase activase (Larrea tridentata)</td>
<td>64450 / 7.38</td>
<td>GLAY DISDDQODITR SFQCELV FAKMGINPIM MSAGELESGN AGEPAK MCALFIN DLDAGAGR EGPPTFDQPK</td>
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</tr>
<tr>
<td>9</td>
<td>S9</td>
<td>Rubber elongation factor protein (REF) (Hevea brasiliensis)</td>
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<td>SLAS SLPGQTK</td>
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<td></td>
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<tr>
<td>10</td>
<td>S10</td>
<td>Pathogen related protein 10 (Vigna radiata)</td>
<td>38582 / 5.04</td>
<td>DADNI IPKAVGSFQS VEIVEGNGGP GTIKKISFVE DGTEK GSLIK AVEAY LLANP</td>
<td>208</td>
<td></td>
</tr>
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Oryza sativa under salinity and other abiotic stresses. RuBP/oxygenase activase catalyzes the carboxylation of RuBisCO, which is the initial step in photosynthetic carbon reduction [15].

In response to salinity and abiotic stress, plants induce a set of genes including Heat Shock Proteins (HSPs) [16]. A number of these proteins are known as molecular chaperons which provide a unique folding-environment to post-translation modification of proteins [17]. Induction of HSPs in our studies indicates that HSP70 plays a great role in plants during salinity stress exposure. Several other studies suggest a similar role of HSP70 under salinity and other stresses [18]. It has been observed that HSP70 maintains protein metabolism [19], proper protein folding [20], mitigates oxidative stress [21] and helps in the proper development of legumes [22].

Spot S9, overexpressed under salinity, is the rubber elongation factor. It was first identified from rubber tree [23] as a long-chain amino acid having a molecular mass of 14.6 kDa and lacks four amino acids cysteine, methionine, histidine and tryptophan.

A pathogen-related protein (Spot S10) also increased its abundance due to salinity. The pathogenesis-related proteins are defined as the proteins induced in pathological or related situations [24]. In our study, PR proteins were induced hence indicating a very important role of PRs in the mung bean plant against salinity. Earlier, PR proteins have shown to be involved in the organization of the Hsp70/Hsp90 multi-protein complex [25]. Possible roles of the identified PRs in the mung bean plant are protein stabilization and turnover as well as HSP-involved signal transduction processes [26].

**CONCLUSION**

Vigna radiata alters proteome against the salinity stress. A set of numerous protein groups was either upregulated or newly induced. The identified proteins play important roles in photosynthesis, stress response and plant growth. A salinity tolerant cultivar was thus equipped with salinity-tolerant mechanisms operating at various levels.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Not applicable.

**HUMAN AND ANIMAL RIGHTS**

No animals/humans were used for studies that are the basis of this research.

**CONSENT FOR PUBLICATION**

Not applicable.

**AVAILABILITY OF DATA AND MATERIALS**

The data that support the findings of this study are available from the corresponding author [Khalid Rehman Ha-keem], upon reasonable request.

**FUNDING**

None.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest, financial or otherwise.
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