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Identification of Salinity Stress Responsive Proteins in *Vigna radiata* Var. radiata (L.) Wilczek Leaves

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ABSTRACT:

Identification of stress responsive proteins assists in understanding of physiological mechanisms behind stress tolerance and prominently in identification of candidate genes for transgenesis that may result increased crop productivity. In the present exploration, the effect of increased salinity on *Vigna radiata* var. radiata (L.) Wilczek in its vegetative stage was studied. The seeds were sown in red, clayey soil in pots and watered with different concentrations of NaCl viz., 0(Control), 50, 100,150, 200mM. Proteomic analysis was made with the leaf tissues of 45 DAS plants that included extraction of proteins, protein assays, 2 Dimensional gel electrophoresis and further study on differentially expressed proteins by Peptide Mass Finger printing and MASCOT search. The analysis revealed the presence of many differentially expressed proteins including glutathione-s-transferase, kunitz type trypsin inhibitors.

Keywords: stress responsive proteins, transgenesis, kunitz type trypsin inhibitors

[I] INTRODUCTION

Crop plants are habitually subjects of abiotic stress such as heat,drought, salinity, cold and freeze, water logging due to current climatic, environmental changes and agricultural practices. Dramatic physiological, biochemical, metabolic changes occur that induce defense mechanisms to respond and tolerate the stress. Stress responsive proteins are synthesized in response to broad range of stress conditions, either as responsive proteins or stress tolerating proteins, that control, coordinate various cellular and metabolic activities during stress and stress response. [11]

Salinity is one of the major abiotic stress that is generally seen and the tribulations are low osmotic

potential of the soil solution, nutritional imbalance in the plants, generation of reactive oxygen species, that ultimately affect crop yield and productivity. Plants endure by adaptive mechanisms operating at all levels of organization taking on subcellular, cellular, tissue and systemic level. The mechanisms, for example, include (i) accretion of intracellular osmolytes and reducing water loss (ii) escalating antiporter and co-porter ion channel activity across the plasma and vacuolar membranes (iii) progress of enzymic and non enzymic antioxidant systems that scavenge free radicals (iv) complex signaling networks and metabolic networks such as protein kinase pathways.[1]

Improvement in levels of tolerance to these stresses by crop plants is an urgent priority for breeding programmes, to enhance the crop output. Generation of stress tolerant crops through transgenesis requires identification of proteins and genes involved in regulatory pathways such that they can be used as the candidates for the process.

The present analysis focuses on identification of proteins that may be involved in stress response or tolerance through predictable proteomic technologies.

[II] MATERIALS AND METHODS

2.1 Seeds and Salinity treatment

Seeds of *Vigna radiata* var. radiata (L.) Wilczek were obtained from Tamil Nadu Agricultural University. Seeds sterilized by 0.01 M HgCl2 solution for 3minutes, washed thoroughly with distilled water were sown in pots. The pots filled with red soil were divided into five groups of three pots each. The pots of first group were used as control, waterd with tap water. The other groups were watered with aqueous solutions of 50mM, 100mM, 150mM and 200mM NaCl respectively. All the pots were watered once in three days to maintain soil moisture. After 7 days of sowing (DAS) the seeds, lessening was done to leave five uniform seedlings in each pot for carrying out tests. Leaves of 45 DAS plants were used for biochemical analyses.

2.2 Protein Extraction

The proteins in the leaves of control and treated leaves were isolated following the technique using phenol ammonium acetate with minor modifications. 1 g of leaves was crushed in liquid N2 with mortar and pestle and suspended in 0.5 mL of extraction buffer (700 mM sucrose, 500 mM Tris–HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% w/v β-mercaptoethanol and 1mM PMSF) by vortexing on ice. An equal volume of phenol saturated-500 mM Tris–HCl, pH 7.5 was added and the mixture was stirred for 30 min on ice and then centrifuged at 5000 g for 30 min at 4 °C. The upper phenol phase was removed and extracted twice with the extraction buffer. Proteins were precipitated from the phenol phase by addition of five volumes of 0.1 M ammonium acetate in methanol, overnight at −20 °C. Precipitated proteins were pelleted by centrifugation (20,000 g, 20min, 4 °C) and washed thrice with ice-cold 0.1 M ammonium acetate in methanol and twice with ice-cold acetone. The resultant pellet was air dried under laminar air flow and solubilized in rehydration or sample loading buffer.

2.3 Sample Preparation for 1D

Sample were made soluble in sample loading buffer (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.1% bromphenol blue, pH 6.8 and 2%V/V Beta mercaptoethanol)) for 30mins by mixing and centrifuged at 10000xg 4^0C temperature to remove insoluble remains and the supernatant was collected for SDS PAGE and preserved at 4^0C.

2.4 SDS PAGE

The separation of proteins by SDS Poly Acrylamide Gel Electrophoresis was performed using a model vertical slab gel (7x 8cm) electrophoresis apparatus (Protean Mini-PROTEAN 3 cell Biorad USA). SDS-polyacrylamide denaturing gels (separating gels (12%) and stacking gels (6%) were prepared and extracted protein with 2X sample buffer (125 mM Tris HCl, 20% Glycerol, 4% SDS, 0.1%

bromphenol blue, pH 6.8 and 2%V/V Beta mercaptoethanol) loaded to the gel after boiling at 95^0C for 3 Mins. Blank wells were loaded with 1X sample buffer. Molecular Weight Marker (BioRad containing MW- Myocin:200kd, Beta Galactosidase:116.3kd,:Phosporylase b:97kd, bovine albumin: 66kd,Ovalbumin:45kd Carbonic anhydrase:31kd Trypsin Inhibitor:21.5kd Beta Lactalbumin 14.4 ,Aprotinin 6kd) was used in the experiment. Constant voltage (8 V/cm) was applied to stacking gel. After the tracking dye attained the separating gel, the voltage was adjusted to 15 V/cm. The gels were then stained by silver staining protocols. Stained gels were captured and noted on Versa Doc 4000 (BioRad).

2.5 Protein quantification

The concentration of proteins in the supernatant was quantified using the Bio-Rad Quick Start kit (Bio-Rad, Hercules, CA), based on the Bradford method. A standard curve was generated using the Serum Albumin Standard Set (Bio-Rad). Equal quantities of protein (200 µg) from each lysate were taken for extraction and for clean up.

2.6 2Dimensional electrophoresis

The proteins were extracted for two dimensional electrophoresis as per the procedure mentioned below. 200 µg of in rehydration buffer sample was diluted with µl of 100 %TCA in water to yield final 25 % concentration. The mixture was incubated overnight at -20^0C and centrifuged at 15 000g, 4^0C for 15 min. The supernatant was removed and 1000 µl of 90 % ice-cold acetone were added to wash the pellet. The sample was incubated at -20^0C for 30 min and centrifuged as above. The acetone containing supernatant was removed and the pellet was air dried. For 2D gel electrophoresis, the protein pellet was suspended in 100 µl of rehydration buffer containing 7 M urea , 2M thiourea,60 mM DTT, 4% w/v CHAPS ampholyte (3-10)and traces of bromophenol blue .The cleaned protein sample was stored frozen at -20^0C until analysis.

Cleaned Protein sample containing 200 µg of protein was diluted to 300 µl rehydration buffer (7M urea,2M thiourea,2% CHAPS,65 mM DTT,0.5% IPG buffer(3-10 pH) and bromophenol blue) and passively rehydrated on 17cm (3-10pH) strip in rehydration tray and overlaid with 3ml of mineral oil to avoid evaporation and crystallization of urea, rehydration lasted for 16 hrs. After rehydration strips were transferred to focusing tray with electrode wicks and isoelectric focusing was performed in a PROTEAN® IEF Cell (Bio-Rad) at optimum strength of 600 V/cm and a 50mA limit/IPG strip.

For second dimension SDS-PAGE, the focused IPG strips were equilibrated in two steps, first in equilibration buffer I containing 20% v/v glycerol, 0.375 MTris–HCl, pH 8.8, 6 MUrea,2% (w/v) SDS, 130 mM DTT followed by a second equilibration step in buffer containing 20% (v/v) glycerol, 0.375 M Tris–HCl, pH 8.8, 6 M Urea, 2% (w/v) SDS, 135 mM iodoacetamide. IPG strips were placed at the top of a 12% SDS-PAGE gel and overlaid with 0.5% agarose in running buffer (50 mM Tris-HCl, pH 6.8, 1.44% lycine and 0.1% SDS). The second dimension was performed using 12% polyacrylamide gels (7 cm×8 cm, 1.5mm thickness) in a Mini Protean 3 (Bio-Rad) at constant 200 V for 4 h 30 min. Thereafter, the gels were visualized by silver staining.

2.7 Protein Visualization and Imaging

Silver staining: MALDI compatible silver staining was carried out by method described by Yan *et al.*, 2000. The gels were immersed in fixative solution (methanol/distilled water/acetic acid, 40/50/10) for one hour. The gels were sensitized by contact to thiosulfate reagent (0.02% Sodium thiosulfate), followed by impregnation with silver nitrate reagent (0.2% silver nitrate and 0.02% of 37 % formaldehyde) for 30 minutes and developed in developing solution (3% sodium carbonate, 0.05% formaldehyde (37%), 0.0005% sodium thiosulfate). The staining reaction was bunged by using 12% acetic acid solution (for 5 minutes) and gels were preserved in same solution. Silver Stained gels were digitalized using VersaDoc™ (Model 4000) ImagingSystem (Bio-

Rad) and analyzed with PDQuest Advanced™ 2-DAnalysis software (version 8.0.1, Bio-Rad).

2.9 Peptide Mass Fingerprinting

Peptide Mass Fingerprinting (PMF) of selected protein spots was done using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and MALDI-TOF/TOF.

In-gel digestion and protein digest preparation: Selected protein spots in the gel were excised manually with precaution for avoidance for contamination. and were chopped into small pieces. They were destained, dehydrated with acetonitrile and then rehydrated with 50 mM ammonium bicarbonate. Proteins in the excised gel pieces were reduced and alkylated with 25 mM dithiothreitol (30 min at 56 °C) and 55 mM iodoacetamide (45 min in the dark at room temperature), respectively. In-gel Digestion was done with 500 ng of trypsin in 50 mM ammonium bicarbonate buffer overnight. Extraction of peptides was carried out using 0.1 % trifloro acetic acid in water (rehydration), followed by dehydration with 100% acetonotrile. For each extraction step, the solution was aspirated, collected, and collated. Three extraction cycles (dehydration and rehydration) were performed per sample. The recovered peptides were stored, in -20^0C.

MALDI target spotting: The sample was spotted directly onto a MALDI target that was pre-spotted with 0.5 µL MALDI matrix (CHCA) using 0.5 µL of an protein digest solution (0.1% trifluoroacetic acid/50% acetonitrile).

MALDI mass spectrometer parameters: The specifications of the Instrument is as follows: ABI 4800 MALDI TOF/TOF analyzer; data acquisition and processing program: 4000 Series Explorer software; MS acquisition in reflector mode positive ion mode; mass range: m/z = 600 – 4000; 400 laser shots per spectrum; minimum S/N = 10 for MS acquisition; 10 strongest precursors chosen for MS/MS; minimum S/N = 30 for MS/MS precursors.

Database correlation analysis search parameters: Protein identification from MS data: Program for data processing: Applied Biosystems GPS Explorer v3.6; search engine: Mascot (Matrix Science, Boston, MA); sample type: gel samples; digestion enzyme: trypsin; species: mouse; I.D focus: biological modifications; database: NCBInr or Swiss-Prot; search engine: type of search: MS and; Mascot (Matrix Science); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±0.3 - 1 Da; maximum missed cleavages: 1-2; variable modifications: oxidation (M), carbamidomethyl (C).

[III] RESULTS

The results of 2 Dimensional Electrophoresis expose many proteins that are expressed differentially in the control and treated. The raw images of 2D gel electrophoresis of the control and treated samples, the master image is shown in figure 1.

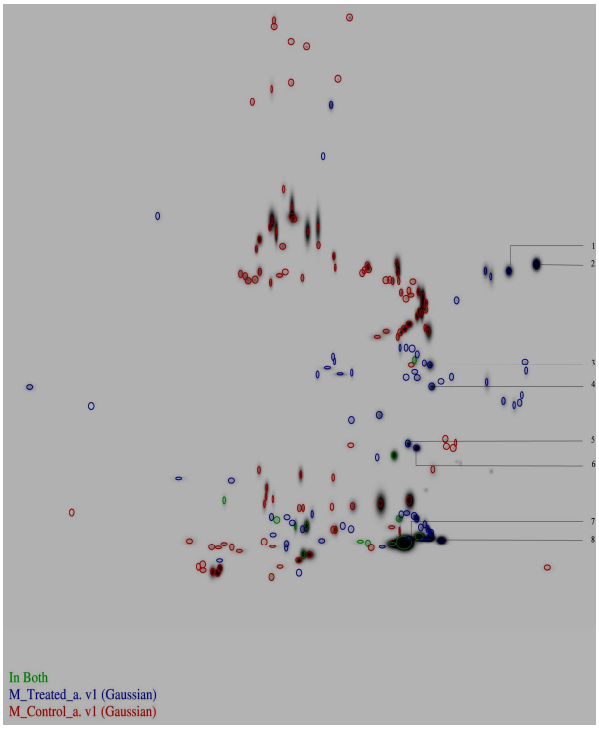

Fig. 1: Master Image of 2DE of control and treated samples overlaid. Spots marked blue indicate proteins found in treated, red indicate proteins found in control and green indicate the proteins seen in both.

The mass spectrometry results of spot marked 4 is shown in figure 2.The Peptide Mass Fingerprinting of spot 4 was identified to be protein subunits similar to Kunitz type trypsin inhibitor β chain. The Mascot search result for Peptide Mass Fingerprint of spot 4 was with score 42. Protein score is -10*Log(P), where P is the probability that the observed match is a random event.

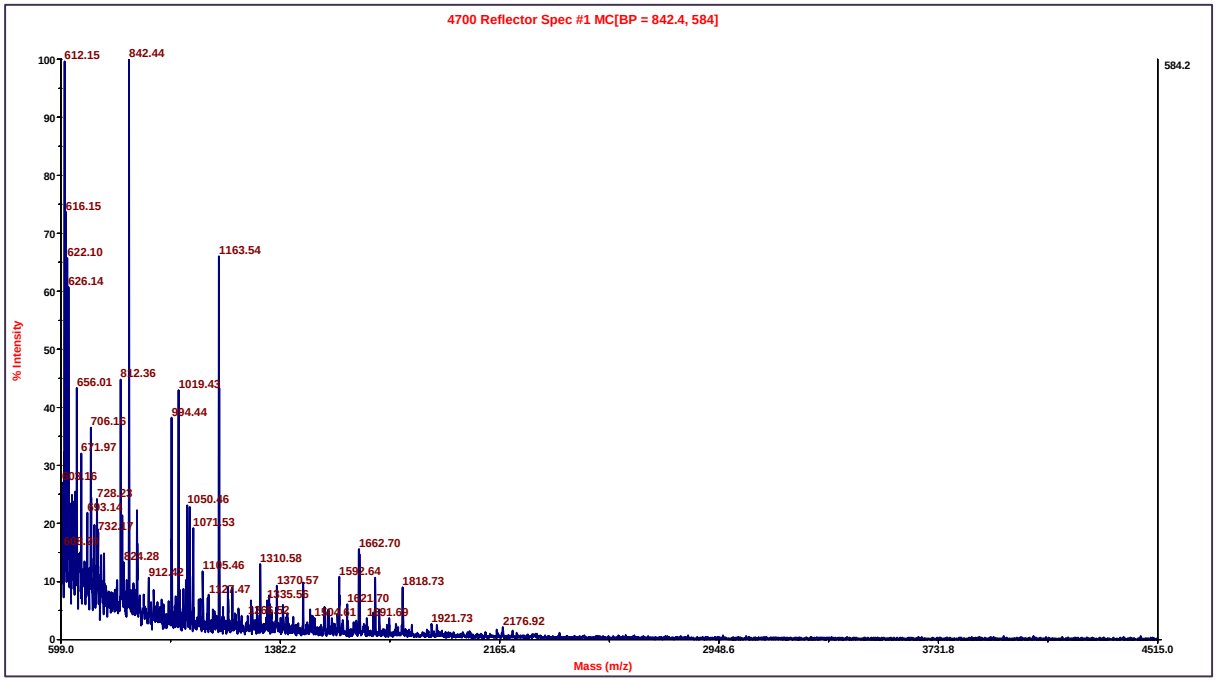

Fig. 2: PMF data of spot 4.

The protein spot 4 has shown maximum similarity to Kunitz type trypsin inhibitor beta chain .

[IV] DISCUSSION

Kunitz-type inhibitors The inhibitors in this family are widespread in plants and have been described in legumes, cereals and in solanaceous species. Kunitz-type PIs are produced under stress The members of this family are mostly active against serine proteases and have been shown to inhibit trypsin, chymotrypsin and subtilisin, but they also inhibit other proteases, including the aspartic protease cathepsin D and the cysteine proteinase papain. Protease inhibitors are key players in the endogenous defense system, as they help regulate and balance protease activities. In plants these inhibitors are also important participants in the exogenous defense. The importance of protease inhibitors has been realized for some time now, and many transgenic plants overexpressing different PIs have been produced with resistance against different pathogenic organisms. This is, however, yet to be fully appreciated, and it can have important consequences beyond their recognized scope. [5]

[V] CONCLUSION

Plants have a developed defence mechanisms that help them in response to stress and tolerate both biotic and abiotic stress. Proteins play key role in these mechanisms that bring about their capacity in stress tolerance through various functions from control of synthesis of regulatory components to maintenance of their concentrations and functions accordingly. Identification of proteins in stress tolerance may help in identification of a suitable target for transgenesis.

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