MONITORING OF SALT TOLERANCE RESPONSES OF DIFFERENT MAIZE INBRED LINES USING PROTEIN PROFILE AND PROTEIN SPECTROFLUORESCENCE

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Salt-affected soils are naturally present in more than 100 countries of the world where many regions are also affected by irrigation induced salinization (Hillel, 2005). The saline soil water inhibits plant growth by an osmotic effect, which reduces the ability of the plant to take up water and by ion excess, which affects the plant cells. Consequently, photosynthetic capacity is reduced and plant senescence is accelerated (Jones, 2003). Food production is limited by human-induced salinity, together with the natural and complex salinity found in soils of most semi-arid regions of the world (Rengasamy, 2010).

To avoid this adverse effect, plant evolves a great variety of adaptive mechanisms, such as osmotic adjustment, selective ion uptake, and cytoplasmic and vacuolar ion compartmentation. Recent biotechnical efforts to improve plant salt stress tolerance have two main strategies, the elucidation of salt stress signaling and effector output determinants that mediate ion homeostasis. However, the presences of numerous salt tolerance determinants and essential evolutionary necessities have limited the development of salt tolerance transgenic plants (Munns, 2005).

Alternatively, there are crop improvement strategies that are based on the use of molecular marker techniques and biotechnology that can be used in conjunction with traditional breeding efforts (Ribaut and Hoisington, 1998). RAPD primer showed general consistency as a marker for many plant genera (Fritsch *et al*., 1993). Genetic variability for salt tolerance was reported in alfalfa (McKimmie and Dobrenz, 1991), Trifolium (Ashraf *et al*., 1987), sunflower (Francois 1996) and maize (Maiti *et al*., 1996; Sharif *et al*., 1999; Khan *et al*., 2003). In maize, RAPD technique proved to be useful to generate genetic marker especially in cases of inbred lines and haploid lines (Beaumont *et al*., 1996). Proteomics is an increasingly ambiguous term that is now being applied to almost any aspect of protein expression, structure and function. Furthermore, the analysis of the plant's proteome is an important amendment to analysis of the genome, because gene expression is altered under salinity stress (Zörb *et al*., 2004). Recently, spectrofluorescent emission can detect phosphorylation state of the protein and trace both changes of protein structure and interactions (Wang *et al*., 2011). In this

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study, we aim to link changes in cellular protein of different maize varieties to their genetic divergence under salt stress condition.

MATERIALS AND METHODS

Plant growth and treatments

Maize inbred lines, inbred line of six maize varieties were used for comparison of salt stress response. These maize varieties have provided by International Maize and Wheat Improvement Center (CIMMYT). The pedigree and origin of these varieties are shown in (Table 1).

Seeds of the maize inbred lines were surface-sterilized for 20 min with 75% ethanol solution, rinsed with sterilized distilled water five times and kept for 4 d at 28°C in sterilized river sand for germination. The seedlings were grown in a greenhouse at 25-31°C under the conditions of a 12 h light period and 60-80% relative humidity. When the seedlings had three full-grown leaves, they were treated by salt stress at 8:00 h by supplementing the nutrient solution with 150 mM NaCl for three successive days. The control seedlings were parallel-treated in the nutrient solution without added NaCl. The stressed and control seedlings were harvested for analysis of gene expression and physiological parameters. The harvested tissue samples were rinsed with sterilized distilled water and frozen in liquid nitrogen.

Apparatus

All fluorescence measurements were carried out on Perkin Elmer L545 fluorescence spectrometer in the range (260-750 nm). SDS-PAGE electrophoresis has been carried on a Mini-Protein II TM Cell (Bio-Rad Richmond, CA, USA). Amplification was performed in a Thermal Reactor (TPersonal, Biometra).

General procedure

The genetic characterization and genetic diversity assessment were conducted using protein and RAPD markers. The extraction of proteins was performed using 0.2 g of leaves as described by Wang *et al*. (2003). Protein crude extract was used for assays and quantification as described by Bradford (1976) using bovine serum albumin as a standard. Proteins were separated by 12% acrylamide gel electrophoresis (SDS-PAGE) using equal (70 µg) protein content and stained by Coommassie Brilliant Blue (Sigma Aldrich) according Hames and Rickwood (1998). The protein marker was prestained protein marker (ThermoSeintific). For spectrofluoresence assay protein has dialyzed against 10% glycerol and 50 mM HEPES, pH 7.0 for 18 hrs. The genomic DNA was isolated from leaf tissue following the CTAB procedure described by Porebski *et al.* (1997). RAPD-PCR amplification was performed with eight RAPD primers as shown in (Table 2). The PCR programme had 36 cycles in which first denaturation was carried out at 94°C for 3 min, segment denaturation at 94°C for 1 min, annealing at 35.5°C for 1 min, extension at 72°C for 2 min and final extension for 3 min at 72°C. RAPD fragments were separated on 1.4% agarose gels in 0.5 x TBE buffer. Presense/absense of protein

fractions or DNA fragments was transformed to binary data (0 and 1). The computing of binary data including coefficients of similarity and UPGMA clustering was performed using NTSYS-pc software (Rohlf, 2000).

Fluorescence emission measurements

Measurement of leaf water soluble protein fluorescence was determined with Perkin Elmer L545 fluorescence spectrometer. 500 µg of total cellular proteins was suspended in 200 µl of 60% (v/v) glycerol, 50 mM HEPES, pH 7.0 and excited using wavelength of 270 nm.

RESULT AND DISCUSSION

RAPD data analysis

RAPD essay can efficiently generate both randomly dispersed markers as well as markers linked to specific genes. Constrain of the technique is its reproducibility, which difficult possibility of interchanging results in and between laboratories. However, the problems related with reproducibility of RAPD can be resolved by rigorous attention to detail, (Jones *et al*., 1998). In this study, PCR amplification of genomic DNA was tested on 13 RAPD primers in two rounds of amplification which of eight primers gave clear and reproducible banding patterns (Fig. 1). PCR amplification with eight RAPD primers gave totally 111 RAPD fragments of different molecular weight ranging from 0.19 to 1.5 kb (Fig. 1). The percentages of polymorphic bands per primer were ranged from 100% to 61.5% with average of 84.6 % of bands were polymorphic (Table 3). The Similarity coefficients were calculated according to dice matrix (Nei and Li, 1979). The genetic coefficient distance for each genotype combination ranged from 0.52 to 0.72 (Table 4). The similarity dendrogram in (Fig. 2) shows that the accessions can be classified into two major groups A and B. The first main cluster (A) contains L1 and L2 accessions that are separated at genetic similarity of 0.65. The second main clusters (B) separated at genetic similarity 0.62 into two main sub clusters. The first main sub cluster (1B) separated into two small sub clusters, 1Ba sub cluster included L3 and L4 at genetic similarity of 0.72 and the 1Bb sub cluster included individual cultivars L5 at genetic similarity of 0.67. The second main sub cluster (2B) included individual cultivars L6 at genetic similarity of 0.62. These results are similar with results of study of 57 elite corn inbred lines where 84% RAPD fragments were polymorphic (Hahn *et al*., 1995). Other result on hybrid lines of maize show similar ratio of polymorphic bands, (Bauer *et al*., 2005). The genetic similarities dendrogram shows that the accessions from the same geographical region were found to have a close genetic relationship.

Protein profile

The molecular mechanism of salt stress tolerance in plant could be elucidated using cDNA microarrays. However, mRNAs may not be transcribed or that changes in the protein level or enzyme activity can occur without any detectable

change in transcript abundance due to translational or other levels of control. Therefore, it is necessary to study the salt stress responses at the protein level (Shunping *et al*., 2005). The protein banding pattern of non-salt treated and salt treated maize leaf is demonstrated in (Fig. 3A). The six lanes represent six maize varieties total cellular protein patterns. The major protein profile of maize leaf under control conditions show protein bands that can categorized in high molecular weight (estimated as 320, 280 and 220 kDa), medium range molecular weight (55, 41, 41 kDa and 36 kDa) and low range molecular weight less than 25 kDa. The high molecular weight bands in maize leaf are not recognized in previous research of maize (Mohamed, 2005). This could because protein profile of plant leaves is significantly influenced by the extraction method (Maldonado, *et al*., 2008). However, under non salt stress different maize varieties show some specific variation. Minor band of 45 kDa did not expressed in varieties of L5 and L6 while another minor band of 23 kDa was not expressed in L6 variety. The differential pattern of salt stress protein is highly related to the plant genotype. Consequently, some plants did not display large differences in protein patterns between control and salt stressed conditions, in other cases the stressed protein SDS PAGE profile show a contrasted profile versus unstressed protein profile (Amini *et al*., 2007; Sohrabi *et al*., 2011). The protein profile patterns emphasize the closely genetic relationship between L5 and L6 variety. Protein bands within the range of 20-45 kDa were isolated from the apoplast infiltrate of maize leaf (Witzel *et al*., 2011).

Under salt stress conditions, the protein banding pattern of maize leaf varieties show absence of high molecular protein. The major protein bands of salt stress conditions are 55 kDa, 45 kDa, 34 kDa, 28 kDa, 24 kDa and 23 kDa. (Fig. 3B). Under salt stress conditions, protein profile of the maize varieties show high homogeneity pattern among the different varieties. Comparing to non-stress protein pattern, the high molecular weight bands were disappeared and the major protein bands were located at the medium molecular weight range. The number of bands of leaf protein extracted from control plants was much higher than of the salt treated plants (Fig. 3A and B). This could be due to the inhibitory effects of salt stress on transcriptional process (Jain *et al*., 1993). The fast breakdown of cellular protein during salt stress might be also due to the increasing activity of acid and alkaline protease (Parida *et al*., 2004).

The major band of 55 kDa is closely related to the major mesophyll protein band of 56 kDa of ribulose-1,5-bisphosphate carboxylase/oxygenase in maize. This chloroplast expressed enzyme is crucial for the fixation of carbon dioxide (Klein and Vernon, 1973; (Bewley and Black, 1994). Many of maize cytosolic cellular protein in maize have molecular weight ranged 45-29 kDa. Most of these proteins are enzymes involved in glyco-

lytic pathway and are necessary for cellular energy conservation (Okamoto *et al*., 2004). Homeostasis and mineral deficiencies has been a consequence of salt stress (Schroeppel-Meyer and Kaiser, 1988). In maize, mineral deficiency leads to increase in chloroplast protein content. The identified maize chloroplast protein show 17.5 kDa for coupling factor, 23 kDa for structure protein, 33 kDa for cytochrome F, 37 kDa coupling factor, 42 kDa for cytochrome b6, 45 kDa for transhydrogenase and 56 kDa for coupling factor and ribulose diphosphate carboxylase (Barr and Crane, 1974). Several proteins within the range of 56 kDa-17 kDa have been identified as apoplastic proteins for biotic stress in maize (Witzel *et al*., 2011).

Spectroscopic analysis

The emission fluorescence of extracted leaf protein samples of control and salt stressed were measured using visible and UV wave length. The emission fluorescence of all sample were recorded at excitation spectra of 270 nm. All maize varieties show fluorescence emission peak at range of 320 to 340 nm however, the intensity of this peak show alternation of intensity for salt stressed and control samples (Fig. 4). Maize varieties L1 and L2 have higher peak intensity of salt stressed leaf protein compared to the control samples. The other maize varieties L3, L4, L5 and L6 show higher peak intensities for the control samples. Other emission peaks were associated with salt stressed protein of samples varieties L2, L3, L4 and 6 with two common peaks at 440 and 490 nm. The maize variety number 6 shows excess emission peak at 550 nm for the control sample. The fluorescence emission of proteins is mainly referred to their contents of aromatic amino acids tryptophan, tyrosine and phenylalanine (Teale, 1960). The main peak of fluorescent emission in all fluorescent patterns of normal and salt stress conditions were at 320 nm except variety L1 which was at 340 nm (Fig. 4). This fluorescent emission value is very close to the emission peak of tryptophan amino acid (Boteva *et al*., 1996; Kabiri *et al*., 2012). The tryptophan residue in protein has emission spectrum a ranged from 310~350 nm should reflect the average environment of the tryptophan. Hence, the variations in tryptophan emission are due to the structure of the protein (Vivian and Callis, 2001). The increase of emission wave length of tryptophan peak in sample 2 could refer as a change in its protein structure comparing to other maize varieties. The fluctuation pattern of peak intensity between salt and control protein samples show higher peak intensity in case of control samples (with exception of varieties L1 and L2). The quenching effect in case of salt stressed samples could be result of protein binding interaction that cause collisional quenching of emission intensity (Eftink, 1991). The increment of emission spectra that associate salt stressed protein samples of varieties L1 and L2 indicate protein changes that prevent quenching effect of protein collision. The other important observation is in case of losing the quenching effect the salt stress protein samples of varieties L1 and L2 show higher peak intensity rather than

the average intensity of control samples. Several data show increase of protein fluorescence intensity as a result of protein phosphorylation process or protein binding with calcium (Miranda *et al*., 2004; VanScyoc*et al*., 2002). Under salt stress, plants activate sensor proteins through protein binding with calcium and phosphorylation of many protein kinases (Du *et al*., 2011). Our data suggest presence of protein phosphorylation and or protein calcium binding in case of salt stressed protein. However, the subsequent effect of emission intensity increment was hidden by the collisional protein effect. Additional peaks of 380 nm, 420 nm and 440 nm were associated with salt stressed samples of varieties L2, L3, L4 and L6. These emission spectra are most likely due to emission spectra of non-pretentious compounds that may co-precipitate during protein preparation. Some maize secondary metabolites show fluorescence spectra close to peaks in our results. Gallic acid, o-Coumaric acid and Cinnamic acid have emission peaks of 382 nm, 426 nm and 420 nm, respectively. These substances have antioxidant effect and research data show their involvement in abiotic resistance of maize plant (Lopez *et al*., 2009; Pál *et al*., 2005; Tuberoso *et al*., 2007). These data may interpret the presence of 380 nm, 420 nm and 440 nm in most spectrofluorescence samples of salt stress under study.

CONCLUSION

According RAPD data the maize varieties under study can grouped into two main groups where varieties L1 and L2 has grouped in one cluster. Results of SDS-PAGE and protein spectrofluorescences show that, the main differences between maize varieties have observed under control condition. However, protein spectrofluoresences data show a very similar behavior of L1 and L2 varieties which are in context with result of RAPD genetic similarities. Protein spectrofluoresence has proved to be a reliable tool for monitoring proteome responses to salt stress condition.

SUMMARY

Seedlings of Six maize inbred lines CML 511, CML 448, CML 444, CML 395, CML 254 and CML 216 were used to analyze salt stress of (150 mM NaCl). The analyses of RAPD results showed 84.6% of polymorphic fragments. Accessions of CML 511 and CML 448 were included in one main cluster with genetic similarity of 0.65. The genetic similarity between CML 444 and CML 395 was 0.72, the other accessions of CML 254 and CML 216 had genetic similarities of 0.67 and 0.62, respectively. Leaf proteins were studied by one-dimensional SDS-PAGE. Under non stress conditions, the minor band of 45 kDa was not expressed in varieties of CML 254 and CML 216 while band of 23 kDa were expressed in all accessions except of CML 216 accession. Under stress conditions high molecular weight bands were disappeared and the major protein bands were located at the medium molecular weight range. Protein spectrofluorescence of leaf protein sam-

ples show main emission peak at about 320 nm in both conditions of salt stress and control. However, the emission intensities showed some variations among different maize lines in alternative conditions of control and salt stress. This might be referred to change in protein interactions. Inbred lines of CML 511 and CML 448 exhibit closely related fluorescence pattern, this is in context of their genetic similarities as has deduced by RAPD results.

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Table (1): Name, pedigree and origin of inbred lines.

Entry	Id. Num.	Acc. Num.	Pedigree	Address	Origin of seed	Observations
L1	28110	25835	CML 511	A11 A0225	TL08A-1903-168	Lowland
L2	28047	25772	CML 448	A10 G0632	AF08A-0903-137	Lowland
L3	28043	25768	CML 444	A10 G0628	AF08A-0903-134	Africa MA/ST
L4	25400	22470	CML 395	A10 G0514	AF08A-0903-121	Africa MA/ST
L5	17593	16424	CML 254	A10 G0120	TL08A-1903-185	Lowland
L6	17123	16394	CML 216	A10 F0623	AF08A-0903-112	Africa MA/ST

Table (2): RAPD primers used in this study which gave clear and reproducible bending.

RAPD primers	5'-3' sequence	RAPD primers	5'-3' sequence
OPD-02	GGACCCAACC	OPE-15	ACGCACAACC
OPB-12	CCTTGACGCA	OPH-17	CACTCTCCTC
OPN-03	GGTACTCCCC	OPD-01	ACCGCGAAGG
OPA-03	AGTCAGCCAC	OPN-10	ACAACTGGGG

Table (3): Total band number and polymorphic bands of eight RAPD primers used with the 6 maize inbred lines.

Primer name	Total No. bands	Polymorphic bands	% of Polymorphism
OPA03	21	16	76.0
OPA07	19	19	100.0
OPD01	13	8	61.5
OPE15	9	8	88.9
OPH17	7	7	100.0
OPN03	13	13	100.0
OPN10	14	11	78.5
OPP12	15	12	80.0

Table (4): Genetic similarity matrix based on RAPD marker.

	L1	L2	L3	L4	L5
L2	0.65				
L3	0.49	0.64			
L4	0.54	0.59	0.71		
L5	0.51	0.58	0.67	0.67	
L6	0.49	0.51	0.55	0.68	0.63

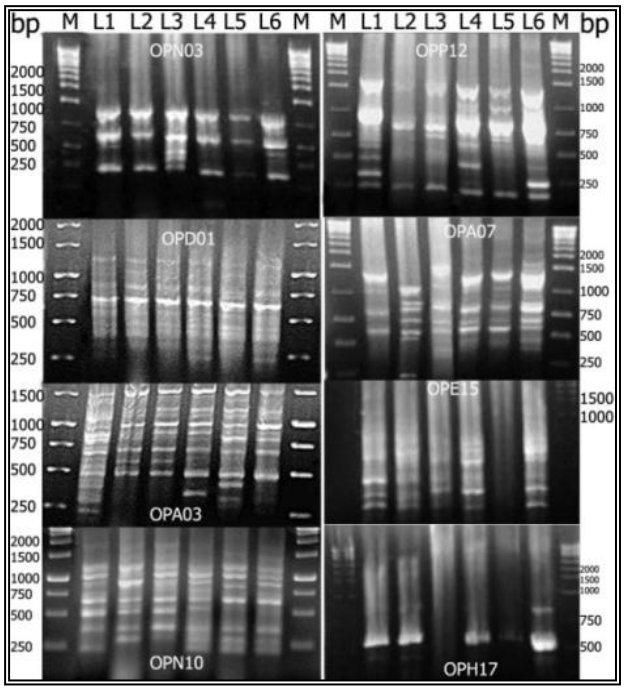

Fig. (1): The random amplified polymorphic DNA (RAPD) profile of maize varieties using the random primers which is listed in Table (2).

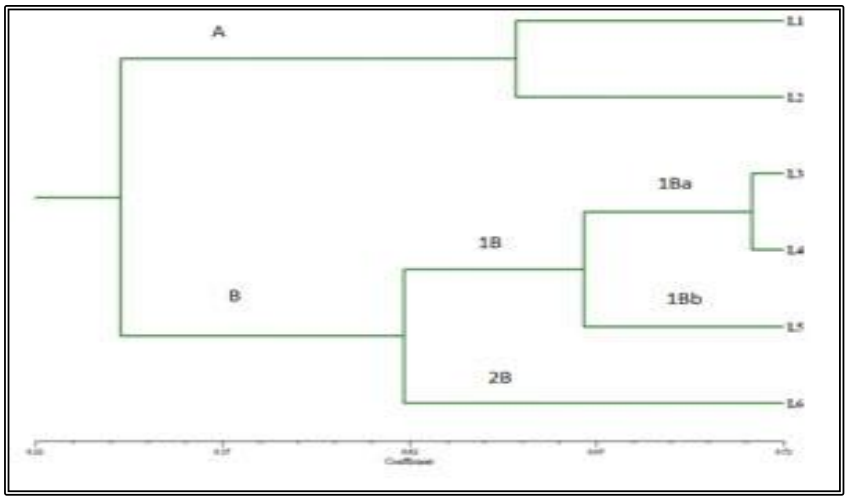

Fig. (2): RAPD based dendogram of investigated maize genotypes based on cluster analysis of Nei and Li's genetic distance (1979).

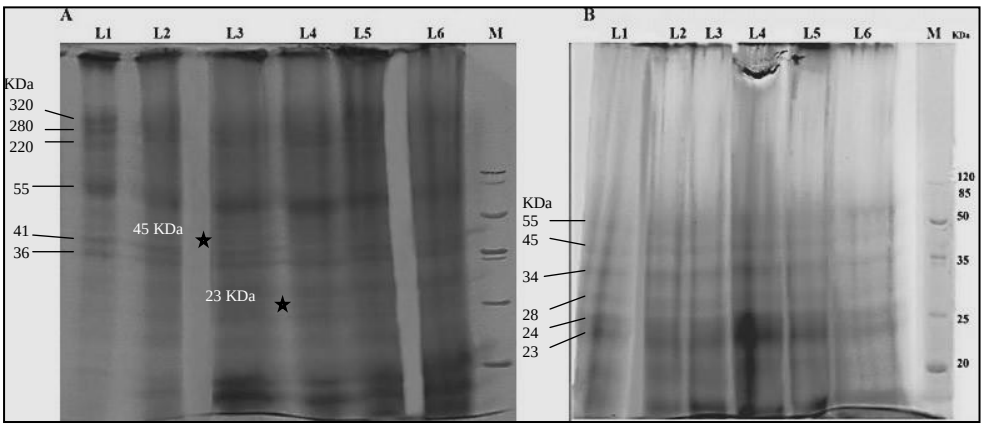

Fig. (3): SDS-PAGE profiles of total leaf protein of maize varieties under control condition (A) and under salt stress condition (B).

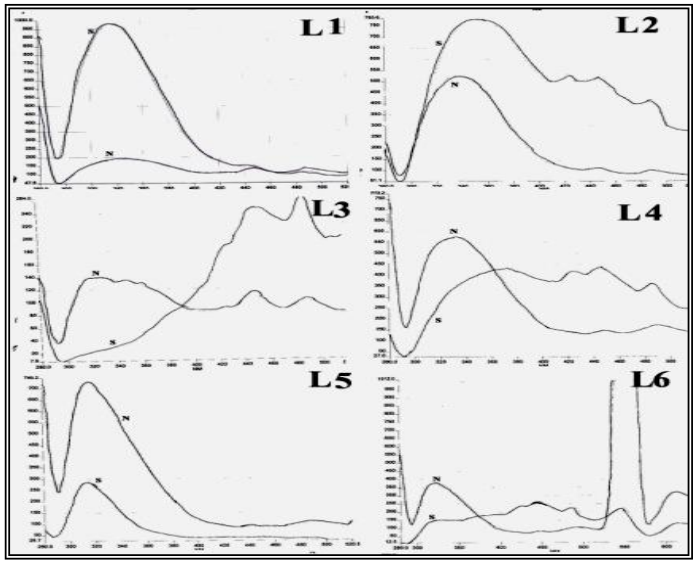

Fig. (4): Fluorescence emissionspectra (excitation: 270 nm) of total leaf protein of the six maize varieties in water. Leaf maize protein under control condition (N) and salt stress condition.