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FUNCTIONAL GENOMICS FOR SALT TOLERANCE IN RICE (*Oryza sativa* L.)

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Rice is one of three cereal crops produced worldwide annually at a level of approximately half a billion tons (FAO, 2004). Rice consumption and therefore demand will increase over the next several decades according to the predicted human population rise. As a limited acreage of intensively cultivated areas is available to increase rice cultivation, both higher yields and expansion of cultivation into salt-affected areas are essential to meet the anticipated rising demand.

In addition to being an important cereal crop for human consumption, it is also becoming clear that rice could play a major role as a good model for cereal genomics. Rice has a genome size considerably smaller than the other major cereals, which is estimated at 420 to 450 megabase pairs (Mbp). Sorghum, maize, barley, and wheat have significantly larger genomes (1000, 3000, 5000, and 17000 Mbp, respectively). The small genome size of rice results in a higher gene density relative to the other cereals. Assuming a total of 30,000 genes in each of the cereal genomes, rice will have on average of one gene approximately every 15 kilobase pairs (Kbp), while, Maize and wheat will have one gene approximately every 100 and 500 Kbp, respectively. This higher gene density in rice makes it an attractive target for cereal gene discovery

efforts and genome sequence analysis (Goff, 1999).

Although genes in rice are present at a higher relative density than in other cereals, they are predicted to be arranged in a similar general order within the genome. Comparisons of the physical and genetic maps of cereal genomes had led to reports that a significant amount of co linearity of gene order exists among the different cereal genomes (Ahn *et al.*, 1993). Accordingly, the use of rice as a model for cereal comparative genomics analysis had been proposed and recently reviewed (Havukkala, 1996).

Salinity, water deficit, chilling and freezing, heat stress, and oxygen deficiency are major abiotic stress factors restricting plant growth (Boyer, 1982; Salisbury and Ross, 1989). Some of which such as high temperature can become stressful in a few minutes; others may take days to weeks such as soil water deficient or even months such as mineral nutrients to become stressful. Salinity can affect any process in the plant life cycle, so that tolerance will involve a complex interplay of many characters. Cloning and characterization of environmental stress-induced genes have contributed greatly to our understanding of the physiological responses of plant cells at the molecular level to different environmental factors. For example, drought and salinity stresses cause a number of physiological and biochemical changes in plants such as closure of stomata decrease in photochemical activities, reduction of CO_2 fixation, accumulation of osmolytes and osmoprotectants, and alteration in carbohydrate metabolism (Tabaeizadeh, 1998).

Differential display (DD) is one of the effective methods for analyzing gene expression in eukaryotic cells and tissues. DD has been widely applied to study changes in mRNA expression induced by temporal developments, biotic and abiotic factors (Liang and Pardee, 1992; Liang *et al.*, 1992; Guimaraes *et al.*, 1995; McCarthy *et al.*, 1995, Hu *et al.*, 1996; Liu and Baird, 2003). This powerful technique simultaneously screens for both up-regulated and down-regulated transcripts in multiple cell populations under different developmental and environmental conditions.

The present study aims to detect and characterize some stress-related cDNAs of rice through, identification of some salt-tolerant rice strains, isolation of some Expressed Sequence Tags (ESTs) related to salt tolerance, cloning & sequencing of isolated ESTs and determining gene functions for each EST using bioinformatics approaches.

MATERIALS AND METHODS

Plant material

Five Egyptian rice cultivars, *Oryza sativa* L., namely; Giza 159, Giza 177, Giza 178, Sakha 102 and Agami M1, used in this study, were kindly provided from Rice Research Department, Field Crops Research Institute, ARC, Giza, Egypt.

Screening experiment to detect salt-tolerant cultivars

In order to assess relative responses to salt stress based on yield-related traits, the five rice cultivars were grown in greenhouse in 36 pots (30x50 cm) in a sand culture in a randomized complete block design with three replications, according to Abdel-Tawab *et al.* (1998). Plants were irrigated with Hoagland solution, every two days with two salt concentration treatments (0 and 10000 ppm NaCl) for 60 days. Data on yield components, related directly to salt stress, i.e., visual rank (VR), plant height (PH), number of shoots/plant (NS), fresh weight/plant (FW) and dry weight/plant (DW), were recorded at the end of the experiment. Data experiment was statistically analyzed according to Steel and Torrie (1980). The differences between means of plants grown under control versus salt stress were compared using t-test.

RNA isolation and mRNA differential display

Seeds of the salt tolerant genotype (Agami M1) were planted in pots containing pre-washed sand and were irrigated with Hoagland solution. Two weeks-old seedling were treated with Hoagland solution containing 10,000 PPM NaCl. Control plants were irrigated with Hoagland solution only. Shoot tissue was harvested at 0 (control), 1 hour and 10 hours after NaCl treatment and frozen in liquid nitrogen. Total RNA was isolated from frozen tissues using an RNeasy kit (Qiagen, USA). DNA contamination was removed using DNase I (DNase- RNase free).

mRNA differential display (Liang and Pardee, 1992) was conducted using RNAimage kit (GenHunter Corp.) according to the manufacture's instruction, except that the anchor primers (T11A, T11G, T11C) were fluorescence-labeled (Metabion Corp.). DD-PCR amplification for each primer pair was performed twice from RNA samples isolated separately at each time point. PCR products were separated on 6% denaturing polyacrylamide gels and visualized using Green laser beam (532 nm) for fluorescence excitation using Typhoon scanner (Amersham, USA). Location of bands of interest exhibiting differential expression patterns were determined using gradient transparent sheets.

Cloning

Differentially displayed cDNA bands were excised from polyacrylamide gels with a scalpel and eluted in 100 µl sterilized water for one hour at room temperature followed by 15 min at 94°C. cDNAs were recovered by ethanol precipitation, redissolved in 20 µl nuclease-free water and 2 µl aliquot was used for reamplification. Two µl of PCR reaction product were sub cloned into pGEM-Teasy vector system (Promega, USA) according to the manufacturer manual.

Northern blot analysis

Analysis of differentially displayed bands was carried out using the corresponding cDNAs as probes on northern blots. Total RNA (10 µg per lane) was denatured for 5 min at 65°C in 50% formamide/6% formaldehyde, electrophoresed at 8 volts/cm for 90 min in 1% agarose gel containing 2% formaldehyde, transferred overnight to positively charged nylon membrane, and bound to the membrane by UV-crosslinking. To make cDNA probes, 40 ng of purified insert were labeled using non-radioactive digoxigenin (DIG), High Prime DNA Labeling and Detection Starter Kit (Boehringer Mannheim, cat no. 1585614). The probe was denatured at 95°C for 5 min and added to RNA blots pre-incubated for 4 hr at 65°C in 4 ml of aqueous hybridization buffer (Sambrook *et al.,* 1989). Blots were hybridized overnight at 65°C, washed once for 15 min in 100 ml 2X SSC (1x SSC is 0.15 M NaCl, 0.015 M Na3-citrate.2 H2O, pH 7.0) at room temperature, followed by sequential 20 min washes at 65°C in 100 ml 2X SSC, 1X SSC, and 0.5X SSC. Blots were exposed to X-ray film for 30 min.

Sequencing and sequence alignments

The cloned cDNA fragments were sequenced using M13 primers with the ABI PRISM Big Dye Terminator (PE Applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). All inserts were sequenced on both strands, and DNA sequences were compared with sequence databases using BLASTN and BLASTX algorithms (Altachul *et al.,* 1997).

RESULTS AND DISCUSSION

Screening experiment

This experiment was designed to select the salt-tolerant genotype based on five yield and yield-related traits, i.e., visual rank (1-5), plant height (cm), number of shoots/plant and fresh and dry weight/plant (g) for the five different rice genotypes. All measurements were taken at 60 days after planting. Mean comparisons in each trait between control (non-saline water) and treatment (10,000 ppm NaCl) for each cultivar were done according to t-test at $P < 0.05$ as shown in Table (1). The results indicated that, Agami M1 cultivar was the most tolerant cultivar against salt stress since no significant difference was observed between its control and treatment in any of the measured traits. Also, Agami M1 was able to continue growing under 10,000 ppm NaCl, while Giza 177 cultivar was the most salt-sensitive as all traits were significantly reduced under salt stress compared with their respective control values. Accordingly, Agami M1 cultivar was chosen for subsequent molecular analysis to detect possible stress-related genes using DD-PCR.

Differential Display

Rice genes whose expression was regulated by salinity stress were studied by differential mRNA display. The

fluorescence-labeled anchor primer (T11A, T11G, T11C) was used to target the polyA tail at the 3` end of stress-related genes, and the random primers (ARP6 and ARP7), were used in an attempt to hit the open reading frames (ORFs) at the 5` end of these genes. Fig. (1) shows DD-PCR results visualized by using Green laser beam (532 nm) for fluorescence excitation using Typhoon high-quality confocal optical imaging system scanner.

A total number of 324 bands were observed along the three DD-gels, 30 of them (9.26%) showed differential amplification between treatments. Depending on the peak optical density (OD), the 30 DD-cDNA fragments under control, 1 h and 10 h salinity treatments were classified into six patterns of expression as shown in Table (2) and Fig. (2). Group 1 included four cDNA fragments (No.; 1, 2, 4 and 17) with up-regulation under 10 h treatment, while normal rate of expression was shown under control as well as 1 h treatment. Group 2 involved seven cDNA fragments (No.; 3, 6, 11, 15, 18, 21 and 27) with up-regulation of corresponding genes after 1 h treatment, while no expression was shown under either control or 10 h treatment. Group 3 included 11 cDNA fragments (No.; 5, 7, 8, 9, 14, 16, 19, 23, 24, 26 and 30) with up-regulation after 1 h treatment, while normal rates of expression were shown for their corresponding genes under either control or 10 h treatment. Group 4 included seven cDNA fragments (No.; 10, 12, 13, 20, 22, 28 and 29) with down-regulation under 1 hr stress treatment and up-regulation under control and 10 hr stress treatment. An opposite trend of expression was shown for group 5, where the cDNA fragment (No.; 25) whose corresponding gene has no expression under control condition, while it was up-regulated under 1 hr treatment and down-regulated under 10 hr treatment.

Cloning and validation of the differential display cDNA clones

The nine cDNA fragments with lengths of more than 100 bp were cloned into pGEM-T Easy vector and sequenced with M13 primers. The data were validated by performing northern blot analyses for a randomly selected transcript (fragment 12). The expression of fragment 12 disappeared after 1 hr treatment, then return to normal at 10 hr (Table 2). This expression pattern was confirmed using northern blot (Fig. 3).

Sequence homologies of the differential display cDNA clones

DNA sequencing reactions were conducted for fragments over 100 bp (1, 2, 11, 12, 13, 20, 21, 22 and 26). Table (3) summarizes the homology search results based on computer analysis using Blast programs from National Center for Biotechnology Information (NCBI).

Fragment 1 showed significant homology with *Oryza sativa* L. (japonica cultivar-group) genomic DNA, chromosome 2 with score = 252 bits, E-value = 3e-64 with results of alignment and

identities equal 177/194 (91%). Location of fragment 1 on the rice chromosome 2 was found to be between 19, 475 and 427 bp to 19, 475 and 620 bp which belongs to a hypothetical protein. Fragment 12 showed significant homology with *Oryza sativa* (japonica cultivar-group) genomic DNA, chromosome 1 with Score = 121 bits, E-value = 6e-25 with results of alignment and Identities equal 61/61 (100%). Location of fragment 12 on the rice chromosome 1 was found to be between 12, 636 and 384 bp to 12, 636 and 968 bp which belongs to ubiquitin / ribosomal protein S27a. Similar results were reported for polyubiquitin gene in maize (Takimoto *et al.*, 1994) and *Arabidopsis thaliana* (Sun and Callis, 1997). Scanning of fragment 13 in the Gene Bank showed significant homology with *Oryza sativa* L. (japonica cultivar-group) genomic DNA, chromosomes 3, 10 and 12 according to the repeatability of high positive hits with *Oryza sativa* L. which shows many similar sequences on chromosomes 3, 10 and 12, also fragment 13 showed some low similarity to proteins from *Gossypium hirsutum*, *Oryza sativa*, *Cicer arietinum, Pinus radiate, Humulus lupulus and Malus domestica*. Due to the short length of fragment 13 (106 bp) it was difficult to identify the exact related gene or location. Fragment 20 showed no significant similarity with *Oryza sativa* L. DNA, but due to the repeatability of low similar hits with *Oryza sativa* L. It is clear that fragment 20 belongs to *Oryza sativa* L. while protein search showed a significant similarity to Ca^{+2}-transporting ATPase-like proteins which are involved in many functions in the cell like cell signaling, cell cycle and ion exchange (Cunningham and Fink, 1994). Scanning of fragment 22 in the Gene Bank showed significant homology with *Oryza sativa* L. (japonica cultivar-group) genomic DNA, chromosome 7 with score = 240 bits, E-value = 1e-60 with results of alignment and identities 121/121 (100%). Fragment 22 was located on the rice chromosome 7 between 23, 196 and 907 to 23, 196 and 787 which did not belong to any identified genes. Also protein alignment did not reveal any significant similarity to any known protein. While, no significant homologies in the gene bank database was found for the fragments (2, 11, 21 and 26).

Finally, it is evident from the aforementioned results that we have isolated some cDNA fragments that resemble some genes that play a role in salt tolerance mechanisms by using florescent differential display technique. For example, ubiqitin is an important component of plant salt-stress response mechanisms (Chinnusamy *et al.*, 2004; Khedr *et al.*, 2003). Also, Ubiqitin was found to play an important role in regulating chromatin structure (David, 1995). In this study we also isolated a fragment which is related to Ca^{+2}-transporting ATPase which is responsible for Ca^{+2} transporters and components of Ca^{+2}-related signal transduction pathways which suggest that Ca^{+2} plays a physiologically relevant role in plant responses to salinity (Tester and Davenport, 2003).

However, this calls for further studies to isolate the full length cDNAs and promoter regions of the genomic sequences corresponding to the stress-regulated clones reported here.

SUMMARY

Five rice cultivars of (*Oryza sativa* L.), were screened for salt tolerance, i.e. Giza 159, Giza 177, Giza 178, Sakha 102 and Agami M1. Agami M1 was chosen as the most tolerant cultivar for salt stress and was used for differential display experiment. Florescent differential display was conducted to compare the differences in gene expression between 1 and 10 h salinity-stressed (10,000 ppm NaCl) and unstressed (control) plants. Out of 324 bands observed, 30 bands (9.26%) showed differential amplification between treatments. The nine relatively longest cDNA fragments (more than 100 bp) were cloned and sequenced. Some fragments showed homology to some cDNAs that play a role in salt tolerance mechanisms like ubiquitin and Ca^{+2}-transporting ATPase. All identified gene-related fragments could play a major role in understanding salt tolerance mechanisms which can be used for isolation of salt related genes (full length) by using isolated cDNAs as probes.

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Table (1): Performance of five rice cultivars for five yield-related traits under salt stress (10,000 ppm NaCl) compared to their respective controls.

Cultivar	Visual rank	Plant height	No. shoots/plant	Fresh weight/plant	Dry weight/plant
Giza 159	7.19*$^+$	4.82*	3.51*	1.83	0.01
Giza 177	5.84*	6.67*	3.74*	3.98*	3.38*
Giza 178	7.43*	2.00*	0.42	2.12	1.22
Sakha 102	7.00*	5.97*	3.06*	1.17	0.03
Agami M1	0.75	1.29	0.29	0.65	0.11

*significance based on t-test, ($P < 0.05$) $^+$ calculated t values

Table (2): Expression patterns of the obtained DD of shoot fragments.

Pattern	Control	1 hour	10 hours	DD fragments
1	normal	normal	up-regulated	1, 2, 4 & 17
2	none	up-regulated	none	3, 6, 11, 15, 18, 21 & 27
3	normal	up-regulated	normal	5, 7, 8, 9, 14, 16, 19, 23, 24, 26 & 30
4	none	up-regulated	up-regulated	25
5	normal	down-regulated	down-regulated	29
6	normal	down-regulated	normal	10, 12, 13, 20, 22 & 28

Table (3): DD-fragments and homology search results.

Frag no.	Molecular size (bp)	Homology search results (GeneBank accession number; E-value)	Function
1	219	Chromosome 2 (AP008208.1; $3e^{-64}$)	Hypothetical protein
2	190	No homology	
11	200	No homology	
12	168	Chromosome 2 (AP008207.1; $6e^{-25}$)	Ubiquitin/ribosomal protein S27
13	106	Chromosome 3, 10 or 12	
20	189	Ca^{2+} transport genes	Ca^{+2} transporting ATPase
21	180	No homology	
22	186	Chromosome 2 (AP008213.1; $1e^{-60}$)	
26	180	No homology	

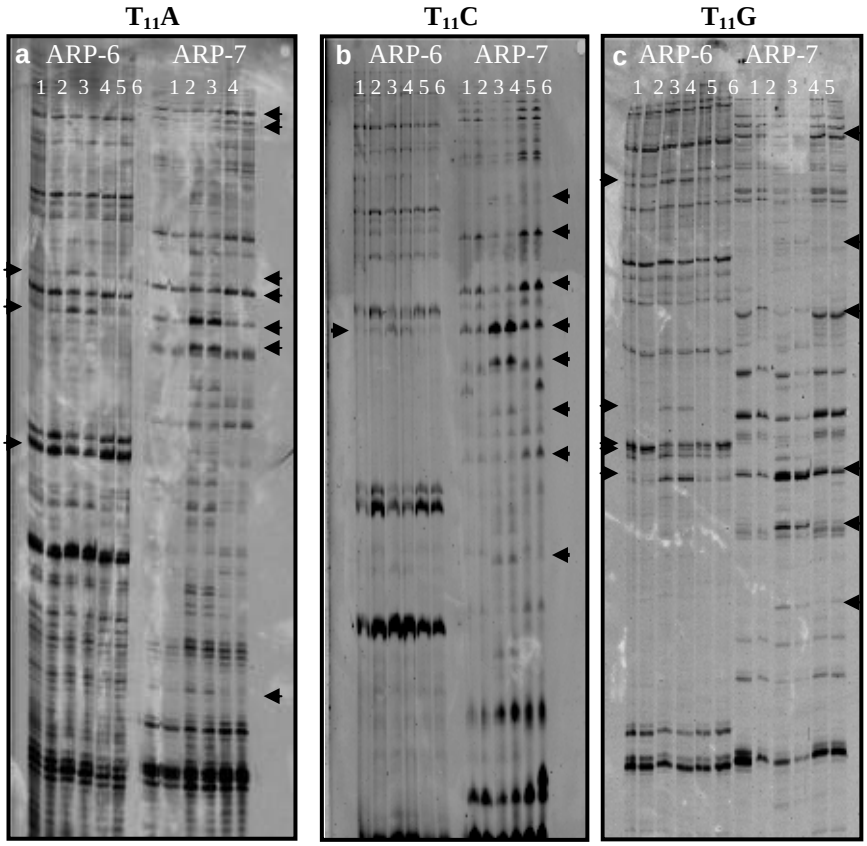

Fig. (1): DD-polyacrylamide gels of shoot cDNAs under control and stress (1 and 10 h) conditions utilizing different primer combinations, a) $T_{11}A$, b) $T_{11}C$ and c) $T_{11}G$. Lanes 1 and 2 (control), lanes 3 and 4 (1-h shoot treatment, 10,000 ppm), lanes 5 and 6 (10-h shoot treatment, 10,000 ppm). Arrows indicate a number of differentially expressed bands on a duplicate basis.

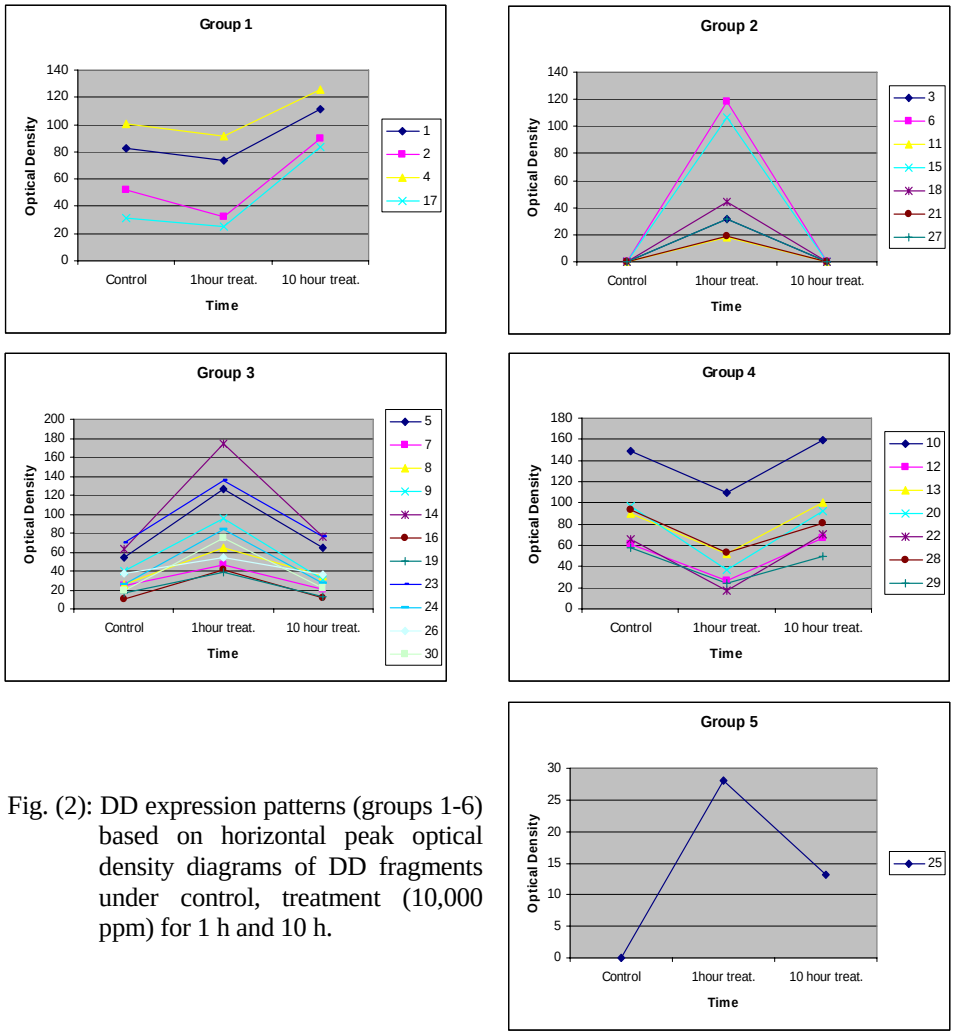

Fig. (2): DD expression patterns (groups 1-6) based on horizontal peak optical density diagrams of DD fragments under control, treatment (10,000 ppm) for 1 h and 10 h.

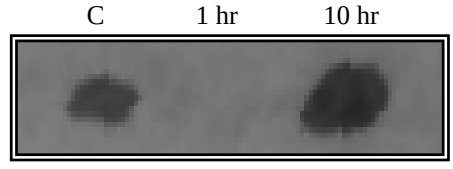

Fig. (3): Verification by Northern blot analysis of salt-induced gene fragment identified by differential display. Probe for fragment 12 was generated from cloned DNA. Total RNA samples from control (C), salt stressed for one hour (1h) and salt stressed for 10 hours (10h) are shown.