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Isolation and expression analysis of salt stress-associated ESTs from contrasting rice cultivars using a PCR-based subtraction method

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Abstract Salt stress adversely affects the growth of rice plants. To understand the molecular basis of salt-stress response, four subtracted cDNA libraries were constructed employing specific NaCl-stressed tissues from salt-tolerant (CSR 27 and Pokkali) and salt-sensitive (Pusa basmati 1) rice cultivars. An efficient PCR-based cDNA subtraction method was employed for the isolation of the salt-stress responsive cDNA clones. In all, 1,266 cDNA clones were isolated in the course of this study, out of which 85 clones were end-sequenced. Database search of the sequenced clones showed that 22 clones were homologous to genes that have earlier been implicated in stress response, 34 clones were novel with respect to their function and six clones showed no homology to sequences in any of the public database. Northern analysis showed that the transcript expression pattern of selected clones was variable amongst the cultivars tested with respect to stress-regulation.

Keywords Contrasting cultivars · Rice · Salt stress · Subtractive cDNA libraries · Transcript analysis

Introduction

High soil salinity, contributed largely by NaCl, is one of the important factors that limit the distribution and productivity of major crop plants, including rice. This is a permanent and increasing agronomic problem in a large portion of crop-growing areas. Rice is the most important food crop of the world. The irrigation schemes in rice fields have accelerated the soil-salination process and raised salt levels in large areas. Substantial yield reduction has been observed in salt-affected rice fields in several parts of India and elsewhere (Widawsky and O'Toole 1990; Khush and Toenniessen 1991). Identification and functional characterization of salt-stress responsive genes may provide clues to understand the complexity of the underlying mechanism(s) of salt stress (Pareek et al. 1997; Zhu et al. 1997; Grover et al. 1999; Cushman and Bohnert 2000; Grover et al. 2001).

Hasegawa et al. (2000) enumerated several processes that are affected upon exposure of plants to salt stress (i.e. carbon metabolism, accumulation of compatible osmolytes, ion partitioning, energy metabolism and growth). Although salt stress causes pleiotropic effects, ion toxicity and hyper-osmotic stress are the primary effects of high salinity. Salt tolerance is considered to be due to effectors that directly modulate stress etiology or attenuate stress effects, and to the regulatory molecules that are involved in stress perception, signal transduction and modulation of the functions of effectors (Hasegawa et al. 2000; Gong et al. 2001; Zhu 2001). Enzymes that catalyze rate-limiting steps in the biosynthesis of compatible osmolytes and proteins, that protect membrane integrity and control water or osmotic homeostasis and scavenging of reactive oxygen species (ROS), are examples of stress tolerance effectors. Regulatory determinants include transcription factors that interact with promoters of osmotic stress-regulated genes [basic leucine zipper motif, MYB, MYC and zinc finger transcription factors including rd22BP1 (MYC), AtMYB2 (MYB), DREBIA and DREB2A (AP2 domain) and ALFIN1 (Zn finger)] and signal pathway intermediates that post-transcriptionally activate effectors [*SOS3*(Ca^{2+}-binding protein), *SOS2* (Suc non-fermenting like) kinase, Ca^{2+}-dependent protein kinases and mitogen-activated protein kinases and *SOS1* (putative plasma membrane Na^+/H^+ antiporter)].

Efforts for improving the tolerance of plants to high salinity by genetic engineering or breeding have not been

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successful to a great extent, due to the lack of mechanistic understanding of genetic complexity and the inherent diversity of the salt stress-response pathways (Flowers and Yeo 1995). Recent developments in the field of structural/functional genomics and proteomics provide ample opportunities for the isolation and characterization of novel salt stress-responsive/associated genes, proteins and pathways. The evolutionary conservation of stress response has shown that functionally analogous stress-associated determinants exist in both unicellular and multicellular organisms. Elucidation of components of stress-response pathways in simpler systems like bacteria and yeast may help in finding overlapping mechanisms in higher plants. High throughput sequencing programs supported by genome-wide transcript profiling has a great potential in isolating differentially expressed and functionally important stress-regulated genes. A global transcript profiling in yeast in response to salt stress, using genome-wide chip analysis, showed that about 7% of the total genes encoded in the yeast genome are induced more than 5-fold after a brief and mild saline shock (Posas et al. 2000). Kawasaki et al. (2001) examined transcript regulation in response to high salinity in the salt-tolerant rice cultivar Pokkali with a microarray employing 1,728 cDNAs from different libraries of salt-stressed tissues. This group found that about 10% of the transcripts in Pokkali were significantly up- or down-regulated within 1 h of salt stress.

Recent studies imply that comparative gene expression analysis is a promising approach for understanding the mechanisms of differential tolerance and susceptibility (Kozian and Kirschbaum 1999). The analysis of differences between two complex genomes holds promise for the discovery of genes that are present, or expressed specifically, in the one and absent from the other system (Lisitsyn et al. 1993). Subtractive cloning has emerged as a powerful technique for this kind of analysis (Diatchenko et al. 1996). In this method, nucleic acid from which one wants to isolate differentially expressed sequences (i.e. the tester) is hybridized to the complementary nucleic acid that is believed to lack the sequences of interest (i.e. the driver) (Sagerström et al. 1997). For removing driver-tester hybrids and excess driver, use of biotinylation and streptavidin or avidin has been shown to be highly effective (Sagerström et al. 1997). Use of PCR allows one to work with a small amount of starting material and to easily perform multiple rounds of subtraction (Sagerström et al. 1997). The potency of the PCR-based technique in conjunction with the use of magnetic beads for making subtracted cDNA libraries has been demonstrated through the isolation of rarely expressed cDNAs (Sagerström et al. 1997). Recently, Gong et al. (2001) reported the construction of PCR-based subtractive libraries. This group carried out repetitive rounds of subtractions followed by nucleotide sequencing and Northern-blot analyses, and identified 84 salt-regulated genes in *Arabidopsis*.

All-India evaluation studies have shown that CSR 27 is a highly salt-tolerant rice cultivar that shows better performance when grown under salty tracts (Mishra and Singh 2000). Pokkali rice is a local selection in India noted for high salt tolerance. This rice type has in fact often been taken as a check in several national and international programs aimed at evolving genotypes combining high salt tolerance and high crop yield (Akbar et al. 1986a, b). Extensive physiological and molecular studies have been carried out employing Pokkali rice for understanding the mechanisms of salt tolerance (Moons et al. 1995; Kawasaki et al. 2001). Pusa basmati 1 (PB 1) is a premier rice type from India combining high yield with a much sought-after "basmati" flavor. In this study, our goal was to study the comparative response of these rice types with respect to their salt-induced transcript regulation. We employed the following four subtractive screenings: (1) Pokkali salt-stressed root (tester) – PB 1 salt-stressed root (driver) (T2D1), (2) CSR 27 salt-stressed root (tester) – PB 1 salt-stressed root (driver) (T4D1) (3) CSR 27 salt-stressed root (tester) – Pokkali salt-stressed root (driver) (T4D2), and (4) Pokkali salt-stressed shoot (tester)-CSR 27 salt-stressed shoot (driver) (T3D5). Several known and unknown cDNA clones that were found to be associated with NaCl stress response in different contrasting rice types are reported in this study.

Materials and methods

Rice seeds obtained from different sources (CSR 27 and Pokkali – Central Soil Salinity Research Institute, Karnal, India; PB 1 – Indian Agricultural Research Institute, New Delhi, India) were washed with mild detergent solution and rinsed with distilled water. Seeds were surface-sterilized by the following treatments: 70% ethanol, 45 s; 1.2% sodium hypochlorite, 20 min and 0.1% mercuric chloride, 10 min. Subsequently, seeds were rinsed thoroughly with distilled water and were placed on a wet cotton pad for 1 week. Uniform-sized seedlings were subjected to 250 mM of NaCl (for up to 96 h) at 28 ± 2 °C in a growth chamber (Conviron, USA) with a light and dark cycle of 14 h and 10 h, respectively. In a separate experiment, 1-week-old seedlings were initially transferred to 150 mM of NaCl for 24 h (for pre-treatment at a sub-lethal stress level) and later to 300 mM of NaCl for 96 h. After stress treatments, seedlings were placed for recovery on distilled water and their growth was examined during the recovery phase.

Total RNA from seedlings treated with 150 mM of NaCl for 24 h was isolated as described by Chomczynski and Sacchi (1987). mRNA was enriched from the total RNA population by using a PolyATract mRNA isolation kit (Promega Inc., USA). First-strand cDNA synthesis was initiated from the enriched mRNA of tester and driver using different poly dT-linker primers (with the sequence 5′ TATAGATCTGCGGCCGCAAGCTTTTTTTTT 3′ for the tester and 5′ GTAATACGACTCACTATAGGGTTTTTTTT 3′ for the driver) and Superscript RNaseH$^-$ reverse transcriptase (Gibco-BRL, USA).

The double-stranded mRNA-cDNA hybrids were converted to a single-stranded cDNA population by digestion of mRNA with RNaseH. First-strand cDNA was purified using Qiaquick columns (Qiagen, Germany), and specific oligonucleotides (with sequences 5′ GCTAGCATATGGGCCCGAATTCC 3′ for the tester and 5′ CCCTTTAGTGAGGGTTAATTTC 3′ for the driver) were ligated at the 3′ end of the respective cDNA populations using T_4 RNA ligase (Roche, Germany). The first-strand cDNA of the driver was amplified using excess of the biotin-labeled forward primer (corresponding to the respective ligated oligonucleotide) and a reverse primer (corresponding to the respective linker-primer). The PCR was performed as follows: at 94 °C for 3 min; for 20 cycles at

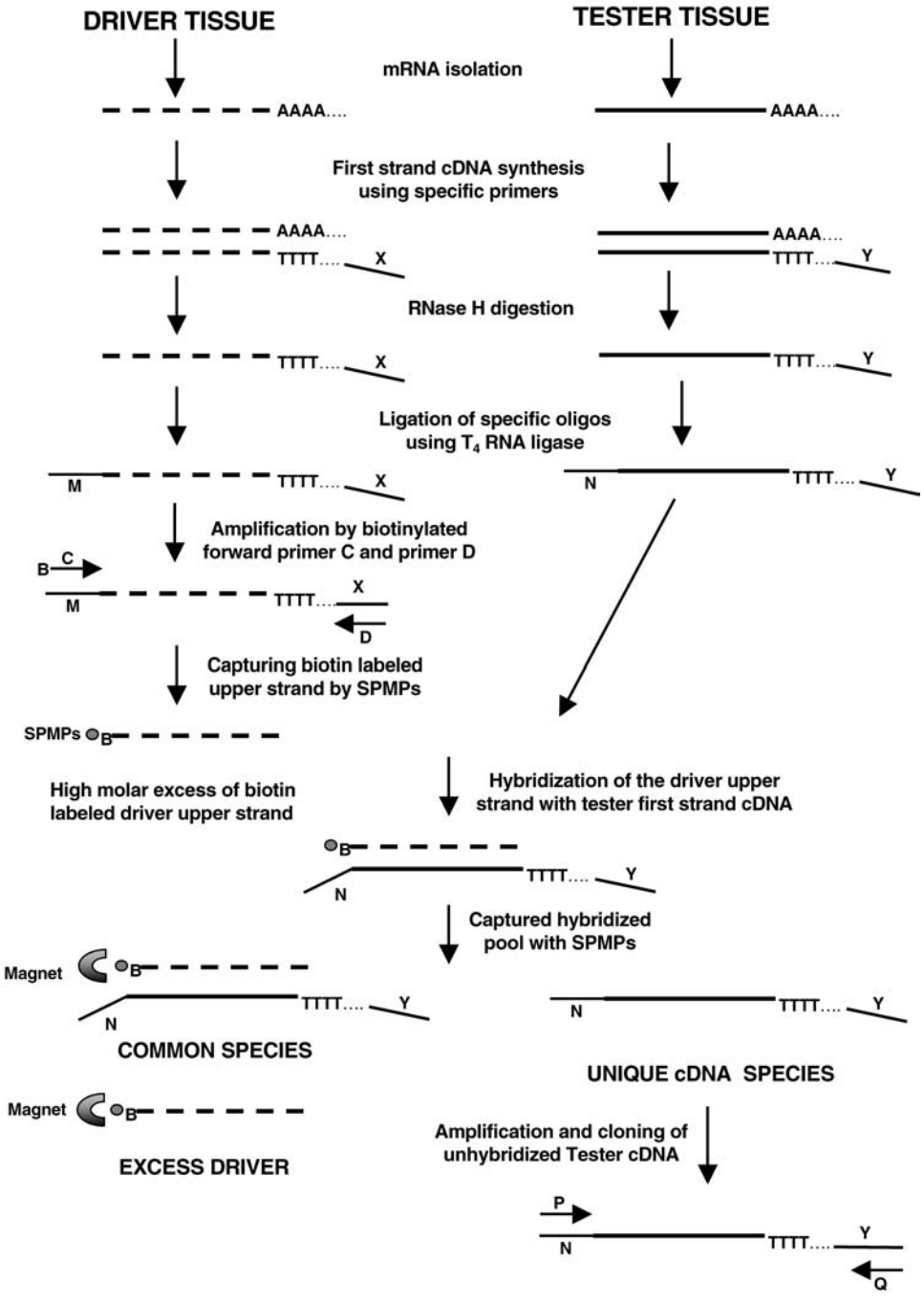

Fig. 1 Steps involved in the construction of subtractive cDNA libraries. mRNA was isolated and cDNA was synthesized using specific primers for the driver (*X*) and tester (*Y*). Specific oligonucleotides (*M* for driver and *N* for tester) were ligated to the first-stand cDNA using T_4 RNA ligase. The driver cDNA population was amplified using the biotinylated forward primer C and primer D to obtain high molar excess of the driver. The biotin-labeled upper strand of the driver was captured after denaturation by streptavidin paramagnetic particles. The biotin-labeled second-strand cDNA of the driver was hybridized with the tester first-strand cDNA. The excess driver and the common cDNA species that hybridized to the driver were removed. The unique species left in the pool were amplified using primers *P* and *Q* and cloned

94 °C for 1 min, 50 °C for 1 min and 72 °C for 4 min; and at 72 °C for 10 min. The double-stranded cDNA of the driver was heat-denatured at 94 °C for 5 min and immediately the biotin-labeled upper strand was captured using streptavidin paramagnetic particles (SPMPs; Roche, Germany). The captured upper strand was hybridized with the first-strand cDNA population of the tester for 16 h at 65 °C. The tester-driver hybrids and excess of the driver were separated from the unhybridized tester cDNA using SPMPs. Tester cDNAs that did not hybridize with the driver were amplified using primers specific to the respective ligated oligonucleotide and linker primers (with the sequence 5′ CGA-T-C-GTA-TACCCGGGCTTAAGG 3′ and 5′ TATAGATCTGCGGCCGCA-AGC 3′ respectively). The PCR was performed as follows: at 94 °C for 3 min; for 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 4 min; and at 72 °C for 10 min. The PCR product was purified using a Qiaquick column (Qiagen, Germany) and digested with *Apa*I and *Not*I (the sites for these enzymes were present in the ligated oligonucleotide and the linker primer, respectively) and cloned in *Apa*I/*Not*I-digested pBCSK (–) (Stratagene, USA) using T_4 DNA ligase (Roche, Germany). The steps involved in construction of subtractive cDNA libraries are shown in Fig. 1.

Following transformation, *Escherichia coli* cells were screened for the insert size by PCR using T_3 and T_7 primers. The DNA sequence of the selected clones was determined by sequencing their 3′ end using T_3 primer either manually by the Thermosequenase Cycle sequencing kit (USB, USA) or by the dye terminator cycle sequencing method (model ABI Prism 3700). Sequence were ana-

lyzed using DNASTAR analysis software. Sequence homologies were examined with the GenBank/EMBL databases using the BLAST program (Altschul et al. 1997).

For Northern analysis, total RNA from 6- to 7-day old uniform-sized rice seedlings (subjected to 150 mM NaCl stress for 24 h) was isolated as described above and 15 μg of total RNA was electrophoresed on a 1% agarose gel made in 1×morpholinopropane sulphonic acid (MOPS) and 1.2% formaldehyde. RNA was transferred to the nylon membrane by capillary action. The membrane was hybridized with radiolabeled PCR amplified fragments of different clones. The amplified fragments were radiolabeled by the random primer extension labeling kit (NEN, USA). Hybridizations were carried out at high stringency (42 °C in the presence of 50% formamide) as described by Sambrook et al. (1989).

Results

As adjudged by the relative effects on the growth of shoot and root axis, the extent of root branching and visual changes in leaf chlorophyll content, it was noted that CSR 27 and Pokkali withstood salt stress to a greater extent than PB 1 (Fig. 2I). It was further observed that the seedlings exposed to high salt concentration survived better when pretreated with mild salt stress (150 mM NaCl, 24 h), as compared to those exposed directly to lethal salt levels (300 mM NaCl, 96 h; Fig. 2II).

In all, 1,266 putative cDNA clones were isolated in this study and their numerical representation was as follows: 333 clones from T2D1, 230 from T4D1, 250 from T4D2 and 463 from T3D5 subtractions. 85 randomly selected clones were sequenced from their 3′ ends. The accession numbers and homology of these clones are presented in Table 1. From the nucleotide sequence (or the corresponding amino-acid sequence) data, it emerged that the salt-stress associated clones in this study were strongly or weakly matched with established stress genes from rice or other plant species, or showed matching with sequences in the genomic database. Some of the clones for which the genomic sequences are available have been annotated while the others have not been annotated as yet. We also found some clones that showed homology to the sequences in the EST database. Finally there were six clones that showed no similarity/homology with sequences in any of the public database (Table 1).

Salt stress-associated transcript expression pattern was analyzed for 45 randomly chosen clones. The transcripts corresponding to several of these clones were found to be constitutive while a few were negatively regulated by salt stress. Clones like T4D2-**216,** T4D2-**165**, T4D2-**50**,T4D2-**66**, T4D2-**96** and T4D1-**5** were positively regulated by salt stress. Importantly, some of these clones exhibited a differential expression pattern in the contrasting cultivars studied. The transcript for T4D2-**216** was not detected in roots of Pokkali (both in control and in stressed conditions) but was induced by salt stress in the roots of CSR 27. Transcripts corresponding to T4D2-**66** and T4D1-**5** were found to be salt inducible in the driver (roots of Pokkali and PB 1, respectively) but were present in constitutively high amounts in both unstressed and stressed root tissues of the tester (CSR 27).

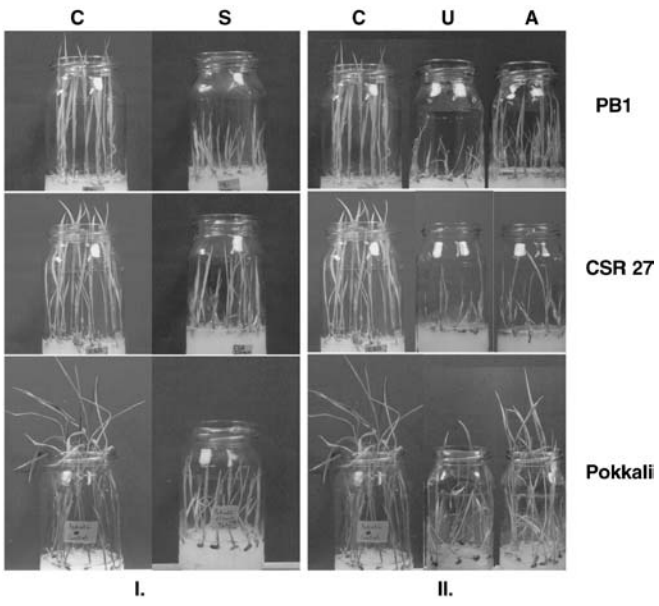

Fig. 2 Effect of salt stress on the growth of rice seedlings. (**I**) Examination of basal salt tolerance. One-week-old seedlings of Pusa basmati 1, CSR 27 and Pokkali rice cultivars were transferred to 250 mM of NaCl for 96 h. Subsequently, seedlings were transferred to distilled water for recovery (*S*). Control seedlings were maintained on distilled water all through (*C*). (**II**) Examination of acquired salt-stress tolerance. One-week-old seedlings of the above rice cultivars were transferred to 150 mM of NaCl for 24 h for adaptation, and then transferred to 300 mM of NaCl for 96 h. Subsequently, seedlings were transferred to distilled water for recovery (*A*). A set of seedlings was directly subjected to the 300-mM NaCl stress for 96 h and then transferred to distilled water (*U*). Another set of seedlings was maintained on distilled water all through (*C*). Plants were photographed after 12 days of recovery. Cultivar names are indicated on the right of the panel

There were certain clones whose expression was not affected by NaCl stress, but a difference in their constitutive levels was observed in the two contrasting cultivars studied. The constitutive level of the T2D1-**288** transcript was detected in the roots of Pokkali (both unstressed and stressed), but this transcript was not detected under similar conditions in the roots of PB 1. The T2D1-**92** transcript was constitutively expressed in both stressed and unstressed tissues of PB 1 and Pokkali roots, but the relative levels of the transcript were higher in roots of Pokkali than PB 1. Details of the expression patterns of selected clones are shown in Fig. 3. Additionally, some clones (i.e. T2D1-**273**, T4D2-**70**) showed barely detectable levels of transcripts.

Discussion

Growth of CSR 27 and Pokkali seedlings was greater in the recovery phase as compared to PB 1 after the application of 250 mM of NaCl for 96 h (Fig. 2I). CSR 27 and Pokkali have been previously shown to be salt-tolerant rice cultivars (Akbar et al. 1986a; Mishra and Singh

Table 1 cDNA clones isolated by subtractive cloning of salt-stressed libraries of rice. *Sequence not submitted

No.	Accession number	Homology
T2D1-10	AJ302302	Ubiquitin fusion protein
T2D1-81	AJ302307	Unknown, homologous to rice dbEST ID-9680170
T2D1-92	AJ302301	*salT* gene
T2D1-99	AJ420731	Unknown, homologous to rice dbEST ID-9108490 (drought-stress library)
T2D1-101	AJ300705	Not annotated, homologous to rice dbEST ID-7009078
T2D1-104	AJ420729	Little homology to the metallothionin gene
T2D1-111	AJ302306	Histone H4
T2D1-117	*	Cyclin-H1 mRNA (interacts/activates CDK-activating kinase)
T2D1-142	AJ420734	Novel
T2D1-157	AJ420730	Unknown, homologous to rice dbEST ID-1972932
T2D1-159	AJ420728	Putative acetyl transferase
T2D1-167	AJ420701	Unknown, rice dbEST ID-8451453
T2D1-185	AJ420702	Cytochrome c oxidase subunit 6b-1
T2D1-198	*	Ribosomal protein 117 (249) gene
T2D1-207	*	Novel, little homology to the ripening-regulated protein
T2D1-221	AJ420733	Unknown, little homology to rice dbEST ID-9108322 (drought-stress root library)
T2D1-247	AJ420735	Unknown, homologous to rice dbEST ID-2463178
T2D1-256	AJ420703	Unknown, homologous to rice dbEST ID-R0992
T2D1-273	AJ302303	EREBP
T2D1-274	AJ302300	Putative tumour supressor
T2D1-276	AJ302304	Unknown, homologous to rice dbEST ID-10994959
T2D1-288	AJ302305	Ribosomal protein R41
T2D1-291	AJ420704	Unknown, homologous to rice dbEST ID-9108490 (drought-stress library), little homology to *Arabidopsis thaliana* transporter-like protein
T2D1-313	*	GRP
T3D5-13	AJ312764	ADP-ribosylation factor gene
T3D5-15	AJ420705	PFK2/Fructose-2, 6,-bisphosphatase
T3D5-19	AJ312765	Not annotated, homologous to rice dbEST ID-9680722
T3D5-25	AJ420706	Unknown, homologous to rice dbEST ID-9684137
T3D5-50	AJ312766	Putative peroxidase isozyme 38 kDa precursor
T3D5-53	AJ312767	Putative H^+ transporting ATPase
T3D5-70	AJ312768	Unknown, homologous to rice dbEST ID-4056931
T3D5-92	AJ420707	60S ribosomal protein
T3D5-284	AJ312769	Unknown, homologous to rice dbEST ID-9671617
T3D5-302	AJ312770	Translationally controlled tumor protein
T3D5-324	AJ312771	Little homology to histone H1B
T3D5-329	*	GRP
T3D5-362	*	GRP
T4D1-5	AJ312756	Novel
T4D1-9	*	L32 ribosomal protein
T4D1-11	AJ420721	Novel
T4D1-15	*	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D1-28	AJ420723	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D1-32	AJ312757	YK426 mRNA induced by GA3
T4D1-34	AJ312758	Little homology to At 4-coumarate co-A ligase (*4CL*)
T4D1-37	AJ420720	Little homology to aspartate aminotransferase
T4D1-38	AJ312759	Little homology to alanine aminotransferases
T4D1-43	AJ420708	4-coumarate co-A ligase
T4D1-49	AJ420718	Ribosomal protein 117 (249)
T4D1-56	AJ312760	Unknown, little homology to *Sorghum bicolor* dbEST ID-4125290 (water stress library), homologous to rice dbEST ID-3106446
T4D1-58	AJ420724	Unknown, homologous to rice dbEST ID-9686348, 1973841
T4D1-61	AJ312761	Unknown, homologous to *Medicago* sp. dbEST ID-8152179 (drought library), homologous to rice dbEST ID-8024712
T4D1-77	AJ420700	EN242 (wound inducible basic protein)
T4D1-82	*	GRP
T4D1-86	*	Little similarity to homologous rice dbEST ID-9021699
T4D1-88	AJ420709	Ribosomal protein L 27
T4D1-98	AJ420710	Not annotated, homologous to rice dbEST ID-8890656
T4D1-99	AJ312762	Little homology to Cu chaperone homolog
T4D1-147	*	YK 426 mRNA induced by GA3
T4D1-144	AJ420711	Zn finger-like protein
T4D1-148	AJ420722	Unknown, homologous to rice dbEST ID-9680170
T4D1-163	AJ420712	Unknown, homologous to rice dbEST ID-3071760 (salt-stressed library)
T4D1-209	AJ312763	No hit in rice EST database, homologous to barley dbEST ID-10859857
T4D1-213	*	Putative alanine aminotransferase
T4D2-50	AJ298288	Not annotated, very little homology to rice dbEST-ID 9109651

Table 1 (continued)

No.	Accession number	Homology
T4D2-55	AJ302310	NADP dependent malic enzyme
T4D2-57	AJ298283	Unknown, homologous to rice dbEST ID-1686112
T4D2-61	AJ420713	16S rRNA gene
T4D2-64	*	PAB1 mRNA for alpha 2 subunit of the 20 S protein
T4D2-66	AJ298284	Unknown, homologous to rice dbEST ID-9678169
T4D2-70	AJ298285	Similar to the ascorbate peroxidase precursor EST from *Mesembryanthemum*
T4D2-72	AJ420714	Little similarity to homologous rice dbEST ID-1983692
T4D2-76	AJ298286	Not annotated, homologous to rice dbEST ID-4689497
T4D2-96	AJ298287	*SalT* gene
T4D2-106	AJ420732	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D2-117	AJ302308	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D2-141	AJ302309	Novel
T4D2-147	AJ420715	YK 426 mRNA induced by GA3
T4D2-152	AJ420716	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D2-154	AJ420726	Nucleotide diphosphate kinase
T4D2-162	AJ420737	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D2-165	AJ420725	Not annotated, homologous to rice dbEST ID-9109651 (drought-stress library)
T4D2-189	AJ420736	Not annotated, homologous to rice dbEST ID-1686112
T4D2-191	AJ420717	Tetratricopeptide repeat protein
T4D2-216	AJ420727	Novel
T4D2-302	AJ420719	Little homology to the Zn-finger transcription factor

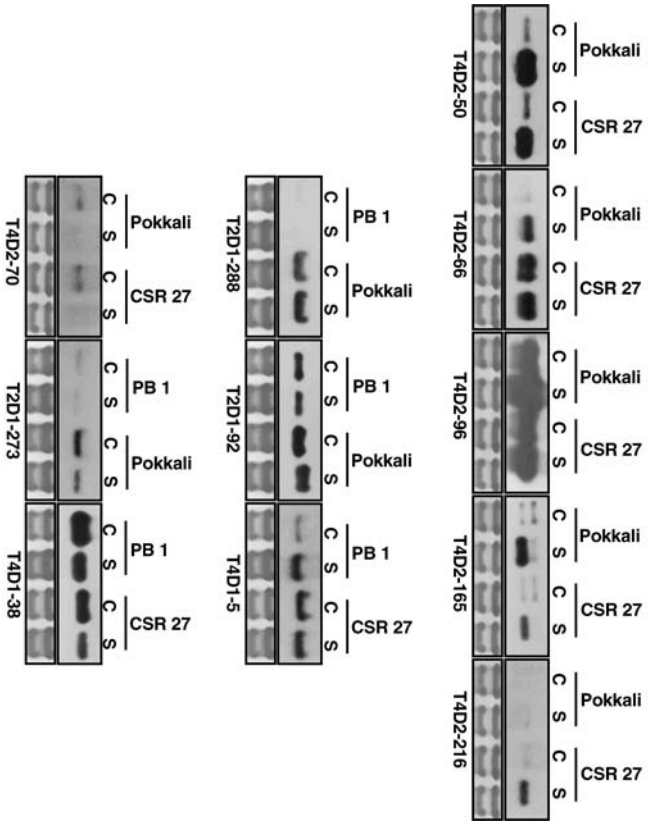

Fig. 3 Northern analyses of selected ESTs; 15 µg of RNA isolated from roots of control (C) and salt-stressed (S) seedlings was resolved on denaturing agarose gel. The membrane was probed with radiolabeled probes of these ESTs. Rice varieties used are indicated at the top and the ESTs at the bottom of each panel. Equal quantities of RNA loading is depicted by comparable intensities of methylene blue-stained bands of rRNA as shown in the panels below each blot

2000). Our results further substantiate higher salt tolerance in these rice cultivars. In general, plants are known to acquire a high level of salt stress tolerance if pretreated with sub-lethal levels of NaCl stress (Amazallag et al. 1990). We also found that pretreatment with 150 mM of NaCl for 24 h could induce increased salt tolerance against subsequent lethal stress of 300 mM of NaCl for 96 h (Fig. 2II). This effect of acquired salt tolerance was seen in all the three cultivars tested. Since the adaptive effects of sub-lethal stress could be attributed to the expression of stress-regulated genes in response to the pretreatment, subsequent experiments were aimed at the isolation of genes that are differentially regulated in response to 24 h of 150 mM NaCl stress in these contrasting rice types. The use of contrasting germplasm is considered to be an effective strategy for the isolation of stress-regulated genes that can possibly be correlated with stress tolerance (Grover et al. 1993; Blomstedt et al. 1998).

Nucleotide and the corresponding amino-acid sequence data revealed that several clones isolated in this study were significantly homologous to salt- or stress-regulated genes/proteins isolated from rice or other plant species (Table 1). These include SalT, glycine-rich RNA binding proteins (GRPs), ADP-ribosylation factor (ARF), NADP dependent malic enzyme, Mub ubiquitin fusion protein, tumour suppressor genes, 4-coumarate co-A ligase, EN 242-wound inducible protein, nucleoside diphosphate kinase (NDPK) and tumor protein. SalT is an organ-specific 15-kDa glycine-rich cytosolic protein of unknown function regulated by salt, dehydration, ABA and proline (Claes et al. 1990; Garcia et al. 1997). GRPs represent a diverse family of genes that are regulated by viral attack, mechanical injury, low temperature, ABA and dehydration. These proteins are thought to

play a regulatory role involving differential stabilization, repression or translation of mRNAs in stressed leaves and desiccating embryos. In addition, selective RNA-binding proteins have been implicated in differential transcript splicing in response to developmental and environmental cues (Bray 1994; Sachetto-Martins et al. 2000). A metabolic shift from the C3 to the CAM mode of carbon assimilation has been well documented under increased salt stress (Höfner et al. 1987; Schmitt et al. 1988). This pathway requires coordinated salt-induced expression of several genes in the pathway. This includes the NADP malic enzyme, which is induced by salt stress (Cushman 1992). The ARF protein is regulated by salt and is involved in salt-stress signalling (Chen et al. 1994; Kiyosue and Shinozaki 1995). This gene was earlier identified as an EST from a salt-stressed cDNA library of rice (Umeda et al. 1994). The transcript corresponding to the NDPK gene is strongly up-regulated by salt stress in Pokkali rice (Kawasaki et al. 2001). Salt induced up-regulation of the translationally controlled tumor protein has also been reported. This protein is believed to play a role in salt-stress signalling via calcium binding (Gong et al. 2001).

We identified clones that were partially homologous to known genes. This category included clones showing homology to the ethylene response-element binding protein (EREBP), alanine aminotransferase, copper chaperone, aspartate aminotransferase, ripening regulated protein, metallothionin and Zn-finger transcription factor. EREBP and Zn-finger proteins are plant transcription factors, which are differentially regulated by a variety of environmental cues and regulate the expression of a large number of stress-associated genes (Kasuga et al. 1999; Kim et al. 2001; Park et al. 2001). It is possible that the isolated ESTs share certain domains with known genes/proteins. Full-length sequencing and characterization of such clones is needed for defining their functionality.

Nineteen clones were appreciably homologous to sequences reported in the genomic database. Out of these, 13 clones have not been annotated while six have been assigned putative functions. The homologs of some of the annotated clones are shown to be salt-regulated. These include clones that correspond to H^+-ATPase (Serrano and Rodriguez-Navarro 2001; Vitart et al. 2001), peroxidase (Botella et al. 1994) and the tricopeptide repeat-containing protein (Kawasaki et al. 2001). The sequences that have not yet been annotated but show homology to clones isolated in this study might have a role in stress response. For instance, the T4D2-**165** sequence showed homology to the nucleotide sequence in the rice genome database. This clone appears to have a stress-related function since Reddy et al. (2002) obtained the corresponding EST (EST ID. 9109651) from a normalized cDNA library constructed from drought-stressed leaves of rice. We further noted in this study that the transcript corresponding to the T4D2-**165** sequence was salt inducible (Fig. 3).

Twenty nine clones isolated in this study showed insignificant homology to any nucleotide (or the corresponding amino-acid) sequence in the existing database, indicating that these represent novel clones. On further analysis, we found that out of these 29 clones, 23 were significantly homologous to specific ESTs in the dbEST libraries. For instance, clones T4D1-**56,** T4D1-**61** and T4D1-**163** were found to be homologous to sequences with ID. 4125290 (from the water-stressed library of *Sorghum bicolor*), ID. 8152179 (from the drought-stressed *Medicago* sp.) and ID. 3071760 (from the salt-stressed rice tissues), respectively. Representation of such clones in our libraries and libraries constructed from stressed tissues is an indication of their putative role in stress response. Further, 14 clones isolated in this study showed homology to genes that have not been directly implicated in stress tolerance. This group included genes for ribosomal proteins, selective GA regulated genes, histones and fructose 6-bis-phosphatase. However, some of the histone variants are induced by water stress and ABA (Wei and O'Connell 1996). Posas et al. (2000) and Kawasaki et al. (2001) have reported the induction of several genes for ribosomal proteins during salt stress in yeast and rice, respectively. It is possible that these proteins may also have some role to play in the salt-adaptation process.

There is a strong correlation between the accumulation of specific genes/proteins and the degree of stress tolerance (Blomstedt et al. 1998). We infer that the increased expression levels of selected clones during salt stress in tolerant cultivars can be associated to their higher salt tolerance. Transcriptional induction of selected genes in response to salt stress has been recognized as an adaptive mechanism of plants against salt stress (Cushman and Bohnert 2000). With the documentation of differentially expressing clones in our study and other related studies (Gong et al. 2001; Kawasaki et al. 2001), it is possible to find newer avenues for engineering salt tolerance in plants.

There are two major points emerging from this study that need further consideration. Firstly, it has been proposed that the transcriptional response against salt stress is an early and transient event (Posas et al. 2000; Kawasaki et al. 2001). Posas et al. (2000) stated that many salt-inducible mRNAs could be missed if the cells are exposed to the stress for too long. As the salt stress duration in the present study was for 24 h, there are chances that we might have missed the early expressing genes. Thus, both the timing and severity of stress need to be taken into account when studying the transcriptional response. Second, the method adopted for the isolation of salt stress-associated cDNA clones in this study showed several false positives. Theoretically, we would expect that all the clones isolated in the present screen should have higher levels of transcripts in the tester as compared to driver samples, but this was not the case. For instance, Northern analysis showed that transcripts corresponding to T4D2-**50** and T4D2-**165** were expressed strongly in the driver samples as well. It is possible that the transcripts corresponding to these clones were present in higher amounts in both the driver and the

tester, which could not be subtracted employing one round of subtraction. It has been proposed that subtractive hybridization techniques do not achieve sufficient enrichment of the sequences that occur only in the tester (the target) partly because of the high complexity of the genomes (Lisitsyn et al. 1993). The increased genomic complexity prevents effective and complete hybridization and thus removal of the driver sequences.

From the data obtained in this study, it is clear that there are several unknown components in the salt-stress response. We infer that cellular responses to salt stress involve coordinated positive and negative regulation of several genes, as has been reported for drought stress (Blomstedt et al. 1998). We believe that when a single type of salt (NaCl), a single concentration (150 mM) and one time period (24 h) revealed such genetic complexity, the salt-stress response under field conditions would be more complex. The novel ESTs found differentially regulated in salt-tolerant and -sensitive cultivars in this study need further characterization. Although the molecular action of such clones remains illusive, their role in stress tolerance seems possible. The presence of considerable information in the public database offers an unmatched opportunity to characterize stress-responsive genes in the future.

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