Salt stress response in rice: genetics, molecular biology, and comparative genomics

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Abstract Significant progress has been made in unraveling the molecular biology of rice in the past two decades. Today, rice stands as a forerunner amongst the cereals in terms of details known on its genetics. Evidence show that salt tolerance in plants is a quantitative trait. Several traditional cultivars, landraces, and wild types of rice like Pokkali, CSR types, and *Porteresia coarctata* appear as promising materials for donation of requisite salt tolerance genes. A large number of quantitative trait loci (QTL) have been identified for salt tolerance in rice through generation of recombinant inbred lines and are being mapped using different types of DNA markers. Salt-tolerant transgenic rice plants have been produced using a host of different genes and transcript profiling by micro- and macroarray-based methods has opened the gates for the discovery of novel salt stress mechanisms in rice, and comparative genomics is turning out to be a critical input in this respect. In this paper, we present a comprehensive review of the genetic, molecular biology, and comparative genomics effort towards the generation of salt-tolerant rice. From the data on comprehensive transcript expression profiling of clones representing salt-stress-associated genes of rice, it is shown that transcriptional and translational machineries are important determinants in controlling salt stress response, and gene expression response in tolerant and susceptible rice plants differs mainly in quantitative terms.

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Salt stress in rice cultivation

Although Na$^+$ is a plant micronutrient, excess Na$^+$ levels are toxic for plant growth. Salinity adversely affects quantity and quality of the crop produce (Gepstein et al. 2006; Blumwald and Grover 2006). About 20% of irrigated agricultural land is considered to be saline (Flowers and Yeo 1995). Salt-affected soils in arid and semi-arid regions of Asia, Africa, and South America cause considerable agronomic problems. In Asia alone, 21.5 million ha of land area is thought to be salt affected (of which 12 million ha is due to saline conditions and the remaining 9.5 million ha is due to alkaline/sodic conditions), with India having 8.6 million ha salt-affected area including 3.4 million ha sodic soils. It is further important to note that salt stress problems in field crops are getting accentuated with the increasing irrigation networks.

Rice is an important food crop for the entire world population. While active efforts are being made to increase rice productivity, a considerable amount of rice biomass for which genetic potential exists in the present-day cultivars is not harvested under field conditions, primarily because of the sensitivity of this crop to various stresses (Widawsky and O'Toole 1990; Shimamoto 1999; Minhas and Grover 1999). Rice is a salt-sensitive crop (Grover and Pental 2003).

Excess salts adversely affect all major metabolic activities in rice including cell wall damage, accumulation of electron-dense proteinaceous particles, plasmolysis, cytoplasmic lysis and damage to ER, accumulation of citrate, malate, and inositol in leaf blades within 1 d of salt treatment, increase in proline levels by 4- to 20-fold, decrease in Fv/Fm ratio, reduction in photosynthesis, and overall decline in germination and seedling growth (Yeo et al. 1985; Lutts et al. 1995; Garcia et al. 1997; Khan et al. 1997; Pareek et al. 1997; Sivakumar et al. 1998), leading ultimately to reduced growth and diminished grain yield. Relatively speaking, rice is considered as more sensitive to salts during early seedling than at reproductive stages (Flowers and Yeo 1981; Lutts et al. 1995). The parameters like high vigor, salt exclusion at the root level, compartmentalization of ions in structural and older tissues, high tissue tolerance, responsive stomata that close within minutes after exposure to salt stress but partially reopen after a period of acclimation, and up-regulation of antioxidant systems, particularly the ascorbate/glutathione pathway of oxidative stress tolerance, are among the major factors that govern the growth-stage-dependent response of rice to salt application.

A large body of evidence indicates that the response of plant cells to high salts is controlled by multiple genes (Bartels and Sunkar 2005; Chinnusamy et al. 2005; Sahi et al. 2006). To maximize the productivity of rice under saline soils, there is an urgent need to look for: (1) sources of genetic variation that can be used for developing new cultivars with greater yield potential and stability over seasons and ecogeographic locations, (2) identification of molecular markers associated with salt stress tolerance genes or QTL conferring tolerance to salt stress conditions for their use in marker-assisted breeding programs, and (3) discovery of genes imparting salt tolerance and their introduction in salt-sensitive rice cultivars (Blumwald and Grover 2006). For reasons such as the availability of well-saturated genetic maps (Harushima et al. 1998; Chen et al. 2002; McCouch et al. 2002), complete sequence information regarding the chloroplast, mitochondrial and nuclear genomes (Kanno and Hirai 1993; Notsu et al. 2002; IRGSP 2005), full-length cDNAs (Kikuchi et al. 2003), full-genome transcription profilings using high-density oligonucleotide tiling microarrays (Li et al. 2006), Tos17 insertion lines and enhancer trap lines (Miyao et al. 2003; Hirochika et al. 2004; Sallaud et al. 2004) and highly-efficient Agrobacterium-mediated genetic transformation (Minhas and Grover 1999; Roy et al. 2000), rice in recent years has emerged as a model species for understanding the cereal genetics (see special issue of Plant Molecular Biology, http://www.springerlink.com/openurl.asp?genre=issue&eissn=1573-5028&volume=59&issue=1).

To facilitate the comparison and the understanding of gene characteristics, genome organization, pathways, and phenotypes in cereals, there are attempts to develop a comparative genome mapping database for grasses using the rice genome as an anchor (http://www.gramene.org) (Jaiswal et al. 2006). We herewith discuss the progress made in understanding the details of the salt stress response using various tools like traditional plant breeding methods, marker-assisted selection-based approach, and transgenic rice production technique, and, finally, through the tools of comparative genomics.

Traditional breeding for augmenting salt tolerance in rice

The generation of salt-tolerant rice varieties through traditional breeding approach can only be realized if the genes for salt tolerance occur in the gene pool of rice species or in its closely related wild species. In a comparison of the relative tolerance of seven wild species and two cultivated species of rice (Oryza sativa and Oryza glaberrima), none of the wild species was found to be as tolerant as the most resistant of the cultivated lines of O. sativa tested (Akbar et al. 1987). According to Flowers et al. (1990), source(s) of salt tolerance is unavailable within the cultivated germplasm of O. sativa. Nonetheless, there are clear indications that some traditional cultivars and landraces of rice (e.g., Pokkali, Nona Bokra, Bura Rata, etc.) are more salt tolerant than many elite cultivars. In fact, Pokkali rice has been frequently used as a donor of salt tolerance genes in breeding programs. 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). The increased salt tolerance of Pokkali is usually attributed to both its capacity to maintain a low Na^+/K^+ ratio in shoot tissues and its faster growth rate under saline conditions. According to Anil et al. (2005), Na^+ is buffered in shoots of Pokkali by intracellular compartmentalization which is a basis of its robust salt stress response. A recombinant inbred population was developed at the International Rice Research Institute (IRRI), Philippines, using Pokkali and IR29 (IR29 is an improved indica cultivar often used as a salt-sensitive standard) (Bonilla et al. 2002). Several other salt-tolerant and salt-sensitive recombinant inbred lines (RILs) have also been identified in screening for salinity tolerance (Gregorio et al. 2002). Genetic studies have revealed that salt tolerance of these varieties is principally due to additive gene effects; however, the underlying molecular mechanism of salt tolerance has barely been experimentally investigated. At the Central Soil Salinity Research Institute (CSSRI), Karnal, India, CSR27 rice type

Table 1 Selected reports on QTL associated with salt tolerance in rice

Trait	Mapping population	Type of mapping population	Number of QTL mapped	Reference
Na^+, K^+ uptake and concentration	Nona Bokra/Pokkali//IR4630-22- 2-5-1-3/IR10167-129-3-4	RILs	16	Flowers et al. (2000)
Na^+, K^+ uptake and concentration	Nona Bokra × Koshihikari	F_2-F_3	11	Lin et al. (2004)
Dry mass, Na^+, K^+ uptake, concentration and ratio	IR4630 × IR15324	RILs	25	Koyama et al. (2001)
Na^+, K^+ absorption		RILs	2	Bonilla et al. (2002)
Growth in saline water	Nipponbare × Kasalath	BIL	27	Takehisa et al. (2004)
K^+/Na^+ homoeostasis	Nona Bokra × Koshihikari	NILs	1	Ren et al. (2005)

has been bred through crossing Nona Bokra and IR 5657-33-2 rice types. CSR27 variety shows high tolerance to sodic (pH 9.6–9.9) and saline [electronic conductivity (EC) up to 9 dS m^{-1}] soils. Another cultivar developed at CSSRI is CSR10 for sodic and inland saline soils. It is derived from the cross M40-431-24-114 and Jaya. CSR10 can withstand highly deteriorated alkaline (pH 9.8–10.2) and inland saline soil (EC 6–10 dS m^{-1}) conditions under transplanted irrigated management system. Other promising rice types identified include Pobbeli (Indonesia), IR2151 (Sri Lanka), PSBRc 48, PSBRc 50, PSBRc 84, PSBRc 86, PSBRc 88, and NSIC 106 (IRRI, Indonesia). *P. coarctata* Tateoka, a native of salt marshes in Southeast Asia and considered a wild relative of *O. sativa*, is found to be highly salt tolerant (Flowers et al. 1990). It is suggested that *P. coarctata* can be used as a parent for evolving better salt-tolerant rice varieties. Majee et al. (2004) showed that L-*myo*-inositol-1-phosphate synthase (MIPS) enzyme (which catalyzes synthesis of inositol) from *P. coarctata* is more salt tolerant than MIPS from *O. sativa* under in vitro conditions of testing. It is further revealed that the structure of *P. coarctata* MIPS is relatively more stable towards high salt concentrations. An introgression of this gene rendered transgenic tobacco capable of growth in 200–300 mM NaCl with retention of ~40–80% of photosynthetic competence with concomitant increased inositol production compared with the unstressed control tobacco plants (Majee et al. 2004). MIPS protein isolated from transgenic tobacco plants also showed salt-tolerant properties in vitro. The same group is presently attempting to study how salt-sensitive rice type will respond if the MIPS protein from *P. coarctata* is transgressed in it, through transgenic methods (A. Lahiri-Majumdar, personal communication).

Finally, the fact remains that the landraces or the wild types discussed above are associated with a host of inherent problems of poor agronomic traits (such as tall plant stature, photo-sensitivity, poor grain quality and low yield).

Therefore, the breeding for increased salt tolerance using such materials remains as a distant possibility.

Molecular markers and QTL linked to rice salt tolerance

Molecular/genetic rice maps have been constructed using F_2 population or RILs derived from varieties that are genetically far apart, such as japonica and indica rice types as parents. Such a combination generates considerably more polymorphism than that between the same subspecies. Because of the ability to generate a large seed supply, RILs have been used to map several QTL in rice (Wang et al. 1994; Tsunematsu et al. 1996; Xiao et al. 1996; Nandi et al. 1997). Doubled haploid (DH) populations have also been used (Lorieux et al. 1996; Yadav et al. 1997); however, extensive use of DH plants is limited due to difficulty in generating sufficient number of plants through anther culture. Backcrosses and other types of crosses have not been widely used in rice gene mapping. McCouch et al. (1988) reported the first molecular genetic map of rice using RFLP technique; various fine maps have since been released using a variety of different markers including simple sequence repeat (SSR), amplified fragment length polymorphism, and random amplified polymorphic DNA (Causse et al. 1994; Kurata et al. 1994; Harushima et al. 1998). These as well as the genomic tools and methods that have become available [such as expressed sequence tags (ESTs) from salt-stressed libraries, complete genome sequence information, development of new bioinformatics tools, expression profiling by microarrays, random and targeted mutagenesis, and complementation and promoter-trapping strategies] provide opportunities to characterize the salt-tolerance-related gene networks in greater depths (Bohnert et al. 2001; Sahi et al. 2006). Prasad et al. (2000a,b) identified seven QTL for seedling traits associ-

ated with salt stress and mapped these to five different chromosomes. Using F_2 population derived from a salt-tolerant japonica rice mutant, M-20, and the sensitive original variety 77-170A, Zhang et al. (1995) mapped a major gene for salt tolerance on chromosome 7. Koyama et al. (2001) reported the chromosomal location of ion transport and selectivity traits that are compatible with agronomic needs. This group showed that QTL for Na^+ and K^+ transport are likely to act through the control of root development and structure and the regulation of membrane-localized transport compartmentalization, respectively. Gregorio et al. (2002) have mapped a major QTL designated *Saltol* on chromosome 1 (flanked by SSR markers RM23 and RM140) using a population generated from a cross between salt-sensitive IR29 and salt-tolerant Pokkali. This QTL accounted for more than 70% of the variation in salt uptake (Bonilla et al. 2002) and is now being mapped to within 1-cM distance using a large set of near isogenic lines (NILs). Pokkali was the source of positive alleles for this major QTL (Bonilla et al. 2002), which accounted for high K^+ and low Na^+ absorptions and low Na^+-to-K^+ ratio under salinity stress (Gregorio et al. 2002). Lin et al. (2004) have identified a major QTL for high shoot K^+ under salinity-stress in the same region of chromosome 1. Ren et al. (2005) have recently made a breakthrough in mapping *SKC1*, which maintains K^+ homeostasis in the salt-tolerant variety Nona Bokra under salt stress. *SKC1* was mapped to chromosome 1. Using finely mapped BC_2F_2 population, the *SKC1* locus was cloned. This group further showed that the *SKC1* locus was able to confer salt tolerance, when transformed into salt-sensitive variety. A detailed analysis showed that SKC1 protein is highly similar to HKT-type transporters. More details on analysis of salt-stress-associated QTL are provided in Table 1.

Salt tolerance in rice through transgenics approach

Salinity imposes water deficit that results from the relatively high solute concentration in the soil, causes ion-specific stresses resulting from altered Na^+/K^+ ratios, and leads to buildup in Na^+ and Cl^- concentrations that are detrimental to growth and other life processes (Flowers 2004; Munns 2005; Vinocur and Altman 2005). Plants respond to salt stress by restricting the uptake of salt and adjust their osmotic pressure by the synthesis of compatible solutes (proline, glycinebetaine, sugars, etc.) (Greenway and Munns 1980) and sequestering salt into the cell vacuoles for the maintenance of low cytosolic Na^+ levels (Glenn et al. 1999; Gepstein et al. 2006; Blumwald and Grover 2006). Transgenic rice plants tolerant to salt stress have been produced taking clues from the above account (Sahi et al. 2006). Xu et al. (1996) generated transgenic rice

(cv Nipponbare) over-expressing barley *hva1* gene (encoding a Late Embryogenesis Abundant, LEA) protein. This group exposed 3-week-old transformed and untransformed seedlings to two rounds of stress (200 mM NaCl for 10 d, followed by a recovery period of 10 d and a second round of 50 mM NaCl stress for 30 d) and noted that the transgenic plants maintained a higher growth rate initially and showed better recovery on removal of stress. Sakamoto et al. (1998) genetically engineered rice with the ability to synthesize glycinebetaine by introducing the *codA* gene (encoding for choline oxidase, obtained from the soil bacterium *Arthrobacter globiformis*). Levels of glycinebetaine were as high as 1 and 5 μmol per gram fresh weight of leaves in two types of transgenic plant in which choline oxidase was targeted to the chloroplasts (ChlCOD plants) and to the cytosol (CytCOD plants), respectively. Although treatment with 0.15 M NaCl inhibited the growth of both wild-type (WT) and transgenic plants, the transgenic plants recovered faster than the wild type plants and resumed normal growth after removal of the salt stress. It was indicated that ChlCOD plants are more tolerant than CytCOD plants to photoinhibition under salt stress (and low-temperature stress). Saijo et al. (2000) generated transgenic rice (cv Notohikari) plants over-expressing a Ca^{2+}-dependent protein kinase (OsCDPK7). The youngest leaves wilted 3 days after salt stress of 200 mM NaCl in WT plants, while transgenic plants exhibited greater tolerance. Mohanty et al. (2002) generated transgenic lines of indica rice Pusa basmati 1 (PB 1) by *Agrobacterium*-mediated transformation with codA gene. In this work, more than 50% of the transgenic plants could survive salt stress and set seed whereas the WT plants failed to recover. Garg et al. (2002) generated transgenic indica rice PB 1, expressing the fusion gene comprising both trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase obtained from *Escherichia coli*. Compared to WT rice, transgenic lines exhibited sustained plant growth, less photo-oxidative damage, and more favorable mineral balance under salt (also drought and low temperature) stress conditions. Jang et al. (2003) reported the generation of trehalose-producing, transgenic japonica rice plants by the introduction of a gene encoding a bifunctional fusion (TPSP) of the trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) of *E. coli*, under the control of the maize ubiquitin promoter. Increased trehalose accumulation in transgenic plants resulted in increased tolerance to drought, salt, and cold. Transgenic R_1 rice overexpressing *p5cs* (encoding pyrroline carboxylate synthase, an enzyme that rate-limits proline biosynthesis) showed increased biomass than the control plants under salt stress and water stress conditions (Zhu et al. 1998). Considering that mechanisms that reduce cytosolic Na^+ concentrations could provide a degree of tolerance to

Table 2 Selective reports on production of salt-tolerant transgenic rice

Gene	Protein	Source	Comments	Reference
AgNHX1	Na$^+$/H$^+$ antiporter gene	Atriplex gmelini	Kinuhikari; CaMV35S promoter; transgenics survived under conditions of 300 mM NaCl for 3 days	Ohta et al. 2002
c-GS2	Chloroplastic glutamine synthetase	Oryza sativa	Kinuhikari; CaMV35S promoter; overexpression reduced the increase of Na$^+$ content at high salinity	Hoshida et al. 2000
ch-codA, cy-codA	Choline oxidase A	Arthrobacter globiformis	Nipponbare; CaMV35S promoter; transgenic plants had high levels of glycinebetaine and grew faster compared to wild types on removal of stress	Sakamoto et al. 1998
codA	Choline oxidase A	Arthrobacter globiformis	PB 1; 50% R1 plants survived after exposure to salt stress for 1 week	Mohanty et al. 2002
hva 1	LEA protein	Hordeum vulgare	Nipponbare; rice actin promoter; transgenic seedlings maintained a higher growth rate during stress and plants showed faster recovery on removal of stress	Xu et al. 1996
Mn-SOD	Superoxide dismutase	Saccharomyces cerevisiae	Sasanishiki; CaMV35S promoter, transgenics showed enhanced tolerance to salt	Tanaka et al. 1999
nhaA	Na$^+$/H$^+$ antiporter gene	Escherichia coli	Zhongzou 321; CaMV35S promoter, transgenics grew faster as compared to the wild types under stress conditions	Wu et al. 2005
Oscdpk7	Calcium-dependent protein kinase	Oryza sativa	Notohikari; CaMV35S promoter; transgenic plants exhibited greater salt tolerance	Saijo et al. 2000
OsNHX1	Na$^+$/H$^+$ antiporter	Oryza sativa	Nipponbare; CaMV35S promoter; transgenic plants showed increased tolerance to salt stress	Fukuda et al. 2004
otsA + otsB	Trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase	Escherichia coli	PB 1; CaMV35S promoter; transgenic plants showed enhanced tolerance both during and after stress and had longer and thicker roots	Garg et al. 2002
SOD2	Na$^+$/H$^+$ antiporter gene	Saccharomyces pombe	Zhonghua no. 11; CaMV35S promoter transgenics had enhanced P-ATPase hydrolytic activity, increased photosynthesis and root proton exportation capacity, reduced ROS generation	Zhao et al. 2006a
SsNHX1	Na$^+$/H$^+$ antiporter gene	Suaeda salsa	Zhonghua no. 11; CaMV35S promoter; transgenic plants increase salt stress resistance, which resulted from Na$^+$ sequestration; plants had an increase in H$^+$-ATPase and H$^+$-PPase activity, reduced ROS generation and increased photosynthesis	Zhao et al. 2006b
TPS + TPP fusion	Trehalose-6-phosphate synthase and T-6-P phosphatase	Escherichia coli	Maize ubiquitin promoter; trehalose levels in seeds and leaves were more	Jang et al. 2003

relatively low NaCl concentrations (Blumwald and Grover 2006), Ohta et al. (2002) engineered salt-sensitive rice cultivar Kinhuikari using the Na$^+$/H$^+$ antiporter gene from Atriplex gmelini (AgNHX1). The activity of the vacuolar-type Na$^+$/H$^+$ antiporter in the transgenic rice plants was eightfold higher than in WT rice plants. Salt tolerance assays followed by non-stress treatments showed that the transgenic plants over-expressing AgNHX1 could survive under conditions of 300 mM NaCl for 3 days while the WT plants could not. Zhao et al. (2006b) have recently generated transgenic rice plants with SsNHX1 from Suaeda salsa which showed markedly enhanced tolerance to salt

stress (and to water deprivation) compared with non-transgenic controls grown under outdoor conditions. More details on rice transgenics produced thus far for increased salt tolerance are presented in Table 2.

Comparative genomics and proteomics

The salt stress response is a quantitative trait, involving activation of a large number of specific genes concomitant to the repression in activity of a large number of housekeeping genes (Sahi et al. 2006). Major alterations

in transcriptional and post-transcriptional activities have been noted to accompany response of plants to salt stress. Micro- and macroarray-based transcriptional profiling provides quantitative information about the expression levels of a large number of genes simultaneously. In higher plants, different EST/cDNA collections have been employed for transcriptional profiling to identify genes whose expression levels change in response to salt (Seki et al. 2001; Kanesaki et al. 2002; Sahi et al. 2003; Kore-Eda et al. 2004; Marin et al. 2004; Sottosanto et al. 2004; Taji et al. 2004; Shiozaki et al. 2005). Transcriptional profiling data from *Arabidopsis thaliana* and *O. sativa* suggest that metabolic readjustment is one of the hallmarks of the salt stress response (Kawasaki et al. 2001; Kreps et al. 2002; Seki et al. 2002; Oono et al. 2003; Rabbani et al. 2003; Sottosanto et al. 2004). From the data obtained, it appears that overall tolerance to high salt levels is due to effectors that directly modulate stress etiology or attenuate stress effects and due to regulatory molecules that are involved in stress perception, signal transduction, and modulation of the effectors' functions (Hasegawa et al. 2000; Sahi et al. 2003). Enzymes that catalyze rate-limiting steps in the biosynthesis of compatible osmolytes and proteins that protect membrane integrity, control water or ion homeostasis, and bring about scavenging of reactive oxygen species (ROS) are the examples of stress tolerance effectors. Regulatory determinants include transcription factors that interact with promoters of osmotic-stress-regulated genes (like rd22BP1, AtMYB2, DREBIA, and DREB2A) and intermediates that post-transcriptionally activate the effectors [SOS3 (Ca^{2+}-binding protein), SOS2 (a Ca^{2+}-dependant protein kinase), and SOS1 (putative plasma membrane Na^+/H^+ antiporter)] (Chinnusamy et al. 2005).

Further progress on the transcript changes in response to salt application has been made using the comparative genomics approach. Comparative stress genomics essentially means that various commonalities and differences in expression patterns of different genes relative to populations that differ in stress tolerance are scored. This approach appears highly valuable for unveiling the key genetic contributors to the complex physiological processes involved in salt tolerance trait (Bressan et al. 2001). The identification of salt-stress-specific changes in gene expression can be achieved by comparing gene expression in non-induced and stress-induced tissues or by comparing genetically different genotypes such as contrasting cultivars (Grover et al. 1993). Taji et al. (2004) noted that a fewer number of genes was induced by 250 mM NaCl stress in *Thellungiella halophila* (salt cress, a wild salt-tolerant relative of *Arabidopsis*), in contrast to *Arabidopsis*, indicating that the stress tolerance of salt cress may be due to the constitutive overexpression of many genes that function in stress tolerance and that are only stress inducible in

Arabidopsis. A larger spectrum of gene expression changes noted between Atnhx1 knockout mutant (Apse et al. 2003) and wild type plants by Sottosanto et al. (2004) showed that salt-sensitive and salt-tolerant *Arabidopsis* phenotypes differ markedly in expression of their genetic machinery. Kawasaki et al. (2001) examined transcript regulation in response to high salinity in the salt-tolerant rice cultivar Pokkali and salt-sensitive rice cultivar IR29 using a micro-array of 1,728 cDNAs from different libraries obtained from salt-stressed tissues. They suggested that the gene expression response to salt in contrasting rice plants is both qualitative as well as quantitative. Shiozaki et al. (2005) recently showed that transcript levels of a large number of genes (including those associated with detoxification, stress response, signal transduction, etc.) undergo pronounced changes upon subjecting seedlings of salt-tolerant rice (Dee-geo-woo-gen) to salt stress. Walia et al. (2005) investigated the response of salt stress in IR29 and salt-tolerant FL478 line (FL478 is RIL obtained from a cross of IR29 and Pokkali) using microarray. This group found that salinity stress induced a large number of genes involved in the flavanoid biosynthesis pathway in IR29 but not in FL478. Cell-wall-related genes were responsive in both genotypes, suggesting that cell wall restructuring is a general adaptive mechanism during salinity stress.

Comparative profiling of salt-stress-response-associated transcripts in Pusa basmati 1, CSR27, and Pokkali rice types

To identify and isolate genes that are differentially regulated by salt stress in rice plants which are extensively grown in India, three indica cultivars, namely, Pusa basmati 1 (PB 1, salt sensitive), CSR 27 (salt tolerant), and Pokkali (salt tolerant), were employed by Sahi et al. (2003). These three rice varieties showed distinctive growth differences upon treating seedlings with NaCl stress (Sahi et al. 2003). All India evaluation studies have shown that CSR 27 is a highly salt-tolerant rice cultivar (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). PB 1 is a premier rice type from India combining high yield with a much sought-after "basmati" flavor. A sensitive and highly efficient polymerase chain reaction (PCR)-based subtraction method employed to construct four subtracted cDNA libraries (namely, T2D1, T4D1, T4D2, and T3D5; see Table 3 for selected details on these libraries) yielded 1,266 cDNA clones (Sahi et al. 2003). Expression profiling of 969 subtracted clones was carried out using macroarray

Table 3 Details of the subtractive libraries constructed and macroarray analysis carried out in this study

Library name	Tester tissue–driver tissue	Total number of clones obtained	Number of clones spotted on the macroarray blot	Characteristics of the library (based on macroarray analysis)
T2D1	Pokkali salt-stressed root–PB 1 salt-stressed root	333	234	cDNA corresponding to Pokkali (control and salt-stressed roots) and PB 1 (control and salt-stressed roots) employed for probing of the macroarray blot; 41 clones up-regulated and 52 clones down-regulated in Pokkali in response to application of salt; 43 clones up-regulated and 60 clones down-regulated in PB 1 in response to application of salt; 39 common clones down-regulated in both tester and driver samples
T4D1	CSR27 salt-stressed root–PB 1 salt-stressed root	230	122	cDNA corresponding to CSR 27 (control and salt-stressed roots) and PB 1 (control and salt-stressed roots) employed for probing of the macroarray blot; 63 clones up-regulated in CSR 27; 29 up-regulated in PB 1; most of the salt-induced up-regulated clones in PB 1 also up-regulated in CSR 27
T4D2	CSR27 salt-stressed root–Pokkali salt-stressed root	250	210	cDNA corresponding to CSR 27 (control and salt-stressed roots) and Pokkali (control and salt-stressed roots) employed for probing of the macroarray blot; in CSR 27, 67 clones up-regulated and 40 clones down-regulated, 29 clones uniquely salt induced and 37 clones uniquely salt repressed; in Pokkali, 92 clones up-regulated and 7 clones down-regulated, 54 clones uniquely salt induced and 4 clones uniquely salt repressed; 38 common clones up-regulated in both CSR 27 and Pokkali
T3D5	Pokkali salt-stressed shoot–CSR27 salt-stressed shoot	463	403	cDNA corresponding to Pokkali (control and salt-stressed shoots) and CSR 27 (control and salt-stressed shoots) employed for probing of the macroarray blot; 26 clones up-regulated and 47 clones down-regulated in Pokkali; 8 clones up-regulated and 44 clones down-regulated in CSR 27; 6 common clones down-regulated in both Pokkali and CSR27

analysis. Separate replicate membranes were probed with radiolabeled total cDNA corresponding to control and salt-stressed roots/shoots of different tester and driver tissues (details shown in Table 3). A representative blot corresponding to the analysis of T2D1 library is shown in Fig. 1. In this set, 234 clones were spotted on the nylon membrane (Fig. 1a). The details of the expression characteristics of specified clones are indicated in Fig. 1b. Salt-up regulated clones, salt-down regulated clones, and clones that are not significantly affected by salt stress are shown by dark blue, light blue, and white colored boxes, respectively, in this representation. Clones that are differentially expressed in the Pokkali and PB 1 are shown by grey colored boxes in Fig. 1b. Further details on expression of various ESTs from this and three other libraries based on macroarray data are shown in Table 5 (annotations for the selected clone numbers shown in Fig. 1b are presented in Table 4 as mentioned below). One hundred eighty-three cDNA clones representing both up- and down-regulation on the basis of macroarray profiles (chosen randomly) were sequenced either manually or by automated sequencing

from 3′ ends. The sequences were annotated using different public databases. Most of the clones could be identified using the KOME database in particular, and their putative functions were assigned. In selected cases, our sequences did not match with any entry in KOME libraries but homologous counterparts were noted in EST databases. Based on the annotation analysis as presented in Table 4, a wide repertoire of genes belonging to different functionality classes (such as signaling molecules, transcriptional machinery, translational machinery, protein turnover and folding, ion transporters and metal binding proteins, and general metabolism) were seen associated with the response of different rice varieties to salt stress. The expression pattern of 77 ESTs (selected based on salt-regulated/cultivar-specific expression patterns as noted in macroarray blots) was further examined by Northern blotting (Fig. 2). Several ESTs were noted to be salt induced in this analysis. This category included putative Zn-finger protein (T4D1-144), subtilisin-chymotrypsin inhibitor (T4D2-165 and T4D2-50), cytosolic glyceraldehyde-3-phosphate dehydrogenase (T4D2-43),

A PB1 Pokkali   **B**
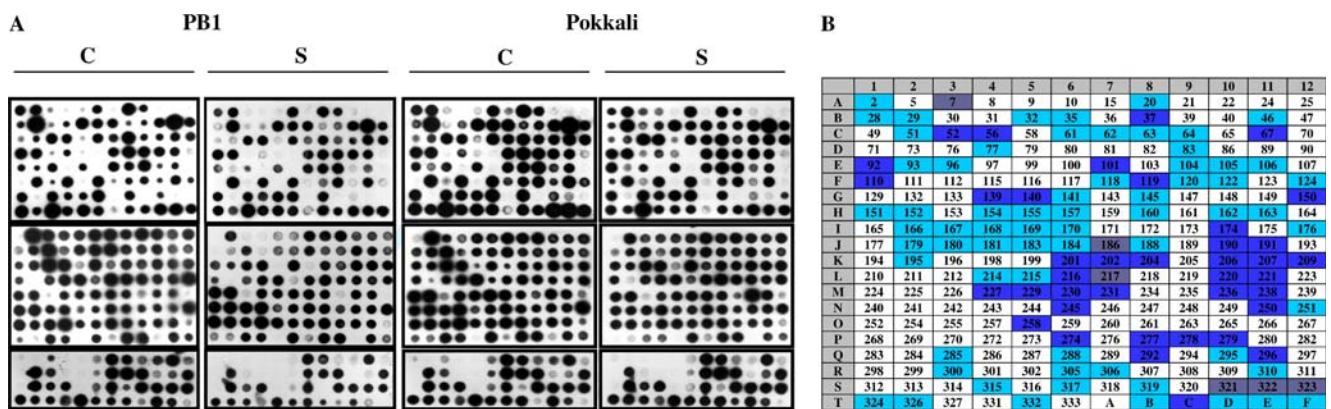
Fig. 1 Macroarray blot showing salt-stress-induced transcript alterations in Pokkali and Pusa basmati 1 roots corresponding to clones of the T2D1 library. Spotted on nylon membranes was 150 ng of clone plasmid DNA. The membranes were probed with radiolabeled total cDNA corresponding to control or salt-stressed (150 mM NaCl, 24 h) roots of PB 1 and control or salt-stressed (150 mM NaCl, 24 h) roots of Pokkali as shown in **a**. Representative up-regulated and down-regulated clones have been marked with *dark blue* and *light blue boxes* in **b**. Clone numbers of clones up-regulated in Pokkali but down-regulated in PB 1 are marked with *grey boxes* in **b**. A, B, C, D, E, and F represent controls from three other libraries constructed in our study (clones D5T3-13, D2T4-66, D2T4-50, D2T4-165, D2T4-216, and T4D1-227, respectively). Rice (*O. sativa* L.; cultivars Pusa basmati 1, CSR 27, and Pokkali) seeds were sown on wet cotton pads placed in plastic trays (Sahi et al. 2003). The seedlings were subjected to salt stress by placing these in beakers containing cotton pads soaked with 150 mM of NaCl solution instead of distilled water. The details on steps followed in the construction of subtractive cDNA libraries are shown in our earlier publication (Sahi et al. 2003). Replicate blots were probed with radiolabeled total cDNA prepared from control and salt-stressed roots/shoots of their respective rice types as shown in Results section. Northern blotting was carried out as per the standard protocol (Sambrook and Russell 2001). Radiolabeled probes were made either using "Megaprime Labeling Kit" (Amersham, UK) or "Random Primer Extension Labeling System" (NEN, USA). αP^{32}-dCTP (BRIT, India) was used as the radiolabeling molecule. The nucleotide and protein sequences were analyzed using DNAStar software. Multiple alignments were done using MegAlign module of DNAStar. The nucleotide and the deduced amino acid sequences were searched for their homology with the previously existing sequences in the NCBI database using the BlastN and BlastP programs (Altschul et al. 1997). For protein domain analysis, sequences were searched against SMART (*S*imple *M*odular *A*rchitectural *R*esearch *T*ool) database (Letunic et al. 2004). The sequences were also searched on rice full-length cDNA consortium database at http://cdna01.dna.affrc.go.jp/cDNA/ (KOME database; Kikuchi et al. 2003)

FAD-linked oxidoreductase (T2D1-173), NAD kinase (T4D2-114), vacuolar ATPase (T4D2-45), ribosomal protein genes (T4D2-66, T2D1-177 and T2D1-288), putative aldolase (T4D1-61), putative serine/threonine kinase (T2D1-99), ubiquitin ligase (T2D1-302), MD2 lipid recognition protein (T2D1-101), putative tumor protein (T3D5-302), *salT*(T4D2-96), ADP ribosylation factor (T3D5-13), etc. Genes showing homology to eukaryotic translation initiation factor (eIF2α) (T4D1-219) and an uncharacterized reteroelement (T4D2-130a) were also noted to be salt induced. Selected ESTs representing genes with unknown functions were found to be salt inducible. On the other hand, selected clones were noted to be down-regulated by salt treatment (such as T2D1-180, T4D1-209, and T4D2-231) or were expressed in comparable amounts in control and salt-treated tissues (such as T4D1-82 and T4D2-68). Clones showing up-regulation and down-regulation of the transcripts in response to salt treatment are shown by ⬆ and ⬇ arrows in Fig. 2, respectively. On the other hand, clones which did not show any marked change upon salt treatment are shown with ⬌ symbol. The clones displaying differential response to salt application between the salt-sensitive PB 1 and salt-tolerant CSR 27 or Pokkali cultivars [such as ubiquitin ligase (T2D1-302)] are shown with bold font of the clone numbers in Fig. 2. On the other hand, clones showing differential transcript levels in response to salt application between the two salt-tolerant cultivars (CSR 27 and Pokkali) [such as putative amino acid transporter (T4D1-163)] are shown with underlining of the clone numbers in Fig. 2. The clones showing constitutive differential expression or salt-induced differential expression in the three rice varieties are summarized in Table 5. In the macroarray blots, homologous cDNA clones were detected using probes corresponding to both tester and driver rice varieties in a given subtraction set (Fig. 1). Thus, contrasting rice genotypes appear to have a similar genetic response to imposed stress in qualitative terms. This implies that the differences in the relative salt response in contrasting genotypes are controlled largely by the differential regulation of several stress-regulated genes. Kawasaki et al. (2001) noted that the stress-sensitive IR29 rice cultivar responded to stress slower than the salt-tolerant rice cultivar Pokkali. Several transcripts were induced within the first 15 min of the imposed stress in Pokkali, while the same took approximately 3 h in the salt-sensitive IR29 rice type (Kawasaki et al. 2001).

From the nucleotide sequence and annotation (Table 4; Sahi et al. 2003), macroarray profiling (Fig. 1), Northern blotting data (Fig. 2; Sahi et al. 2003), and the published

Table 4 Classification of various ESTs obtained in this study in different functionality classes based on annotation analysis

Functionality class	Clone details
Signaling machinery	
ADP-ribosylation factor	T3D5-13 (*AJ312764*;**AK122007**)
EF-hand-containing protein	T4D2-120 (*AJ853694*; **No hit**)
Rab GDP dissociation inhibitor (GDI2)	T4D1-152 (*AJ853669*; **No hit**)
Seceretory protein	T4D2-89 (*AJ853684*; **AK101249**)
Serine/threonine kinase	T2D1-99 (*AJ420731*; **AK065576**); T2D1-291(*AJ420704*; **AK065576**)
Transcriptional machinery	
MADS23 mRNA	T2D1-92 (*AJ302301*; **AK063114**)
Zn-finger-containing protein	T2D1-247 (*AJ853658***AK067971**); T2D1-256 (*AJ420703*; **AK100301**); T4D1-144 (*AJ420711*; **AK061911**); T4D2-140 (*AJ853701*; **AK120945**)
EREBP-like protein	T2D1-273 (*AJ302303*; **AK100301**)
DNA-dependent RNA polymerase II 23-kDa polypeptide	T4D2-77 (*AJ853682*; **AK059963**); T4D2-227 (*AJ864571*; **AK059963**)
Viviparous 1 protein	T4D2-108 (*AJ853688*; **AK119780**)
bHLH transcription factor	T4D2-212 (*AJ853704*; **AK063498**)
Pirin-like protein	T4D2-231 (*AJ853707*; **No hit**)
Translational machinery	
16S ribosomal RNA gene	T4D2-61, T4D2-109 (*AJ420713*; **AK108725**)
eIf2α	T4D1-219 (*AJ853673*; **AK105818**)
GCN2	T2D1-36 (**AK072226**)
Glycine-rich RNA binding protein	T2D1-207 (*AJ853650***AK112034**); T2D1-217 (*AJ853652*; **AK070016**); T2D1-219, T2D1-313, T3D5-329, T3D5-362, T4D1-82, T4D1-157, T4D2-34, T4D2-113 (*AJ853651*; **AK070016**)
Retrotransposon	T4D2-130a (**AK065448**)
Ribosomal protein 117 (249 gene)	T2D1-218, T2D1-261 (*AJ853653*; **AK121994**)
Ribosomal proteins	
L9	T4D1-32, T4D1-147 (*AJ312757*; **AK064936**)
L10	T2D1-274 (*AJ302300*; **AK066128**)
L17	T2D1-141, T2D1-171, T2D1-198, T2D1-243, T2D1-303 (*AJ853644*; **AK120324**)
L23	T4D1-56 (*AJ312760*; **AK121994**)
L27	T4D1-88 (*AJ420709*; **AK121593**)
L32	T4D1-9, T4D2-66 (*AJ298284*; **AK063151**)
L38	T4D1-173 (*AJ853672*; **AK073724**)
S21	T2D1-251 (*AJ853634*; **AK073724**)
R41	T2D1-288 (*AJ302305*; **AK105083**)
Small nuclear ribonucleoprotein Sm-D3	T3D5-70 (*AJ312768*; **AK061558**)
Protein turnover, folding, and protein–protein interactions	
α-2 subunit of 20S protein	T4D2-64 (**No hit**)
α-COP homolog	T4D2-189 (*AJ420736*; **AK105672**)
F-box protein	T2D1-176 (**AK070881**)
Membrane protein with WD-40 repeat	T4D2-121 (*AJ853695*; **AK111777**)
Peptidyl prolyl isomerase	T4D1-98 (*AJ420710*; **AK061127**)
OsHsp90	T4D2-178 (*AJ864569*; **AK064780**)
RING-finger-containing ubiquitin ligase	T2D1-302 (*AJ853662*; **AK067254**)
RUB1 gene	T4D2-224 (*AJ864570*; **AK121590**)
Subtilisin-chymotrypsin inhibitor 2	T2D1-133, T2D1-186, T2D1-223, T2D1-255, T2D1-260, T4D1-015, T4D1-28, T4D2-98, T4D2-106, T4D2-117, T4D2-129, T4D2-152, T4D2-162, T4D2-165, T4D2-185, T4D2-195 (*AJ853639*; **AK062495**)
Tetratricopeptide repeat protein	T4D2-191 (*AJ420717*; **No hit**); T4D2-76 (*AJ298286*; **No hit**)
Ubiquitin-associated protein	T3D5-25 (*AJ420706*; **AK060817**)
Ubiquitin-conjugating enzyme	T4D2-119 (*AJ853693*; **AK099284**)
Ubiquitin fusion protein	T2D1-10 (*AJ302302*; **AK061988**)
Ion transporters and metal-binding proteins	
AAA-type ATPase	T4D1-227 (*AJ853674*; AK065505)
ATP synthase like protein	T2D1-150 (**AK120641**); T4D2-45 (*AJ853678*; **AK120641**)

Table 4 (continued)

Functionality class	Clone details
Blue copper binding protein	T2D1-47 (**AK060044**); T2D1-81, T2D1-238, T4D1-148 (*AJ302307*; **AK063639**)
Copper homeostasis factor/copper chaperone	T2D1-221(*AJ853654*; **AK062378**); T4D1-99 (*AJ312762*; **No hit**)
Membrane protein	T2D1-177 (**AK065186**)
Metallothionin	T2D1-20, T2D1-104, T2D1-180, T2D1-196 (*AJ853636*; **AK103445**)
General metabolism	
3-Methyladenine-DNA-glycosylase I	T4D2-131 (*AJ853699*; **AK065590**)
4-Coumarate CoA ligase	T4D1-34, T4D1-43 (*AJ420708*; **No hit**)
5′-Nucleotidase	T3D5-19 (*AJ312765*; **AK065179**)
Acetyl transferase	T2D1-159 (*AJ420728*; **AK069226**)
Actin-like protein	T2D1-163 (**AK068991**)
Alanine aminotransferase	T4D1-38 (*AJ312759*; **AK121994**); T4D1-213 (*AJ864568*; **AK067732**)
Aldolase	T4D1-61 (*AJ312761*; **AK105687**)
Amino acid transporter	T4D1-163 (*AJ420712*; **AK071044**)
Anaerobically inducible early gene 2 (*aie2*)	T4D2-137 (*AJ853700*; **AK120264**)
Ascorbate peroxidase precursor	T4D2-70 (*AJ298285*; **AK058770**)
Aspartate aminotransferase	T4D1-37 (*AJ420720*; **AK105687**)
Auxin efflux carrier protein	T4D1-209 (*AJ312763*; **AK069848**)
β-Adaptin-like protein	T4D2-223 (*AJ853706*; **AK061897**)
Class III chitinase	T4D2-138 (**AK059767**)
Co-atomer α-subunit	T4D2-57 (*AJ298283*; **AK105672**)
Cyclin H1	T2D1-117 (*AJ864562*; **AK105468**)
Cysteine synthase	T2D1-145 (*AJ864561*; **No hit**)
Cytochrome *c* oxidase subunit 6b-1	T2D1-185 (*AJ420702*; **AK120174**)
Cytosolic GAPDH	T4D2-43 (*AJ853677*; **AK099086**)
Dynamin4-like protein	T4D2-128 (*AJ853697*; **AK061703**), T4D2-192 (*AJ853702*; **AK073186**)
EN242 (wound-inducible basic protein)	T4D1-77 (*AJ420700*; **AK073415**)
Exostosin-like protein	T2D1-161 (*AJ853642*; **AK073335**), T4D2-197 (*AJ853702*)
Expressed unknown protein	T2D1-63 (**No hit**)
FAD-binding-domain-containing protein	T2D1-276 (*AJ302304*; **AK105866**)
FAD-linked oxidoreductase	T2D1-172; T2D1-173 (**AK105866**)
Fah1	T2D1-242 (*AJ853656*; **AK067847**)
Histones	
H4	T2D1-111 (*AJ302306*; **AK058741**)
H2B	T3D5-324 (*AJ312771*; **No hit**)
Hypothetical protein with fascin domain	T2D1-142 (*AJ853640*; **AK106415**)
Ids4-like protein	T4D2-2, T4D2-221 (*AJ853675*)
Lysine decarboxylase	T2D1-215 (*AJ853651*; **AK062595**)
Mannosyl transferase	T2D1-259 (*AJ853660*, **AK069661**)
MD-2-related lipid-recognition protein	T2D1-101 (**AK059284**)
NADP-dependent malic enzyme	T4D2-55 (*AJ302310*; **AK072842**)
NADPH HC-toxin reductase	T2D1-64 (**AK063834**)
Nucleotide diphosphate kinase	T2D1-35, T4D2-114; T4D2-154 (*AJ853690*; **AK121796**)
Pathogenesis-related protein WIN2	T2D1-89, T4D2-130a (**AK121127**)
Pectin glucuronyltransferase	T4D2-88 (*AJ853683*; **AK073335**)
Peroxidase	T2D1-169 (*AJ853643*; **AK107853**)
Peroxidase precursor	T3D5-50 (*AJ312766*; **AK065793**)
PFK2/fructose 2,6 bisphosphatase	T3D5-15 (*AJ420705*; **AK069285**)
Phenylalanine ammonia lyase	T2D1-201 (*AJ853649*; **AK058306**)
Plastidic ATP sulfurylase	T2D1-105 (**AK099593**)
Putative barbendase protein	T4D1-172 (*AJ853671*; **AK120879**)
SalT gene	T4D2-96 (*AJ298287*; **AK062520**)
ScoE-like protein of maize	T2D1-199 (*AJ853648;***No hit**)
Signal peptidase	T3D5-284 (*AJ312769*; **No hit***)*
Sugar transporter	T4D2-122 (*AJ853696*; **No hit**)

Table 4 (continued)

Functionality class	Clone details
Thionin-like protein	T2D1-307(**No hit**)
Thioredoxin-like protein	T2D1-37, T2D1-39, T2D1-157 (*AJ853641*; **AK101877**)
Translationally controlled tumor protein	T3D5-302 (*AJ312770*; **AK105453**)
Transmembrane protein	T4D2-99 (*AJ853686*; **AK107853**)
Ubiquinol cytochrome *c*-reductase	T4D2-46 (*AJ853679*; **AK121368**)
WIN2 protein	T4D2-130 (No hit)
Unknown/hypothetical proteins	T4D2-115 (*AJ853692*; **AK103332**), T4D2-141 (*AJ302309*; **AK067857**), T2D1-32 (**AK070520**), T2D1-62 (**AK107076**), T2D1-167 (*AJ864563*; **AK120490**), T4D1-11 (*AJ853664*; **AK066700**), T4D1-98 (*AJ420710*; **AK061127**), T4D1-5 (*AJ312756*; **AK070149**), T4D2-216 (*AJ420727*; **AK070014**)

Accession numbers obtained in the present work are shown in italicized font. Accession numbers of the matching hits in KOME database are shown in bold font

literature, the following cellular processes appear relevant to the salt stress response of contrasting rice varieties:

Signaling processes Different molecules have been shown to act as signals in the stress-associated signal transduction processes, and changes in cytosolic Ca^{2+} concentrations have been shown to act as a second messenger in salt stress (Chinnusamy et al. 2005). Kawasaki et al. (2001) observed that ESTs encoding Ca^{2+}-dependent protein kinases were up-regulated during initial periods of salt stress in Pokkali rice. Salt-induced up-regulation of translationally controlled tumor protein has been reported. This protein is believed to play a role in salt stress signaling via Ca^{2+} binding (Gong et al. 2001). Isolation of cDNA clones encoding for EF-hand-containing proteins and Ca^{2+}-binding proteins further substantiated the role(s) of Ca^{2+} in salt stress response. Clones like T3D5-13, T2D1-99, and T4D2-120 represent some of the signaling molecules identified (Table 4). It has been considered that most of the signaling components are induced and expressed within the initial time points of stress (Kawasaki et al. 2001). However, an EST corresponding to a putative serine/threonine protein kinase (T2D1-99) was noted at 24 h of salt exposure. A cDNA clone encoding a GDP inhibitor protein (T4D1-152) was also identified, suggesting that proteins binding GDP/GTP could play putative roles in salt-stress response in plants.

Transcriptional processes RNA metabolism including varied transcriptional as well as post-transcriptional events appears as an important and well-conserved component of stress response in yeast, animal, and plant cells. The regulation of processes associated with RNA synthesis has been reported under diverse abiotic stresses (Sahi et al. 2003; Agarwal and Grover 2005). The activation of the transcription machinery to regulate salt-dependent gene expression requires the induction of specific transcription factors (TFs) as well as RNA polymerases (Estruch 2000). A microarray analysis in *Arabidopsis* showed that the

levels of several TFs changed in response to environmental stresses (Chen et al. 2002).Several regulatory proteins have also been similarly identified. Clones showing homology to Zn-finger-containing TFs are found to be redundant in salt-stress-related gene expression profile studies. For instance, T4D1-144 (homologous to Zn-finger gene) was induced by salt stress (Fig. 2). Other clones obtained in similar kind of studies include ethylene response element binding protein (EREBP), basic helix loop helix (bHLH), and MADS (*MCM1*, agamous, deficiens, serum response factor) box containing transcription factors. bHLH and MYB transcription factors have been reported to function as transcriptional activators of ABA signaling in plants and were implicated in stress-regulated gene expression (Abe et al. 2003). Cooper et al. (2003) showed that MADS box TFs are important components of salt stress networking in plants. Pirin-like protein identified as an interacting molecule of nuclear factorI/CCAAT box TF (NFI/CTF1) stimulates RNA-polymerase-II-driven transcription in humans (Wendler et al. 1997). A representation of EST corresponding to pirin-like protein (T4D2-231) and DNA-dependent RNA polymerase II (T4D2-77 and T4D2-227) in salt-stressed libraries suggests that salt stress might regulate homologous events in the transcription machinery in plants.

Translational processes Regulation of the translational machinery is considered an integral component in the cellular stress response (Wood and Oliver 1999). It has been indicated that ribosomal proteins are not only central to translational efficiency but have pleiotropic effects and have extra-ribosomal functions (Wool 1996). The polysomal retention of transcripts for ribosomal proteins RPS14, RPS16, and RPL23 has been correlated to desiccation response in *Tortula ruralis* (Wood et al. 2000). Ribosomal protein genes *Gmrps6*, *Gmrps13*, and *Gmrpl37* were found responsive to low temperature stress in soybean (Kim et al. 2004). *Arabidopsis* plants mutated for ribosomal
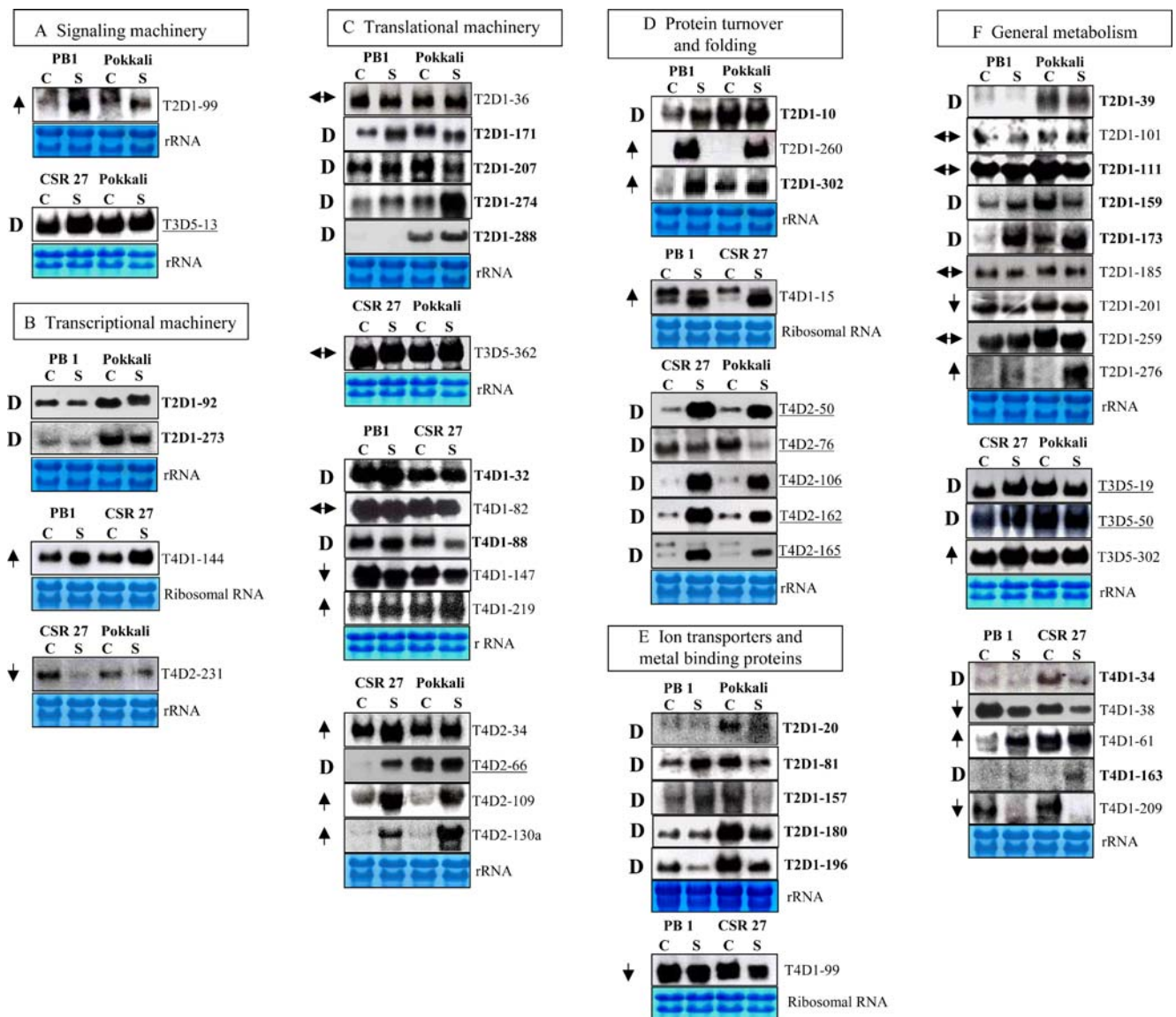
Fig. 2 Expression profiling of transcripts for selected clones by Northern blot analysis. PB 1, CSR 27, and Pokkali seedlings were salt stressed (150 mM NaCl, 24 h). An amount of 15 μg of total RNA isolated from root (in the case of analysis for the clones of T2D1, T4D1, and T4D2 libraries; see text and Table 3 for more details) or shoot tissues (in the case of analysis for the clones of T3D5 library; see text and Table 3 for more details) was resolved on 1% denaturing agarose gel. The blots were probed with PCR-amplified DNA fragment for the corresponding clones. *C* and *S* depict RNA from unstressed control and salt-stressed tissues, respectively. Methylene-blue-stained bands of rRNA depict equal RNA loading. ▲, ▼, ◄►, and *D* indicate clones showing increased transcript expression in tester and driver tissues, decreased transcript expression in tester and driver tissues, comparable transcript expression in tester and driver tissues, and differential transcript expression between tester and driver tissues, respectively. The *clone numbers* of the clones showing differential transcript expression between the salt sensitive PB 1 and salt tolerant cultivar (CSR27 or Pokkali) or within the two salt tolerant cultivars (CSR27 and Pokkali) are shown with *bold font* and with *underlining*, respectively. The annotations for the respective clone numbers are shown in Table 4

gene *S27* displayed normal phenotype under control conditions but the same was noted to be indispensable for *Arabidopsis* plants under genotoxic conditions (Revenkova et al. 1999). Several different ribosomal genes are noted to be stress-regulated (like T2D1-81, T2D1-171, T2D1-288, and T4D2-66 in Figs. 1 and 2).

Apart from ribosomal proteins, clones associated with different other aspects of RNA metabolism were also found to be associated with salt stress (like T2D1-207, T2D1-217, T2D1-219, T2D1-313, T3D5-329, T3D5-362, T4D1-86, T4D1-157, T4D2-34, and T4D2-113 in Figs. 1 and 2). RNA binding proteins, mostly glycine-rich (GR) RNA

Fig. 2 (continued)
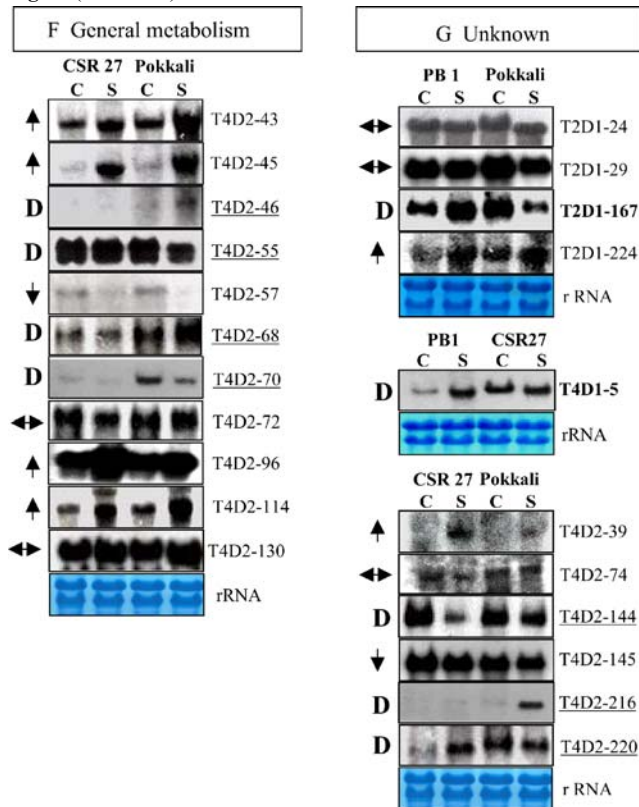
binding proteins (RBPs) (GR–RBPs) are highly repetitive in stress related expression profiles (Sahi et al. 2006). Clones corresponding to small nuclear ribonucleoprotein (T3D5-70) and retrotransposon (T4D2-130a) were noted. EST encoding the retrotransposon was found to be salt

inducible (Fig. 2). The active transcription of plant retrotransposons under diverse stress conditions would suggest their involvement in the stress response (Takeda et al. 2001). Phosphorylation of eIf2α by eIf2α kinase(s) is considered to be a key step in regulating stress-dependent translation in eukaryotes (Anderson and Kedersha 2002). cDNA encoding eIf2α (T4D1-219) was noted to be salt induced (Fig. 2). This protein has been reported to have a bearing on selected translation of stress-associated genes in several systems (Harding et al. 2002). GCN2 (eIf2α kinase) was also found to be stress-regulated (T2D1-36; Fig. 2). The latter protein has been shown to be implicated in salt toxicity in yeast (Goossens et al. 2001).

Protein turnover and folding Protein degradation during stress is a highly conserved and regulated phenomenon in all the organisms reported so far (Vierstra and Callis 1999). Genes encoding proteins like ubiquitin ligase, polyubiquitin, proteasome subunit, and protease inhibitor are known to be expressed in the cDNA libraries of stressed tissues (Mahalingam et al. 2003). Increased transcript levels of a gene encoding a putative ubiquitin fusion protein (T2D1-10) were seen. It was observed that E3 ubiquitin ligases and the RING (Really Interesting New Gene) finger proteins are key components of the ubiquitin proteasome pathway (Freemont 2000; Taji et al. 2004), and RING finger proteins are thought to be involved in rapid degradation of regulatory proteins (Lee et al. 2001). Several polyubiquitin and related to ubiquitin (RUB) [encoding genes in our screen (such as T2D1-10, T3D5-25, and T4D2-224)] appear to be associated with salt stress response (Figs. 1

Table 5 Details of the clones that showed differential transcript expression in Pusa basmati1 (PB 1), CSR 27, and Pokkali rice types

Group A: salt-stress-induced differences	
High in Pokkali than PB 1	FAD-linked oxidoreductase (T2D1-173), MADS23 protein (T2D1-92), subtilisin-chymotrypsin inhibitor (T2D1-260), thioredoxin-like protein (T2D1-39)
High in CSR27 than PB 1	Amino acid transporter (T4D1-163), hypothetical protein (T4D1-5), subtilisin-chymotrypsin inhibitor (T4D1-15), Zn-finger protein (T4D1-144)
High in PB 1 than Pokkali/CSR27	Unknown protein (T2D1-167), YK426 mRNA (T4D1-32)
Differential between Pokkali and CSR27	Hypothetical protein (T4D2-216), nucleotide diphosphate kinase (T4D2-114), subtilisin-chymotrypsin inhibitor (T4D2-106), ubiquinol cytochrome *c*-reductase (T4D2-46), unknown protein (T4D2-144), vacolar ATP synthase 22-kDa proteolipid subunit (T4D2-45)
Group B: constitutive differences	
High in Pokkali than PB 1	Mannosyl transferase (T2D1-259), EREBP like protein (T2D1-273), ribosomal protein R41 (T2D1-288), MADS23 protein (T2D1-92), acetyl transferase (T2D1-159), ubiquitin fusion protein (T2D1-10), RING-finger-containing ubiquitin ligase (T2D1-302), ribosomal protein L27 (T4D1-88)
High in CSR 27 than PB 1	
High in PB 1 than Pokkali/CSR 27	Metallothionin-like protein (T2D1-280), 4-coumarate CoA ligase (T4D1-34)
Differential between Pokkali and CSR 27	Ascorbate peroxidase precursor (T4D2-70), ribosomal protein L32 (T4D2-66), subtilisin-chymotrypsin inhibitor (T4D2-50)
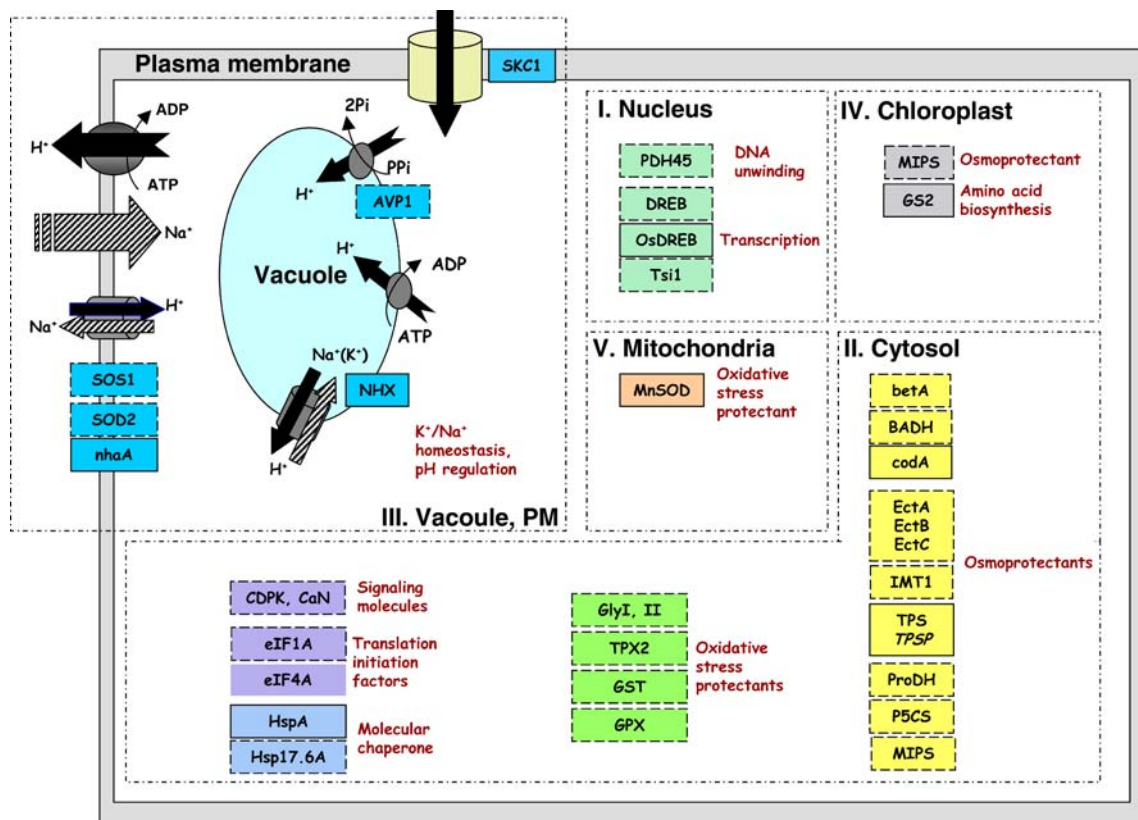
Fig. 3 Schematic representation of mechanisms associated with salt tolerance phenotype in different cellular compartments of the plant cell. For validating the functionality of proteins shown in *solid boxes* in salt response, transgenic rice plants have been raised. Proteins shown in *broken boxes* represent examples where the transgenic host employed is other than rice. Evidence for the involvement of proteins not shown in solid or broken boxes in salt response has been obtained using varied other approaches (such as biochemical experiments, transcript/protein expression studies, mutant analysis, or yeast complementation assays). Involvement of SKC1 in salt tolerance was initially suggested on the basis of QTL analysis and, later on, it was functionally validated by producing transgenic rice plants. *DREB*, dehydration responsive element binding protein (Kasuga et al. 1999; Dubouzet et al. 2003); *PDH45*, pea DNA helicase 45 (Sanan-Mishra et al. 2005); *Tsi1*, tobacco stress-induced gene 1 (Park et al. 2001); *BADH*, betaine aldehyde dehydrogenase (Holmstrom et al. 2000; Jia et al. 2002; Yilmaz and Bulow 2002); *CaN*, calcineurin (Pardo et al. 1998); *codA*, choline oxidase (Lilius et al. 1996; Hayashi et al. 1997; Sakamoto et al. 1998; Alia et al. 1999; Prasad et al. 2000a, b); *CDPK*, calcium-dependent protein kinase (Saijo et al. 2000); *ectA, ectB*, and *ectC*, L-2,4-diaminobutyric acid acetyltransferase, L-2, 4-diaminobu-

tyric acid transaminase, and L-ectoine synthase (Nakayama et al. 2000); *eIF1A*, eukaryotic translation initiation factor 1A (Rausell et al. 2003); *eIF4A*, eukaryotic translation initiation factor 4A (Montero-Lomeli et al. 2002); *Gly I* and *II*, glyoxylase I and II (Veena et al. 1999; Singla-Pareek et al. 2003); *GST*, glutathione-*S*-transferase (Roxas et al. 1997); *GPX*, glutathione peroxidase (Roxas et al. 1997); *HspA* (Asadulghani et al. 2004); *Hsp17.6A* (Sun et al. 2001); *IMT1*, *myo*-inositol *O*-methyl transferase (Vernon et al. 1993); *P5CS*, pyrroline-5-carboxylate synthetase (Kishore et al. 1995); *ProDH*, proline dehydrogenase (Nanjo et al. 1999); *SOD2*, sodium 2 (Gao et al. 2003); *TPS*, trehalose-6-phosphate synthase; *TPSP*, TPS phosphatase (Garg et al. 2002; Jang et al. 2003); *TPX2*, tomato peroxidase 2 (Amaya et al. 1999); *AVP1*, vacuolar H⁺ pyrophosphatase (Gaxiola et al. 2001; Park et al. 2005); *NHX1*, Na⁺/H⁺ antiporter (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001; Ohta et al. 2002; Fukuda et al. 2004; Wang et al. 2004; Wu et al. 2004; Xue et al. 2004; Yin et al. 2004; He et al. 2005; Lu et al. 2005; Wu et al. 2005); *SKC1*, HKT type (Ren et al. 2005); *SOS1*, salt overly sensitive 1 (Shi et al. 2003); *GS2*, glutamine synthetase (Hoshida et al. 2000); *MIPS*, L-*myo*-inositol 1-phosphate synthase (Majee et al. 2004); *MnSOD*, Mn-superoxide dismutase (Tanaka et al. 1999)

and 2). Ubiquitin has been noted to be involved with vegetative desiccation tolerance in plants (O' Mahony and Oliver 1999).

It has been shown recently that salt stress induces wound-related genes, suggesting that salt and wounding stresses are closely linked (Dombrowski 2003). Bowman–Birk protease inhibitors, WRKY transcription factors, chitinase, EN242, and other PR proteins have been shown to be associated with salt stress (Dombrowski 2003; Sottosanto et al. 2004).

At the transcription level, AtMYB102 has been identified as a key regulatory component, which integrates osmotic stress and wounding responses (Denekamp and Smeekens 2003). Clones coding for subtilisin-chymotrypsin protease inhibitors were amongst the abundant clones in salt-stressed root libraries (clones like T2D1-46, T2D1-133, T2D1-186, T2D1-223, T2D1-260, T4D1-15, T4D1-28, T4D2-50, and T4D2-106). Clones corresponding to the wheat WIN2 (T2D1-89 and T4D2-130), chitinase (T4D2-138), and

EN242 gene (T4D1-77) were also obtained. As cDNA clones coding for proteins promoting proteolysis as well as inhibiting protein degradation were enriched in cDNA libraries, future work on the characterization of these proteins may reveal the intriguing complexity in co-coordination of protein metabolism under stress.

HSPs that help in proper protein folding or prevention of stress-induced folding problems (Grover 2002) have been reported to be expressed under salt stress (Asadulghani et al. 2004). *Synechocystis* cells mutated for a small molecular mass HSP were noted to be salt-sensitive and displayed severe protein folding aberrations under moderate salt stress (Asadulghani et al. 2004).EST clone corresponding to *hsp90* (T4D2-178) was induced by salt stress in macroarray analysis. Hsp90 proteins have been shown to be expressed under salt stress in rice (Pareek et al. 1995).

Ion transporters and metal-binding proteins Ion transporters have received a great deal of attention because of their crucial role in salt tolerance and potassium nutrition (Blumwald and Grover 2006). Ion homeostasis during salt stress is affected by sodium fluxes, transport, and compartmentalization. Clones homologous to vacuolar H^+-ATPase (T4D1-227), vacuolar ATP synthase (T4D2-45 and T2D1-150), and various other uncharacterized proteins with putative transmembrane domains (T2D1-177 and T4D2-99) were notably present in salt stress cDNA libraries.

Metal-binding ESTs were also abundant in salt stress libraries (T2D1-20, T2D1-47, T4D1-99, etc.). Metallothionins were the most enriched among these (T2D1-20, T2D1-104, and T2D1-180). Other metal-binding proteins included copper binding proteins and copper chaperones (T2D1-47, T2D1-81, T2D1-221, T2D1-238, and T4D1-99). Metallothionins have been found in several other salt stress libraries (Kawasaki et al. 2001; Reddy et al. 2002) and have also been implicated in leaf senescence and cell death, and the induction of genes encoding copper binding proteins was reported in the response of *Arabidopsis* to salt (Sottosanto et al. 2004).

General metabolism Stress leads to an oxidative stress response due to the accumulation of hydrogen peroxide, hydroxyl radicals, and superoxide anions, resulting in perturbance of the overall cellular metabolism (Mittler 2002). Salt stress cDNA libraries were rich in genes encoding peroxidases (T2D1-169 and T3D5-50), ascorbate peroxidase (T4D2-70), FAD-linked oxidoreductases (T2D1-172 and T2D1-173), and thioredoxins (T2D1-37, T2D1-39 and T2D1-157). Clones associated with senescence including cell cycle control and death such as cyclin H1 (T2D1-117), cytochrome *c* oxidase (T2D1-185), and tumor suppressor genes (T3D5-302) were also noted. The T3D5-302 and T2D1-276 clones were induced by salt treatment. EST clone

homologous to 3-methyladenine DNA glycosylaseI gene (T4D2-131), which was reported to be involved with DNA repair pathway (Santerre and Britt 1994), was obtained.

Genes encoding proteins involved in amino acid metabolism (T2D1-145, T2D1-201, T2D1-215, T4D1-37, T4D1-38, T4D1-163, T4D1-213, and T4D2-68), mannosyltransferase (T2D1-259), glucosyltransferase (T4D2-88), fructose-2, 6,-bisphosphatase (T3D5-15), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (T4D2-43), NADP-dependent maleic enzyme (T4D2-55), and nucleoside diphospho kinase (NDPK1; T4D2-114 and T2D1-35) were noted to be abundant. The transcript corresponding to the *NDPK* gene was strongly up-regulated by salt stress in Pokkali rice (Kawasaki et al. 2001). Levels of T4D2-55 transcript in roots of both CSR27 and Pokkali were regulated by salt treatment. Upon salt treatment, this transcript was down-regulated in case of Pokkali but not in CSR27. Furthermore, clones related to photosynthesis (T2D1-37, T2D1-157 ,and T2D1-185), respiration (T4D1-161, T3D5-15, and T4D2-46), carbohydrate (T2D1-259, T4D2-88, and T4D2-122) and fat and lipid metabolism (T2D1-101 and T4D1-34) were obtained. According to Kawasaki et al. (2001), genes encoding proteins associated with cellular homeostasis such as those belonging to the TCA cycle, respiration, cellular biogenesis and DNA repair showed a distinct decline in the more sensitive IR29 rice but not in Pokkali rice type.

Clones like exostosin (T2D1-161), phragmoplastin, lipid-recognition protein (T2D1-101), cysteine synthase (T2D1-145), protein-containing domains like fascin (T2D1-142), TPR (tricopeptide repeat) (T4D2-191), and WD-40 (T4D2-120) were noted. *SalT* was detected in one of the subtracted libraries (T4D2-96). *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). cDNA encoding for putative membrane protein (T4D1-5) was detected to be salt inducible in PB 1 while it was constitutively expressed in CSR 27. Proteins with membrane spanning or lipid interacting domains could play significant roles in stress response (Urao et al. 1999).

One of the primary effects of salt stress is the dehydration effect, which is associated with the decrease in water potential in the extracellular solution (Munns 2005). This effect would increase the capacity of the cells to produce osmoregulants/compatible solutes (Munns 2005). An increased accumulation of proline and trehalose has been reported during salt stress in rice (Garcia et al. 1997). However, ESTs corresponding to synthesis of osmolytes were largely not represented in subtracted rice cDNA libraries, suggesting that osmolyte synthesis under salt stress may be a highly conserved salt stress response amongst different rice types.

Clones expressed differentially in contrasting rice varieties Several transcripts that appeared to be expressed at

higher levels in Pokkali but at lower levels in PB 1 were identified (such as FAD-linked oxidoreductase, MADS23 protein, subtilisin-chymotrypsin inhibitor, and thioredoxin-like protein; Table 5). Specific differences in transcriptional regulation were also apparent between Pokkali and CSR 27 rice varieties (such as nucleotide diphosphate kinase, subtilisin-chymotrypsin inhibitor, ubiquinol cytochrome *c*-reductase, and vacaular ATP synthase 22-kDa proteolipid subunit; Table 5). The transcript levels of selective clones were noted to be constitutively higher in Pokkali/CSR 27 compared to PB 1 (such as mannosyl transferase, EREBP-like protein, ribosomal protein R41, MADS23 protein, acetyl transferase, ubiquitin fusion protein, RING-finger-containing ubiquitin ligase, and subtilisin-chymotrypsin inhibitor; Table 5). Kawasaki et al. (2001) observed that a gene-encoding translation elongation factor-1α was up-regulated during initial periods of salt stress in Pokkali but was not induced at similar time points in the salt-sensitive IR29. This observation emphasized that tolerant cultivars are possibly more efficient in restructuring their translational machinery during salt stress and are able to respond faster to the imposed stress. A set of clones (T2D1-81, T2D1-157, T2D1-159, and T2D1-167) showed higher constitutive expression in Pokkali compared to PB 1 (Fig. 2). NaCl treatment notably caused up-regulation of these clones in PB 1 but down-regulation in Pokkali. Clone T2D1-288 encoding for R41 ribosomal protein was salt-up-regulated in Pokkali. The transcripts corresponding to this clone were not detected in PB 1. Three highly expressed clones isolated from Pokkali, namely, T2D1-273 (EREBP-like protein), T2D1-274 (ribosomal protein L10), and T2D1-276 (FAD-binding-domain-containing protein), showed not only high levels in CSR 27 but also differential expression in CSR 27 as compared to PB 1. Some of the clones that expressed differentially in these rice varieties (e.g., T4D2-220 and T4D2-144) have not yet been annotated.

The abundance of stress-regulated and transcriptional- and translational-associated genes in salt stress libraries (such as bHLH, EREBP, Zn-finger protein, GCN2, eIf2α etc.) indicate that cells undergo an adjustment to re-program their metabolism to survive under stress conditions. According to Sahi et al. (2006), there appears to be a coordinated action of *trans*-acting factors, ribosomal proteins, RNA binding proteins, translation initiation and elongation factors along with several accessory proteins for regulating stress-associated processes. Induction of specific ribosomal genes, translation initiation factors and elongation factors during salt stress in yeast, *Arabidopsis* and rice in recent studies (Sahi et al. 2006; this work) indicates that alterations in these cellular components represent a conserved stress response. From the above account, it is indicated that gene expression response in tolerant and susceptible plants differs mainly in quantitative terms.

Further analysis of the salt stress-related cDNA clones in terms of transcript and protein expression may shed light on requisite promoters and interacting protein partners that are critical in controlling salt stress response.

Final comments

It is important to note that in spite of the high salt sensitivity, rice is the only cereal that is recommended as a desalinization crop because of its ability to grow well under flooded conditions, and because the standing water in rice fields can help leach the salts from the topsoil to a level low enough for subsequent crops (Bhumbla and Abrol 1978). This and also the fact that rice is an important food crop, increasing its salt tolerance has enormous implications. From the progress made thus far, several directions are emerging that may yield useful genes in breeding for rice salt tolerance. Strong evidence exist in showing that members of protein families involved in osmoregulation, ion transport and protection from oxidative damage are critical in governing high salt tolerance (Fig. 3). Recent work provides indications (through the abundance of stress-regulated and transcriptional- and translational-associated genes in salt stress libraries) that cells undergo an adjustment to re-program their metabolism to survive under stress conditions, and thus genes involved in this reprograming may as well turn out to be important players in controlling salt stress response. For discovery of novel salt-stress-tolerance-related genes, comparative genomics is clearly turning out to be an important tool. There are several attempts to unravel how Pokkali, *Porteresia*, and other related rice types are endowed with high salt stress tolerance using comparative genomics approach. Novel salt-stress-tolerant genes thus emerging from different directions of research need to be validated in future attempts. To undertake this, it is important that knockout mutants of rice for each and every individual gene are produced as has been done in the case of yeast and *Arabidopsis*. Such attempts are, in fact, in progress (http://www.iris.irri.org/IRFGC/). Gene knockout mutants may also help to validate the cellular functions of QTL identified by mapping. Furthermore, transgenic rice production has reached the stage where routine experiments with candidate genes are possible. With the requisite mutants and the optimized transformation protocols, validating the candidate genes for salt tolerance in rice appears a clear possibility. Finally, the above discussion has mainly emphasized transcript analysis. It is imperative that protein data is obtained in parallel. In recent years, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique in conjunction with automated gel scanning and computer-assisted analysis has contributed greatly to the development of protein database (Dubey and Grover 2001; Komatsu et al. 2003). Abbasi and Komatsu (2004), using this approach to examine changes in

protein expression in response to salt stress in rice seedlings, have reported that specific proteins expressed in defined regions of rice show a coordinated response to salt stress. Kong-ngern et al. (2005) analyzed the response of salt stress in leaf sheaths of rice seedlings. This group identified 13 different proteins which were induced and ten different proteins which were down-regulated by salt stress. The effect of salt stress on rice leaf lamina proteins has recently been analyzed by Parker et al. (2006). The highly optimized 2D-PAGE protocol used in this study resulted in separation of ~2,500 protein species of which 32 were observed to be significantly regulated by salinity. Tandem mass spectrometry analysis showed that rubisco activase and ferritin are increased predominantly during salt stress. However, information on salt stress-associated protein changes in various other tissues at varied growth stages and at the whole plant level is far from complete; this aspect therefore merits further research.

While the above account of salt stress response has mainly been addressed on rice plant, a host of other plant species are being analyzed to unravel the salt stress biology. Among the crop species, salt stress response has been analyzed in appreciable details in barley, tomato, and wheat (Forster et al. 2000; Francki and Appels 2002; Colmer et al. 2005; Kawaura et al. 2006; Walia et al. 2006). Model plants like *Arabidopsis* and tobacco have particularly been exploited with the aim of testing the role(s) of candidate genes in governing salt stress tolerance using transgenic approach (Tarczynski et al. 1993; Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001; Shi et al. 2003). Work on halophyte plants like salt cress (*T. halophila*) has been useful in comparative-genomics-based studies. Put together, it reflects that the understanding of the molecular events in plant salt stress response is making a fast progress (see March 2006 issue of *Journal of Experimental Botany* for more articles on plant salt stress biology, http://jbx. oxfordjournals.org/current.dtl#RESEARCH_PAPERS_ PLANTS_AND_SALINITY).

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