Raising salinity tolerant rice: recent progress and future perspectives

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ABSTRACT

With the rapid growth in population consuming rice as staple food and the deteriorating soil and water quality around the globe, there is an urgent need to understand the response of this important crop towards these environmental abuses. With the ultimate goal to raise rice plant with better suitability towards rapidly changing environmental inputs, intensive efforts are on worldwide employing physiological, biochemical and molecular tools to perform this task. In this regard, efforts of plant breeders need to be duly acknowledged as several salinity tolerant varieties have reached the farmers field. Parallel efforts from molecular biologists have yielded relevant knowledge related to perturbations in gene expression and proteins during stress. Employing transgenic technology, functional validation of various target genes involved in diverse processes such as signaling, transcription, ion homeostasis, antioxidant defense etc for enhanced salinity stress tolerance has been attempted in various model systems and some of them have been extended to crop plant rice too. However, the fact remains that these transgenic plants showing improved performance towards salinity stress are yet to move from 'lab to the land'. Pondering this, we propose that future efforts should be channelized more towards multigene engineering that may enable the taming of this multigene controlled trait. Recent technological achievements such as the whole genome sequencing of rice is leading to a shift from single gene based studies to genome wide analysis that may prove to be a boon in re-defining salt stress responsive targets. **[Physiol. Mol. Biol. Plants 2008; 14(1&2) : 137-154]** *E-mail : sneh@icgeb.res.in*

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Most crops growing under field conditions are often exposed to various abiotic stresses such as high or low temperature, drought and salinity, which influence plant metabolism directly or indirectly, thereby affecting plant growth, development, and finally productivity. Amongst these, salinity is one of the major factors limiting the production of cereal crops especially rice. Rice has an exceptional agricultural importance, as this is the staple food crop. However, rice is highly sensitive to salt, posing a major challenge for maintaining world food supplies. Thus, there is an urgent need to raise varieties that can, not only withstand high levels of salt but can also maintain optimum yield levels.

In general, rice shows variability in sensitivity towards excess salinity at various developmental stages during its life cycle. It is considered relatively tolerant to salinity at the germination stage while the young seedling stage and early reproductive stages, i.e. panicle initiation and pollination are the most salinity-sensitive growth stages, directly affecting the crop yield (Heenan *et al*., 1988; Khatun *et al*., 1995; Zeng *et al*., 2001). Also, rice yields are fast approaching a theoretical limit set by the crop's own efficiency in harvesting sunlight and using its energy to make carbohydrates. So to feed the world, there is a need to re-engineer rice to harvest better yields (Surridge, 2002). There are two ways to achieve this goal, first is to increase the photosynthetic efficiency and the second is to minimize the crop losses due to various environmental stresses. Considering these facts, there have been several efforts to develop stress tolerant plants via available tools and technologies in last decade. Recently, a number of excellent reviews have been published on the abiotic stress response and tolerance mechanisms and transgenic technology for developing crop plants tolerant to abiotic stresses (Singla-Pareek *et al.*, 2001; Zhu, 2001; 2002; Sairam and Tyagi, 2004; Yamaguchi and Blumwald, 2005; Rodriguez *et al.,* 2005; Vinocur and Altman, 2005; Bajaj and Mohanty, 2005; Sahi *et al.,* 2006; Gao *et al.,* 2007; Viz and Tyagi 2007; Kathuria

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et al., 2007). Availability of the genome sequence of rice is proving to be an additional benefit to look for the suitable target genes that can be employed for selected gene manipulation to achieve tolerance against various abiotic stresses in rice (Goff *et al.*, 2002). In the recent past, genome-wide analysis of an array of protein families has given insight into the architecture of various genes as well as their responsiveness towards various environmental abuses (http://mpss.udel.edu/rice/). Hence, in the future such studies may help to understand how various genes ultimately contribute towards the ability of rice to adjust to the stressful environment. Here, we review recent efforts aiming towards better understanding rice adaptation to salinity stress and to highlight efforts to integrate results of advances in physiology and molecular biology into rice breeding programs. We have also made an attempt to compile different strategies, which have been attempted by various scientific groups to select genes and their use in transformation for developing salt tolerant transgenic rice plants.

Development of salt tolerant rice

Several promising approaches have been put forth to obtain salt-tolerant rice cultivars. Screening of preexisting genotypes has been attempted to explore the natural genetic variability. Several salt tolerant lines have been also developed employing the breeding approaches. Lately, generation of transgenic plants (to introduce novel genes or to alter expression levels of the existing genes) with improved tolerance to salinity stress has also been a method of choice in this endeavour. We have attempted to present the successful reports where some of these approaches have been used. It has not been our objective to present all the reports, but pick up selected ones which may serve as a representative example only.

Screening of preexisting salt-tolerant genotypes of rice

The rice genotypes are known to have natural genetic variability for salt tolerance, but the tolerance levels are not very high (Xie *et al.,* 2000). In past, extensive efforts have been made to identify differential sensitivity of rice plants to salinity at different growth stages using several screening criterions.

Physiological characters determining salinity resistance at the seedling stage have been studied in detail (Yeo *et al.,* 1990). A relationship between potassium and sodium uptake by the rice plant and performance under salinity has been established (Rajarathinam *et al.,* 1988; Pandey and Srivastava, 1991;

Bohra and Dorffling, 1993). Asch and coworkers (2000) have screened 21 rice genotypes for salt tolerance based on leaf K^+/Na^+ ratio. Recently, Natarajan and coworkers (2005) have grouped rice genotypes for salinity tolerance based on grain yield and Na^+/K^+ ratio under coastal environment. The rice accessions from the high yielding and tolerant group recorded a lower value for the Na^+/K^+ ratio and higher value of grain yield. Zeng *et al.* (2003) have evaluated twelve genotypes of rice for salinity tolerance on the basis of physiological characters. Leaf area index (LAI) was shown to play an important role in contributing salt tolerance as defined by grain yield although altered source-sink relations under salt stress may also be involved. Various agronomic parameters have been established for the screening for salinity tolerance (Zeng and Shannon, 2000a,b; Counce and Wells 1990; Gravois and McNew, 1993). Twelve rice genotypes have been evaluated for salt tolerance based on various agronomic parameters. Wide genotypic differences were observed for relative salt tolerance in terms of spikelet number per panicle and tiller number per plant. Spikelet and tiller numbers contributed most of the variations to seed yield under salinity among parameters investigated when data were averaged across all genotypes (Zeng *et al.,* 2002).

Conventional Breeding Approaches for Enhancing Salt Tolerance in rice

The major aim of rice breeding program is to integrate the desired agronomic characteristics with an increasing level of tolerance to salinity (Boyer, 1982). Native varietal tolerance has long been exploited to characterize the difference between salt-sensitive and salt tolerant cultivars (Winicov, 1998). By selecting individual physiological traits contributing to salt tolerance and combining them in breeding programs could make possible to increase resistance to salt beyond the existing phenotypic range (Khatun *et al.,* 1995). Due to these efforts, considerable success has been obtained in raising rice genotypes with improved tolerance towards salinity (Malmberg and McIndoo, 1984; Zapata and Aldemita, 1986; Miah *et al.* 1996; Satish *et al.,* 1997; Das *et al.,* 2000). Other promising salt tolerant rice types include Pobbeli, IR2151, Pokkali and Nona Bokra, which have been used in several breeding programs to develop salt tolerant rice genotypes.

Conventional breeding techniques *viz.,* interspecific hybridization, screening and recurrent selection have been made use of (Rivelli *et al.,* 2002; Wei *et al.,* 2003; Zheng *et al.,* 2003). Senadhira *et al.* (2002) have developed 79 di-haploid lines in rice through anther culture of the cross of two indica breeding lines (IR5657-

33-2 x IR4630-22-2-5-1-3). The cross was designed to combine the high yielding ability of IR5657-33-2 with the salinity tolerance of IR4630-22-2-5-1-3. Out of several lines showing desirable traits such as high yield, salinity tolerance, early maturity, good plant type, and resistance to pests and diseases, IR51500-AC11-1 line performed better in the trials conducted under saline conditions. Later, this line was named as PSBRc50 "Bicol" and recommended for commercial cultivation in salt affected rice lands. This was the first F_1 AC-derived line from an indica/indica cross to be released as a cultivar for cultivation in saline prone areas.

Induced mutagenesis is also a widely accepted breeding strategy for changing plant characters (Yoshida, 1962; Rutger, 1992). Lee *et al.* (2003) have selected and characterized radiation-induced salinity tolerant line of rice. Two lines among 350 M_3 lines were more tolerant and exhibited improved plant height, panicle length, tiller number, spikelet number and greater yield than those of the original variety. RAPD markers were developed for both salt tolerant lines. From DNA-hybridization experiment, it appeared that the fragment arose from the middle repetitive copy sequences. The transcript involved in the marker showed a higher expression in the salt tolerant lines than the sensitive lines. The salt tolerant lines would be useful as a resource developing salt tolerant rice through breeding. Shylaraj and Sasidharan (2005) have released a promising 'Mahsuri' mutant VTL 5, using induced mutagenesis, which was suitable for cultivation in the coastal saline ecosystem of Kerala. This variety had multiple tolerances to abiotic stresses such as salinity, acidity and submergence.

Within India, Central Soil Salinity Research Institute (CSSRI, Karnal, India) has been actively engaged in developing improved salinity tolerant lines. CSR10, the first salt-tolerant early maturing rice variety of the country, is capable to withstand highly alkaline (pH 9.8-10.2) and saline conditions (ECe, 6-10 dsm^{-1}) under transplanted irrigated management system. Subsequently, CSSRI, Karnal has released several other salt-tolerant varieties such as CSR13, CSR27 and CSR30 (for details refer http://www.plantstress.com/admine/file/salt_karnal.htm). Efforts are still on to further improve the rice varieties with better adaptability towards saline and sodic soils without yield penalty.

The low success in breeding programs for the development of salt tolerance rice is partially due to the low selection efficiency using overall agronomic characters, lack of effective evaluation methods for salt tolerance among genotypes, and the complexity of salinity tolerance phenotypes among genotypes.

Approaches based on selected gene manipulations

In recent years, our understanding about how plants perceive the salinity stress and respond to it has advanced rapidly. These advancements have been made at physiological, biochemical as well as molecular level. Most importantly, a number of genes induced in response to salinity have been identified from a range of organisms adapted to stressful environment and transferred into crop plants in order to improve stress-tolerance (Cushman and Bohnert, 2000; Apse and Blumwald, 2002; Zhu, 2002; Bajaj and Mohanty, 2005; Singla-Pareek *et al.*, 2007a). The attempts made to engineer rice for enhancing salinity tolerance have been discussed in the following text and are summarized in the form of Table I.

Targeting Signal transduction machinery

To survive the adverse environmental conditions, plants have developed elaborate mechanisms to perceive external signals and to manifest adaptive responses with proper physiological and morphological changes. At the molecular level, the perception of extracellular stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades (Xiong and Yang, 2003). Various components of salt stress signal transduction pathway have been identified and studied (Zhu, 2002). Plants perceive salt stress through both ionic (Na^+) and osmotic stress signals. Excess Na^+ can be sensed either on the surface of the plasma membrane by a transmembrane protein or within the cell by membrane proteins or Na^+ sensitive enzymes (Urao *et al.,* 1999; Zhu, 2003; Pareek *et al.,* 2006). Salt stress signal transduction pathways can be divided into three major signaling types: (i) osmotic/oxidative stress signaling that uses mitogen-activated protein (MAP) kinase modules, involves the generation of ROS scavenging enzymes and antioxidant compounds as well as osmolytes; (ii) Ca^{2+} dependent signaling that lead to the activation of late embryogenesis abundant (LEA) type genes, involves the production of stress responsive proteins mostly of undefined functions and (iii) Ca^{2+} dependent salt overly sensitive (SOS) signaling that regulate ion homeostasis. It involves the SOS pathway, which is specific to ionic stress (Rodriguez *et al.*, 2005). Potential roles of these signaling pathway components in abiotic stress tolerance have been shown using various plant species.

MAP Kinase Signaling Pathway

MAP-kinase cascade is one of the well-characterized intracellular signaling modules, and it is highly

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Table 1. Genetic enrichment of salt stress tolerance in transgenic rice.

Gene	Encoding protein	Gene source	Target rice variety	Phenotype	Reference
Targeting signal transduction machinery					
HVA1	LEA protein	*Hordeum vulgare*	Nipponbare	Salinity and water deficit tolerance	Xu, *et al.,* 1996
OsCDPK7	CDPK	*Oryza sativa*	Notohikari	Improved performance under salinity, drought and cold	Saijo *et al.,* 2000
HVA1	LEA protein	*Hordeum vulgare*	Pusa Basmati 1	Salinity and water deficit tolerance	Rohila *et al.,* 2002
OsMAPK5	MAP kinase (OsMAPK5a)	*Oryza sativa*	Nipponbare	Improved performance under salinity, drought and low temperature	Xiong and Yang, 2003
CNAtr	Calcineurin	Mouse	Xiushui 04	Salt stress tolerance	Ma *et al.,* 2005
Targeting transcription factor machinery					
CBF3 and *ABF3*	Transcription factors	*Arabidopsis thaliana*	Nakdong	Salinity, drought and moderate low temperature stress tolerance	Oh *et al.,* 2005
MYB2	MYB transcription factor	*Arabidopsis thaliana*	TNG-67	Salinity tolerance	Malik and Wu, 2005
DREB family genes	Transcription factors	*OsDREB1A & OsDREB1B from Oryza sativa and DREB1A, DREB1B & DREB1C* from *Arabidopsis thaliana*	Kita-ake	Salinity, drought and cold tolerance	Ito *et al.,* 2006
SNAC1	NAC transcription factor	*Oryza sativa*	Nipponbare	Salinity and drought tolerance	Hu *et al.,* 2006
HvCBF4	CBF transcription factor	*Hordeum vulgare*	–	Salinity, drought, and cold tolerance	Oh. *et al.,* 2007
Targeting antioxidant machinery					
MnSOD	SOD	*Saccharomyces cerevisiae*	Nakdong	Salinity tolerance	Tanaka *et al.,* 1999
GS	Glutamine synthetase	*Oryza sativa*	Kinuhikari	Salinity tolerance	Hoshida *et al.,* 2000
GST & CAT	Glutathione S-transferase and Catalase	*Suaeda salsa*	Zhonghua No. 11	Salinity and oxidative stress tolerance	Zhao and Zhang, 2006
Cu/Zn-SodI	SOD	*Avicennia marina*	Pusa Basmati-1	Salinity, drought and oxidative stress tolerance	Prashanth *et al.,* 2007

Table 1. Continued ....

Gene	Encoding protein	Gene source	Target rice variety	Phenotype	Reference
katE	Catalase	*Escherichia coli*	Nipponbare	Salt tolerance	Nagamiya *et al.,* 2007
glyII	Glyoxalase II	*Oryza sativa*	Pusa Basmati-1	Salinity and methylglyoxal tolerance	Singla-Pareek *et al.,* 2007
Targeting osmotic homeostasis machinery					
codA	Choline oxidase	*Arthrobacter globiformis*	Nipponbare	Salt and cold stress tolerance	Sakamoto *et al.,* 1998
P5CS	Δ^1-pyrroline-5-carboxylate synthetase	*Vigna aconitifolia*	Nipponbare	Salinity and drought stress tolerance	Zhu *et al.,* 1998
ADC	Arginine decarboxylase	*Avena sativa*	TNG-67	Salt stress tolerance	Roy and Wu, 2001
codA	Choline oxidase	*Arthrobacter globiformis*	Pusa Basmati-1	Salt stress tolerance	Mohanty *et al.,* 2002
TPS & TPP	Trehalose-6-phosphate synthase & trehalose-6-phosphate phosphatase	*E. coli*	Pusa Basmati-1	Salt and drought tolerance	Garg *et al.,* 2002
SAMDC	S-adenosyl methionine decarboxylase	*Tritordeum*	TNG-67	Salt stress tolerance	Roy and Wu, 2002
TPS & TPP	Trehalose-6-phosphate synthase & trehalose-6-phosphate phosphatase	*E. coli*	Nakdong	Salt, drought and cold tolerance	Jang *et al.,* 2003
P5CS	Δ^1-pyrroline-5-carboxylate synthetase	*Vigna aconitifolia*	Kenfong	Salt and water deficit stress tolerance	Su and Wu, 2004
codA	Choline oxidase	*Arthrobacter pascens*	TNG-67	Salt stress tolerance	Su *et al.,* 2006
Targeting ion homeostasis machinery					
AgNHX1	Vacuolar Na^+/H^+ antiporter	*Atriplex gmelini*	Kinuhikari	Salt tolerance	Ohta *et al.,* 2002
OsNHX1	Vacuolar Na^+/H^+ antiporter	*Oryza sativa*	Nipponbare	Salt tolerance	Fukuda *et al.,* 2004

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Table 1. Continued ....

Gene	Encoding protein	Gene source	Target rice variety	Phenotype	Reference
SsNHX1	Vacuolar Na$^+$/H$^+$ antiporter	*Suaeda salsa*	Zhonghua-11	Salt and water deficit tolerance	Zhao *et al.,* 2006
SOD2	Plasma membrane Na$^+$/H$^+$ antiporter	*Schizosaccharomyces pombe*	Zhonghua-11	Salt tolerance	Zhao *et al.,* 2006
PgNHX1	Vacuolar Na$^+$/H$^+$ antiporter	*Pennisetum glaucum*	Pusa Basmati-1	Salt tolerance	Verma *et al.,* 2007
OsKAT1	Shaker potassium channel	*Oryza sativa*	Nipponbare	Salt tolerance	Obata *et al.,* 2007

conserved among eukaryotes (Hirt, 1997; Kultz, 1998). In plants, the components of the MAPK cascade have been identified and characterized from several species and shown to be involved in stress signaling, including cold and drought (Jonak *et al.,* 1996), salinity (Munnik *et al.,* 1999; Kiegerl *et al.,* 2000), wounding (Seo *et al.,* 1995; Usami *et al.,* 1995; Zhang and Klessig 1998) and during plant-pathogen interaction (Ligterink *et al.,* 1997; Droillard *et al.,* 2000). Most of the understanding of this cascade comes from extensive studies in dicotyledonous species. But during last few years, several genes of MAPK cascade have been identified and characterized from rice. OsBWMK1, OsMSRMK2, OsMAP1, OsMSRMK3 and OsMAPK4 genes have been shown to be up-regulated during salinity stress in rice (Agrawal *et al.,* 2003). Recently, computational analysis identified 16 rice MAPKs and classified into four distinct phylogenetic subgroups (Liu and Xue, 2007). Despite several genes of MAPK cascade have been identified and characterized from rice, only one gene, OsMAPK5 has been over-expressed in rice. OsMAPK5 has been shown to be induced by abscisic acid, wounding, drought, salinity and low temperature. Overexpression of OsMAPK5 under the control of CaMV35S promoter provided tolerance to abiotic stresses such as drought, salinity and low temperature in rice cv Nipponbare (Xiong and Yang, 2003).

Calcium dependent signaling

In plant cells, Ca^{2+} acts as secondary messenger in signal transduction pathways. A wide range of stimuli are known to evoke cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) level in plant cells, which suggests role of $[Ca^{2+}]_c$ in diverse signal transduction pathways (Sanders *et al.,* 1999). To respond appropriately to a specific ($[Ca^{2+}]_c$ perturbation, a cell must activate a unique combination of Ca^{2+}-binding proteins. These ($[Ca^{2+}]_c$ sensors include calmodulin (CaM), CaM-like proteins, calcineurin B-like (CBL) proteins and Ca^{2+}-dependent protein kinases (CDPKs).
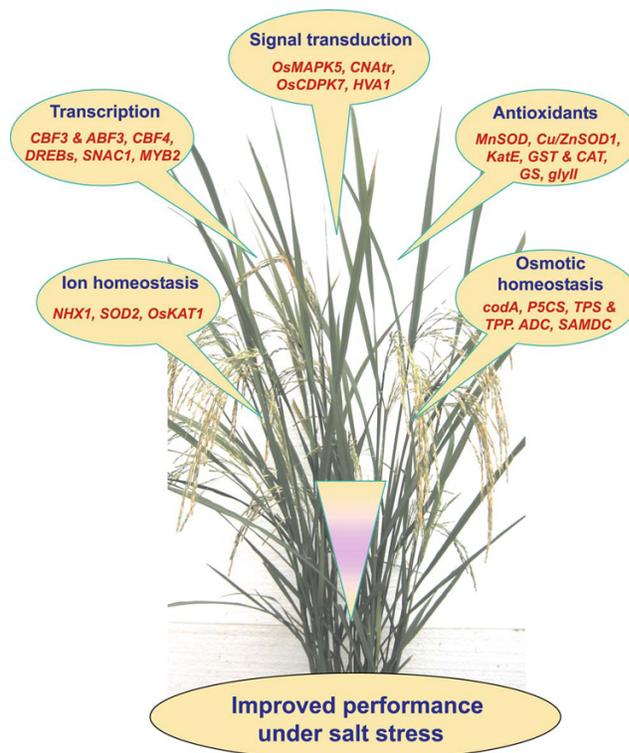

Fig. 1. Cartoon summarizing the reports where genetic engineering of rice plant has been attempted employing genes belonging to diverse cellular functions. These transgenic plants showed improved tolerance to salinity stress.

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Many of these proteins bind Ca^{2+} using a helix-loop-helix structure termed 'EF hand', which binds a single Ca^{2+} molecule with high affinity (Strynadka and James, 1989).

CaM is found in the apoplast and in the cytosol, ER and nucleus of plant cells. CaM has been implicated in Ca^{2+}-dependent responses to light, gravity, mechanical stress, phytohormones, pathogens, osmotic stress, salinity, heavy metals, xenobiotics, anoxia, oxidative stress, heat shock and chilling (Zielinski, 1998; Snedden and Fromm, 2001; Reddy, 2001; Rudd and Franklin-Tong, 2001; Fasano *et al.,* 2002). Genome-wide identification and analyses of the rice calmodulin and related potential calcium sensor proteins indicated that there are 243 proteins in the rice genome that possibly have 'EF-hand' motifs and 37 CaMs and related potential calcium sensor proteins. The functions of most proteins encoded by these genes are still unknown (Boonburapong and Buaboocha, 2007). However, in *Arabidopsis* one of the isoforms of CaM was found to bind to a transcription factor MYB2, enhancing its DNA binding activity. Overexpression of this isoform of CaM in *Arabidopsis* upregulated the transcription of MYB2 regulated genes including P5CS1, a rate-limiting enzyme in proline biosynthesis (Yoo *et al.,* 2005).

Beside CBL, calcineurin (CaN) itself has been shown to have multiple functions in cells including regulation of ion homeostasis. CaN is a Ca^{2+} and calmodulin-dependent serine/threonine phosphatase that, in yeast, is an integral intermediate of a salt stress signal transduction pathway that affects NaCl tolerance through the regulation of Na^+ influx and efflux (Mendoza *et al.,* 1996). A truncated form of the catalytic subunit and the regulatory subunit of yeast CaN were co-expressed in transgenic tobacco plants to reconstitute *in vivo* a constitutively active phosphatase. Transgenic lines expressing activated CaN exhibited substantial NaCl tolerance (Pardo *et al.,* 1998). In a recent study, a truncated form of the catalytic subunit of mouse CaN was expressed in rice and found that transgenic plants had higher salt tolerance than the non-transgenic plants. Transgenic rice plants were also able to grow and fertilize normally in the field. Protective mechanism of CaN against salinity stress was through inhibition of Na^+ accumulation in root cells (Ma *et al.,* 2005).

Calcium-dependent protein kinase (CDPK) is one of the most widely studied Ca^+ binding proteins. CDPKs have been identified throughout the plant kingdom and in some protozoans, but not in animals. CDPKs are activated by the binding of Ca^+ to their calmodulin-like domain, but are not stimulated by calmodulins. It has been reported that CDPKs from various plants are induced by a variety of stimuli, including hormones and/or stresses, elicitors and light (Asano *et al.,* 2005). A rice gene encoding CDPK (OsCDPK7) was over-expressed in rice and found that transgenic plants were more tolerant to salt, drought and cold than the non-transgenic plants (Saijo *et al.*, 2000). Recently, genome-wide analysis of rice CDPKs identified 29 CDPK genes and eight closely related kinase genes, including five CDPK-related kinases (CRKs), one calcium and calmodulin-dependent protein kinase (CCaMK) and two phosphoenolpyruvate (PEP) carboxylase kinase-related kinases (PEPRKs). RNA gel blot analysis showed that most of the rice CDPK genes exhibited tissue specific expression. Studies using transgenic plants have revealed the biological function of a few CDPK genes in higher plants. The biological function of majority of the CDPK genes and their target proteins in higher plants is still unclear (Asano *et al.,* 2005).

Late embryogenesis abundant (LEA) proteins, which are ubiquitous in plants, accumulate during the late stage of seed formation and in vegetative tissues under drought, heat, cold, and salt stress conditions or with abscisic acid (ABA) application (Sivamani *et al.,* 2000). Pathways leading to the activation of LEA-type genes including the dehydration responsive element (DRE)/C-repeat (CRT) class of stress-responsive genes may be different from the pathways regulating osmolyte production. The activation of LEA-type genes may actually represent damage repair pathways (Xiong and Zhu, 2002). A LEA protein gene, *HVA1,* from barley (*Hordeum vulgare* L.) has been shown to confer tolerance to water deficit and salt stress in japonica rice cv Nipponbare (Xu, *et al.,* 1996; Babu *et al.,* 2004) and in indica rice cv Pusa Basmati 1 (Rohila *et al.,* 2002). Wheat LEA genes, PMA80 and PMA1959 conferred dehydration tolerance in transgenic rice (Cheng *et al.,* 2002). Recently, a LEA gene Rab16a from the indica rice Pokkali was shown to confer salt tolerance in transgenic tobacco plants (RoyChoudhury, 2007).

Calcium-dependent SOS signaling

SOS signaling appears to be relatively specific for the ionic aspect of salt stress and is calcium dependent. The targets of this type of signaling are ion transporters that control ion homeostasis under salt stress. The SOS pathway is triggered by excessive amounts of extracellular or intracellular Na^+, which elicits a cytoplasmic Ca^+ signal. The enhanced Ca^+ levels are sensed by SOS3 (calcium sensor) that activates SOS2 (serine/threonine protein kinase). Subsequently, SOS2 and SOS3 together activate SOS1 (a plasma membrane Na^+/H^+ antiporter). Recently, it has been shown that SOS

salt tolerance pathway also operates in rice and shares a high degree of structural and functional conservation with Arabidopsis (Martinez-Atienza *et al.,* 2007).

Targeting Transcription Machinery

Transcription factors (TFs) are key regulatory proteins that enhance or repress the transcriptional rate of their target genes by binding to specific promoter regions (i.e. cis-acting elements) upon activation or de-activation of upstream signaling cascades. TFs thus constitute master control elements of dynamic transcriptional networks. TFs have fundamental roles in almost all biological processes (development, growth and response to environmental factors). Plant genomes contain a large number of transcription factors; for example, *Arabidopsis* and rice dedicates 7.5% and 4.0% of their genome coding for 2304 and 2516 TFs, respectively. (Riano-Pachon *et al.,* 2007). In *Arabidopsis,* transcription factors belonging to various subfamilies such as DREB1A and DREB2A of AP2 family (Liu *et al.*, 1998), AREB1, AREB2 and AREB3 of bZIP family (Uno *et al.*, 2000), Atmyb2, CpMYB10 and BOS1 of MYB family (Urao *et al.,* 1993; Mengiste *et al.,* 2003; Villalobos *et al.,* 2004), RD26, ANAC019, ANAC055 and ANAC072 of NAC family (Fujita *et al.,* 2004; Tran *et al.,* 2004), and zinc finger proteins such as AZF1, AZF2, AZF3, STZ and ZPT2-3 (Sugano *et al.,* 2003; Sakamoto *et al.,* 2004) have been implicated in plant stress responses. Multiple TFs required for transcriptome reprogramming under abiotic stresses have been identified and functionally analyzed. Some of them have been well studied in rice, for example, DREBs (dehydration-responsive element-binding protein) / CBF (C-repeat-binding factor) and NACs (NAM, ATAF, and CUC), which have been briefly discussed in the following section.

DREBs/CBFs

The DRE (dehydration-responsive element)/CRT (C-repeat) was identified as a cis-acting element regulating gene expression in response to dehydration (salt, drought and cold stresses) in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994). TFs DREB1/CBF1-3, CBF4 and DREB2, belonging to the ERF/AP2 family, were reported to bind to DRE/CRT elements (Stockinger *et al.,* 1997; Liu *et al.,* 1998; Haake *et al.,* 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Five cDNAs of DREB homologs (OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D and OsDREB2A) have been isolated from rice (Dubouzet *et al.,* 2003). Overexpression of DREB/CBF from wheat (Shen *et al.,* 2003) and *Arabidopsis* (Lee *et al.,* 2004) was attempted in rice, but the level of stress tolerance could not be achieved and transgenic plants

showed growth retardation under normal conditions. This phenotype could be corrected and abiotic stress tolerance be achieved in rice by overexpressing *Arabidopsis* CBF3/DREB1 (CBF3) and ABF3 gene under the control of constitutive maize ubiquitin promoter (*Oh et al.,* 2005). Recently, several transgenic rice lines were generated over-expressing *OsDREB1A* and *OsDREB1B* from rice and *DREB1A, DREB1B* and *DREB1C* from *Arabidopsis* under the control of constitutive ubiquitin promoter. The transgenic plants were found to be more tolerant to salt, low temperature and drought conditions, but their growth was retarded under normal conditions. However, stress inducible expression recovered normal vegetative growth, which suggests that fine-tuning of expression of these genes is required (Ito *et al.,* 2006). Recently, overexpression of CBF4 from barley has been shown to confer drought, salinity and low temperature tolerance in transgenic rice without stunting growth (Oh *et al.,* 2007).

NAC genes

The NAC domain was originally characterized from consensus sequences from petunia NAM and from *Arabidopsis* ATAF1, ATAF2, and CUC2. The NAC gene family members encode one of the largest families of plant-specific TFs and are expressed in various developmental stages, tissues and environmental factors. Most of the NAC proteins contain a highly conserved N-terminal DNA-binding domain, a nuclear localization signal sequence, and a variable C-terminal domain. There are 75 and 105 putative NAC genes in rice and *Arabidopsis* genomes, respectively (Ooka *et al.,* 2003). Recently, a NAC gene *SNAC1* (stress-responsive NAC1) was isolated and characterized in rice. *SNAC1* was induced by drought and salinity predominantly in guard cells. *SNAC1* over-expressing transgenic rice plants showed significantly improved drought resistance under field conditions and strong tolerance to salt stress (Hu *et al.,* 2006).

Other transcription factors

Although multiple TFs have been well characterized in various plant species, transcriptional reprogramming under salt/drought stress is not fully understood. In rice, many transcription factors, including zinc finger, NAC, bHLH, Myb and WRKY were also identified to be induced by salt and drought stresses. An intronless gene *OSISAP1* from rice encoding a zinc-finger protein, which is inducible by different types of stresses, namely cold, desiccation, salt, submergence and heavy metals as well as injury was isolated and characterized (Mukhopadhyay *et al.,* 2004). Over-expression of this gene in tobacco

conferred cold, dehydration and salt tolerance at the seed germination/seedling stages. Thus *OSISAP1* gene could be a potential candidate for engineering stress tolerance in crop plants. Over-expression of *Arabidopsis* MYB2 gene in japonica rice under the control of ABA inducible promoter conferred salt stress tolerance in transgenic plants (Malik and Wu, 2005). Beside these, recently, a cDNA array containing 753 putative rice TFs was generated and the transcript profiles of these genes under drought and salinity stresses and abscisic acid treatment at seedling stage in rice was analyzed. About 80% of these TFs showed detectable levels of transcript. A total of 18 up-regulated transcription factors and 29 down-regulated TFs were detected with 2.0 to 20.5 folds changes in at least one stress treatment (Wu *et al.,* 2006). These novel stress-responsive transcription factors provided new opportunities to study the regulation of gene expression in plants under stress conditions.

Targeting Antioxidant machinery

A common consequence of a wide range of environmental stresses (such as high and low temperature, drought, salinity, UV or ozone stress, pathogen infections, herbicide action and oxygen shortage) is the enhanced production of reactive oxygen species (ROS) within several sub-cellular compartments of the plant cell. The reduction of oxygen to water provides the energy necessary for the metabolism of the organisms, but its reduction is a mixed blessing. When ROS are incompletely reduced, they can be extremely reactive and oxidize biological molecules such as DNA, proteins and lipids (Breusegem *et al.,* 2001). To control the level of ROS and to protect cells under stress conditions, plant tissues contain several enzymes scavenging ROS (superoxide dismutase, catalase, peroxidase and glutathione peroxidase), detoxifying lipid peroxidation products (glutathione S-transferase, phospholipid-hydroperoxide glutathione peroxidase, and ascorbate peroxidase) and a network of low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds and tocopherols). In addition, a whole array of enzymes is needed for the regeneration of the active forms of the antioxidants (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) (Blokhina *et al.,* 2003). Further, enhancement of photorespiration by overexpression of glutamine synthetase has been shown to confer salt tolerance in transgenic rice plants (Hoshida *et al.,* 2000).

Several attempts have been made to develop stress tolerant transgenic plants by over-expression of genes encoding any of these antioxidants. In this section, we bring out the reports where these antioxidants have been tested directly or indirectly as suitable candidate genes for developing salt stress tolerant crops with special emphasis to rice. Enhanced formation of ROS under stress conditions induces both protective responses and cellular damage. In the ROS scavenging process, SOD is the first line of defense, which converts superoxide radical to H_2O_2 (Scandalios, 1993). Overexpression of various isoforms of SOD was found to act as a safe guard against high temperature (Gupta *et al.,* 1993a), low temperature (Gupta *et al.,* 1993b; McKersie *et al.,* 1993; 1999, 2000), salt (Badawi *et al.,* 2004; Wang *et al.,* 2004), drought (McKersie *et al.,* 1996; Badawi *et al.,* 2004; Wang *et al.,* 2005a) and ozone induced cellular damage (Van Camp *et al.,* 1996) in various plant species. But, there are only few reports where over-expression of SOD in rice has been attempted. Tanaka *et al.,* (1999) reported that over-expression of a yeast mitochondrial Mn-SOD conferred salt tolerance in transgenic rice plants. Wang *et al.,* (2005a) overexpressed Mn-SOD and showed that transgenic plants were more tolerant to PEG stress. In a recent report, Prashanth *et al.,* (2007) have cloned a cDNA encoding cytosolic Cu/Zn-SOD from the mangrove plant *Avicennia marina.* Overexpression of this gene in *indica* rice cultivar Pusa Basmati 1 conferred salt and drought tolerance to transgenic plants. These reports suggest SOD as a potential candidate to engineer plants for multiple stress tolerance.

The intracellular level of H_2O_2 is regulated by a wide range of enzymes, the most important being catalase (Willekens *et al.,* 1995) and peroxidases. Overexpression of wheat catalase has been shown to confer chilling stress tolerance in transgenic rice plants (Matsumura *et al.,* 2002). Recently, Nagamiya *et al.,* (2007) have introduced *katE* a catalase gene from *E. coli* into japonica rice cultivar, Nipponbare and showed that transgenic rice plants were able to grow for more than 14 days in the presence of 250 mM NaCl, and were able to form flower and produce seeds in the presence of 100 mM NaCl. Increased activity of ascorbate peroxidase in response to environmental stresses such as salinity, chilling, metal toxicity, drought, heat etc. has been reported in different plant species which suggests its possible role in eliminating H_2O_2 from cells. Overexpression of ascorbate peroxidase has been attempted in various plant species to achieve tolerance against salt (Wang *et al.,* 2005b), chilling (Kornyeyev *et al.,* 2003; Wang *et al.,* 2005b), high temperature (Shi *et al.,* 2001), drought (Yan *et al.,* 2003) and oxidative stress (Wang *et al.,* 1999). Recently, Lu *et al.,* (2007) developed transgenic *Arabidopsis* plants over-expressing two rice cytosolic APXs (OsAPXa and OsAPXb) and reported that transgenic plants exhibited increased tolerance to salt stress than wild type plants.

In another recent report, simultaneous overexpression of three genes of antioxidant defense pathway viz., Cu/Zn-SOD, APX and dehydroascorbate reductase (DHAR) in chloroplast of tobacco plants was reported. Transgenic plants were found to exhibit very high tolerance to oxidative stress induced by paraquat and salinity stress than wild type plants (Lee *et al.,* 2007).

Glutathione S-transferase (GST) has been shown to confer tolerance in various plants against oxidative and salt stress (Katsuhara *et al.,* 2005; Zhao and Zhang, 2006). Zhao and Zhang (2006) have developed transgenic rice plants expressing glutathione S-transferase (GST) and catalase from *Suaeda salsa.* Transgenic plants exhibited higher tolerance against salt and oxidative stresses. It has been well established in literature that glutathione (GSH) plays an important role in antioxidant defense system in plants. An increase in glutathione synthesis as well as GSH/GSSG ratio has been shown to be related to stress tolerance (Tausz *et al.,* 2004). The glyoxalase pathway involving glyoxalase I (gly I) and glyoxalase II (gly II) enzymes has been shown to be required for glutathione-based detoxification of methylglyoxal (MG). In our own laboratory we have developed transgenic tobacco plants over-expressing *glyI* and *glyII* and showed that transgenic plants had much higher salinity tolerance and heavy metal tolerance than non-transgenic plants (Singla-Pareek *et al.,* 2003, 2006). Recently, we showed that over-expression of rice *gly II* gene in rice conferred tolerance to toxic levels of methylglyoxal and NaCl compared with non-transgenic plants (Singla-Pareek *et al.,* 2007b). Pyramiding of multiple genes of antioxidant defense pathway could be a useful strategy to develop transgenic rice plants, which might be tolerant to multiple stresses, because of their ability to scavenge ROS efficiently.

Targeting Osmotic homeostasis machinery

Osmotic stresses such as salinity and drought stresses result in cellular dehydration. Plants accumulate osmotically active compounds called osmolytes in order to lower the osmotic potential. These osmolytes serve as osmoprotectants and stabilize biomolecules under stress conditions and can be referred to as compatible solutes because they do not apparently interfere with the normal cellular metabolism. Sugars, polyols, amino acids and diverse substances are synthesized and accumulated intracellularly to balance the osmotic potential of the cell. Overproduction of various osmolytes has been tested in rice to achieve abiotic stress tolerance that has been discussed in the following text.

Glycine betaine (*N,N,N*-trimethylglycine hereafter GB) is a quaternary ammonium compound that occurs naturally in a wide range of plants, animals and microorganisms (Rhodes and Hanson, 1993). In most organisms, GB is synthesized as a result of two-step oxidation of choline via betaine aldehyde, a toxic intermediate. In several higher plants, the relevant enzymes are choline monooxygenase (CMO) - a ferredoxin-dependent soluble Rieske-type protein, and betaine aldehyde dehydrogenase (BADH) - a soluble NAD$^+$-dependent enzyme (Weigel *et al.,* 1986; Brouquisse *et al.,* 1989). In mammalian cells and microorganisms such as *E. coli,* GB is synthesized by choline dehydrogenase (CDH) - a membrane bound oxygen-dependent enzyme, in combination with BADH (Wilken *et al.,* 1970; Landfald and Strom, 1986). In contrast to each of these two pathways that involved two enzymes, the biosynthesis of GB is catalysed by a single flavoenzyme choline oxidase (COD) in certain microorganisms such as the soil bacterium *Arthrobacter globiformis* (Ikuta *et al.,* 1977). To achieve tolerance against various abiotic stresses, overexpression of BADH (Moghaieb *et al.,* 2000; Kumar *et al.,* 2004; Yang *et al.,* 2005), CDH (Lilius *et al.,* 1996; Quan *et al.,* 2004) has been carried out in several plant species. Overexpression of *coda* gene encoding COD has been most widely attempted to develop abiotic stress tolerant transgenic plants (Hayashi *et al.,* 1997; Alia *et al.,* 1998; Huang *et al.,* 2000, Sulpice *et al.,* 2003; Parvanova *et al.,* 2004; Prasad and Pardha-Saradhi, 2004). Transgenic rice, tolerant to salt stress has also been developed by the overexpression of *codA* by various groups (Sakamoto *et al.,* 1998; Mohanty *et al.,* 2002; Su *et al.,* 2006).

Proline is another one of the most widely exploited molecule for the development of abiotic stress tolerant transgenic plants. In organisms ranging from bacteria to higher plants, there is a strong correlation between increased cellular proline levels and the capacity to survive both water deficit and the effects of high environmental salinity. The proline is synthesized from glutamate via two intermediates- glutamic-γ-semialdehyde and Δ^1-pyrroline-5-carboxylate. The first step is catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS), and is rate limiting (Delauney and Verma, 1993). Overexpression of *P5CS* has been attempted in various plant species and transgenic plants were reported to exhibit tolerance against drought (De Ronde *et al.,* 2001; 2004; Molinari *et al.,* 2004; *Yamada et al.,* 2005; Molinari *et al.,* 2007), salinity (Kavi Kishor *et al.,* 1995; Hamida-Sayari *et al.,* 2005). In rice, overexpression of *P5CS* conferred drought and salinity tolerance to the transgenic plants (Zhu *et al.,* 1998; Su and Wu, 2004).

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Trehalose, a non-reducing disaccharide of glucose plays an important physiological role as an abiotic stress protectant in a large number of organisms, including bacteria, yeast and invertebrates. Trehalose has been shown to stabilize dehydrated enzymes, proteins and lipid membranes efficiently, as well as protect biological structures from damage during desiccation. In the plant kingdom, most species do not seem to accumulate detectable amounts of trehalose, with the exception of highly desiccation-tolerant resurrection plant, *Selaginella lepidophylla* (Wingler, 2002). In bacteria and yeast, trehalose is synthesized in a two-step process: trehalose-6-phosphate is first formed from UDP-glucose and glucose-6-phosphate in a reaction catalyzed by trehalose-6-phosphate synthase (TPS). Trehalose-6-phosphate is then converted to trehalose by trehalose-6-phosphate phosphatase (TPP) (Goddijn and van Dun, 1999). Overproduction of trehalose by overexpressing *E. coli* TPS and TPP fusion gene in rice under the control of ABA inducible (Garg *et al.,* 2002) and maize ubiquitin promoters (Jang *et al.,* 2003) conferred drought and salinity stress tolerance.

Among nitrogenous osmolytes, proline and polyamines (PAs) are the most important. PAs are low-molecular weight polycations, and are present in all living organisms. The key enzymes in polyamine biosynthesis are arginine decarboxylase (ADC), ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC). Overproduction of polyamines was achieved by the over-expression of ADC (Roy and Wu, 2001) and SAMDC (Roy and Wu, 2002) in rice under the control of ABA inducible promoter and it was shown that transgenic plants had higher salinity tolerance as compared to non-transgenic plants.

Targeting Ionic homeostasis machinery

Another strategy for achieving greater tolerance is to help plants re-establish homeostasis in stressful environments. Various ion transporters are the terminal determinants of ionic homeostasis. Because excess Na^+ inhibits many cellular enzymes, it is important to prevent Na^+ accumulating to a high level in the cytoplasm or in organelles other than the vacuole (Zhu, 2001). It has been reported that in salt-tolerant plants, the compartmentalization of Na^+ into vacuoles through the operation of a vacuolar Na^+/H^+ antiporter provides an efficient mechanism to avert the deleterious effects of Na^+ (and chloride) accumulated in the vacuole to drive water into the cells (Glenn *et al.,* 1999). It has been reported that endogenous levels of Na^+/H^+ antiporter remain low in salt sensitive plants such as rice even

when subjected to salt stress (Fukuda *et al.,* 1998). Orthologs of Na^+/H^+ antiporter genes have been isolated from both glycophytes and halophytes (Hasegawa *et al.,* 2000; Munns, 2005). Overexpression of *Arabidopsis thaliana AtNHX1* conferred enhanced salt tolerance in *Arabidopsis* (Apse *et al.,* 1999), tomato (Zhang and Blumwald, 2001), *Brassica napus* (Zhang *et al.,* 2001) *Triticum aestivum* (Xue *et al.,* 2004) and *Zea mays* (Xiao-Yan *et al.,* 2004). Overexpression of rice NHX (*OsNHX1*) (Fukuda *et al.,* 2004), *Suaeda salsa* NHX (*SsNHX1*) (Zhao *et al.,* 2006), *Atriplex gmelini* NHX (*AgNHX1*) (Ohta *et al.,* 2002) and *Pennisetum glaucum* NHX (*PgNHX1*) (Verma *et al.,* 2007) in rice has been attempted and found that transgenic plants exhibited higher salt tolerance than non-transgenic plants. Beside NHX, overexpression of a plasma membrane Na^+/H^+ antiporter *SOD2* from yeast (*Schizosaccharomyces pombe*) increased salt tolerance in transgenic rice (Zhao *et al.,* 2006). Recently, a rice homolog of Shaker family K^+ channel KAT1 (OsKAT1) has been identified from full-length cDNA expression library of japonica rice cv. Nipponbare. OsKAT1 was shown to suppress the salt-sensitive phenotype of yeast mutant, which lacks a major component of Na^+ efflux. Overexpression of *OsKAT1* in rice conferred salt stress tolerance in transgenic rice (Obata *et al.,* 2007).

CONCLUSIONS AND FUTURE PERSPECTIVES

Several candidate genes targeting various protective pathways pertaining to salt stress tolerance mechanism(s) have been identified and validated using model systems such as Arabidopsis and tobacco. However, only handful of genes has so far been functionally validated in transgenic rice for its improved performance under salinity stress (Figure 1). It is clear from the transcriptome analysis that a wide array of genes gets up regulated in response to salinity stress (Pareek *et al.,* 2007). However, the question that which are the best candidate gene(s) related to salinity stress tolerance improvement is still baffling plant scientists. Identifying and validating novel genes in rice that will significantly improve its performance under salinity stress and thus possibly have great impact on the yield must be taken as the prime challenge. Under field conditions, crops can be exposed to multiple stress factors simultaneously and thus, it would be important to look for those genes that can confer tolerance to multiple abiotic stresses to ensure agricultural sustainability in soils with multiple unfavorable edaphic factors. Since salt stress tolerance of plants is a complex trait and involves multiple physiological, biochemical mechanisms and a wide array of genes, future work on improvement

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of rice for salinity stress tolerance should involve stacking of various genes. This can be done either with respect to combining multiple genes targeting a single protective pathway or by combining key regulatory genes of diverse protective pathways. Of additional importance and benefit, would be to employ strong stress regulated promoters to drive the expression of the transgenes for several obvious reasons. Future efforts for developing salinity tolerant rice should take into account all these aspects.

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