



Full Length Article

Identification of New Alleles in Salt Tolerant Rice Germplasm Lines through Phenotypic and Genotypic Screening

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Abstract

The present study investigated eight rice lines (Rupsal, Nagalmutha, Polai, Ravana, Marishal, Talmugra, Kamini and Raspanjar) collected from coastal region of eastern India for salinity tolerance through phenotypic and genotypic screening. Among these, three rice lines as highly tolerant (Talmugra, Marishal and Kamini), three tolerant (Rupsal, Polai and Raspanjar) and two moderately tolerant (Ravana and Nagalmutha) to salt stress were identified in phenotypic screening. Pokkali was categorized as tolerant under salinity condition (12 EC dS m⁻¹). In PCR screening using microsatellite (SSR) markers located within Saltol locus, we documented new allelic pattern in selected highly tolerant and tolerant genotypes with RM8094 marker as compared to Pokkali. Besides, another marker RM10694 was found to associate with selection of salinity tolerant genotypes similar to Pokkali. In gene expression studies, no significant difference linked with abscisic acid (ABA), calcium-dependent proteins kinase (CDPK), ionic and osmotic signaling pathways in salinity tolerant genotypes was found as compared to sensitive line (IR29). Induction of AP37 gene expression differentiated Kamini and Marishal genotypes from other tolerant and sensitive lines. The chlorophyll and protein contents were maintained in highly tolerant genotypes (Marishal, Kamini and Talmugra) as compared to sensitive one (IR29). Among agronomic traits, selected rice lines recorded to have significant morphological features such as plant height, tiller number, leaf length and width in order to tolerate salt stress. Genetic diversity analysis revealed that, highly tolerant genotypes showed distant relationship with Pokkali at genetic level. © 2016 Friends Science Publishers

Keywords: Rice lines; Salt stress; Microsatellite (SSR) marker; Protein analysis; Gene expression

Introduction

Soil salinization is a growing problem for agriculture worldwide. In Asia, out of 21.5 m ha of salt affected land area, about 12 m ha is saline and 9.5 m ha sodic. Salt accumulation in arable soils is mainly derived from irrigation water containing trace amounts of sodium chloride (NaCl) (Tester and Davenport, 2003). Salt stress also is a major problem for rainfed agriculture in coastal areas because of seawater ingress during high tide and the rising shallow saline groundwater, particularly during the dry season. Increased soil salt concentration (NaCl) decrease the ability of a plant to take up water. Both Na⁺ and Cl⁻ negatively affect plant growth by impairing metabolic processes and decreasing photosynthetic efficiency through the occurrence of osmotic stress and accumulating ionic Na⁺ stress (Maser *et al.*, 2002). In this case, plants enact mechanisms to mitigate osmotic stress by reducing water loss while maximizing water uptake by minimizing the harmful effects by compartmentalization of Na⁺ into vacuoles (Munns and Tester, 2008). In addition, plants

exhibit many physiological responses to osmotic stress such as rapid rise in Ca²⁺ levels within seconds of exposure to NaCl or mannitol (Knight *et al.*, 1997), cytosolic downstream abscisic acid (ABA) production (Wang *et al.*, 2011) and structural changes in the plasma membrane/cell wall to alter the water diffusibility into the cells. Other secondary messengers are also induced by salt stress, for example, expression of calcium-dependent proteins kinases (CDPK) (Boudsocq and Sheen, 2013) and calcineurin B-like proteins (CBLs) with CBL-interacting protein kinases (CIPKs) (Weinl and Kudla, 2008) to activate downstream protein and gene transcription. In gene regulation, transcription factors such as basic leucine zipper (bZIP) (Yang *et al.*, 2009), WRKY (Jiang and Deyholos, 2009), Apetala2/ETHYLENE RESPONSE FACTOR (AP2/ERF) (Kasuga *et al.*, 1999), MYB (Cui *et al.*, 2013), basic helix-loop-helix (bHLH) (Jiang *et al.*, 2009) and NAC (Tran *et al.*, 2004) regulate the expression levels of various genes that ultimately influence salt tolerance of plants. Furthermore, for enhancing salinity tolerance, Na⁺ transporters such as HKTs, NHXs and ROS (reactive

oxygen species) scavengers play major roles in salt homeostasis and positively influence the capacity of the plant to deal with elevated salinity. The accumulation of organic osmolytes such as proline, glycine-betaine, sugar alcohols, polyamines and proteins from the late embryogenesis abundant (LEA) super family, plays a key role in maintaining the low intracellular osmotic potential of plants and in preventing the harmful effects of salinity stress (Verslues *et al.*, 2006).

Naturally, some traditional cultivars and lines of rice are more tolerant than elite cultivars to salt stress and considered to be good source of tolerance traits. Generally, rice is considered to be salt sensitive and genetic variation exist for salt tolerance at critical stages in the cultivated gene pool (Moradi *et al.*, 2003). One example of a traditional and tolerant genotype to high salinity is the Indian landrace Pokkali and has been frequently used as a donor of salt-tolerance trait in breeding programs. In this genotype, the salt tolerance is usually attributed to both its capacity to maintain a low Na⁺ to K⁺ ratio in shoot tissue and its faster growth rate under saline condition. FL478, which is one of RILs derived from Pokkali and IR29 appears to be a good candidate for salinity stress tolerance in rice particularly at the seedling stage of growth (Bonilla *et al.*, 2002). However, Moradi *et al.* (2003) has suggested that salt tolerance at the seedling and reproductive stages is only weakly associated, therefore, pyramiding of significant traits at both stages is required for developing salt tolerant rice cultivars. Additionally, an anticipated effect of global warming is an increase in salt affected area and severity of salt stress, both in coastal and inland ecosystems. In coastal areas, an increase in salt intrusion has already been observed in some of the low-lying deltas such as in South Bangladesh, Vietnam and Myanmar. In inland areas, salt deposition is expected to increase as a consequence of increased evapotranspiration and water shortage with rising temperatures. Moreover, the earth's climate is predicted to still warm by an average of 2–4°C by the end of the 21st century due to both anthropogenic and natural factors (IPCC, 2007; Eitzinger *et al.*, 2010). In this context, rice breeders are being insisted for the need of further enhancement in tolerance or combination of more than one trait in rice cultivars in order to increase rice production under changing climate for fulfilling rapidly growing food demands of increasing human population. Here, eight rice germplasm lines (Rupsal, Nagalmutha, Ravana, Marishal, Polai, Talmugra, Kamini and Raspanjar) collected from coastal region of eastern India were analysed for new alleles for salinity tolerance through phenotypic and genotypic screening.

Materials and Methods

Plant Materials

In this study, eight rice lines (Rupsal, Nagalmutha, Polai, Ravana, Marishal, Talmugra, Kamini, Raspanjar) collected

from coastal region of eastern India including salinity tolerant (Pokkali) and sensitive check (IR29) were used.

Phenotypic Screening

Pre-germinated seeds were sown in holes on styrofoam floats with a net bottom suspended on trays filled with Yoshida nutrient solution (Yoshida *et al.*, 1976). Seedling of 5-days old were salinized with NaCl to EC 6 dS m⁻¹ in Yoshida nutrient solution for 5 days and then to EC 12 dS m⁻¹ until the final scoring. Pokkali (tolerant) and IR29 (sensitive) were used as checks. The pH of the nutrient solution was adjusted daily to 5.0 and the culture solution was replaced weekly. At 15 days after treatment (DAT), entries were scored based on visual symptoms using IRRIs standard evaluation scale (SES) for rice, with ratings from 1 (highly tolerant) to 9 (highly sensitive) (Thomson *et al.*, 2010).

Genomic DNA Extraction and PCR Amplification

A crude DNA suitable for PCR screening was prepared using a simplified miniscale procedure (Lang, 2002). PCR amplification was performed using genomic DNA of eight rice lines along with tolerant (Pokkali) and sensitive (IR29) checks using SSR markers (RM493, RM8094, AP3206, RM1287, RM10694, RM10748, RM562, RM7075 and RM3412) located within Saltol locus (Thomson *et al.*, 2010).

RNA Extraction and Reverse-transcriptase (RT)-PCR

For gene expression analysis, total RNA was extracted from leaves of highly tolerant lines, Talmugra, Marishal, Kamini, Pokkali and IR29 under salinity condition (on 5th DAT) using TRIzol according to manufacturer's instructions. RNA pellet was dissolved in RNase free water and stored at –20°C. Then, synthesis of cDNA was done using SuperScriptTM III Reverse Transcriptase according to manufacturer's protocol (Invitrogen, California, USA) in a reaction mixture containing 50–75 ng RNA with the final volume completed to 20 µL using RNase free water. PCR amplification was carried out at 56°C (annealing temperature) using cDNA with primer sequences of stress related genes (*RAB16A*, *LEA3*, *LIP9*, *Salt*, *AP37*, *AP59* and *DREB1A* (dehydration-responsive element) (Fukao *et al.*, 2011), *OsDREB2A* (Dubouzet *et al.*, 2003) and *OsCDPK7* (Calcium-dependent protein kinase) (Saijo *et al.*, 2000). In another study, gene expression analysis of *OsSOS1*, *OsCIPK24*, and *OsCBL4* genes of salt overly sensitive (SOS) pathway and Na⁺/K⁺ exchanger gene family, *OsHKT1;5* (Yang *et al.*, 2012) in shoot and root tissues of rice lines of highly tolerant (*Kamini*, *Talmugra* and *Marishal*) and tolerance reaction (*Polai*) was executed in presence of *Pokkali* and IR29 under salinity condition (EC 12 dS m⁻¹) at 5th DAT. RNA extraction and cDNA synthesis were done as mentioned above. The sequence of primers and their accession number are given in Table 1.

Table 1: List of primers used in reverse transcriptase (RT)-PCR and their sequences and accession number

Primer	Sequence (5'-3')		Accession No.
	Forward	Reverse	
<i>RAB16A</i>	CATGGACAAGATCAAGGAGAAGC	CTTATTATTCAGGAAGGTGACGTGG	LOC_Os11g26790
<i>LEA3</i>	GCCGTGAATGATTTCCCTTTG	CACACCCGTCAGAAATCCTCC	LOC_Os05g46480
<i>LIP9</i>	TGGAATTTGGAAGTGTTTGGC	CCCACACGAAACACAAACTTC	LOC_Os02g44870
<i>SalT</i>	TGGATTCTTTGGAAGGTCTGG	TTGACCACTGGGAATCAAGG	LOC_Os01g24710
<i>AP37</i>	TCCGATGTTTTGGTCCTCTG	TCCACGGTTTAGTCCATCTCATC	LOC_Os01g58420
<i>AP59</i>	GGTGATTTAGCCATCTTTGTGCG	TCGTACATTTCTTGGAGCAG	LOC_Os02g43790
<i>DREB1A</i>	GGGGAATTCATGTGCGGGATCAAGCAGGAGATG	GGGGATCCTAGTAGCTCCAGAGTGGGAC	AF300970
<i>OsDREB2A</i>	ATCGCGGCCGCATGGAGCGGGGGAGGGGAG	GGGGATCCTACTCTAATAGGAGAAAAGGCT	AF300971
<i>OsCDPK7</i>	ACATCGTCATGGAGCTCTGCGCC	GAGCTACGTAATATGGGCTCCG	AB042550
<i>OsSOS1</i>	CTCCGTGTCATAGAATCGC	ATACTACTCAAGTGGGTCAATACC	AY785147
<i>OsCIPK24</i>	AAGAAGCGGTGGGGAGGT	GCGGTGGTTGAGGATGGTGT	AK102220
<i>OsCBL4</i>	GGCATCGTTCCGATTTTAC	GAGATTCGCCCTTTCTGCTGTT	AK101368
<i>OsHKT1;5</i>	TGCCACCTTACACCACTTTCG	TGCCATACGCACTGATAACCTC	DQ148410

Chlorophyll Pigment Estimation

Chlorophyll estimation was done for highly tolerant rice lines (Talmugra, Marishal, Kamini) in the presence of Pokkali and IR29 at 0, 3rd- and 6th- day of salinity condition (12 EC dS m⁻¹, NaCl). For this, total chlorophyll content was extracted from 1 g of leaf sample using 80% acetone and kept for 48 h at room temperature. The amount of chlorophyll present in the leaf extract (mg *Chl* g⁻¹) was calculated using the following equations (Arnon, 1949):

$$\text{For mg } Chl \text{ a/g} = \frac{12.7 (A_{663}) - 2.69(A_{645}) \times V}{1000 \times W} \quad (1)$$

$$\text{For total } Chl \text{ /g} = \frac{22.9 (A_{665}) - 4.68(A_{645}) \times V}{1000 \times W} \quad (2)$$

$$\text{For mg } Chl \text{ b /g} = \frac{20.2 (A_{645}) - 8.02(A_{663}) \times V}{1000 \times W} \quad (3)$$

Where, A is the absorbance at specific wavelengths; V is the Final volume of chlorophyll extract in 80% Acetone and W is the fresh weight of tissue extract.

Protein Profile

Protein analysis was done (NaCl, 12 EC dS m⁻¹) at 5-DAT for highly tolerant rice lines (Talmugra, Marishal, Kamini), Pokkali and IR29 maintained in plastic tray. Leaf material from each genotype were ground in liquid nitrogen and homogenated with phosphate buffer containing 1 mM Dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF). Protein solution were collected after centrifugation for 15, 000 rpm at 4°C for 20 min. The protein was estimated according to Bradford method with bovine serum albumin (BSA) as standard (Bradford, 1976). Then, the protein sample (100 µg) was loaded along with protein ladder in 10% acrylamide gel and the gel was run for 6 h at 50 mA. The gel was stained with coomassive brilliant blue (CBB) solution for 2 h and destained with solution containing methanol and acetic acid (1:1 ratio). Gels were documented (SYNGENE, UK) and the intensive protein bands were recorded.

Characterization of Agronomic Traits

Seeds of eight rice lines, Pokkali and IR29 were kept at 50°C for 5 d to break dormancy and then dipped in 70% alcohol for 2 min and washed properly with distilled water. Seeds were then dipped in 2% Clorox for 30 min and washed properly with distilled water. Seeds were kept in an incubator at 35°C for 7 days under dark condition and germinated seeds were planted in trays for two weeks. Two week old seedlings were then transplanted in the paddy field according to the randomized complete block design with three replicates and 5 plants per replicate with 15 cm × 20 cm spacing in rice field of CRRI, Cuttack. Fertilizers were applied at rates of Urea 50 kg h⁻¹, TSP 62.5kg/ha, MOP 50kg/ha at sowing time. Urea at a rate of 37.5 kg/ha was also applied as top dressing after 2 and 7 weeks of planting. The rice yield, yield components and other characteristics were determined according to the method of Standard Evaluation System for Rice (IRRI, 1988). For evaluation of agronomic traits of rice genotypes in field condition, following parameters were taken: for plant height (cm), plant height was measured from the base of the plant to the top of the latest spikelet on the panicle, excluding awn; for leaf blade length (cm), leaf length was measured from the leaf base to the leaf tip of the fully expanded leaves; for leaf blade width (cm), length was measured at the widest point of the leaf; for number of tillers per plant, tillers were counted at the maturity stage; for panicle length (cm), panicle length was measured from the base of the lowest spikelet to the tip of the latest spikelet on the panicle, excluding awn; for number of spikelets per panicle, total number of spikelets were counted in sampled panicles; for panicle weight (g), panicles of five plants of each rice line was weighed; for 1000 grain weight (g), 1000 grains were counted from five plants of each line and weighed; for Seed length (mm) and Seed width (mm), they were measured using Venire calliper; and for number of sterile seed/panicle, unfilled grains were counted in sampled panicles. Data were made in LSD (P=0.05) value using SAS programme (SAS, 1982).



Fig. 1: Survival of highly tolerant rice lines in healthy condition under salinity condition
a-Talmugra; b-Kamini; c-Marishal

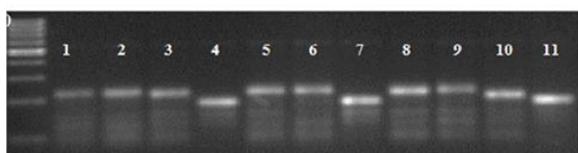


Fig. 2: PCR amplification in eight rice landraces with RM8094 marker
Lane: 1&2-Pokkali, 3-Rupsal, 4-Nagalmutha, 5-Polai, 6-Talmugra, 7-Ravana, 8-Marishal, 9-Kamini, 10-Raspanjar, 11-IR29

Genetic Diversity Analysis

For studying genetic diversity among eight rice genotypes, Pokkali and IR29, 56 SSR markers across 1–12 chromosomes were used and the details of primers are given in Table 2. Based on PCR banding pattern, these genotypes were grouped using Dice similarity coefficient index according to the UPGMA method (Nei *et al.*, 1983).

Statistical Analysis

In phenotypic screening, visual symptoms of rice seedlings was done using IRRI's standard evaluation scale (SES) for rice, with ratings from 1 (highly tolerant) to 9 (highly sensitive) (Thomson *et al.*, 2010). Protein analysis was done according to Bradford method with BSA as standard (Bradford, 1976). In genetic diversity analysis, clustering of rice lines was done using dice similarity coefficient index according to the UPGMA method (Nei *et al.*, 1983). In characterization of agronomic traits, data were made in LSD ($P=0.05$) value using SAS programme (SAS, 1982).

Results

Phenotypic and PCR Screening

Under salinity, among eight rice lines, Marishal, Kamini

and Talmugra as highly tolerant (score 1), Rupsal, Polai, Raspanjar with Pokkali as tolerant (score 3), Nagalmutha and Ravana as moderately tolerant (score 5) and IR29 as sensitive (score 7) were categorized (Fig.1).

With SSR markers, maximum number of rice genotypes were found to possess allele similar to Pokkali with RM10748 (8 genotypes) followed by 5 genotypes (Polai, Talmugra, Ravana, Nagalmutha and Raspanjar) with AP3206, 5 genotypes (Polai, Marishal, Talmugra, Kamini, Rupsal and Raspanjar) with RM10694, 4 genotypes (Marishal, Talmugra, Ravana and Nagalmutha) with RM3412, 4 genotypes (Marishal, Kamini, Rupsal and Raspanjar) with RM562, 4 genotypes (Polai, Talmugra, Rupsal and Raspanjar) with RM7075) and 1 genotype (Rupsal) with RM493 (Table 3). But, none of these genotypes possessed Pokkali alleles with RM8094 marker. For this marker, rice lines were categorized as highly tolerant and had alleles around 205 base pair (bp) length when compared to Pokkali (210bp) and IR29 (195bp) (Fig. 2).

Reverse Transcriptase (RT)-PCR

In gene expression analysis, expression of *RAB16A*, *LEA3*, *LIP9*, *Salt*, *AP37*, *AP59*, *DREB1A* and *OsDREB2A* genes was detected in Talmugra, Marishal, Kamini, Pokkali and IR29 under salinity (Fig. 3). However, expression of these genes was insignificant when compared to IR29. But, expression of *AP37* gene was found to induce only in Kamini and Marishal genotypes. There was no significance in gene expression of *OsSOS1*, *OsCIPK24*, *OsCBL4* and *OsHKT1;5* in shoot and root parts of Marishal, Kamini, Talmugra and Pokkali when compared to IR29 (Fig. 4).

Chlorophyll Contents

Under salinity stress, the optical density (OD) value of *Chl a* and *b* and total chlorophyll content was higher at 6th day in Marishal, Kamini, Talmugra and Pokkali when compared to IR29. However, there was no significant difference in OD values of *Chl a*, *b* and total chlorophyll among genotypes at 0 and 3rd day (Fig. 5).

Protein Contents

In protein analysis of highly tolerant genotypes under salinity stress, we found the maintenance of protein level at 125, 72, 42, 33, 18 and 15 kDa when compared to sensitive check IR29 (Fig. 6).

Agronomic Traits

Among eight rice lines, Pokkali and IR29, had the highest and lowest mean value of each trait as follows: Talmugra and Ravana for plant height; Raspanjar and Marishal for leaf length; Talmugra and Nagalmutha and Marishal for leaf width; Rupsal and IR29 for number of tillers per plant;

Table 4: Mean values of five plants for each trait of ten rice lines

Rice lines	PH (cm)	LL (cm)	LW. (cm)	No. of tiller/plant	of PL (cm)	No. spikelet/panicle	of PW (g)	SL (mm)	SW (mm)	No. of sterile Seed/panicle	1000 grain wt. (g)
Pokkali	150.8 ^c	70.4 ^a	1.1 ^d	7.4 ^e	30.0 ^c	218.4 ^d	4.1 ^e	9.0 ^{cd}	3.6 ^b	53.4 ^c	27.8 ^d
Rupsal	131.6 ^e	75.6 ^b	1.0 ^d	11.6 ^a	28.0 ^d	206.8 ^f	3.2 ⁱ	9.6 ^c	3.0 ^c	75.4 ^a	25.4 ^e
Nagalmutha	137.0 ^f	64.3 ^c	0.8 ^f	10.0 ^{abc}	22.4 ^f	213.2 ^e	3.4 ^{gh}	10.4 ^b	3.0 ^c	33.4 ^d	23.3 ^e
Polai	207.0 ^b	72.7 ^d	1.0 ^d	9.0 ^{bcd}	26.5 ^e	251.2 ^b	7.3 ^a	11.6 ^a	4.0 ^a	10.6 ^b	34.7 ^b
Marishal	177.0 ^c	53.3 ^e	0.8 ^f	8.0 ^{cde}	38.0 ^a	271.4 ^a	4.6 ^d	7.8 ^e	4.0 ^a	31.2 ^e	20.2 ^f
Ravana	127.0 ^g	54.7 ^f	0.9 ^e	6.2 ^{eig}	22.0 ^f	115.4 ⁱ	2.3 ⁱ	10.6 ^b	3.6 ^b	12.8 ^g	24.2 ^f
Talmugra	217.0 ^a	62.1 ^g	1.5 ^b	6.8 ^{eig}	28.0 ^d	181.2 ^g	5.4 ^c	9.6 ^c	4.0 ^a	9.0 ^f	33.1 ^b
Kamini	157.0 ^d	68.0 ^b	1.0 ^d	10.2 ^{ab}	31.4 ^b	175.2 ^h	3.6 ^f	6.6 ⁱ	3.2 ^c	23.2 ^f	17.3 ^j
Raspanjar	157.0 ^d	76.5 ⁱ	1.2 ^c	9.4 ^{bcd}	9.4 ^g	125.4 ^j	3.2 ^{hi}	8.4 ^e	4.0 ^a	4.6 ^g	30.4 ^c
IR29	107.0 ^h	64.3 ^j	1.0 ^d	5.6 ^{ef}	5.6 ⁱ	158.8 ^j	3.5 ^{ef}	10.6 ^b	3.2 ^c	10.2 ^{hi}	24.0 ^f

In a column, means followed by a common letter are not significantly different at $P=0.05$

PH-plant height; LL-leaf length; LW-leaf width; PL-panicle length; PW-panicle weight; SL-seed length; SW-seed width

Marishal and Raspanjar for panicle length; Marishal and Ravana for number of spikelet per panicle; Polai and Ravana for panicle weight; Polai and Kamini for seed length; Polai, Marishal, Ravana, Rupsal and Nagalmutha for seed width; Rupsal and Raspanjar for number of sterile kernels per panicle; Polai and Kamini for 1000 kernel weight and number of sterile spikelet in Rupsal and Raspanjar (Table 4).

Genetic Diversity

Eight lines grouped into two main clusters in which Marishal and Kamini were in one cluster and rest of rice lines including Pokkali and IR29 in another cluster (Fig. 7). In major cluster, Pokkali grouped into sub-cluster along with moderately tolerant genotype, Nagalmutha and Ravana, whereas IR29 grouped with Raspanjar in a separate sub-cluster. And, Rupsal, Polai and Talmugra grouped into sub-cluster separately.

Discussion

In centers of diversity, rice lines are products of natural evolution brought about by the interaction of both abiotic and biotic factors. By this evolutionary process, rice lines have been identified for their attribution of useful traits which play significant role in rice improvement against many abiotic stresses such as FR13A for submergence tolerance (Vergara and Mazaredo, 1975) and Pokkali for salt tolerance (Bonilla *et al.*, 2002). However, so far identified gene/QTLs are not associated with tolerance to all stages of crop. For example, *Saltol* locus is associated with seedling stage tolerance and not associated at reproductive stage. Therefore, it is suggested that pyramiding of significant traits/alleles at both stages is required for developing salt tolerant rice cultivars (Moradi *et al.*, 2003). Besides, present global warming by increasing temperature necessitates identification of source of rice lines with more efficient alleles in order to tolerate adverse effects of climate change (IPCC, 2007; Eitzinger *et al.*, 2010). In this study, three rice lines (Marishal, Kamini and Talmugra) were

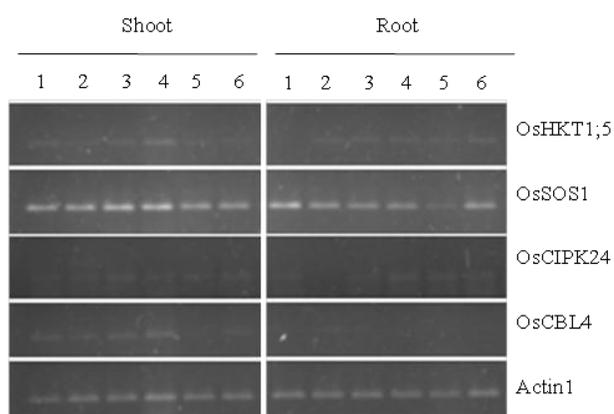


Fig. 4: Gene expression analysis through reverse transcriptase (RT) PCR under salinity condition. Lane1-Pokkali; 2-IR29; 3-Polai; 4-Marishal; 5-Talmugra; 6-Kamini

selected as highly tolerant under salinity (12 dS m^{-1}). These rice lines survived in healthy condition without any stress symptom until completion of experiment when compared to Pokkali and other tolerant lines (Fig. 1). In tolerant lines (Rupsal, Polai and Raspanjar), the stress symptoms were observed in older leaves after one week of stress. In moderately tolerant lines (Ravana and Nagalmutha), most of the leaves started to dry after one week, and in IR29, all leaves completely dried within one week. In the PCR screening, all SSR markers did not show salinity tolerant rice line except RM8094 and RM10694 markers. Because rice lines categorized as moderately tolerant showed to harbour same type of alleles with these markers i.e., Ravana and Nagalmutha (moderately tolerant) as well as Polai, Talmugra, Kamini, Marishal, Rupsal and Raspanjar (tolerant) have had same type of alleles with RM10748, AP3206 and RM3412 markers. The markers RM562, RM7075 and RM493 helped to identify Pokkali alleles but not in all tolerant lines i.e. RM493 marker revealed Pokkali allele only in Rupsal and not in other tolerant lines. In this study, RM10694 marker played a significant role in identification of tolerant rice lines with Pokkali alleles.

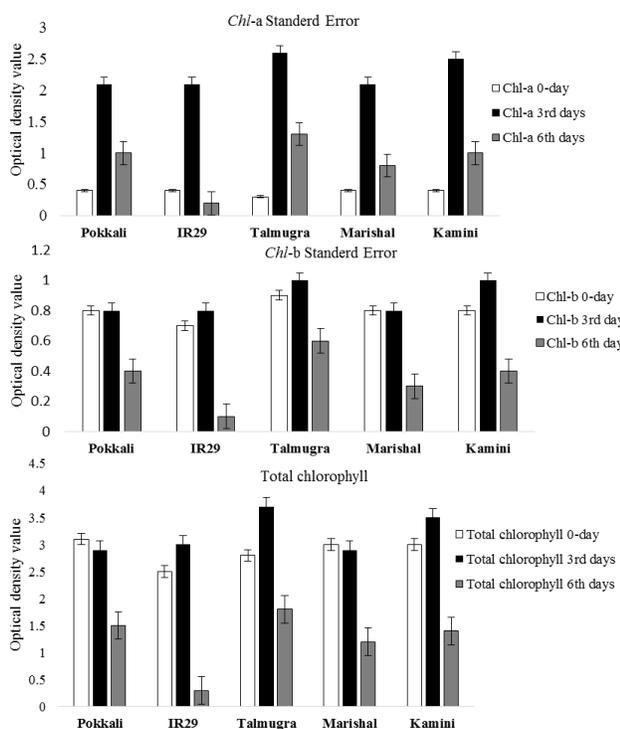


Fig. 5: The level of *Chl a* and *b* and total chlorophyll content in tolerant and sensitive rice lines at different day intervals (0, 3, 6 days) under salinity condition

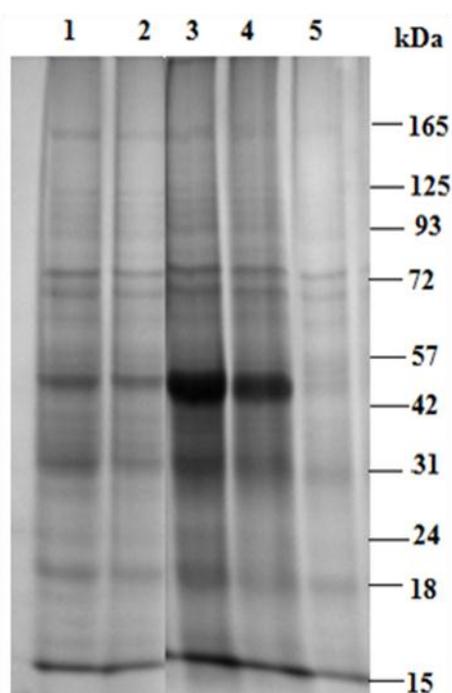


Fig. 6: Protein analysis
Increased and decreased level of protein content in tolerant and sensitive genotypes
1: Kamini, 2: Marishal, 3: Talmugra, 4: Pokkali, 5: IR29 under salt stress condition

Hence, this marker will help to identify rice lines with Pokkali allele in screening process. Likewise, another marker RM8094 differentiated tolerant lines from moderately tolerant including IR29. With this marker, highly tolerant and tolerant lines harboured a new allelic pattern that differs from Pokkali and IR29 (Fig. 2). In a previous study, inconsistency in selection of salinity tolerant lines using RM3412 and RM493 markers was observed. But, the rice lines having Pokkali allele with RM8094 marker showed highly tolerance or tolerance reaction under salinity stress (Mohammadi-Nejad *et al.*, 2008). Recently, Thomson *et al.* (2010) reported the significance of RM8094 in selection of tolerant rice line in breeding programme. Interestingly, in present study, with this marker, rice lines categorized as highly tolerant or tolerant showed to have non-Pokkali alleles. This indicates that RM8094 can also be used to identify new allelic source of salinity tolerance in rice germplasms. Moreover, the level of chlorophyll and protein content was maintained in highly tolerant genotypes (Marishal, Kamini and Talmugra) when compared to sensitive genotype (IR29). This indicates the rice seedlings are not affected severely by salt stress because of synthesis of chlorophyll pigments, DNA and RNA (Ranjbarfordoei *et al.*, 2000; Ashraf and Foolad, 2007).

In gene expression, expression of stress-related genes (*RAB16A*, *LEA3*, *LIP9*, *SalT*, *AP37*, *AP59*, *DREB1A*, *OsDREB2A* and *OsCDPK7*) was found in both tolerant and sensitive genotypes. Expression of these genes has been reported in rice lines under drought, salinity and cold condition (Claes *et al.*, 1990; Chen *et al.*, 2008; Oh *et al.*, 2009; Matsukura *et al.*, 2010; Hirayama and Shinozaki, 2010; Fukao *et al.*, 2011). Recently, Fukao *et al.* (2011) demonstrated expression of these stress-related genes in rice lines having SUB1 locus but not in non-SUB1 lines. But, in present study, we found no significant difference between salinity tolerant and sensitive lines in gene expression. However, expression of AP37 gene only in Kamini and Marishal genotypes was found under salinity condition but not in tolerant genotypes. Expression of AP37 gene belongs to AP2/ERF groups VIIIa, which enhances recovery process in plants from water deficit (Hirayama and Shinozaki, 2010). Moreover, no expression of *OsCDPK7* gene in none of these genotypes under salt stress condition was observed. Previously, it has been reported that *OsCDPK7* is induced by cold and drought/salt tolerance in rice but in distinct pathways (Saijo *et al.*, 2000). However, in shoot and root part of rice lines, expression of *OsSOS1* gene was strong when compared to other genes (*OsCIPK24*, *OsCBL4* and *OsHKT1;5*) associated with osmotic and ionic stress signaling pathway but no significant difference between salinity tolerant and sensitive genotype was found. *SOS1* is reported for in controlling long-distance Na⁺ transport from root to shoot and retrieving Na⁺ from the xylem stream under severe salt stress (Shi *et al.*, 2002).

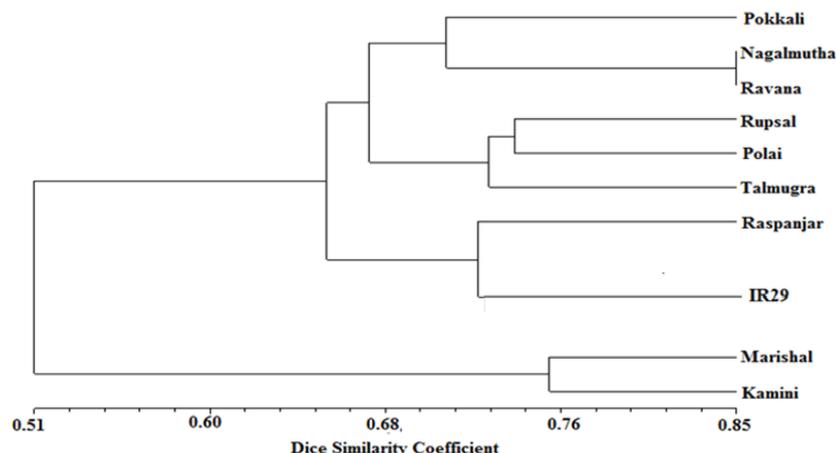


Fig. 7: Clustering of eight rice lines, Pokkali and IR29 based on PCR banding pattern obtained with 56 SSR markers across 12 chromosomes using Dice similarity coefficient was created according to the UPGMA method

Moreover, plant morphological characters also play an important role in salinity tolerance of rice lines by processing compartmentalization of salt ions under salinity. Rice lines with more tillers and spikelet numbers, shoot and root length are associated with salinity tolerance (Zeng and Shannon, 2000; Thomson *et al.*, 2010). In this study, we observed more number of tillers in Marishal, Polai, Rupsal, Raspanjar, Kamini and also Nagalmutha when compared to Pokkali as well as spikelets in Marishal and Polai. In case of plant height, Talmugra, Polai, Marishal, Raspanjar and Kamini were taller than Pokkali. Additionally, in selection process of tolerant genotypes, rice lines with more genetic diversity is essential to develop a good variety (Senguttuvel *et al.*, 2010). We found rice genotypes selected as highly tolerant (Marishal and Kamini) have distant genetic relationship with Pokkali as well as other tolerant genotypes. At the same time, Ravana and Nagalmutha have close genetic relationship with Pokkali. Other tolerant genotypes also have separated from Pokkali at sub-cluster level.

Conclusion

Three rice lines (Talmugra, Marishal and Kamini) were identified more salinity tolerant than Pokkali. Also, a new allelic pattern in these lines is documented with RM8094 marker but not similar to Pokkali. Moreover, the maintenance of chlorophyll and protein level in selected lines was significant rather than Pokkali. Although, there was no significant difference in expression of genes linked with abscisic acid (ABA), CDPK, ionic and osmotic signaling pathways in salinity tolerant genotypes was found when compared to sensitive line (IR29). Induction of *AP37* gene expression associated with retrieval process during water deficit in plants and only in Kamini and Marishal genotypes under salinity condition but not in Pokkali. Besides, significant morphological features such as more tiller, spikelet and plant height associate with salinity

tolerance were found in these rice lines as compared to Pokkali. Similarly, difference between selected rice lines and Pokkali at genetic level was also found. Therefore, identified salinity tolerant lines with genetic variability can be amenable to genetic manipulation to further enhance salinity tolerance in rice lines. In future, these selected rice lines will be subjected to further screening for reproductive stage tolerance.

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