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Identification of salt tolerant rice lines among interspecific BILs developed by crossing *Oryza sativa* × *O. rufipogon* and *O. sativa* × *O. nivara*

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Abstract

Salinity is one of the major abiotic stresses, which affects growth and yield potential of rice. Therefore, there is an urgent need to develop rice cultivars that show tolerance towards salinity stress. Elite varieties (KMR3 and Swarna) have been crossed with wild species (*O. rufipogon* and *O. nivara*) for generating high yielding back cross inbred lines (ILs). These ILs are rich repository for screening their tolerance towards dosage-dependent salinity stress. Here in this study, 15 ILs were screened for their tolerance towards salinity stress under different salinity regime (0, 50, 100,150 and 200 mM NaCl) using various physiological and biochemical traits. ILs were categorized into sensitive (SSIL) and tolerant (STIL) types based on their seed germination and seedling growth response under different salinity treatment. There was a noticeable differential accumulation pattern of Na^+ across different ILs ranging from no accumulation in STILs (K467, K463 and K478) to accumulation at different levels in both STIL (K458) and SSIL (K40). Tolerance to salinity in STILs could be mediated either through Na^+ exclusion from roots (K467, K463 and K478) or its compartmentalization in vacuoles (K458). On the contrary, accumulation of toxic levels of Na^+ by K40 could be attributed to its sensitivity to salinity stress. Further, SSILs and STILs were grown to maturity under field conditions for evaluating the responses of their different agronomic traits to salinity stress. During growth at 100 mM NaCl there was a significant attenuation in yield potential of SSILs compared with STILs (S166, S3-1 and S14). Together, studies at germination, seedling and maturity stages revealed consistency across STILs and SSILs towards their response to salinity stress. Contrasting STILs and SSILs identified from this study are useful repository for dissecting the intricacies involved in tolerance towards salinity stress using comprehensive omics approach.

Keywords: Growth, introgression, ions, salinity, wild rice, yield.
Abbreviations: AICRIP_all India coordinated rice improvement project; Mha_million hectare; Mt_metric ton; ANOVA_analysis of variance; Chl_chlorophyll; DW_dry weight; GP_germination percentage; ILs_introgression lines; K_KMR3 and S_Swarna; LSM_ least square mean; NaCl_sodium chloride; ROC_reduction over control; RWC_relative water content; SAS_statistical analysis system; SSI_salt stress susceptibility index; SSIL_salt sensitive introgression line; STIL_salt tolerant introgression line.

Introduction

Rice (*Oryza sativa*) is one of the most important cereal crops in tropical and temperate regions. It is a staple food for 2/3rd of population in the world (FAO, 2013). India ranks first in area (42.4 Mha) and second in production (87.6 Mt) accounting for 27.5% and 14.5% of the global share, respectively. Around 6% of the world's total land area and 20% of irrigated land is affected by high salinity (FAO, 2008). In India, 9.04 million hectares of rice growing area is affected by salinity leading to considerable loss of grain yield. Salinity stress induces multitude of adverse morphophysiological and biochemical changes ranging from water deficit due to greater osmolarity of soil solution, cellular damage caused by increased cellular accumulation of Na^+, enhanced lipid peroxidation, increased production of reactive oxygen species like superoxide radicals, hydrogen peroxide and hydroxyl radicals. These eventually affect plant growth and development and lead to considerable loss of grain yield (Munns et al., 2006). Rice is more prone to salinity stress at seedling and flowering stages resulting in biomass reduction and sterility (Lee et al., 2013). Plants have evolved adaptive strategies for conferring tolerance to salinity that could be categorized into three distinct mechanisms i.e., (1) exclusion of Na^+ from roots in soil (Yeo et al,. 1990) (2) accumulation of Na^+ and Cl^- in to vacuoles or partitioning accompanied by the accumulation of K^+ or organic solutes inside cytoplasm which mostly occurs in halophytes and (3) maintaining high K^+/Na^+ ratio in cytoplasm (Flowers and Colmer, 2008). Wild rice species *O. rufipogon* and *O. nivara* (2n=24, AA), are the progenitors of cultivated rice (*O. sativa* L.), and have been used as potential sources of novel genetic variation for increasing yield (Marri et al., 2005, Swamy et al., 2012; Xu et al., 2012). Introgression lines derived from *O. rufipogon*, and *O. nivara* crosses with *O. sativa* possess QTLs and beneficial to overcome the adverse effects for higher yield ptential (Swamy et al., 2014). For instance, *O. rufipogon* alleles have contributed to 74% of the QTLs, among which nine QTLs were novel (Marri et al., 2005). Some of the high yielding

backcross inbred lines or introgression lines (ILs) of *O. sativa* x *O. rufipogon* and *O. sativa* x *O. nivara* exhibited tolerance to water stress and/or nutrient (N, P and S) deficiency in field conditions **(**deVicente and Tanksley, 1993; Xiao et al., 1996, 1998; McCouch et al., 2007; Sudhakar et al., 2012). There are no comprehensive studies available on salinity stress responses on various morphophysiological attributes in the stable and high yielding ILs used. These studies are pivotal for delineating the various ILs into salinity tolerant or sensitive types before subjecting them to stringent evaluation using various cost prohibitive omic technologies like transcriptomic, proteomics and metabolomics.

In the present work, we evaluated 15 yield selected ILs derived from *O. rufipogon* (WR120, IC 22015) and *O. nivara* (IRGC 81832 and 81848) with KMR3 and Swarna as recurrent *O. sativa* parents, respectively (Marri et al., 2005; Kaladhar et al., 2008, Swamy et al., 2012, Sudhakar et al., 2012) for their salt tolerance. However, it is not necessary that they themselves be salt tolerant just as yield has been increased using wild species which are themselves not high yielding (Swamy and Sarla, 2008; Nevo et al., 2010). We report the concentration dependent effects of salinity stress on various morphophysiological, agronomic and biochemical traits during seed germination, vegetative and reproductive growth of these high yielding ILs at different developmental stages. The study revealed extensive genetic variability in these ILs with respect to their responses to salinity stress.

Results

Effect of NaCl stress on seed germination

Under control condition, KMR3 and Swarna showed 97% germination whereas their ILs exhibited slight variation (93 to 100%). Germination of all genotypes at 50 mM NaCl was comparable with the control. A less significant difference was observed in germination % at 100 mM NaCl in KMR3, Swarna and their ILs (K467, K242, K463, K40, K215, K458, K478, K50-13, S231, S14, S75, S248 and S166) (Table. 1, 4A) while, a few ILs (K198, S3-1) revealed lower per cent germination values. Highest germination percentage was observed in K50-13 and K478 consistently and lowest in K198 under 150 mM NaCl conditions. Overall germination at 150 mM NaCl was quite consistent and significant among all the ILs. Noticeably, per cent germination of Swarna and several ILs viz., K242, K40, K458, K478, K50-13, S14, S75, S248 and S166, remained unaffected even under high salinity of 200 mM NaCl. High salinity treatment resulted in reduced germination in KMR3, K198, K467, K463, K215, S231 and S3-1. ANOVA results clearly showed variations in salinity stress mediated seed germination responses in KMR3, Swarna and their ILs (Table. 1).

Total dry weight (TDW) of control and treated seedlings of KMR3, Swarna and their ILs were analyzed. Among KMR3 ILs TDW ranged from 22% (K198) to 88% (K242) and Swarna ILs between 6% (S231) and 73% (S3-1) under control condition. Majority of the ILs showed reduction over control (ROC) in TDW under different salt treatments. There was 100% ROC in TDW in K215 under 200 mM NaCl conditions. However, other KMR3 ILs K467, K463, K458 and K50-13 and Swarna ILs S75 and S248 exhibited consistency in TDW even under high salt stress conditions (Table. 2). The ILs exhibited significantly varied content of chlorophyll in response to salt stress. It was increased sharply among few ILs such as K198, K463 and K215 of KMR3 and S75 of Swarna under 50 mM NaCl conditions and reduced thereafter in higher salt regimes. On the contrary, chlorophyll increased more drastically in K467, K242, K458, K478 and S14 under 100 mM NaCl conditions. These ILs expressed differently under higher salt concentrations of 150 and 200 mM, except for K463 and Swarna which showed same level of expression while, decreased level of chlorophyll was observed in K40, K458 and S14. Noticeably, K50-13 exhibited almost similar level of chlorophyll throughout in all concentrations of salt. Reduction of chlorophyll content in 200 mM salt stress was significantly prominent in K198 of KMR3 and S231, S14, S248 and S166 of Swarna (Table. 2).

Potassium (K^+) content among KMR3 ILs ranged from 17% (K242) to 40% (K215) and 10% (S14) to 47% (S166) in Swarna ILs, more than their respective parents, under control conditions. K^+ content remained almost same in 50 mM NaCl treated ILs. Though, a marginal increase of only 10% was observed in KMR3, but a highly significant increase of 24% (K478) and 364% (K215) in KMR3 ILs and 18% (S166) to 262% (S14) in Swarna ILs at 100 mM salt conditions was observed (Table 3). Conversely, a decrease of more than 50% in K^+ content from 100 mM to 150 mM NaCl was observed in all the ILs of KMR3 and Swarna except for K215. K^+ content increase was significantly higher in 200 mM NaCl treated KMR3 ILs K463, K215, K458 and K478 and Swarna ILs S3-1 and S14 (Table. 2).

Altered chloride (Cl^-) and sodium (Na^+) content in physiology of plant under salt stress is quite common. Chloride content in KMR3, Swarna and their ILs was consistent under control conditions while, an increase over control in all genotypes was significant in all other higher concentrations of NaCl. An increase of Cl^- content by increased salt stress was significant in KMR3 and K198 (Table. 2). Maximum Cl^- content was recorded at 100 mM NaCl (about 2 fold) and decreased at 200 mM NaCl. Cl^- level at 150 mM NaCl was not affected much except in K458. At 200 mM NaCl, the trend of increased accumulation of Cl^- content was shown by most of the ILs with exceptions in K458, K50-13, S75 and S3-1. Like-wise, sodium (Na^+) content in KMR3 ILs (K215, K467, K242, K463, K50-13) showed significant increase between 20% and 40% while, Swarna ILs (S14, S3-1) between 20% and 77%, under control conditions. KMR3, its ILs such as K198, K215, K242 and Swarna showed low values for TDW, chlorophyll content and also exhibited ≥10 folds increase in Na^+ in 200 mM NaCl stress, thus they were categorized into salt sensitive introgression lines (SSILs). On the contrary, K467, K463, K458, K478, K40, K50-13, S14, S231, S3-1, S75, S248 and S166 were categorized as salt tolerant introgression lines (STILs) for their overall performance (Table. 2).

Least square means (LSM) analysis of different treatments (0, 50, 100, 150 and 200mM NaCl) at germination stage (Table. 4A) revealed significant differences among ILs for TDW and ionic (Na^+, K^+ and Cl^-) profile. Also, a significant

Table 1. Dosage dependent effects of salinity on morpho-physiological responses of the seedlings of introgression lines developed from *O. sativa x O. rufipogon* var *indica* rice.

Source	DF	Germination percentage		Dry Weight		Chlorophyll		Na+ concentration		K+ concentration		Cl- concentration	
		MSS	Pr>F	MSS	Pr>F	MSS	Pr>F	MSS	Pr>F	MSS	Pr>F	MSS	Pr>F
Variety	16	23.49	<0.0001	0.0076	<0.0001	23.49	<0.0001	4.15	<0.0001	2.35	<0.0001	9.46	<0.0001
Treatment	2	19.20	<0.0001	0.1251	<0.0001	19.20	<0.0001	91.15	<0.0001	8.11	<0.0001	44.65	<0.0001
Variety X Treatment	32	7.30	<0.0001	0.0029	<0.0001	7.30	<0.0001	2.69	<0.0001	1.65	<0.0001	5.76	<0.0001
Error	102	0.86		0.0000		0.86		0.11		0.02		0.02	

Analysis of variance (ANOVA) of morpho-physiological traits in seedlings at different salt concentrations was analyzed and documented. Data were documented 3 days after sowing for per cent germination and for other physiological traits (DW, chlorophyll content and ionomic profiling) were performed 10 - day old seedlings. Values represent *n*=3 replicates of 10 seedlings each for ANOVA; MSS - Mean Sum of Squares; DF - Degrees of Freedom; Pr - Probability; F - F Ratio.

Table 2. Least Square Means (LSM) analysis for different traits at germination stage. Values with different letters (superscript) indicate that the LSM differ significantly ($P \leq 0.05$).

Genotype	Dry weight	Chlorophyll	Na+	K+	Cl-	Category
KMR3	0.091^{F}	2.275^{F}	2.173^{C}	0.781^{EFG}	4.550^{C}	SSIL
K198	0.085^{F}	2.313^{F}	3.680^{A}	0.678^{FG}	5.150^{A}	SSIL
K467	0.104^{E}	4.319^{CD}	1.048^{I}	0.625^{G}	2.241^{I}	STIL
K242	0.149^{A}	6.632^{A}	1.812^{CD}	0.686^{FG}	3.200^{E}	SSIL
K463	0.140^{AB}	5.236^{BC}	1.247^{GHI}	0.995^{D}	2.577^{FGH}	STIL
K40	0.119^{D}	3.629^{DE}	1.299^{GHI}	0.726^{FG}	3.266^{E}	SSIL
K215	0.106^{E}	4.326^{CD}	1.957^{CD}	2.190^{A}	2.488^{H}	SSIL
K458	0.131^{BC}	2.722^{EF}	1.307^{FGHI}	1.219^{C}	1.033^{J}	STIL
K478	0.130^{BC}	3.795^{DE}	1.338^{EFGHI}	0.955^{DE}	3.088^{E}	STIL
K50-13	0.105^{E}	5.306^{BC}	1.161^{HI}	0.818^{DEF}	2.237^{I}	STIL
Swarna	0.104^{E}	5.245^{BC}	2.763^{B}	0.609^{G}	4.844^{B}	SSIL
S75	0.104^{E}	6.822^{A}	1.234^{GHI}	0.601^{G}	3.544^{D}	STIL
S248	0.053^{G}	6.984^{A}	1.047^{I}	0.843^{DEF}	2.588^{FGH}	SSIL
S166	0.050^{G}	5.349^{BC}	1.562^{DEFGH}	0.756^{FG}	2.677^{FG}	STIL
S231	0.144^{A}	6.728^{A}	1.591^{DEFG}	0.766^{FG}	2.566^{GH}	SSIL
S3-1	129.188^{CD}	6.097^{AB}	1.749^{CDE}	1.536^{B}	3.266^{E}	STIL
S14	81.577^{F}	6.804^{A}	1.733^{DEF}	2.222^{A}	2.755^{F}	STIL

LG – Letter Group, [a]Genotype with LG involving same letter are not significant different. Tukey's test used for testing the difference of Least Square Means; Letter A as well as letter A along with other letters (AB, etc) represent highest performing genotypes.

Table 3. Temporal study of treatment of NaCl on various morpho-physiological traits of IL at seedling stage.

Source	DF	Chlorophyll		Dry weight		Proline		Na+		K+		Cl-	
		MSS	Pr>F	MSS	Pr>F	MSS	Pr>F	MSS	Pr>F	MSS	Pr>F	MSS	Pr>F
Variety	6	257.42	<0.0001	0.0098	<0.0001	3.89	<0.0001	3.02	<0.0001	0.67	<0.0001	0.88	<0.0001
Treatment	2	251.32	<0.0001	0.0146	<0.0001	6.60	<0.0001	17.88	<0.0001	2.04	<0.0001	2.04	<0.0001
Variety X Treatment	12	9.15	<0.0001	0.0041	<0.0001	1.58	<0.0001	2.22	<0.0001	0.17	<0.0001	0.13	<0.0001
Error	42	0.06		0.00007		0.008		0.04		0.006		0.001	

Analysis of variance (ANOVA) of morpho-physiological traits in seedlings at different salt concentrations was analyzed and documented. Data *n*=3 replicates of 10 seedlings each. Seedlings were exposed to 150 mM NaCl for salinity treatment and samples were drawn at 0, 3 and 24 h. ; MSS - Mean Sum of Squares; DF - Degrees of Freedom; Pr - Probability; F - F Ratio.

Table 4. Least Square Means (LSM) analysis for three different treatments at (A) germination and (B) seedling stages. The means are computed over all ILs for each treatment.

A: Germination Stage

NaCl (mM)	Germination percentage	Total dry weight	Chlorophyll	Na+	K+	Cl-
0	79.433^{A}	0.1961^{A}	5.2710^{C}	0.2779^{D}	0.5486^{C}	2.0000^{D}
50	79.101^{A}	0.1907^{AB}	9.6150^{A}	1.4047^{C}	0.4645^{D}	3.7037^{B}
100	73.726^{B}	0.1909^{AB}	9.4569^{A}	1.8219^{B}	1.3961^{A}	3.9166^{A}
150	71.121^{B}	0.1887^{B}	6.7188^{B}	2.9340^{A}	1.3885^{A}	3.1555^{C}
200	70.851^{B}	0.1523^{C}	4.8104^{C}	2.9185^{A}	1.2097^{B}	3.1333^{C}

B: Seedling Stage

NaCl (mM)	Total dry weight	Chlorophyll	Proline	Na+	K+	Cl-
0	0.204^{A}	13.836^{A}	1.010^{C}	1.528^{C}	0.333^{C}	0.492^{C}
150 (3 h)	0.192^{B}	12.921^{B}	1.711^{B}	2.494^{B}	0.687^{B}	0.588^{B}
150 (24 h)	0.153^{C}	7.439^{C}	2.119^{A}	3.374^{A}	0.956^{A}	1.074^{A}

Tukey's test used for testing the difference of Least Square Means; Letter A as well as letter A along with other letters (AB, etc) represent highest performing genotypes.

Table 5. Least Square Means analysis for different traits atseedling stage. Values with different letters (superscript) indicate that the LSM differ significantly ($P \leq 0.05$).

Genotypes	Chlorophyll	Total dry weight	Proline	Na+	K+	Cl-	RWC
KMR3	6.411^{F}	0.161^{D}	1.071^{E}	2.850^{B}	0.272^{D}	0.924^{B}	43.194^{C}
K478	10.992^{D}	0.214^{A}	2.667^{A}	2.457^{C}	0.765^{BC}	0.254^{E}	48.139^{B}
K463	22.045^{A}	0.206^{AB}	2.309^{B}	1.725^{E}	0.797^{BC}	0.670^{C}	50.661^{A}
K198	6.222^{F}	0.172^{C}	1.048^{E}	2.855^{B}	0.280^{D}	0.983^{A}	38.166^{E}
K50-13	14.021^{B}	0.208^{AB}	2.033^{B}	1.777^{E}	0.773^{BC}	0.266^{E}	48.188^{B}
Swarna	12.457^{C}	0.123^{E}	1.365^{D}	3.332^{A}	0.701^{C}	0.940^{AB}	31.887^{F}
S166	9.303^{E}	0.198^{B}	1.771^{C}	1.918^{DE}	0.826^{B}	0.940^{AB}	31.988^{F}
S75	12.361^{C}	0.207^{AB}	1.063^{E}	2.122^{D}	0.971^{A}	0.316^{D}	40.072^{D}

LG – Letter Group, [a]Genotype with LG involving same letter do not differ significantly. Tukey's test used for testing the difference of Least Square Means; Letter A as well as letter A along with other letters (AB, etc) represent highest performing genotypes.

Table 6. Grain yield and salt susceptibility index at 100 and 150mM NaCl grown plants in soil pot condition.

Genotypes	Grain yield (GY)				Salt susceptibility index (SSI)		
	Control	100mM	150mM	LS Means	100 mM	150 mM	LS Means
KMR3	56.00	23.13	14.00	30.12^{D}	0.05	0.64	-0.20^{B}
K463	56.00	31.40	19.00	35.60^{AB}	-0.29	0.46	-0.41^{BC}
K40	55.00	24.00	15.00	31.44^{CD}	-0.01	0.57	-0.36^{BC}
K478	56.00	35.00	19.33	37.26^{A}	-0.06	0.27	-0.16^{B}
K50-13	38.50	20.80	15.00	25.03^{E}	-0.25	0.39	-0.53^{C}
Swarna	42.00	15.00	10.00	21.67^{F}	-0.69	0.32	0.44^{A}
S75	45.00	32.00	20.00	32.55^{C}	-0.56	0.50	0.45^{A}
S166	47.33	32.25	21.00	34.50^{B}	-0.57	0.30	0.40^{A}
S3-1	44.50	31.00	15.50	30.33^{D}	-0.61	0.45	0.45^{A}
S14	37.20	24.00	16.00	25.24^{E}	-0.49	0.32	0.39^{A}

Genotype with letter group (superscript) involving same letter do not differ significantly. Tukey's test used for testing difference of Least Square Means.

difference was observed in germination percentage in all genotypes under 100 mM, 150 mM and 200 mM NaCl over control and also in chlorophyll content in 100mM and 150 mM NaCl treatments over control

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Temporal effects of salinity stress on SSILs and STILs

Temporal effects (0 h, 3 h and 24 h) of salinity (150 mM NaCl) on different physiological traits of SSILs and STILs were evaluated at seedling stage (Table. 3). Total dry weight was not changed much in STILs (K478, K463, S166 and S75) under 150 mM NaCl at 3 and 24 h while, salt stress showed a significant difference in SSILs (KMR3, K198 and Swarna). In SSILs dry weight was 10–17% reduced after 3 h of 150 mM NaCl treatment. When treatment was extended for 24h there was about 40% ROC among the SSILs. Effect of salt stress was more pronounced in Swarna that showed 90% reductions in TDW. To analyze further, relative water content (RWC) was measured at 24 h after salt treatment. Percentage of relative water content increased to 22% in K478 whereas, 15% and 27% ROC was recorded in K463 and S166, respectively. Reduction in RWC was highly significant in SSILs than in STILs (Table 5).

Chlorophyll content in STILs and SSILs was not affected significantly under short-term (3 h at 150 mM NaCl) salt stress whereas, a considerable decline was observed among ILs after 24 h of 150 mM NaCl treatment, in which it was more accentuated across the members of SSILs when compared with members of STILs (Table 5). Interestingly, maximum reduction was recorded in SSIL K198 with increasing concentrations of salt stress. Further, proline content was measured at seedling stage in short-listed genotypes (SSILs and STILs) to evaluate them at 3 h and 24 h in 150 mM NaCl conditions. Under control conditions, STILs, S75 showed lowest (0.30 mg/g dry weight) and K463 highest (1.95 mg/g dry weight) levels of proline accumulation whereas, SSILs showed a wide range of variations from 0.63 mg/g dry weight (KMR3) to 1.18 mg/g dry weight (Swarna). A significant increase in proline content was observed in STILs (K478, S166 and S75) and SSILs (KMR3, Swarna and K198) under 3 h salt treatments except in K463 that showed only 20% reduction over control. After prolonged salinity treatment (24 h 150 mM NaCl), an elevated proline accumulation of 1.4 fold in Swarna and 7.7 fold in S75 was recorded (Table. 5).

Ionic profile (K^+, Cl^- and Na^+) in SSILs and STILs was investigated to understand temporal effects (0 h, 3 h and 24 h at 150 mM NaCl)) of salinity at seedling stage. K^+ content increased in STILs with rising concentrations of salt stress.

Genotypes KMR3 and K198 showed consistency in K^+ concentration at 3 h salt stress in comparison to control while, higher (4–8 folds) content was observed among K478, K463, S166, S75 and Swarna. A two fold increase over control was noticed in KMR3 and K198 after 24 h salt treatment. Members of SSILs revealed an increase in K^+ content after 24 h of treatment with NaCl as compared to 3 h treatment. However, no specific trend in temporal accumulation of K^+ was observed across members of STILs and SSILs. Effects of salt stress on Na^+ at 3 h as well as 24 h was not significant in K478 and K463 over control. Swarna ILs S166 and S75 showed two folds and SSILs with three folds higher accumulation of Na^+ at 24 h salt treatment. These results prominently showed that members of SSILs tend to accumulate more Na^+ during prolonged exposure to salt stress. Chloride (Cl^-) content among STILs and SSILs ranged from two (K478 and S75) to three (S166) fold under control conditions. There was no significant difference in Cl^- content of members of STILs and SSILs under short-term salt exposure (3 h). However, Cl^- content increased to two folds in KMR3, K198 and Swarna after 24 h of NaCl stress. Least square means analysis for different treatments (0 mM NaCl, 150 mM NaCl at 3 h and 150 mM NaCl at 24 h) in Table 4B revealed significant differences among genotypes for all traits.

Performance at reproductive stage

Germination and seedling stage screened and selected introgression lines (ILs) were grown under normal conditions for 30 days. They were transferred to pots containing soil saturated with 0, 100 and 150 mM NaCl. Seedlings grown under these conditions for 30 more days were scored for phenotypic traits (IRRI score scale) and then grown to maturity for grain yield.

KMR3 IL, K478 showed no inhibitory effect on its growth performance in 100 and 150 mM salt stress (data not shown). There was no adverse effect on the growth of S166 at 100 mM NaCl and it scored 7 at 150 mM NaCl conditions. Other members of STILs (K463 and S75) were comparatively stable at 100 while the response worsened at 150 mM NaCl conditions. Relatively, effect of salinity stress on phenotypic traits of SSILs (KMR3, K198 and Swarna) could be perceived at 100 that got accentuated at 150 mM NaCl. Further, members of SSILs and STILs were evaluated for their yield potential during growth at 100 and 150 mM NaCl. Although salinity treatments at 100 and 150 mM NaCl resulted in reduction of grain yield of all members of STILs and SSILs, the effect was more aggravated in members of SSILs. Lowest percentage of ROC was recorded in K478 with 37.50%, followed by K463 (43.93%) and K50-13 (45.97%) under 100 mM salt stress. Similarly, ROC was lowest in S75 (28.89%) followed by S3-1 (30.34%), S166

(31.86%) and S14 (35.48%) among Swarna ILs under 100 mM NaCl stress. Highest yield reduction of 64.29% was prominent in Swarna, followed by KMR3 (58.70%) and K40 (56.36%) under 100 mM NaCl stress conditions. This trend remained same under 150 mM NaCl conditions with 76.19%, 75% and 72.73% in Swarna, KMR3 and K40, respectively. ROC of yield in 150 mM NaCl was less among Swarna ILs viz., S75 (55.56%), S166 (55.63%), S14 (56.99%) and highest in S3-1 with 65.17%. Among KMR3 ILs., lowest ROC in yield was observed for K50-13 (61.04%) followed by K478 (65.48%) and K463 (66.07%) under 150 mM NaCl conditions. KMR3 IL, K198 failed to survive after 85 days at 150 mM NaCl stress (Table. 6).

Discussion

Wild rice is a rich reservoir for new genes/alleles for salt tolerance (Zhang and Xie 2014). The ILs derived from 3 wild accessions were introgression of wild genes/alleles into popular rice varieties KMR3 and Swarna. An effort was made to determine underlying mechanism between morpho-physiological traits, ionic profile (Na^+, K^+, Cl^+) and salt tolerance with high yielding lines.

Morpho-physiological responses at early growth stages after salinity stress

Germination responses of different ILs under different concentrations of NaCl are vital during later developmental stages. Among the lines tested, there were significant variations in their responses towards salinity treatment. For instance, per cent seed germination of Swarna and several ILs (K242, K458, K478, K50-13, S14, S75 and S166) remained unaffected even when grown under high salinity (200 mM NaCl) condition. Thus, 3 of these lines K478, S75, S166 are categorized as salt tolerant line. Other agronomic traits were also evaluated to complement the germination studies. Various lines were further assessed for their chlorophyll content. Salinity stress triggered significant attenuation in content of chlorophyll in all the members (Swarna, KMR3 and K198) of SSILs. A similar loss of chlorophyll was reported in olive plant under saline conditions (Ben Ahmad et al., 2011). Therefore, the use of this physiological trait could be a potentially quick indicator for salt susceptibility/tolerance when screening large populations. However, more exhaustive and stringent studies are required to use it for predicting salt tolerance accurately. It is logical to assume that higher chlorophyll content during salinity stress means higher photosynthetic rate that would be commensurate with the level of biomass. In the present study, Swarna and KMR3 showed reduced chlorophyll content and also significant reductions in total dry weight during salinity stress. However, correlation between the dry weight and chlorophyll content was not always linear. For instance, the chlorophyll content increased during salinity stress at 200mM NaCl amongst K242 and K463 yet there was significant reduction in their TDW. Therefore, it is apparent that the effect of salinity stress on these two different traits may not be always positively correlated.

Based on germination stage stress responses, the ILs were categorized into two different groups i.e., salt tolerant lines (STILs) and salt sensitive lines (SSILs). However, at germination stage relatively weak correlation was observed across different traits among STILs and SSILs. Thus, the SSILs (KMR3, K198 and Swarna) and STILs (K463, K478, S75, S166, S3-1) were evaluated for their degree of tolerance during temporal growth under different salinity regime. Interestingly, a comparative growth analysis at 200 mM NaCl revealed a growth inhibition in Swarna due to salt stress mediated by accentuated loss of water but K463 showed increased dry weight. A highly significant difference was noticed for all the growth parameters (root-shoot length) in salinity over genotypes used. A report on Pokkali showed a less inhibited seedling growth in this salt tolerant variety than that of the salt susceptible IR-29 (Lee et al., 2003).

Plants maintain their internal water potential to sustain turgor pressure and water for growth and development. Relative water content (RWC) is an important parameter to determine water potential and salt tolerance in rice (Duangjai et al., 2004). RWC decreased in all the members of SSILs but STILs showed consistency even under salt stress conditions. In this study, K478 increased its RWC indicating that this line has an ability to maintain its internal turgor pressure or osmotic effect even under high salinity. Salinity stress triggers the synthesis of compatible solutes like proline, glycine betaine, choline and O-sulphate (Phang et al., 2008). In the present study, proline content was found to increase in all the ILs except in recurrent parents. Accumulation of proline under abiotic stress protects the plants by maintaining continuous water adsorption and/or stabilization of protein and membranes against destabilizing effect of abiotic stress which leads to cellular water depletion (Parida and Das, 2004). Munns and Tester (2008) reported that proline protects higher plants against salt/osmotic stresses by protecting the photosynthetic apparatus. In this study, proline concentration was positively correlated with chlorophyll content at seedling stage indicating that proline contributed to maintain chlorophyll level under salt stress. Few reports showed that the increase in proline concentration was more prominent in sensitive rice genotypes (Moradi and Ismail, 2007) contrary to our study where maximum increase in proline was in S166.

In *Triticum monococcum*, different lines have different combination of tolerant mechanism like Na^+ exclusion, Na^+ tissue tolerance and osmotic tolerance (Rajendran et al., 2009). In our study, it was found that Na^+ concentration increased in SSILs (K198, K40 and K215) but did not change much in STILs (K467, K463 and K478) indicating this as an important trait in delineating tolerant and susceptible lines. On the other hand, in Swarna ILs (S166, S3-1, S14 and S75), the Na^+ concentration increased invariably at all levels of NaCl. At young seedling stage also Na^+ concentration did not change in STILs (K478 and K463). The other ILs except K478 and K463 showed increased Na^+ with increase in NaCl concentrations. It can thus be concluded that K478 and K463 have salt exclusion mechanism for salt tolerance while all others including Swarna ILs which are tolerant to NaCl have tissue tolerance ability. Swarna IL, S75 managed to control Cl^- level but not Na^+/K^+ ratio and so it was STIL. We hypothesized that *SOS1* may be active for Na^+ extrusion, hence maintaining K^+/Na^+ ratio through roots in K478 and K463 as in Arabidopsis roots (Shabala et al., 2005).

Seedlings grown in saline conditions showed different visual symptoms of salt injury. Symptoms are prominent on the first and second leaves with leaf rolling, brownish and whitish color of leaf tip, drying of leaves and also reduced root length, stunted growth and stem thickness leading to complete cessation of growth and finally deterioration of seedlings (Gregorio, 1997). Damage of leaves was attributed to Na^+ accumulation in the shoot in extremely high salt stress (Lin et al., 2004). The chloride (Cl^-) exclusion is also required in plants for salinity tolerance. Most species can 'exclude' Cl^- and Na^+ up to 90 to 98 % (Munns, 2005), but salt tolerance is high by efficient sequestration of Cl^- and Na^+

in vacuoles to prevent toxic level accumulation in the cytoplasm like S75 that maintained Cl^- level under salt stress conditions.

Effect of salt stress at later growth stages

Salinity tolerance among genotypes at seedling and reproductive stages was weakly correlated (Moradi and Ismail, 2007). Agronomic traits of SSILs and STILs were recorded in terms of biomass, panicle weight, panicle length (data not shown) and grain yield. STILs S3-1, K478, K50-13 exhibited least reduction in yield under salt stress and were thus considered tolerant on an overall basis (at different growth stages). Statistical analysis depicted a strong correlation in K463 between dry weight and grain yield. Most remarkable feature is level of Na^+ at 100, 150 and 200 mM NaCl stress. KMR3 IL, K478 maintained Na^+ and Cl^- concentrations at all the above salinity levels. STIL K458 could maintain its Cl^- concentration even under high salt stress and reduced Na^+ (50%) at 200 mM NaCl. A positive correlation with low Na^+ content and high proline accumulation was prominent in S75. An unchanged Na^+ concentration at 100, 150 and 200 mM NaCl and lower Cl^- content at 200 mM NaCl indicated that the K50-13 has an ability to maintain low level of Cl^- at higher saline condition. Highest Na^+ and Cl^- content and reduced dry weight in K198 at seedling stage was recorded. Thus, K198 is identified as the most sensitive IL at vegetative and reproductive stage.

Introgression lines are most useful resource not only for further basic studies on salt tolerance and allele mining from the wild species but also of applied value. The STILs (S166, K463, K467, K458 and K50-13) were included in National Saline Alkaline Screening Nursery (NSASN), Alkaline and Inland Saline Tolerant Variety Trial (AL&ISTVT) and Coastal Saline Tolerant Variety Trial (CSTVT) of All India Coordinated Rice Improvement Project (AICRIP) at Directorate of Rice Research (DRR), Hyderabad during 2010-2011 (NSASN) and 2011-2012 (AL & ISTVT and CSTVT) to test in multi-location field trials. The pH and EC varied at these locations from 5.1 to 9.9 and from 0.96 dS/m to 11.5 dS/m, respectively. K463 showed 17% increase in yield over the national check CSR36 in alkaline areas and K467 (27%), K458 (16%), K50-13 (24%) increase over the national check CST 7-1 in coastal saline areas. They were promoted to second year of multi-location testing in 2011 (DRR Annual Progress Report, 2010). K463 and K50-13 were further promoted to third year of testing in 2012 (DRR Annual Progress Report, 2011). After 4th year testing in 2013, K50-13 (IET 21943) was identified for release in coastal saline areas of West Bengal (DRR Annual Progress Report, 2011). Hence, selected STILs (K467, K458 and K50-13) were validated in field condition also for their salt tolerance and both KMR3 x *O. rufipogon* and Swarna x *O. nivara* ILs were found to be more salt tolerant than their recurring parent KMR3 and Swarna except K198. Thus, our study demonstrated complexity of response to NaCl stress from germination to seedling stage in terms of Na^+, K^+, Cl^- and proline.

Materials and Methods

Plant materials

Many high yielding stable introgression lines were derived from advanced backcross populations (BC_2F_{6-7}) of three crosses (1) KMR3 (IR58025A) X *O. rufipogon*, (2) Swarna (IRGC81848) X *O. nivara* and (3) Swarna (IRGC81832) X *O. nivara* (Marri et al., 2005; Kaladhar et al., 2008; Swamy et al., 2012). We used 15 high yielding ILs (ILs with 2 to 20% SSR loci introgressed from wild parent – data not shown) to screen, identify and compare possible salt responsive mechanisms associated among the lines with their respective parents, at three different stages viz., germination, seedling and reproductive stage .

Germination stage

Seeds of 15 ILs (KMR3 ILs - K198, K467, K242, K463, K40, K215, K458, K478 and K50-13 and Swarna ILs - S231, S3-1, S14, S75, S248 and S166) and their respective recurrent parents were sterilized with 70% ethanol and blot dried. Seeds (30) were sown and incubated in three petri plates (50 mm diameter x 9 mm height lined with blotting paper) for each genotype with 5 ml Hoagland's medium containing different concentrations of NaCl viz., 0 (control) mM, 50 mM, 100 mM, 150 mM and 200 mM (30 seeds/5 ml Hoagland's media with respective concentration of NaCl/petri plate/genotype with three such replicates). Such an arrangement of three biological replications (three plates) was maintained per genotype and seed germination was recorded at every alternate day till the end of experiment (10 day). Germination percentage, shoot and root length was analyzed for each plate (30 seeds) and recorded the overall mean of three replications. Further, 10 days old seedlings grown under different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM and 200 mM) were screened and analyzed for total dry weight (TDW), total chlorophyll and ionic (Na^+, K^+ and Cl^-) content as mentioned in next section.

Seedling stage

Six introgression lines (KMR3 ILs – K478, K463, K50-13 and K198 and Swarna ILs – S166 and S75) and their respective recurrent parents were short-listed based on their overall performance at germination stage. They were further analyzed for salt tolerance and to understand the mechanisms involved at seedling stage. Since each IL exhibited a clear response at germination stage under 150 mM NaCl concentrations hence, the genotypes were sown and grown in normal Hoagland's medium for 14 days continuously (30 seeds/petriplate/genotypes for three replicates at early seedling stage) and exposed to 150 mM NaCl for two different time periods (3 h and 24 h) separately. Further, fresh seedlings were used to analyze the content of chlorophyll, proline and relative water content (RWC) as explained below and seedlings were oven dried at 65°C for 48 h to record dry weight (DW) measurements

Quantification of Chlorophyll content

Chlorophyll content was measured in leaves of both germination and seedling stages plants. Fresh weight of seedlings were taken and homogenized separately for control and treated samples. Acetone (80%) was used for homogenization and incubated at -20°C overnight. After incubation period, collection was centrifuged at 12,000 rpm for 10-15 min and supernatant was taken and absorbance was taken at 663 and 645 (Palta, 1990). Final values were calculated for 1 g of fresh weight (FW) and expressed in mg/g FW units.

Proline content

Seedlings after 14 day exposed to 150 mM NaCl stressed for 3 h and 24 h were used for proline quantification. Briefly, samples were weighed (~0.05 g), homogenized in 1 ml of 3 % aqueous sulfo-salicylic acid. Extract was centrifuged at 8000 rpm for 12 min and supernatant was mixed with 1 ml ninhydrin and glacial acetic acid (1:1). Mixture was heated to 100°C in a water bath for 1 h and reaction was stopped immediately by placing the tubes in ice. An equal volume of toluene was added to vortex for 15 to 20 s and observed for chromophore formed, which was aspirated from the aqueous phase. Absorbance was recorded at 520 nm and the readings were calculated by using standard graph of L-proline (Bates et al., 1973). Final values were expressed in mg/g DW.

Relative water content (RWC)

Seedlings were weighed (FW), dipped in sterile water to incubate overnight and turgid weight (TW) was recorded (Duangjai et al., 2004) were further oven dried at 65^0C for 48 h for dry weight (DW). Percentage of relative water content (RWC) was calculated using the formula: RWC= (Fresh Weight-Dry Weight) / (Turgid Weight-Dry Weight)*100

Ion analysis

Leaf tissue of salt treated and un-treated ILs were oven dried at 38°C for 48 h and cut into small pieces, weighed and transferred into test tubes. Further, 5 ml aliquots of cocktail of acids *i.e.,* conc. sulphuric acid, conc. nitric acid and perchloric acid (5:10:5) was added to each samples. Samples were digested for 2 h and cooled. Mixture was then filtered through Whatman filter paper. Sodium (Na^+) and potassium (K^+) were analyzed using Flame photometer. Values we are calculated and expressed in mMol/g DW. Chloride (Cl^-) ions were quantified by colorimetric titration method. For the analysis of chloride, sample mixture using potassium dichromate ($K_2Cr_2O_7$) as an indicator was titrated against 0.05N silver nitrate ($AgNO_3$), till the solution turned brick red, as an end point and values were recorded in units per g DW.

Maturity stage

Based on overall performance at early seedling and vegetative stages under salinity stress, eight ILs were selected to measure grain yield in pots under saline conditions. Mature seedlings (22 days) grown in soil pot were transferred to plastic pots filled with eight kilograms of dried soil each. All such plastic pots were placed in water-filled pits to maintain soil moisture level uniformly. With a control batch, two different salt treatments i.e., 100 mM (10 dS/m) and 150 mM (15 dS/m) NaCl were stringently maintained by measuring electrical conductivity (EC), periodically. Each pot had four plants and four replications for each IL were retained. Observations were made at vegetative stage (data not shown) and grain yield was recorded. For estimation of salt stress susceptibility index (SSI), grain yield per plant was evaluated under control, 100 mM and 150 mM NaCl. SSI was calculated according to Fischer and Maurer (1978) using the formula: SSI = (1-Ys/Yp) / D
where, Ys= mean grain yield of a genotype under stress; Yp= mean grain yield of the same genotype without stress; D (stress intensity) = 1- (mean Ys of all genotypes/ mean Yp of all genotypes).

Statistical analysis

A two-way cross classification with interaction model was fitted to the performance of different traits measured at germination (experiment 1) and seedling stages (experiment 2). Treatment, variety and treatment × variety interaction effects were treated as fixed effects in the model with errors assumed to be normally and independently distributed with zero mean with constant variance. Under SAS 9.2, *proc GLM* was used to carry out ANOVA for different traits. Least Square (LS) Means analysis was also performed to identify the high performance group of varieties as well as treatment × variety interactions. An *arcsine* transformation was applied on trait – 'germination percentage', using the formula *ASIN* [*SQRT* (y_{ijk}/100)) × (180/π)], to normalize the performance. However, in case of 100% and 0% germination, the Bartlett's correction was applied on transformation using formula 100 × (1 – (1/(4*n*))) and (1/(4*n*)) respectively and *n* (=30) is number of plants on which percentage germination was measured.

Conclusion

Conclusively different mechanism for salinity tolerance can be predicted for KMR3 and Swarna ILs. KMR3 ILs (K463 and K478) have ability to exclude Na^+ while Swarna ILs (S166, S3-1, S14 and S75) were efficient in compartmentalization of Na^+ in leaf tissue. All the above STILs could also maintain consistent level of chlorophyll under salinity stress condition. K478 was unique in having osmotic tolerance as well due to higher accumulation of an osmolyte proline and maintaining osmotic potential inside cells. However, K198 is highly sensitive because accumulation of Na^+ and Cl^- to toxic level. Grain yield of Swarna ILs (S75, S166) and KMR3 ILs (K50-13, K463 and K478) were least affected by salt stress. On the whole, a strong positive correlation was observed between germination, seedling and yield under salt stress among STILs. Our study endorsed that high yielding introgression lines derived using wild species are a valuable resource for salinity tolerance even though the wild accession used may not itself be salt tolerant.

Competing interest

We declare no competing interest.

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