Phenotyping of salt stress tolerance in F$_2$ population derived from salt tolerant Thai rice 'Jao Khao'

Susinya Habila[1,2], Teerapong Buaboocha[3,4], Monnat Pongpanich[4,5,6], Duangjai Suriya-Arunroj[7], Meechai Siangliw[8] and Supachitra Chadchawan[1]*

[1]*Center of Excellence in Environment and Plant Physiology, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand*
[2]*Department of Plant Science and Biotechnology, Faculty of Natural Science, University of Jos, Nigeria*
[3]*Molecular Crop Research Unit, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand*
[4]*Omics Sciences Center, Faculty of Science, Chulalongkorn University, Bangkok, Thailand*
[5]*Department of Mathematics and Computer Science, Faculty Science, Chulalongkorn University, Bangkok, Thailand*
[6]*Age-related Inflammation and Degeneration Research Unit, Chulalongkorn University, Bangkok, Thailand*
[7]*Rice Department, Ministry of Agriculture and Cooperation, Bangkok, Thailand*
[8]*National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, Thailand*

*Corresponding author: s_chadchawan@hotmail.com; Supachitra.c@chula.ac.th

ABSTRACT

Rice is susceptible to salt stress at seedling and reproductive stages. In order to identify the specific region in the genome conferring salt tolerance trait, F$_2$ population of the Thai landrace salt tolerant variety, 'Jao Khao' and the salt 20 susceptible line, IR29, was generated. Based on the phenotype of 'Jao Khao' under salt stress condition, it is clustered with 'Pokkali', which is the salt tolerant standard cultivar. The phenotyping of 600 F$_2$ individuals was performed in the hydroponic system. Two-week old seedlings were treated with stepwise salt stress using NaCl solution to reach 12 dS.m^{-1} for 12 days. The F$_2$ members showed a high level of distribution in salt injury score determined according to the standard salt injury evaluation system (SES), cell membrane stability (CMS), relative water content (RWC), whole plant fresh weight, plant height and root length. SES distribution tended to show the random distribution, while CMS and RWC had bimodal distribution. The whole plant fresh weight after 12 days of salt stress displayed right skewed distribution. For the plant height and root length, both values displayed normal distribution. All of the parameters show correlation to one another. SES shows the strong negative correlation with CMS (-0.9), RWC (-0.9), and whole plant fresh weight (-0.8), but it shows less negative correlation with plant height (-0.6) and root length (-0.5). This F$_2$ population can be used to identify the salt tolerance regions from 'Jao Khao' via bulk-segregant analysis in the future.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major food crops in the world (Chauhan *et al*., 2017). Its importance in food security cannot be overemphasized. The growth, development, and yield of the rice plant are affected by both biotic and abiotic stresses (Hoang *et al*., 2016). Salt stress is among the abiotic stresses reported to have affected rice plants at different growth and developmental stages (Hoang *et al*., 2016). Tolerance to salt stress or salinity in rice is dependent upon the developmental stage. The tolerance of rice crops to salt stress has been reported to be better at the germination and vegetative or tillering stages, but highly sensitive at the seedling and early reproductive or booting stages (Zeng *et al*., 2001). Pearson *et al*. (1966) also reported that rice plants were more susceptible to salt stress at the seedling stage and their tolerant ability increased as the plants developed until the early reproductive stage where they became susceptible again.

Salt stress induces osmotic and ionic stresses in plants. Under osmotic stress conditions, salt concentration around the root tissues is elevated, while ionic stress is attained when Na$^+$ concentration within the plant tissues is higher than the normal concentration (Munns & Tester, 2008). The osmotic and ionic stresses usually lead to the disruption of many

metabolic processes and cell deaths (Munns & Tester, 2008). Salinity causes the increase in leaf temperature and inhibits cell elongation, resulting in stomatal closure (Rajendran *et al.*, 2009; Sirault *et al.*, 2009), and these occur independently from the salt accumulation in the shoots. In rice, the salinity decreases seed germination (Shobbar *et al.*, 2012), retards growth and seedling establishment (Lutts *et al.*, 1996), causes the destruction of the chloroplast (Yamane *et al.*, 2008), decreases photosynthetic ability (Moradi & Ismail, 2007) and inhibits seed set and grain yields (Asch & Wopereis, 2001). Rice has different mechanisms to cope with the adverse effects of salinity on its growth and development, such as osmolyte accumulation, ion homeostasis and compartmentalization, and an antioxidant defense system (Hoang *et al.*, 2016).

Researchers have intensified efforts on the identification and characterization of salt-tolerant genes in crop plants by using molecular marker technology in order to develop salt-tolerant varieties. Some of the technologies include quantitative trait loci (QTLs) mapping for any trait of interest (Abhayawickrama *et al.*, 2020; Chattopadhyay *et al.*, 2021; Krishnamurthy *et al.*, 2020; Pundir *et al.*, 2021; Yang *et al.*, 2013; Zhang *et al.*, 2020), genome-wide association studies (GWAS) and transcriptome (RNA-Seq) analysis (Chandran *et al.*, 2019; Chen *et al.*, 2017; Chutimanukul *et al.*, 2021; Lei *et al.*, 2020; Lekklar *et al.*, 2019a; Wang *et al.*, 2018; Warraich *et al.*, 2020; Yu *et al.*, 2017). Several genetic regions harboring salt-tolerant genes have been identified by these approaches.

Although high-throughput sequencing cost has decreased dramatically, sequencing a large population is still costly. Bulk-segregant analysis (BSA) offers an alternative approach that is efficient and cost-effective. BSA is one of the methods used for the rapid discovery of molecular markers for specific traits of interest (Giovannoni *et al.*, 1991; Michelmore *et al.*, 1991). In BSA-based identifications of quantitative trait loci, DNA bulks are made from two extreme ends with contrasting phenotypes among the members of a population (Becker *et al.*, 2011). Since the advent of the BSA approach, several genomic regions harboring genes of particular interests have been identified in plants and other organisms (Arikit *et al.*, 2019; Han *et al.*, 2015; Itoh *et al.*, 2019; Sun *et al.*, 2018; Thakur *et al.*, 2021; Tragoonrung *et al.*, 1996; Venuprasad *et al.*, 2009; Vikram *et al.*, 2012; Yang *et al.*, 2017; Yang *et al.*, 2021; Zhao *et al.*, 2021). All these studies have shown that bulk-segregant analysis is a good approach that could be used to identify and map quantitative trait loci for traits of interest.

'Jao Khao' is one of the Thai landrace rice varieties showing the salt-tolerant phenotypes at seedling (Habila *et al.*, 2022 and flowering stages (Lekklar *et al.*, 2019b). In order to develop the salt-tolerant rice cultivar by using the genetic contribution from the Thai landrace, the determination of the specific salt tolerance region in the genome is required. In this research we created the F$_2$ population, having 'Jao Khao', Thai salt-tolerant variety, and IR29, the salt susceptible line as the parental lines in order to use these materials for bulk-segregant analysis to identify the important region(s) for salt tolerance in the future.

MATERIALS AND METHODS
Plant materials and experimental condition

The F$_2$ population of Thai rice (*Oryza sativa* L.) obtained from a cross between a Thai rice variety with salt tolerance ability, 'Jao Khao', and a standard salt-sensitive variety, IR29 were provided by Nakhon Ratchasima Rice Research Center. Six hundred seeds from one of the F$_2$ progeny were sterilized and incubated for 3 days at 60°C for 24 hours before they were germinated in sterile plates. Seven days old seedlings were transplanted into a half-strength WP nutrients solution (Vajrabhaya & Vajrabhaya, 1991). At fourteen days old, the rice seedlings were administered full strength WP nutrients solution and evaluated under salt stress condition in a stepwise manner. Firstly, the rice seedlings were treated with 75 mM NaCl (9 dS.m^{-1}) for six days, then the salt concentration was increased to 100 mM (12 dS.m^{-1}) and the seedlings were treated for another six days. Physiological traits and growth parameters were evaluated after 12 days of salt stress treatment.

Evaluation of the salt responsive traits under salt stress condition

The phenotypes of the F$_2$ population under salt stress were evaluated at the end of the experiment (12 days of salt stress exposure), except for the standard salt injury evaluation system (SES) that was recorded daily. The SES of each F$_2$ population was determined daily according to Gregorio *et al.* (1997). The leaf relative water content (RWC) for each member of the F$_2$ population was determined by using the youngest fully expanded leaf from each seedling. The fresh weight (FW) of each sampled leaf piece was measured using a digital weighing balance (Mettler Toledo (Model ML204/01), Switzerland) and kept in empty Eppendorf tubes. One ml of distilled water was added into the tube containing the leaf sample and kept in the dark for 24 hours before the turgid weight (TW)

of each leaf sample was measured. The leaf samples were thereafter oven-dried at 60°C to a constant dry weight (DW). The relative water content of each F_2 progeny was calculated using the formula described by Sade *et al*., 2015 as shown: RWC (%) = [(FW–DW)/(TW–DW) x 100].

Cell membrane stability (CMS) was evaluated according to the method descried by Blum & Ebercon, 1981 as modified by Naghashzadeh, 2014. Leaf samples from the middle portion of the youngest fully expanded leaf of each F_2 progeny were collected at the end of the experiment, immediately weighed, and cut into pieces. About 0.01 gram of the leaf pieces were put into 15 ml tubes containing 10 ml distilled water and kept at room temperature for about 2 hours. The initial electrical conductivity (EC_1) of each leaf sample in the respective tubes was measured using Electroconductivity Meter (S230 Mettler Toledo, Switzerland). The leaf samples were then boiled in a water bath at 100°C for 15 minutes before the final electrical conductivity (EC_2) of each sample was measured and recorded. The cell membrane stability (CMS) evaluated as the percentage of the injury was calculated as: CMS = 100 x [1 – (EC_1/EC_2)]. Growth parameters including plant height and root length, and the plant's total fresh weight were measured at the end of the experiment immediately by using a ruler and the Mettler Toledo (Model ML204/01), Switzerland, digital weighing balance respectively.

The salt responsive phenotypes of the 104 accessions together with the standard salt tolerant and susceptible checks, 'Pokkali' and IR29, were evaluated at the reproductive stage by Lekklar *et al*. (2019a). Briefly, the experiment was laid out in a randomized complete block design with four replications. The rice seedlings were grown in a hydroponic solution (WP nutrient solution) until they were 21 days old. The seedlings were transplanted into pots containing 5 kg of soil until harvest. When the rice plants developed to the heading stage, salt stress was applied by addition of 150 mM NaCl to the soil in order to cause the salinity stress at 8-9 dS.m^{-1} for 9 days. After that, salt was washed out by tap water, until the soil EC was less than 2 dS.m^{-1}. Then, they were grown until seed harvest. For the control experiment, the plants were treated with tap water instead of NaCl solution at the same time. Yield-related traits like the number of tillers per plant, TIL; the number of panicles per plant, PAN; the number of filled grains per plant, FG; and the number of unfilled grains per plant, UFG were all measured. The measurement was carried out for both accessions grown under normal and stress conditions. The ratio of their performance under stress to their performance under normal conditions was used to generate the stability indices for each accession.

Clustering of rice phenotypes

Based on the phenotypic data from Lekklar *et al*. (2019a), the stability indices of tiller number, panicle number, number of filled grains, and number of unfilled grains, calculated from the ratio of the values obtained from stressed plants and those obtained from normal-grown plants, were used for hierarchical clustering analysis of the Euclidian distances between the stability indices (value from stressed plants/value from normal-grown plants) using 119 JMP software with Ward's method.

Statistical analysis

The salt responsive traits or phenotypic data of the F_2 populations were subjected to distribution curve using R 'ggplot2' package. Pearson pairwise correlation analysis was carried to determine the relationships among the salt responsive traits, and the coefficient of correlation plots were constructed using an R 'corrplot' package.

RESULTS
'Jao Khoa' showed salt tolerance phenotype of maintenance of productivity after salt stress at booting stage

Based on clustering analysis, 104 rice varieties, including 'Pokkali', the salt tolerance standard cultivar, and IR29, the salt susceptible standard line, 'Jao Khao' rice shows the closest relationship to 'Pokkali' (Figure 1). 'Jao Khao' was the only cultivar that had more than 50 % increase in tiller number after salt stress treatment (supplementary Table S1), suggesting the recovery ability after salt stress. Therefore, 'Jao Khao' was selected to be the parental cultivar to cross with the salt susceptible line IR29 to generate the population for bulk segregant analysis.

### Evaluation of the phenotypes of F_2 population under salt stress condition

The 600 F2 lines, together with the parental lines, 'Jao Khao' and IR29, and the standard salt tolerant variety, 'Pokkali', were grown under NaCl-stress conditions. The salt responsive traits, reflecting salt tolerant ability, were collected. The salt injury score collected according to the standard evaluation system (SES) used by The International Rice Research Institute (IRRI) by Gregorio *et al*. (1997) ranged from 1–9 (Figure 2 A). Most of the F_2 population had the

SES at 9, representing the most susceptible to salt stress. However, more than 70 plants from 600 lines had a SES of 1, showing the tolerant phenotype. However, the distribution of SES values was more like the uniform distribution in the F_2 population.
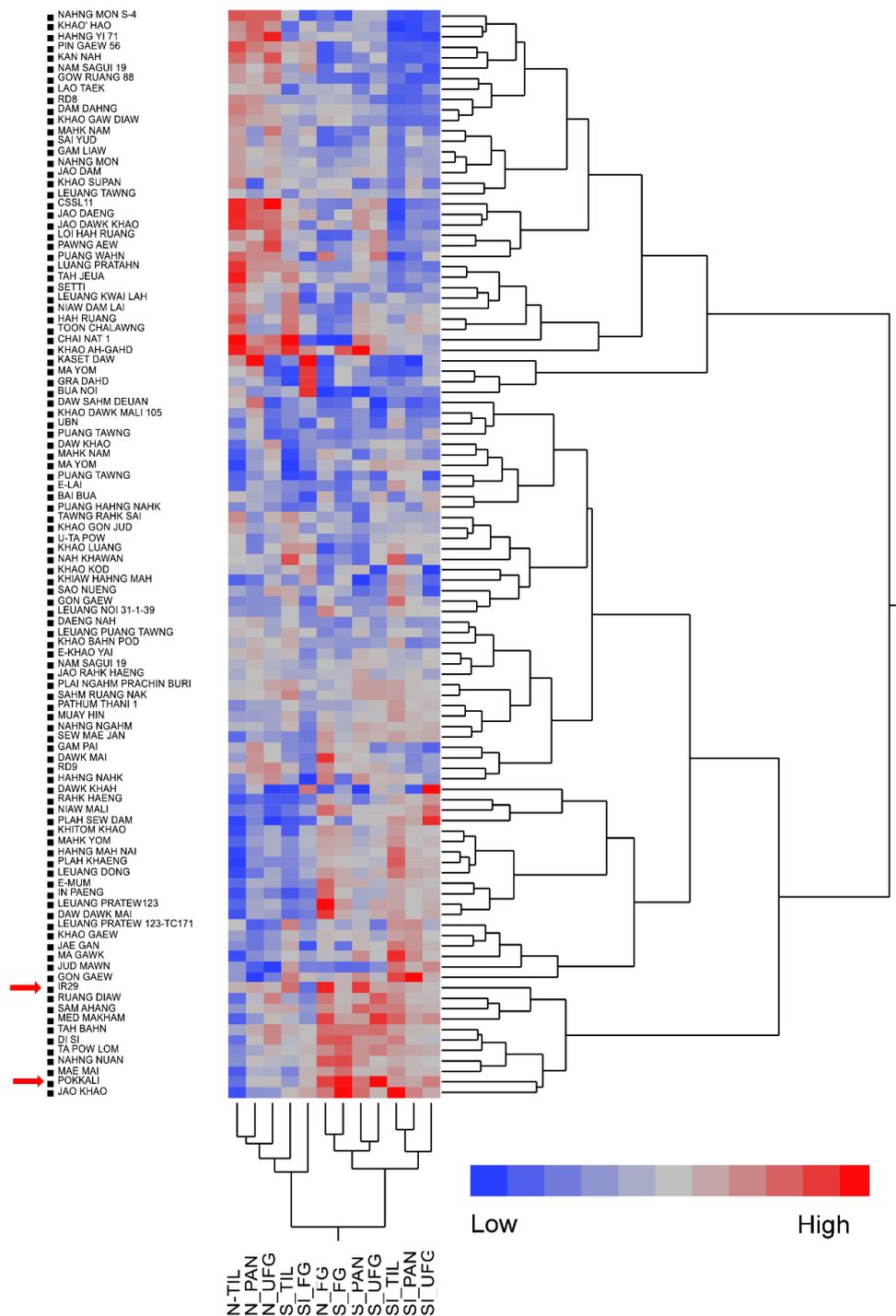
Figure 1 Cluster analysis of the phenotypes of 104 rice cultivars, including salt tolerance rice, 'Pokkali' and salt susceptible rice, IR29, indicated by red arrows. N-TIL: Tiller number in normal grown condition, N_PAN: Panicle number in normal grown condition, N_FG: Filled grain number in normal grown condition, N_UFG: Unfilled grain number in normal grown condition, S_TIL: Tiller number in salt stress condition, S_PAN: Panicle number in salt stress condition, S_FG: Filled grain number in salt stress condition, S_UFG: Unfilled grain number in salt stress condition, SI_TIL: Stability index of tiller number, SI_PAN: Stability index of panicle number, SI_FG: Stability index of filled grain number, SI_UFG: Stability index of unfilled grain number.
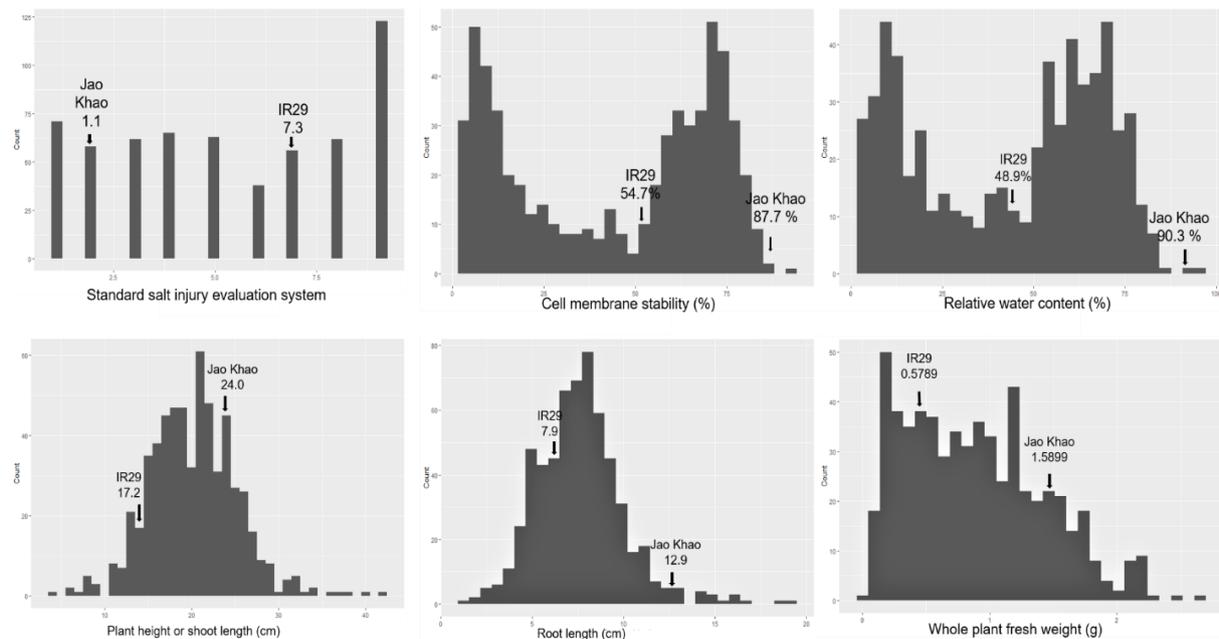
Figure 2 Frequency distribution of six salt responsive traits related to salt tolerance in the 600 F$_2$ progeny obtained from a cross between 'Jao Khao' and IR29.

The bimodal distribution was detected in the distribution of cell membrane stability (CMS) and relative water content (RWC) values of the F$_2$ population (Fig. 2B and 2C). The percentage of cell membrane stability (CMS) represents the damage caused by salt stress on the membrane (Bajji *et al*., 2002; Farooq & Azam, 2006). Based on the CMS histogram, the F$_2$ population can be divided into 2 groups: high CMS (more than 50%) and low CMS (less than 50%). The median of the high CMS group was 65.5%, while the median of low CMS was 14.3% (Fig. 2B). The average CMS of the whole F$_2$ population was 44.0%. The CMS value of parental lines, 'Jao Khao', was 88.7%, and IR29 was 54.7%. The value representing the water status in plants under salt stress conditions is relative water content (RWC) (%). Plants with the ability to conserve water under stress have a higher possibility of surviving or getting fewer effects from stress. In this experiment, we determined the RWC of leaves after salt stress for 12 days. This response in the F$_2$ population can also be distinguished into 2 groups: seedlings with a high RWC 150 (more than 50%) and seedlings with a low RWC (less than 50%). The median of the high RWC group is 65.5%, while the low RWC group has a median of 14.3% (Fig. 2C). The average RWC of the whole F$_2$ population was

43.4%. The RWC values of the parental lines, 'Jao Khao' was 90.3% and IR29 was 48.9%.

The distribution of the whole plant's fresh weight (WFW) after 12 days of salt stress was shown to have a right-skewed distribution (Fig. 2D). The fresh weight of F$_2$ seedlings ranged from 0.02 to 2.67 g/plant. The mean fresh weight was 0.89 g/plant while the median was 0.83 g/plant. The fresh weight of 'Jao Khao' was 1.59 g/plant. IR29's fresh weight was about one third of that of 'Jao Khao', which was only 0.58 g/plant.

Plant height (PH) or shoot length and root length (RL) values of the F$_2$ population resembled normal distribution (Fig. 2E and 2F). The plant height ranged from 4.6 to 50.0 cm and mean plant height in the F$_2$ population was about 20 cm. After growing under salt stress for 12 days, F$_2$ root length varied from 1.1 cm to 19.0 cm. The median and mean root length in the F$_2$ population was 7.6 cm.

Based on these salt-responsive phenotypes in the F$_2$ population, the Pearson correlation coefficients of these parameters were calculated as shown in Table 1. As expected, SES had a significantly strong negative (red-colored cycles) association with the other traits (CMS, RWC, WFW, PH, and RL). The other traits, on the other hand, had a significantly strong positive relationship (blue colored cycles) (Table 1 and Figure 3).

Table 1 Pearson correlation coefficients of six salt responsive traits evaluated at the seedling stage in the F_2 population.

	SES	RWC (%)	CMS (%)	WFW (g)	SL (cm)
RWC (%)	-0.898**				
CMS (%)	-0.885**	0.954**			
WFW (g)	-0.805**	0.754**	0.748**		
PH (cm)	-0.552**	0.550**	0.514**	0.671**	
RL (cm)	-0.495**	0.518**	0.469**	0.559**	0.716**

** Correlation is significant at the 0.01 level. SES, standard salt injury evaluation system; CMS, cell membrane stability; RWC, relative water content; WFW, whole plant fresh weight; PH, plant height and RL, root length.
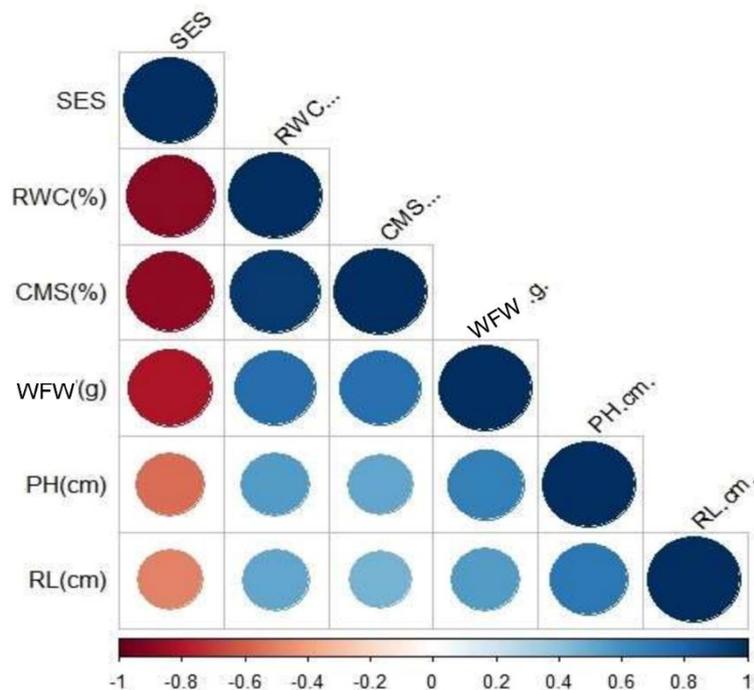
Figure 3 Corrplot of the Pearson correlation coefficients of six traits associated with salt tolerance at the seedling stage of the 600 F_2 population. SES, standard salt injury evaluation system; CMS, cell membrane stability; RWC, relative water content; WFW, whole plant fresh weight; PH, plant height and RL, root length.

DISCUSSION

Salinity tolerance is needed in order to tackle the decline in rice yield faced by farmers as a result of the increasing salt stress in rice fields (Hoang *et al.*, 2016). The identification of genomic regions or genes involved in salinity tolerance could be of great help to plant breeders and farmers at large. The clustering analysis of the yield related parameters during the reproductive stage clustered 'Jao Khao' and 'Pokkali,' the typical salt tolerance check, together, indicating that they may respond to salt stress in the same way. 'Pokkali' is said to have resisted salt stress better than sensitive varieties by effectively lowering the Na^+/K^+ ratio (Suriya-arunroj *et al.*, 2005). Though, at reproductive stage, the salt responsive phenotypes of

'Jao Khao' were clustered in the same group as 'Pokkali' (Fig. 1), their salt tolerance genetic regions may be different. A phylogenetic study of single nucleotide polymorphisms (SNPs) from 164 salt-tolerant genes from 'Jao Khao', 'Pokkali' and the other salt-tolerant Thai rice cultivars (Habila *et al.*, 2021) shows that the various salt-tolerant genomic regions in 'Jao Khao' might be different from those in 'Pokkali' and should be further investigated to understand the salt tolerant mechanisms and develop the marker(s) for salt tolerant rice breeding program in the case that 'Jao Khao' is used to contribute the salt tolerant gene (s) in the breeding program.

In this present study, the F_2 population members were evaluated under stress conditions by

focusing on six salt-responsive traits (SES, CMS, RWC, WFW, PH, and RL). They responded differently to salt stress by showing a wide range of phenotypic variations based on the parameters measured. These phenotypic variations among the F_2 population could possibly be attributed to genetic variations within the population.

SES of the F_2 population of 'Jao Khao' and IR29 cross showed random distribution (Fig. 2A). The more susceptible lines than IR29 and the more tolerant lines than 'Jao Khao' were detected, suggesting the transgressive segregation that the recombination of the genes from these two parental lines possibly gave the stronger salt stress responsive phenotypes. These also suggested that the regulation of this trait (SES) is caused by multiple genes with complex interactions.

Both CMS and RWC traits in the F_2 population revealed a bimodal distribution. These data suggest that these traits are regulated by a couple of genes. These datasets could be carried out for further F_2 bulked-segregant analysis. CMS is the parameter that is widely used to determine salt tolerance in various plant species, such as rice (Lekklar *et al*., 2019a), wheat (Farooq and Azam, 2006), tomato (Moradi *et al*., 2021). Salt toxicity can cause cell membrane damage, leading to an increase in ion and electrolyte permeability. The ability to repair or maintain its integrity mainly depends on the composition of the plasma membrane (Shahid *et al*., 2020). RWC is also another parameter to determine salt tolerance (Nguyen *et al*., 2021). It was reported to be well correlated with CMS (Shahid *et al*., 2020). This is also consistent with our study that showed that CMS and RWC in the F_2 population were well correlated.

The WFW of the F_2 population showed a right-skewed distribution. The parental lines, IR29 and 'Jao Khao' are different in size. IR29 is much smaller than 'Jao Khao'. The segregation of WFW in F_2 distribution indicated multiple gene regulation in plant size under salt stress. Plant height and root length responses of the F_2 population display continuous segregation under salt stress, suggesting that both are quantitative traits controlled by multiple genes, which is consistent with other studies in rice (Wang *et al*., 2010; Tiwari *et al*., 2016). PH was shown to have a significant correlation with grain yield per plant under salt stress conditions (Tiwari *et al*., 2016). The analysis for salt tolerance phenotypes of these growth parameters should be compared to the responses in normal grown condition. Unfortunately, it cannot be done in the F_2 population as the genotype of each F_2 plant is different from one another. For CMS and

RWC, these values were presented by percentage of the unaffected system. Therefore, these two parameters can directly be used for bulk-segregant analysis to determine the salt tolerance region contributed by 'Jao Khao' rice in the future.

ACKNOWLEDGEMENTS

REFERENCES

Abhayawickrama B, Gimhani D, Kottearachchi N, Herath V. Utilization of SNP-based highly saturated molecular map of a RIL population for the detection of QTLs and mining of candidate genes for 233 salinity tolerance in rice. J Agric Sci. 2020;15:345 – 361.

Arikit S, Wanchana S, Khanthong S, Saensuk C, Thianthavon T, Vanavichit A, Toojinda T. QTL-seq identifies cooked grain elongation QTLs near soluble starch synthase and starch branching enzymes in rice (*Oryza sativa* L.). Sci Rep. 2019:9:1–10.

Asch F, Wopereis MC. Responses of field-grown irrigated rice cultivars to varying levels of floodwater salinity in a semi-arid environment. Field Crops Res. 2001;70:127–137.

Bajji M, Kinet JM, Lutts S. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul. 2002:36:61–70.

Becker A, Chao DY, Zhang X, Salt DE, Baxter I. Bulk segregant analysis using single nucleotide polymorphism microarrays. PloS One. 2011;6:e15993.

Blum A, Ebercon A. Cell membrane stability as a measure of drought and heat tolerance in wheat. Crop Sci. 1981;21:43–47.

Chandran AKN, Kim JW, Yoo YH, Park HL, Kim YJ, Cho MH, Jung KH. Transcriptome analysis of rice-seedling roots under soil–salt stress using RNA-Seq method. Plant Biotechnol Rep. 2019;13:567–578.

Chattopadhyay K, Mohanty SK, Vijayan J, Marndi BC, Sarkar A, Molla KA, Chakraborty K, Ray S, Sarkar RK. Genetic dissection of component traits for salinity tolerance at reproductive stage in rice. Plant Mol Biol Rep. 2021;39:386–402.

Chauhan BS, Jabran K, Mahajan G. Rice production worldwide. Springer Nature Switzerland. 2017. eBook.

Chen G, Liu C, Gao Z, Zhang Y, Jiang H, Zhu L, Ren D, Yu L, Xu G, Qian Q. OsHAK1, a high-affinity potassium transporter, positively regulates responses to drought stress in rice. Front Plant Sci. 2017;8:1885.

Chutimanukul P, Saputro TB, Mahaprom P, Plaimas K, Comai L, Buaboocha T, Siangliw M, Toojinda T, Chadchawan S. Combining genome and gene co-expression network analyses for the identification of genes potentially regulating salt tolerance in rice. Front Plant Sci. 2021;12:704549.

Farooq S, Azam F. Cell membrane stability technique for screening salt tolerant wheat genotypes. J. Plant Physiol. 2006;163;629–637.

Giovannoni JJ, Wing RA, Ganal MW, Tanksley SD. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Res. 1991;19(23):6553–6568.

Gregorio G, Senadhira D, Mendoza R. Screening rice for salinity tolerance. 1997;Vol. 22. IRRI discussion paper series.

Habila S, Khunpolwattana N, Chantarachot T, Buaboocha T, Comai L, Chadchawan S, Pongpanich M. Salt stress responses and SNP-based phylogenetic analysis of Thai rice landraces. Plant Genome. 2021 2022;e20189

Han Y, Lv P, Hou S, Li S, Ji G, Ma X, Du R, Liu G. Combining next generation sequencing with bulked segregant analysis to fine map a stem moisture locus in sorghum (*Sorghum bicolor* L. Moench). PloS One. 2015;10:e0127065.

Hoang TML, Tran TN, Nguyen TKT, Williams B, Wurm P, Bellairs S, Mundree S. Improvement of salinity stress tolerance in rice: challenges and opportunities. Agronomy.2016;6:54.

Itoh N, Segawa T, Tamiru M, Abe A, Sakamoto S, Uemura A, Oikawa K, Kutsuzawa H, Koga H, Imamura T. Next-generation sequencing-based bulked segregant analysis for QTL mapping in the heterozygous species *Brassica rapa*. Theor Appl Genet. 2019;132:2913–2925.

Krishnamurthy S, Pundir P, Warraich AS, Rathor S, Lokeshkumar B, Singh NK, Sharma PC. Introgressed saltol QTL lines improves the salinity tolerance in rice at seedling stage. Front Plant Sci. 2020;11:833.

Lei L, Zheng H, Bi Y, Yang L, Liu H, Wang J, Sun J, Zhao H, Li X, Li J. Identification of a major QTL and candidate gene analysis of salt tolerance at the bud burst stage in rice (Oryza sativa L.) using QTL-Seq and RNA-Seq. Rice. 2020;13:1–14.

Lekklar C, Pongpanich M, Suriya-Arunroj D, Chinpongpanich A, Tsai H, Comai L, Chadchawan S, Buaboocha T. Genome-wide association study for salinity tolerance at the flowering stage in a panel of rice accessions from Thailand. BMC Genomics. 2019;20:1–18.

Lekklar C, Suriya-Arunroj D, Pongpanich M, Comai L, Kositsup B, Chadchawan S, Buaboocha T. Comparative genomic analysis of rice with contrasting photosynthesis and grain production under salt stress. Genes. 2019;10:562.

Lutts S, Kinet J, Bouharmont J. Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (Oryza sativa L.) cultivars differing in salinity resistance. Plant Growth Regul. 1996;19:207–218.

Michelmore RW, Paran I, Kesseli R. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. PNAS. 1991;88:9828–9832.

Moradi F, Ismail AM. Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann Bot. 2007;99:1161–1173.

Moradi M, Dehghani H, Ravari SZ. Genetics of physiological and agronomical traits linked to salinity tolerance in tomato. Crop Pasture Sci. 2021;72:280–290.

Munns R, Tester M. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 2008;59:651–681.

Naghashzadeh M. Response of relative water content and cell membrane stability to mycorrhizal biofertilizer in maize. eJBio. 2014;10:68–72.

Nguyen HTT, Das Bhowmik S, Long H, Cheng Y, Mundree S, Hoang LTM. Rapid accumulation of proline enhances salinity tolerance in Australian wild rice *Oryza australiensis* Domin. Plants. 2021;10:2044.

Pearson GA, Ayers A, Eberhard D. Relative salt tolerance of rice during germination and early seedling development. Soil Sci. 1966;102:151–156.

Pundir P, Devi A, Krishnamurthy S, Sharma PC, Vinaykumar N. QTLs in salt rice variety CSR10 reveals salinity tolerance at reproductive stage. Acta Physiol Plant. 2021;43:1–15.

Rajendran K, Tester M, Roy SJ. Quantifying the three main components of salinity tolerance in cereals. Plant Cell Environ. 2009;32:237–249.

Sade N, Galkin E, Moshelion M. Measuring Arabidopsis, tomato and barley leaf relative water content (RWC). Bio-protoc. 2015;5:e1451-e1451.

Shahid MA, Sarkhosh A, Khan N, Balal RM, Ali S, Rossi L, Gómez C, Mattson N, Nasim W, Garcia-Sanchez F. Insights into the physiological and biochemical impacts of salt stress on plant growth and development. Agronomy. 2020;10:938.

Shobbar MS, Azhari O, Shobbar ZS, Niknam V, Askari H, Pessarakli M, Ebrahimzadeh H. Comparative analysis of some physiological responses of rice seedlings to cold, salt, and drought stresses. J. Plant Nutr. 2012;35:1037–1052.

Sirault XR, James RA, Furbank RT. A new screening method for osmotic component of salinity tolerance in cereals using infrared thermography. Funct Plant Biol. 2009;36:970–977.

Sun J, Yang L, Wang J, Liu H, Zheng H, Xie D, Zhang M, Feng M, Jia Y, Zhao H. Identification of a cold-tolerant locus in rice (*Oryza sativa* L.) using bulked segregant analysis with a next-generation sequencing strategy. Rice. 2018;11:1–12.

Suriya-arunroj D, Supapoj N, Vanavichit A, Toojinda T. Screening and selection for physiological characters contributing to salinity tolerance in rice. Agric. and Nat. Res. 2005;39;174-185.

Thakur VJS, Ponnuswamy R, Singh AK, Shankar VG, Srinivasa D. Molecular tagging of Rf genes for the fertility restoration of WA-CMS system by bulk segregant analysis in rice. Indian J Genet. 2021;81:43–49.

Tiwari S, Sl K, Kumar V, Singh B, Rao A, Mithra SVA, Rai V, Singh AK, Singh NK. Mapping QTLs for salt tolerance in rice (*Oryza sativa* L.) by bulked segregant analysis of recombinant inbred lines using 50K SNP chip. PloS One. 2016;11:e0153610.

Tragoonrung S, Sheng J, Vanavichit A. Tagging an aromatic gene in lowland rice using bulk segregant analysis. In Rice Genetics III. 1996. In 2 Parts. pp. 613–618.

Vajrabhaya M, Vajrabhaya T. (1991). Somaclonal variation for salt tolerance in rice. In In: Bajaj Y.P.S. (eds) Rice. Biotechnology in Agriculture and Forestry. Springer. 1991. Vol14. pp. 368-382.

Venuprasad R, Dalid C, Del Valle M, Zhao D, Espiritu M, Cruz MS, Amante M, Kumar A, Atlin G. Identification and characterization of large-effect quantitative trait loci for grain yield under lowland drought stress in rice using bulk-segregant analysis. Theor Appl Genet. 2009;120:177–190.

Vikram P, Swamy BM, Dixit S, Ahmed H, Cruz MS, Singh AK, Ye G, Kumar A. Bulk segregant analysis: An effective approach for mapping consistent-effect drought grain yield QTLs in rice. Field Crops Res. 2012;134:185–192.

Wang J, Zhu J, Zhang Y, Fan F, Li W, Wang F, Zhong W, Wang C, Yang J. Comparative transcriptome analysis reveals molecular response to salinity stress of salt-tolerant and sensitive genotypes of indica rice at seedling stage. Sci Rep. 2018;8:1–13.

Wang ZF, Wang JF, Bao Ym., Wu YY, Xuan S, Zhang HS. Inheritance of rice seed germination ability under salt stress. Rice Sci. 2010;17:105–110.

Warraich AS, Krishnamurthy S, Sooch BS, Vinaykumar N, Dushyanthkumar B, Bose J, Sharma PC. Rice GWAS reveals key genomic regions essential for salinity tolerance at reproductive stage. Acta Physiol Plant. 2020;42:1–15.

Yamane K, Kawasaki M, Taniguchi M, Miyake H. Correlation between chloroplast ultrastructure and chlorophyll fluorescence characteristics in the leaves of rice (*Oryza sativa* L.) grown under salinity. Plant Prod Sci. 2008;11:139–145.

Yang L, Lei L, Li P, Wang J, Wang C, Yang F, Chen J, Liu H, Zheng H, Xin W. Identification of candidate genes conferring cold tolerance to rice (Oryza sativa L.) at the bud-bursting stage using bulk segregant analysis sequencing and linkage mapping. Frontiers Plant Sci. 2021;12:402.

Yang X, Xia X, Zhang Z, Nong B, Zeng Y, Xiong F, Wu Y, Gao J, Deng G, Li D. (2017). QTL mapping by whole genome re-sequencing and analysis of candidate genes for nitrogen use efficiency in rice. Frontiers Plant Sci. 2017;8:1634.

Yang Z, Huang D, Tang W, Zheng Y, Liang K, Cutler AJ, Wu W. Mapping of quantitative trait loci underlying cold tolerance in rice seedlings via high-throughput sequencing of pooled extremes. PloS One. 2013;8:e68433.

Yu J, Zao W, He Q, Kim TS, Park YJ. Genome-wide association study and gene set analysis for understanding candidate genes involved in salt tolerance at the rice seedling stage. Mol Genet Genom. 2017;292:1391–1403.

Zeng L, Shannon MC, Lesch SM. (2001). Timing of salinity stress affects rice growth and yield components. Agric Water Manag. 2001;48:191–206.

Zhang Y, Ponce KS, Meng L, Chakraborty P, Zhao Q, Guo L, Gao Z, Leng Y, Ye G. QTL identification for salt tolerance related traits at the seedling stage in indica rice using a multi-parent advanced generation intercross (MAGIC) population. Plant Growth Regul. 2020;92:365–373.

Zhao H, Zheng Y, Bai F, Liu Y, Deng S, Liu X, Wang L. Bulked segregant analysis coupled with whole-genome sequencing (BSA-Seq) and identification of a novel locus, qGL3. 5, that regulates grain length. Research Square; 2021. DOI: 10.21203/rs.3.rs-263682/v1.