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MAPPING QUANTITATIVE TRAIT LOCI (QTLS) FOR SALT TOLERANCE IN RICE (*ORYZA SATIVA*) USING RFLPS

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Abstract

Recombinant inbred (RI) lines derived from a cross between Tesanai 2 (moderately salt tolerant) and CB (salt sensitive) through single seed descent procedure were used to identify RFLP markers linked to QTLs involved in salinity tolerance. The RI population (F_8) was evaluated for six different parameters using Yoshida's modified nutrient solution at an EC level of 12 dSm^{-1}. The genotyping of 96 RI lines utilized 74 RFLP markers that revealed polymorphism. A linkage map was constructed from 12 linkage groups based on RI segregation data. The map covered 1349.5 cM of the rice genome with an average distance of 18.24cM between marker loci. Based on regression ANOVA (P = 0.05), one marker locus was found significantly associated with seedling survival days, 14 with dry shoot weight, 8 with dry root weight, 2 with shoot Na^+ and 3 each with K^+ and Na^+/K^+ ratio. The proportion of phenotypic variation explained by each QTLs ranged from 4% to 15%. Common QTLs were observed for different traits. The result explains much of the transgressive variation for the most characters measured.

Introduction

Rice is the staple food of more than half of the world's population. The production and planting area of this important crop is greatly affected by soil salinity. Salt tolerance is a quantitative, multigenic character and is often a composite response of the integrated biological system. Traditionally, the genetic system controlling a quantitative trait has been investigated by statistical models. The attempts to resolve quantitative trait into their individual genetic components were initially limited by a lack of polymorphic markers covering a large part of the genome. In recent years, techniques such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPDs) and amplified fragment length polymorphism (AFLPs) have begun to play an important role in the genetic analysis of quantitative variation. Using molecular linkage genetic maps along with the suitable statistical computer programmes, it is now possible to estimate the number of loci controlling genetic variation in a segregating population. These loci can also be characterized with regard to their map position in the genome, gene action, phenotypic effect and pleiotropic effect.

Since the introduction of molecular markers, RFLPs in particular, QTLs have been mapped in different crop species e.g., tomato (Monforte *et al*., 1997), maize (Veldoom *et al*., 1994), and barley (Takeda & Mano, 1997). In rice four molecular linkage maps have been developed by independent research groups (McCouch *et al*., 1988; Saito *et al*., 1991; Causse *et al*., 1994; Kurata *et al*., 1994). These detailed maps have facilitated analyses of quantitative trait loci (QTLs) controlling several traits such as yield related traits (Xiao *et al*., 1996); blast resistance (Wang *et al*., 1994); heading date (Yano *et al*., 1997); root morphology (Price & Tomos, 1997), seed dormancy (Lin *et al*., 1998) and allelopathic effect of rice (Ebana *et al*., 2001).

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QTLs for salt tolerance in rice have been identified by Zhang *et al*., 1995. The present study was initiated to get more information on mapping genes for salt tolerance in rice . The objectives were: (1) to analyze the response of RI lines of rice to salt stress (2) to identify QTLs associated with salt tolerance using RFLP markers and (3) to estimate the genetic effect of individual QTLs towards genetic variation. The dissection of such a complex trait by means of QTL mapping approach will be of great significance to understand the mechanism of salt tolerance and to develop salt tolerant varieties.

Materials and Methods

Plant material: A population of 96 recombinant inbred (RI) lines derived from a cross between Tesanai 2 and CB (indica/indica) through single seed descent method was used for this investigation. The maternal parent Tesanai 2 (TSA) has middle level tolerance whereas CB is very susceptible to salinity.

Evaluation for salt tolerance: The recombinant inbred (RI) lines along with their parents were evaluated for salt tolerance in a green house having temperature of 30^{o} C at Okinawa Subtropical Research Station, Japan. Two indica varieties IR28 and Pokalli previously identified as susceptible and tolerant, respectively were used as check varieties. Ten seeds from each variety and RI population were placed in a hole of thin styrofoam sheet with a nylon net bottom which floated on modified Yoshida's nutrient solution. Seventeen days old seedlings were salinized to an EC level of 12 dSm^{-1} by NaCl. The solution was renewed after four days and its pH was maintained every alternate day at 5.8 by adding either 1M KOH or HCl. Seedling survival days were recorded in days from seeding to death. When a seedling was completely yellow and no more green tissue was evident, it was considered as dead. After harvesting, plants were dried in an oven at 75^{o}C for three days before taking measurements on dry shoot and root weight. The experiment was repeated three times and observations were recorded on seedling survival days, dry shoot weight, dry root weight, shoot $Na^{+}$and K^{+} concentration. The Na^{+} / K^{+} ratio was also calculated.

Restriction fragment length polymorphism (RFLP) survey: Total genomic DNA of parents and RI lines was extracted and utilized for RFLP analysis. Six μg DNA of parents and each RI line was digested by three restriction enzymes (*EcoR1*, *EcoRV* and *Hind 111*) at 37^{o}C overnight. Spermidine (40mM) was added to promote complete digestion. Total digest of 30 μl was loaded per lane on a 0.9% agarose gel in TAE buffer and electrophoresed overnight at 30 V. Southern blotting was carried out to transfer digested DNA onto Hybond N^{+} membrane (Amersham) by capillary transfer method using 0.4% NaOH.

A total of 165 RFLP markers were used which were provided by the courtesy of Rice Genome Project Tsukuba, Japan. The probes were amplified by the PCR and labelled with thermostable alkaline phosphatase enzyme by using the Gene Images Alkphos Direct labelling and detection Kit (Amersham LIFE SCIENCE). The membranes were hybridized with alkaline phosphatase labelled probes at 55^{o}C overnight in a hybridization oven (Techne). Signals were detected by CDP-Star chemiluminescent detection reagent and autoradiographed on X-ray film for 2 hours. Autoradiograms were scored visually and numerical values were assigned to Tesanai 2 (1) and CB (2) homozygote alleles. The blots were reprobed about 20 times.

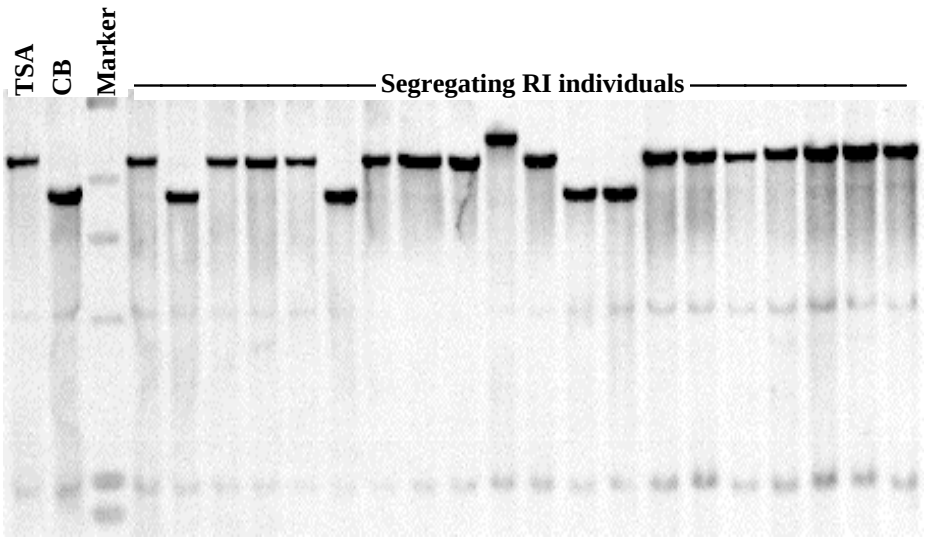

Fig. 1. Segregation of RFLP marker R2289 (Chrom.5) in RI population. DNA of parents and R1population was digested with Hind 111. The first two lanes are parental DNA (Tesanai 2 and CB) followed by DNA from R1 population (part of a segregation population).

QTL analysis: QTL detection was based on the regression of line mean values on the value attributed to the polymorphic marker in the RI population. Simple linear regression function of qGene (Macintosh v/2.16) was used to identify association of RFLP markers with the traits relevant to salt tolerance. A significant correlation at $P< 0.05$ was interpreted as indicating linkage of the QTL to the marker locus. The proportion of the phenotypic variation explained by individual marker loci was determined by the R^2 values.

Results

RFLP in parents: A total of 165 RFLP markers randomly distributed on 12 chromosomes of rice were chosen to scan for the polymorphism between parents. Of these 129 markers were found polymorphic with one or three enzymes. The overall polymorphism detected by three enzymes was 78.18%. Altogether 74 RFLP markers were successfully mapped on a population. Autoradiogram showing segregation of RFLP marker R2289 (chromosome 5) in a population is illustrated in Fig. 1.

Phenotypic evaluation for salt tolerance: The analysis of variance revealed highly significant ($P < 0.01$) differences among RI lines for the most traits measured with the exception of shoot Na^+ and K^+ concentration. The frequency distribution of all the traits measured is given in Fig. 2. Tesanai 2 was less tolerant than Pokalli (resistant check) which has mean survival days of 37.78 ± 2.14 and range values from 35.33 to 39.33 days.

The average dry shoot weight (mg) observed for Tesanai 2 was 330.33 ± 35.36 which is almost double than CB (178.33 ± 26.84). A wider range was observed (156.33 to 446.94) for this trait in the RI population. There was significant difference between mean dry root weight of Tesanai 2 (94.33 ± 12.90) and CB (39 ± 5.57). The RI population has mean value of 75.53 ± 14.98.

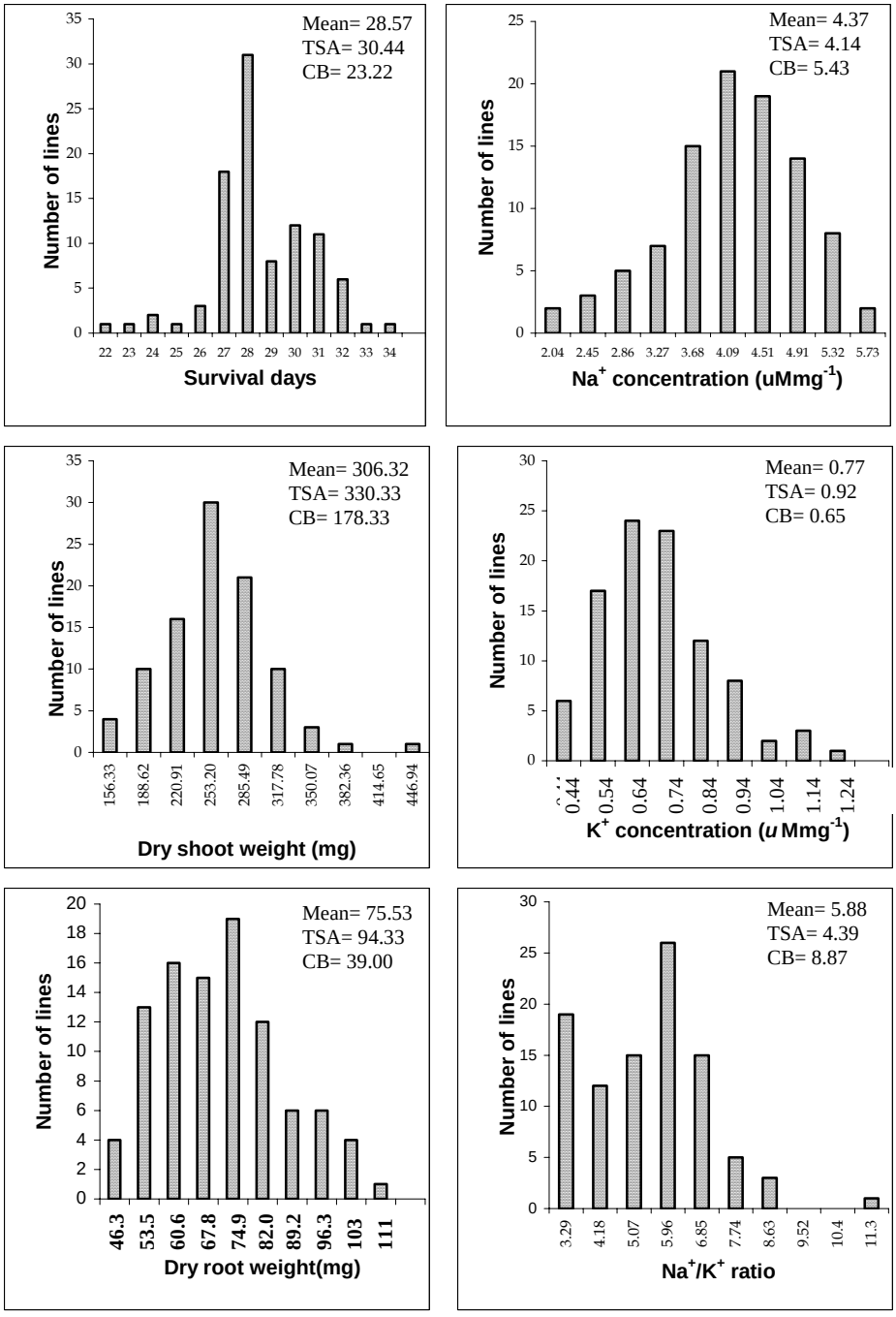

Fig. 2. Frequency distribution of phenotypes for each trait for the 96 recombinant inbred lines (F8) derived from Tesanai2 and CB. The values indicated on the x-axis are the lower limit of each group.

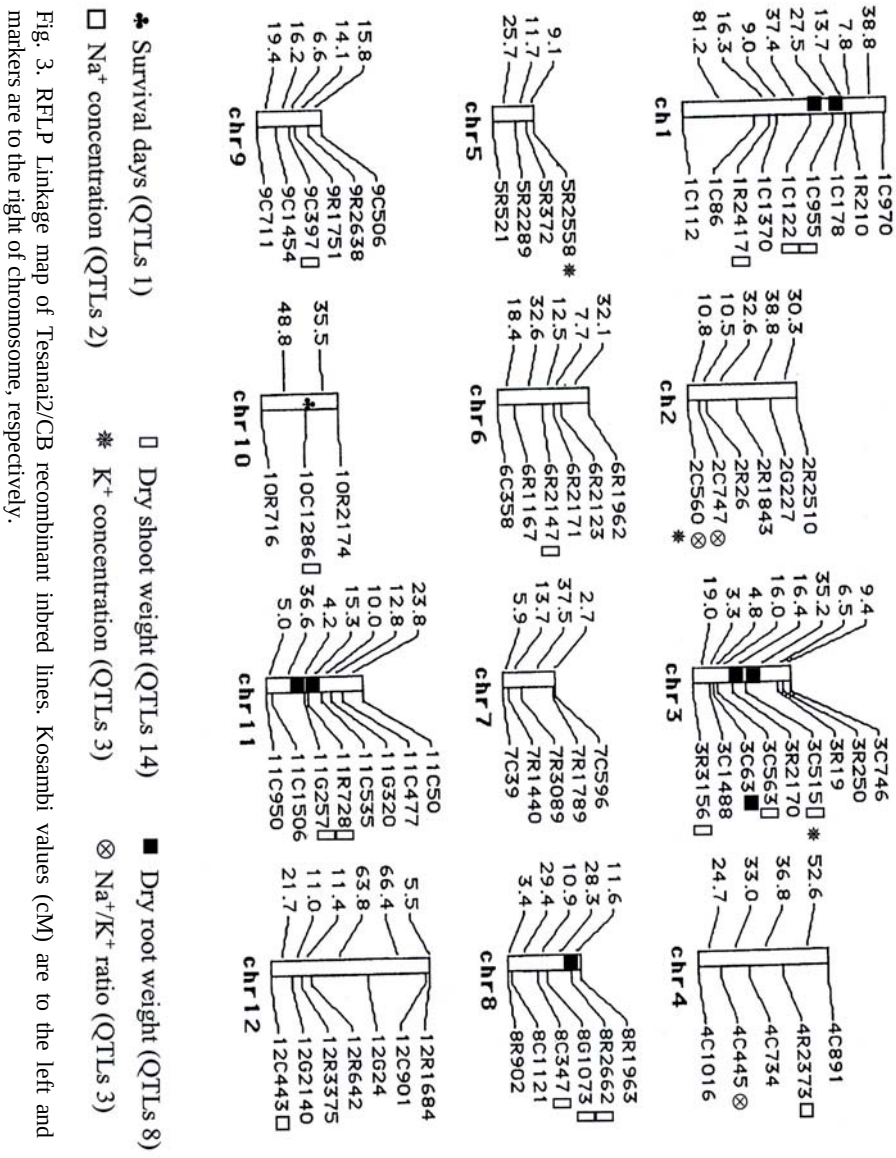

Fig. 3. RFLP Linkage map of Tesanai2/CB recombinant inbred lines. Kosambi values (cM) are to the left and markers are to the right of chromosome, respectively.

Salt uptake was expressed in terms of shoot Na^+ concentration. The differences between the two parents were non-significant for this trait. The range of values for Na^+ uptake in the RI lines was from 2.04 to 5.80 uMmg. When K uptake in shoots was considered it was found that Tesanai 2 is comparatively more efficient (0.92 ± 0.24) than CB ($0.65 + 0.12$). The mean Na^+/K^+ ratio among RI population was 5.88 ± 1.53 (range: $3.29 + 11.35$). Pokalli which is considered tolerant to salinity has low Na^+/K^+ ratio as compared to susceptible check IR 28 ($11.14 + 1.24$).

Table 1. RFLP marker loci associated with survival days, dry shoot weight, dry root weight, Na^+ concentration, K^+ concentration and Na^+/K^+ ratio as indicated by regression ANOVA.

Traits	Marker[a]	Chrom.	F-value	P-value	R^2 (%)[b]	DPC[c]
Survival days	C1286	10	4.53	0.0360	5.60	T
Dry shoot weight	C122	1	14.3	0.0003	14.20	C
	R2417	1	4.49	0.0214	6.00	C
	C955	1	4.93	0.0290	5.40	C
	R2662	8	6.76	0.0112	8.20	T
	G1073	8	5.39	0.0230	6.70	T
	C347	8	4.23	0.0436	5.90	T
	R3156	3	6.20	0.0146	6.70	C
	C515	3	4.53	0.0362	5.00	C
	C63	3	4.46	0.0377	5.10	C
	R728	11	6.56	0.0124	8.00	T
	G257	11	5.94	0.0175	8.30	T
	C1286	10	6.36	0.0138	7.70	T
	R2147	6	4.54	0.0366	6.00	T
	C397	9	4.09	0.0464	4.70	T
Dry root weight	G257	11	10.9	0.0016	14.3	T
	R728	11	8.88	0.0039	10.6	T
	C563	3	5.42	0.233	6.00	C
	C515	3	5.39	0.0226	5.90	C
	C63	3	4.97	0.0285	5.60	C
	C955	1	9.92	0.0022	10.3	C
	C122	1	4.05	0.0473	4.50	C
	R2662	8	5.36	0.0233	6.60	T
Na^+ concentration	C443	12	11.9	0.0010	15.0	T
	R2373	4	4.75	0.032	5.20	C
K^+ concentration	C560	2	5.28	0.0240	5.80	C
	C515	3	3.99	0.0490	4.40	C
	R2558	5	3.97	0.0490	4.50	T
Na^+/K^+ ratio	C560	2	7.47	0.0070	8.00	T
	C445	4	5.42	0.0220	5.90	T
	C747	2	4.09	0.4670	5.10	T

[a] Nearest marker locus of QTLs

[b] Co-efficient of determination, the percent of phenotypic variation explained by the individual marker

[c] Direction of phenotypic effect, T and C indicate Tesanai 2 and CB allele increased that values, respectively.

Table 2. Correlation co-efficient (r) among traits in 96 recombinant inbred (RI) lines derived from a cross between Tesanai 2 and CB.

Traits	1	2	3	4	5
Survival days (1)					
Dry shoot weight (2)	0.398^{***}				
Dry root weight (3)	0.257^{*}	0.612^{***}			
Shoot Na^+ (4)	-0.323^{**}	- 0.147	- 0.081		
Shoot K^+ (5)	0.429^{***}	0.051	0.165	0.064	
Na^+/K^+ ratio (6)	-0.515^{***}	- 0.080	- 0.101	0.0651^{***}	-0.634^{***}

*, **, *** Significant at P = 0.05, P = 0.01 and P = 0.001, respectively.

Correlation among traits: The correlation between traits was computed by regressing phenotypic values of one trait on those of another traits. Seedling survival days were significantly and positively correlated with dry shoot weight, root weight and K^+ uptake (r = 0.398, 0.257, 0.429, respectively) in this population (Table 2). However, it was negatively correlated with Na^+ uptake (r = - 0.323) and Na^+/K^+ ratio (r = -0.515). A strong positive and highly significant correlation was observed between dry shoot weight and root weight (r = 0.612). Dry shoot and root weight showed low correlation with Na^+, K^+ concentration and Na^+/K^+ ratio.

Construction of RFLP linkage map: A linkage map of 74 marker loci was constructed (Fig. 3) based on TSA/CB population using MAPMAKER computer program (Lander *et al*., 1987). The map covered 1349.5 cM of the rice genome with an average distance of 18.24 cM between marker loci. The average intervals are smaller than 20 cM in our map so they are suitable for QTL mapping (Lander & Botstein, 1989).

Identification of markers linked to QTLs

Seedling survival days: Simple linear regression identified one marker locus significantly associated with seedling survival days at 0.05 threshold level. This QTL was located in the vicinity of C1286 on chromosome 10 (Table 1) and explained 5.6% of the total phenotypic variance.

Dry shoot weight: Fourteen putative QTLs were identified for dry shoot weight based on regression ANOVA. One marker locus was found significantly associated with dry shoot weight at 0.01 threshold and other 13 markers were linked at 0.05 threshold level. The observed phenotypic variation explained by these markers ranged from 4.7 to 14.2%.

These QTLs were located in the vicinity of C122, R2417 and C955 on chromosome 1; R2662, G1073 and C347 on chromosome 8; R3156, C515 and C63 on chromosome 3; R728 and G257 on chromosome 11; C1286, R2147 and C397 on chromosome 10, 6 and 9, respectively. All these genomic regions cumulatively explained 97.9% of the observed phenotypic variance in dry shoot weight.

Dry root weight: Eight QTLs were identified for dry root weight based on regression ANOVA. These QTLs were located in the vicinity of G257 and R728 on chromosome 11; C563, C515 and C 63 on chromosome 3; C955 and C122 on chromosome 1, and R2662 on chromosome 8. The percent of phenotypic variation explained by individual QTL ranged from 4.5 to 14.3%. These QTLs collectively explained 63.8% of the total variation. Increased in the traits were conditioned by Tesanai 2 at some QTLs and CB alleles at others.

Na^+ concentration in shoot: Two QTLs were identified which significantly influenced Na^+ uptake. The locus C 443 (chromosome 12) accounted for 15% of the total variation. The other locus (R2373) explained 5.2% variation.

K^+ concentration in shoot: Simple linear regression analysis identified three markers significantly associated with K^+ uptake at 0.05 threshold level. Individually these markers accounted for 4.4 to 5.8% of the total phenotypic variation for this trait.

Na^+/K^+ ratio: Three putative QTLs associated with Na^+/K^+ ratio was detected based on regression ANOVA. These QTLs were located in the vicinity of C560 and C747 on chromosome 2, and C445 on chromosome 4. The percent of phenotypic variation explained by each QTL ranged from 5.1 to 8.0%. These QTLs cumulatively accounted for 19% of the total variation. All these QTLs were derived from Tesanai 2.

Discussion

Salt tolerance has been studied in rice for many years, but to date the mechanism remains largely unknown. The reason is likely to be the complex nature of the effect of salt on plants. This makes it difficult for a breeder to select superior genotypes based on phenotypic evaluation alone. The development of DNA markers and suitable statistical methods allow breeders to select individuals carrying target genes in a segregating population based on linked markers rather than on their phenotypes. Therefore, the populations can be screened at any growth stage and in various environments.

A total of 74 RFLP markers randomly distributed on 12 rice chromosomes were utilized to identify QTLs for salinity tolerance and related traits. Using 0.05 probability level, one quantitative trait loci (QTLs) was found associated with seedling survival days, fourteen QTLs with dry shoot weight, eight with dry root weight, two with shoot Na^+ and three each with shoot K^+ and Na^+/K^+ ratio. The proportion of phenotypic variation explained by each QTL ranged from 4.4 to 15%. Tesanai 2 allele increased overall salt tolerance. When two or more QTLs were found to affect a trait, increased in the trait were conditioned by Tesanai 2 allele at some loci and CB allele at others. These result suggest that though the CB parent is phenotypically poor for most of the traits measured, however, it contains genes that are capable of contributing positively.

Correlation analysis between traits measured revealed that seedling survival days was positively correlated with shoot dry weight ($r = 0.398$), root dry weight ($r = 0.257$) and K^+ uptake ($r = 0.429$). However, an inverse relation was observed with Na^+ ($r = -0.323$) and Na^+/K^+ ratio (-0.515) suggesting that low Na^+ and Na^+/K^+ ratio is a good parameter that can be used to quantify the degree of salinity tolerance (Gregorio & Senadhira, 1996). More shoot weight is an indication of vigorous plant growth which acts to dilute the salt within the tissue and appeared to be a reliable measure for salt tolerance. (Yeo *et al*., 1990). In indicas, Ponnamperuma (1984) found that the concentration of K^+ ion in shoots which plays an important role in activating enzymes and in stomatal function , correlated well with salinity tolerance. Higher correlation of dry shoot weight with root weight ($r = 0.612$) suggest that these traits are interrelated. Low correlation coefficient of shoot dry weight and root dry weight with Na^+, K^+, and Na^+/K^+ ratio indicates weak relationship with these traits.

The statistical threshold is most important in QTL analysis because the number of QTLs would be different when using different threshold. We used 0.05 threshold level to detect maximum QTLs controlling traits related to salinity. The low threshold used here was chosen to maximize the identification of chromosomal regions that may be associated with the control of salinity tolerance and in the light of a relatively small population size. Most of the threshold employed in published QTL analysis have been between LOD 2.0 and 3.0 in MAPMAKER/QTL and between 0.05 to 0.01 in ANOVA (Yano & Sasaki, 1997)

On the basis of regression ANOVA, one QTL was found for seedling survival days on chromosome 10. A relatively small number of the QTL detected seems not to fit to such a complex trait, as it is believed that salt tolerance is controlled by many genes having similar, small and cumulating effect on phenotype. This may be due to the small

population size: the larger the population size, the more likely the effect of lesser QTL will reach statistical threshold (Tanksley, 1993). The other possible explanation is the large environmental effect on this trait, the less likely a QTL be detected.

We successfully detected 14 chromosomal regions controlling dry shoot weight, which collectively explained 97.9% of the total phenotypic variation. Amongst these, 11 QTLs were located on chromosome 1, 3, 8 and 11. It is worth noting that QTLs on chromosome 1 and 8 was in the form of block, which indicates that parental genotypes had alleles with different gene effect at these loci.

Several physiological characters which contribute to salt tolerance in rice have been identified, these include 1) reduced salt transport to shoots, 2) tolerance to salt with in the tissue (tissue tolerance). In this study we assessed the feasibility of mapping QTL for Na^+ and K^+ transport to shoots. Two QTLs for Na^+ and three for K^+ uptake were detected in this population. The alleles from Tesanai 2 increased the values at some loci while CB at others. Tesanai 2, being a moderately salt tolerant variety accumulates reasonable amount of Na^+ ions in shoots. This indicates that tissue tolerance mechanism might be operating in this variety rather than ion exclusion. The analysis of variance revealed non-significant differences among RI individuals. This was not unexpected in a population derived from parents that do not differ significantly in terms of Na^+ and K^+ uptake. To detect QTLs for Na and K, it is suggested that mapping population should be made between parents that significantly differ in sodium uptake and tolerance of Na^+ with in the leaf tissue.

Transgression is defined genetically as the appearance of individuals in segregating populations that fall beyond the parental phenotypes. In this population lines having phenotypic values greater than the higher parents and lesser than the lower parent were observed for almost all the traits measured. For those traits for which two or more significant QTLs were detected, both parents were found to possess QTL alleles which increased phenotypic values. The occurrence of such transgression is accumulation in certain progeny of complementary alleles at multiple loci inherited from the two parents. Veldoom *et al*., (1994) and Xiao *et al*., (1996) demonstrated that correlated traits often have QTLs mapping to the same chromosomal location. The similar trend was observed in this study. For example, dry shoot weight and root weight was highly correlated ($r = 0.612$) and had QTL with large effect which were found approximately at the same map location. Similarly, seedling survival days has positive correlation with dry shoot weight ($r= 0.398$) and had QTLs common at chromosome 10. Trait correlation may result from either pleiotropic effects of single genes or from tight linkage of several genes controlling the traits. This suggest that QTLs affecting dry shoot weight is closely linked with the QTLs affecting dry root weight in that region.

The QTLs, which have been identified, certainly do not comprise the entire set of genes which effect the traits under study, but only a subset of genes, mainly because of the limited number of polymorphic markers scored through the genome. Once QTLs are identified, a saturated map of the genomic area of interest would provide further knowledge of the precise map location, genetic effects of each QTLs and will expedite manipulation of the QTLs themselves.

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