

Studies on identification of putative quantitative trait loci for alkaline tolerance in rice (*Oryza sativa* L.)

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(Received: June 12, 2009; Accepted: July 15, 2009)

ABSTRACT

In the present investigation an attempts has been made to identify the putative genomic regions/ Quantitative trait loci governing alkaline tolerance in CSR 27. A Substantial variation was observed for salt injury scores and Na uptake. Salt injury scores of the F₃ population ranged from 4.00 to 9.00 with a mean value of 5.99. Na uptake values ranged from 1.25 to 2.50 mmol/g dry weights with a mean of 1.29 mmol/g dry weight. The frequency distribution of mean phenotypic values of the two traits clearly indicated that it is not major genes but poly genes that are controlling the trait expression showing continuous segregation of genes. A total of 208 SSR primers were used for polymorphism survey of parental cultivars. Of the 208 microsatellites tested, 120 SSRs amplified well scorable fragments between parents. However on repetition of polymorphic markers between parents and selective genotyping of polymorphic SSRs in tolerant and sensitive F₂ genotypes resulted in further narrowing down of SSRs to 45 which yielded clear cut SSR loci. Percentage polymorphism was found to be 21.6 between parents. A set of 190 DNA samples of F₂ genotypes were screened with 45 polymorphic SSRs. The QTLS and their effects for salt injury scores and Na uptake were obtained using QTL cartographer version 2.5 employing composite interval mapping (CIM) methods.

Key words: *Oryza sativa* L., putative quantitative trait loci, alkalinity tolerance.

INTRODUCTION

Rice is a major staple food crop of world's population. In the past few decades although rice production has been doubled due to the introduction of high yielding rice varieties and is still insufficient to cater to the needs of ever increasing global human population which is expected to increase at the rate of 1% per annum. (Khush, 1997). On the other hand biotic and abiotic stresses affect rice production (Khush, 1997). Salt stress is immense and growing day by day. Actually 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated conditions of sodicity (434 million ha)

(FAO, 2005). In India alkaline and saline soils occupies more than 9 million hectares and is rapidly increasing.

Among abiotic stresses, salt stress is the second most stress limiting rice yields. Salt stresses include coastal saline, inland saline and alkaline stresses. Salt stress is complex and greatly affects plant growth and development and is one of the major constraints for rice crop production. In Asia, saline soils are a major problem in China, India, Thailand, Indonesia and Australia. To limit the effect of salt in plant productivity, amelioration and utilization of salt-affected soils are needed. Two approaches are used to solve the problem: by using

technical approaches (water and soil management) and biological approaches. Financial difficulties as well as the environmental injuries caused by desalinization programs resulted in the much dependency on biological approach, *i.e.*, use of tolerant cultivars with inherent ability to grow in salt stress areas.

Salinity is generally defined as the presence of excessive amounts of soluble salt that hinders or affects the normal functions needed for plant growth. It is measured in terms of electric conductivity (EC_e), or of the exchangeable Na⁺ percentage (ESP) or with the Na⁺ absorption ratio (SAR) and pH of saturated soil paste extract. Therefore saline soils are those with EC_e more than 4 dSm⁻¹ equivalent to 40 mM NaCl, ESP less than 15 % and a pH below 8.5 (Waisel, 1972; Abrol, 1986; Szabolcsi, 1994). The present paper reports the identification of putative quantitative trait loci for alkalinity tolerance in Rice (*Oryza sativa* L.).

MATERIAL AND METHODS

In the present study, two high yielding *indica* cultivars have been used as experimental material *viz.*, BPT 5204 (Alkaline sensitive) and CSR 27 (Alkaline tolerant). BPT 5204 was obtained from Acharya N.G. Ranga Agricultural University (ANGRAU), Hyderabad whereas CSR 27 from Central Soil Salinity Research Institute (CSSRI), Karnal, Haryana.

A cross was made between plants of BPT 5204, a semi dwarf, high yielding widely grown mega variety with long duration (150 days) and medium slender grain but sensitive to alkaline stress and CSR 27, a fine grain, high yielding long duration (135-140 days) salt tolerant variety released by CVRC for alkaline (pH 9.6 to 9.9) and coastal saline soils (EC_e upto 10 dSm⁻¹); derived from Nona Bokra/IR 5657-33-2. It has a morphological marker of purple stigma. This variety possess a higher tissue tolerance capacity, K⁺ mining ability and Na⁺ exclusion mechanisms of tolerance.

Sodic Microplot Screening

The performance of two rice varieties, BPT 5204 (BPT 5204) and CSR 27 was studied in sodic microplots (pH 9.7) at CSSRI, Karnal, Haryana.

Tolerant variety CSR 27 showed much better survival, plant growth as compared to sensitive parent (BPT 5204). Sensitive variety was characterized by higher reduction in growth, and survival.

The parents, CSR 27, BPT 5204; and F₃ families (9 plants/family) were grown in sodic micro plots under high pH (9.7) at CSSRI, Karnal, Haryana. The individual seedlings in each F₃ progenies were scored on 1-9 scale on the basis of visual symptoms of leaf injury following Standard Evaluation System for Rice (SES, 1996). The average scores were calculated. Similarly a quantitative measure of Na uptake were determined by atomic absorption spectrophotometer, seedling wise in each of the F₃ family, averaged and used for testing normal distribution.

Protocol for DNA Isolation

This procedure involves the steps like leaf sample collection, preparation of CTAB buffer, grinding of leaf sample and extraction of genomic DNA. The seeds were placed over a moistened blotting paper in a Petri plates and kept in an incubator maintained at 25-35°C temperature. Water in the Petri plates was changed regularly. The seedlings were harvested after 7-10 days and used for isolation of DNA. The protocol is as follows:

1. About 2cm leaf sample was taken and cut it into small pieces and placed in the well of a spot plate.
2. The leaf pieces were crushed after adding 400µl of extraction buffer using a sterilized blunt ended glass rod. After ensuring complete grinding of leaf pieces, 400µl more of extraction buffer was added.
3. The entire content was transferred in to a 1.5 ml eppendorf tube using the micropipette tip of 1 ml capacity.
4. Equal volume of (800µl) chloroform was added; the contents were mixed well by inversion for about 5 min and centrifuged at 13000 rpm for 15min at room temperature.
5. The supernatant was transferred in to a fresh centrifuge tube without disturbing the intermediate layer of insoluble proteins.
6. Equal volume of chilled isoproponol was added to the supernatant and kept at 4°C for 10 min.

7. The contents were mixed gently and centrifuged at 13000 rpm for 10 min at room temperature.
8. The supernatant was discarded and the DNA pellet was washed with 100 µl of 70% ethanol. The contents were centrifuged at 13000 rpm for 5 minutes at room temperature.
9. The supernatant was discarded and the DNA pellet was air-dried at room temperature for 2-3 hours.
10. Then, the DNA pellet was dissolved in 50-100 µl of TE buffer.

Quantification of DNA

The quality and quantity of extracted genomic DNA was checked by agarose gel electrophoresis (0.8% agarose) with standard sample or by using Nanodrop Spectrophotometer. Clear and intact bands in the agarose gel indicate the high quality of the isolated DNA. A minimum concentration of 10-20 ng/µl of genomic DNA is required for PCR amplification. The genomic DNA was diluted with TE buffer to get approximately 20 ng/ml concentration, which can be directly used for PCR.

Polymerase Chain Reaction (PCR)

In the present study the PCR amplification of isolated DNA was carried out by preparing 8.0 µl of the master mix (sterile distilled water 5.3 ml, 10X buffer 1.0 µl, dNTP's (2.5 mM) 0.5 ml, forward primer (1.25 picomoles) 0.5 µl, reverse primer (1.25 picomoles) 0.5 µl and Taq DNA polymerase (3U/µl) 0.2 µl) in a sterile 1.5 µl eppendorf tube and dispensed into each well of the PCR plate. Then, 2.0 µl of the each DNA sample was added to the respective wells to make a final reaction volume of 10 µl. The PCR plate was then set up in a programmable thermal cycler (Eppendorf PCR machine) for DNA amplification. Temperature

specifications for the proper amplification of isolated DNA are Initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 7 min and cooling at 4°C.

Agarose Gel Electrophoresis

The amplified DNA was separated by using 3% agarose gel electrophoresis. For this the electrophoresis tank was filled with sufficient amount of 0.5X TBE Buffer. About 12 gm of agarose was melted using 400 ml of 0.5X TBE to make 3% gel. Ethidium Bromide (12 µl) was added to the melted gel and mixed thoroughly. Appropriate combs were placed in the slots given in the casting plate so that wells were formed. The solidified gel was shifted to the gel tank containing 0.5X TBE buffer so that it should be completely immersed in the buffer, and carefully removes the combs. Then, the PCR product was loaded in to the well after adding 1 µl tracking dye (Bromophenol Blue). The lid of the gel tank was closed and the electrodes were connected to the power pack. The DNA samples were electrophoresed at 160 V for 1 hour. After the tracking dye reached two third (2/3) distance, the gel was exposed at 300 nm wavelength of UV light and documented using SYNGENE Gel Documentation Unit (Genesnap).

RESULTS AND DISCUSSION

Parental Screening

The parents as well as F₃ population were grown in sodic microplots under controlled conditions (pH 9.7) and subjected to alkalinity stress at CSSRI, Karnal, Haryana. VSR 156 served as alkaline sensitive and CSR 36 as alkaline tolerant checks. The two parental varieties, BPT 5204 and CSR 27 used differ significantly for their response to alkalinity stress and Na uptake. BPT 5204 had a

Table 1: Mean phenotypic performance of parents and F₃ population of the Cross CSR 27 X BPT 5204 under sodic microplots (pH 9.7)

Traits	Parents		F ₃ Population		
	CSR 27	BPT 5204	Mean	Standard deviation	Range
Salt injury scores	4.0	9.0	5.99	0.99	2.0-9.0
Na uptake	2.50	1.25	1.29	0.02	0.18-2.57

score of 9.0 whereas CSR 27 had a score of 4.0 in the scale of 1-9 for alkalinity tolerance. BPT 5204 showed Na uptake of 1.25 mmol/g dry weight and CSR 27 had 2.50 mmol/g dry weight. In this way parents showed differences with respect to phenotypic expression of traits under study.

Phenotyping in F₃ population

Substantial variation was observed for salt injury scores and Na uptake. Salt injury scores of the F₃ population ranged from 4.00 to 9.00 with a mean value of 5.99. Na uptake values ranged from

1.25 to 2.50 mmol/g dry weights with a mean of 1.29 mmol/g dry weight (Table 1).

The frequency distribution of mean phenotypic values of the two traits clearly indicated that it is not major genes but poly genes that are controlling the trait expression showing continuous segregation of genes. (Fig. 1-2).

Also transgressive segregants were observed for salt injury scores and sodium uptake traits for which most of the lines had phenotypic

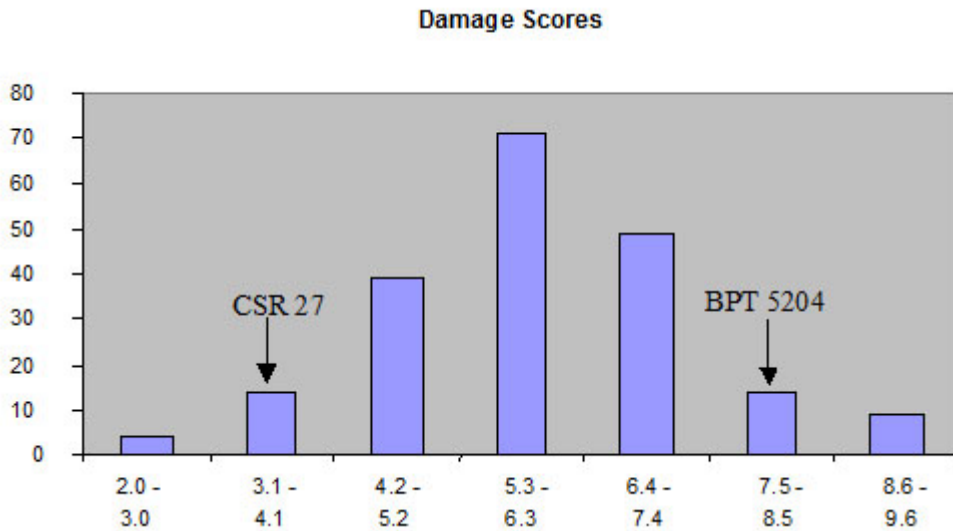


Fig. 1: Frequency distribution of F₃ families for salt injury scores

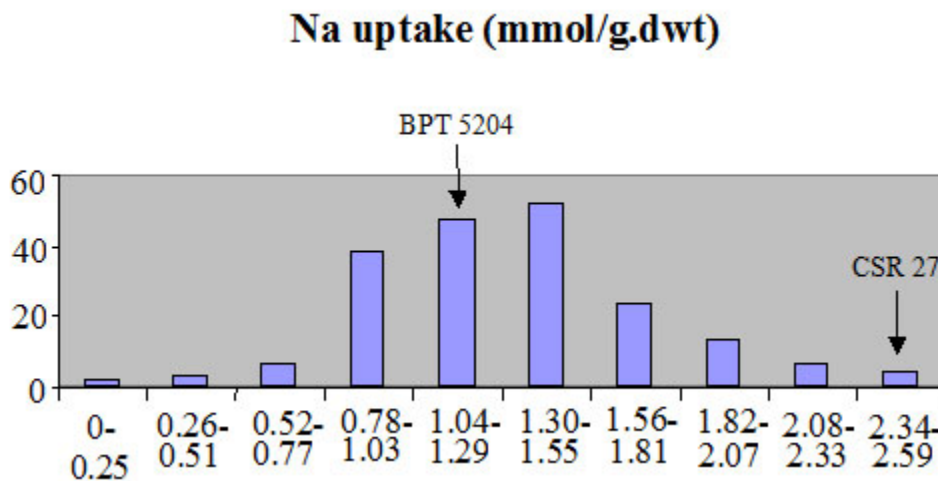


Fig. 2: Frequency distribution of F₃ families for Na uptake

values exceeding parental limits in some of the F_3 progenies. Such a distribution indicated the involvement of minor gene with phenotypic values greater than that of parents. Transgressive segregants tended towards salt tolerant CSR 27 parent whereas for sodium uptake it was towards BPT 5204, salt sensitive parent. These transgressive segregants might have resulted due to accumulation of different favorable genes conferring salt tolerance or development of new combinations of stress related genes from the parents.

Screening for polymorphism

A total of 208 SSR primers were used for polymorphism survey of parental cultivars. Of the 208 microsatellites tested, 120 SSRs amplified well scorable fragments between parents. However on repetition of polymorphic markers between parents and selective genotyping of polymorphic SSRs in

tolerant and sensitive F_2 genotypes, resulted in further narrowing down of SSRs to 45 which yielded clearcut SSR loci. Percentage polymorphism was found to be 21.6 between parents (Table 2) A few of the primer pairs amplified monomorphic fragments which could not distinguish the parents.

Genotyping of F_2 population using SSRs

A set of 190 DNA samples of F_2 genotypes were screened with 45 polymorphic SSRs. A representative gel image of a DNA banding profile obtained using RM 21770 in a F_2 population was presented in Fig 3.

Segregation of SSR loci

SSR markers are considered to behave as codominant markers and are expected to segregate in a ratio of 1:2:1 (1 homozygote with fragment of CSR 27: 2 heterozygotes with

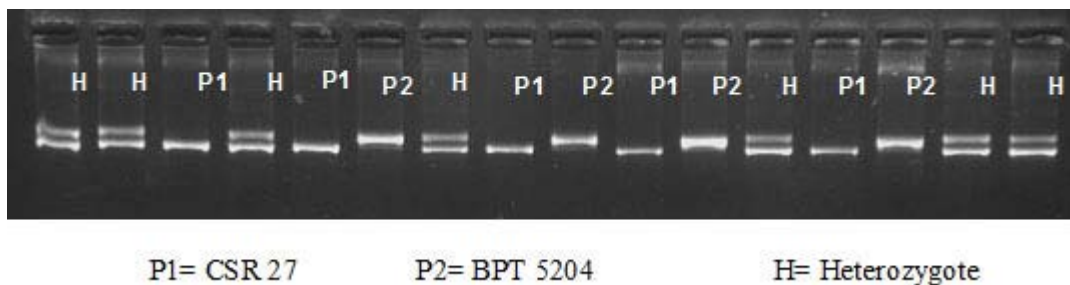


Fig. 3: Segregation of RM 21770 marker locus in $F_{2,3}$ mapping Population of CSR 27 × BPT 5204

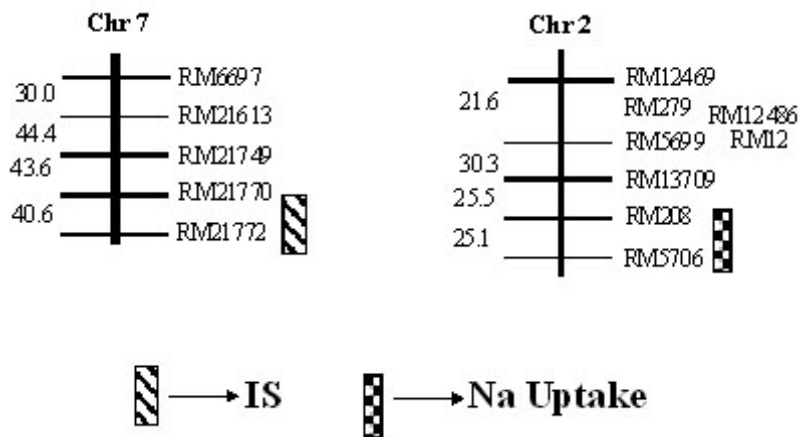


Fig. 4: Linkage map showing chromosomal location of putative QTLs detected for alkalinity tolerance in $F_{2,3}$ mapping population of CSR 27 × BPT 5204. Marker names are listed on right hand side and the distances (cM) on the left of the Chromosome

fragments of both CSR 27 and BPT 5204: 1 homozygote with fragment of BPT 5204). The distribution of majority of these polymorphic loci were more or less in the expected frequency.

Identification of putative QTLs

The QTLs and their effects for salt injury scores and Na uptake were obtained using QTL cartographer version 2.5 employing composite interval mapping (CIM) methods. A logarithm of odds (LOD) score of 2.0 was used as the threshold for the identification of putative QTLs in a given genomic region. The total phenotypic variation and the additive effect of each QTL explained by salt tolerance were also calculated.

A total of two significant QTLs each associated with salt injury scores and Na uptake were detected. A QTL was detected with a LOD threshold of 6.06 between the markers RM 21770-RM 21772 on chromosome 7 for salt injury scores showing a negative additive effect (-0.50) of CSR 27 allele. This QTL explained 22% of phenotypic variance (R^2) of the trait. Another QTL with a LOD threshold of 4.8, R^2 (%) of 14 was detected within a marker interval of RM 208-RM 5076 on chromosome 2 with -0.35 additive effect (Fig. 4).

Earlier studies reported salt tolerance trait to be controlled by poly genes with high environmental influence. The complex nature of the trait contributed by several physiological mechanisms made the breeding programs cumbersome. With the advent of molecular marker technology, the DNA markers became handy to plant breeders in tackling the trait with much ease. Particularly microsatellite markers or simple sequence repeats (SSRs) which are being widely-used as molecular markers for finger printing germplasm to assess genetic diversity, pedigree

analysis, evolutionary studies and genome mapping (Yang *et al.*, 1994; McCouch *et al.*, 1997, Garland *et al.*, 1999). Rice microsatellites have been demonstrated to be polymorphic between (Akagi *et al.*, 1997; Chen *et al.*, 1997) and within rice populations. Microsatellite markers have been used effectively to map QTLs associated with salt tolerance (Lang *et al.*, 2000 and 2001; Singh *et al.*, 2007).

A few attempts have already been made to identify QTLs associated with salinity tolerance in rice. A major gene for salt tolerance was mapped on chromosome 7, using F_2 population derived from a salt-tolerant japonica rice mutant, M-20 and the sensitive original variety 77-170 (Zhang *et al.* 1995). Seven QTLs for seedling traits associated with salt tolerance were identified and were mapped to five different chromosomes (Prasad *et al.*, 2000). The QTLs associated with different mechanisms of salinity tolerance in rice independently govern the uptake of Na and K and Na:K selectivity and are mapped on different chromosomes (Koyama *et al.* 2001).

CONCLUSION

In the present investigation an attempt has been made to identify putative genomic regions/ Quantitative trait loci governing alkaline tolerance in CSR 27. Although considerable progress has been made in saline tolerance through both conventional breeding and molecular marker approaches, studies on alkaline tolerance seems to be limited in literature in rice. Hence we made an effort to study genetic polymorphism, genetic base and identification of putative genomic regions or quantitative trait loci controlling alkaline tolerance using molecular markers, specifically simple sequence repeats.

The quantitative tolerance to alkalinity has been studied in $F_{2,3}$ mapping population derived from samba mahsuri (alkaline sensitive) and CSR 27 (alkaline tolerant). The F_3 progenies have been evaluated under controlled conditions in sodic microplots. Observations have been recorded on physiological parameters *viz.*, Na uptake and salt injury scores. Frequency distribution of salt injury scores and Na uptake displayed a normal

Table 2: Pattern of polymorphism between parents

CSR 27 Vs BPT 5204	
Polymorphic SSRs	4520
Total SSRs	208
% polymorphism	21.6

distribution indicating minor genes each explaining a relatively small amount of phenotypic variance associated with them. The recovery of transgressive segregants further confirmed the polygenic control of the trait. Therefore quantitative inheritance involving poly genes has been established.

The parental survey of polymorphism has been made between parents using DNA based codominant Simple sequence repeats. Parents were found to be genetically polymorphic with respect to physiological traits under study. Simple sequence

repeats were employed to detect genomic locations /quantitative trait loci (QTL). QTL analysis of 45 SSRs spanning 12 chromosomes using composite interval mapping method has detected two significant putative QTLs for salt injury scores and Na uptake traits, one each on chromosome 7 and 2 respectively. The positive alleles came from CSR 27, the donor parent. However there lies scope to use a few more SSRS to identify meaningful QTLs with more phenotypic variance in order to use them in marker assisted selection of alkaline tolerance trait.

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