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MOLECULAR MAPPING AND MARKER-ASSISTED SELECTION FOR SALT TOLERANCE IN RICE (*Oryza sativa* L.)

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ABSTRACT

Salinity stress is the major constraint in rice production. In view of this, the mapping and marker-assisted selection for salt tolerance genes in rice have been conducted. SSR technique combined with selective genotyping was used to salt tolerance in rice. Salt tolerance cultivar AS996 was crossed to IR50404 and 229 recombinant inbred lines (RILs) were produced by single seed descent. The BC_2F_2 from the cross of IR64 / OMCS2000 was mapped with 34 markers to construct a framework map and a total length of 148.6 cM was recovered two target chromosomes (1, and 8). IR 64 / OMCS 2000 //I R 64 and 217 BC_2F_2 were analysed. Regression analyses based on SSR allele class differences were performed. Highly significant associations were detected at the SSR locus RM223 on chromosome 8. To examine the power of the identified SSR markers in predicting phenotype of the salt locus, we determined the genotypes of 93 improved varieties at RM223 locus. The results indicated an accuracy of more than 95 % in identifying the resistant plants which was similar to that using RM223. DNA survey on OM4498, OM5900 will be useful for the selection of parents in breeding programs aimed at transferring these genes from one varieties background to another and use in marker-assisted selection.

Key Words: Marker-assisted selection, RAPD, PCR, SSR.

INTRODUCTION

Rice (*Oryza sativa*) is sensitivity to salinity, which affects one fifth of irrigated land worldwide. Breeding for salt tolerance has been reviewed by several workers. Progress in salinity tolerance breeding is slow due to the following aspects as limited knowledge in the genetics of tolerance, complexity of the several tolerance mechanisms involved, inadequate screening techniques, low selection efficiency and poor understanding of salinity and environmental interactions. Through recent developments in molecular marker analysis, it is now feasible to analyze both the simply inherited as well as quantitative traits, and then identify individual genes controlling the trait of interest. Molecular markers could be used to tag quantitative trait loci and to evaluate their contributions to the phenotype by selecting for favorable alleles at these loci in a marker-assisted selection scheme aiming to accelerate genetic advance. Advanced backcross QTL analysis can be used to evaluate mapped donor introgression in the genetic background of an elite recurrent parent. For rice, QTLs for salt tolerance have been developed through microsatellites and RFLPs with 108 markers based on recombinant inbred lines derived from the cross between Tenasai and CB (Lang *et al.* 1999).

This study aims at:

(1) Investigating the genetic basis of salinity tolerance using microsatellite technique to BC_2F_2 from two groups: IR64 and OMCS2000, IR50404 / AS996

(2) Screening the BC populations for salinity tolerance under greenhouse

(3) Tagging microsatellite markers linked to salinity tolerance.

(4) Determining whether enhancing QTLs from 227 lines of IR64/ OMCS 2000, 229 lines of AS996 / IR50404 would be

detected under salt tolerance

MATERIALS AND METHODS

Plant materials

Two genotypes differing for salt stress namely AS996 (tolerant) and IR64 (susceptible), and a set of 96 BC_2F_2 derived from these parents were used in the present study. The BC_2F_2 population was developed at Cuu Long Delta Rice Research Institute.

OMCS2000 was crossed to the high protein cultivar. Subsequently F_1 was selfed and backcrossed to their respective parents to produce the F_2 and backcross generations. The improved rice cultivars were crossed to IR64 and F_1 hybrids were backcrossed to the elite cultivars. The promising BC_1 plants selected for desirable phenotypic traits were backcrossed to the elite cultivars to generate BC_2 plants. 219 BC_2F_2 were evaluated in screen for salt tolerance at 12 dS/m. Transgressed segregants were observed for tolerant and susceptible genotypes. The BC_2F_2 family was screened using microsatellite markers.

Evaluation of parents and BC_2F_2 for salt stress

1. Salinization of nutrient solution

Salinize nutrient solution by adding NaCl while stirring up to the desired EC (3 and 6 g NaCl / lit) nutrient solution gives an EC of 6 and 12 dS/m, respectively. Fill up the trays with this solution high enough to touch the nylon net bottom of styrofoam. The effective culture solution needed per tray is about 5-10 liters.

2. Management of nutrient solution

The maintenance of the nutrient solution is very important. Considerable attention has to be given in adjusting the pH.

Monitoring and maintaining the pH of the culture solution is very critical because this checks balance of the available nutrients. Significant deviation of culture solution pH from 5.0 will make some nutrients toxic and others deficient, thus a reliable pH meter and its regular calibration are essential (Lang *et al.* 2001).

Due to adding ammonium and nitrate, pH will decrease during the first few days because ammonium ion is favorably absorbed by the plan nitrate ion.

The pH will then increase when ammonium ion is depleted and more nitrate ion taken up by the plants. The increase of pH in the nutrient solution could be used as an indicator that nitrogen source starts to be deficient. Change the nutrient solution every 7 days.

Sometimes algae could be seen during tested so that pH would be adjusted twice a day.

In the case of evaporation and transpiration there will be loss of solution volume in the trays. Make up the volume with distilled water every 3 days

3. Handling of seedling and salinization

Tested seeds have to be heat treated for 5 days in a conversion oven set at 40^{o}C to break seed dormancy. Proper breaking of the seed dormancy is very essential in this screening technique. Delay in germination of some entries will like make these entries more sensitive to salt stress. Seedling vigor has great advantage at this point since salinization occur at very early seedling stage. After breaking the dormancy, surface sterilize seeds were treated by fungicide and rinsed well with distilled water. Place sterilized seeds in petri dishes with moistened filter papers and incubate at 30^{o}C for 48 h to germinate. Sow two pregerminated seeds per hole on the styrofoam seedling float. The radicle should be inserted through nylon mesh. During this process, the radicle may be damaged and this damage may not be visible.

There are adequate nutrients in the endosperm for the seedlings to grow normally for 3-4 days. After three days, when seedlings are well established, replace and rinse with distilled water with salinized nutrient solution. Initial salinity is kept at EC = 6 dS /m. Three days later, increase salinity to EC = 12 dS / m by adding NaCl to the nutrient solution.

Renew the solution every 8 days and maintain the pH at 5.0 daily.

Test entries can be rated at 16 and 18 days after initial salinization.

6. Evaluation of salt tolerance through SD

(survival day) (Lang *et al*. 2000): When the seedlings were grown for 17 days, they were transferred into the solution containing NaCl to EC level of 12 dS/ m. Seedling survival days was recorded in days from seedling to death. The solution was changed by fresh one every days and pH was monitored at 5.0.

In the screening phase of the experiment, it is essential to include a control (susceptible and resistant), for example, using Pokkali as resistant genotype and IR29 as susceptible one.

For the performance test, agronomic characteristics such as plant height, panicle length, tillers per hill, spikelets per panicle, grain yield were investigated and compared with those of the original plant under saline field trials' condition with three replications per lines. Evaluations of lines were done at maturity. Analysis of variance and mean comparisons of the data from field trials was implemented.

CTAB extraction DNA

Grind 4 to 6 g tissue in liquid nitrogen, and transfer power to 50 ml tube (10-15 ml leaf power). The finer the grind the higher the yield, but take care that the tissue does not thaw.

Add 20 ml 2X CTAB extraction buffer (preheated to 65^{o}C) to the leaf power, mix thoroughly and incubate at 65^{o}C for 30 min (up to 1 hour). Remove tubes from water bath and let cool briefly. Add 20 ml 24:1 $CHCl_3$: isoamyl alcohol. Incubate at room temperature with moderate shaking for 20min. Centrifuge at 3000 rpm for 30 min at 4^{o}C. Transfer supernatant (upper phase) to 50ml tube with filtering through micracloth. Add 1 vol (20ml) isopropyl alcohol, mix by inversion. Incubate at room temperature or colder (-20 ^{o}C) for 1 hour to overnight.

Centrifuge at 3000 rpm for 30 min at 4^{o}C.

Drain supernatant away from pellet, wash pellet with 70% EtOH, air dry pellets and dissolve nucleic acids in 5 ml TE. Place tubes at 65^{o}C to help dissolution.

Add 1/10 vol 3M NaAOc (500 µl) and 2 vol Absolute EtaOH (10-11ml). Incubate at –20^{o}C for one hour (or longer: at this step, it is possible to leave the samples overnight at –20^{o}C)

Centrifuge at 3000rpm for 30 min, wash with 70% EtOH, air dry, dissolve in 500 µl TE, transfer microfuge tube, rinse 50 ml tube with 200 µl TE, collect wash rest of solution.

SSR marker genotyping and the field experiment were conducted.

Evaluation of fragment patterns

Amplification products were obtained using RM223 primer, developed by University of Cornell, USA.

Forward primer: 5'- acagtatccaaggccctgg-3'

Reverse primer: 5'- cacgtgagacaaagacggag-3'

Construction of SSR map and assignment of linked groups to chromosomes

To construct the SSR map and to assign the linked groups to chromosomes, a set of SSR markers, which were present in the test population, were first identified. Linkage groups were ordered using MAPMARKER (Lander *et al*. 1987). Linkage group were reconfirmed using the "GROUP" Map units (cM) were derived using the Kosambi function (Kosambi 1944)

RESULTS & DISCUSSION

Phenotypic variation in salinity tolerance

Figure 1 shows a continuous and normal distribution of phenotypic salinity reaction among the BC_2F_2. This showed a good recombination for salinity reaction in the population. For dry shoot weight (St.wt) and dry root weight (Rt.wt), large differences were noticed in the population. St.wt ranged from 45mg to 110mg and Rt.wt from 200mg to 350mg.

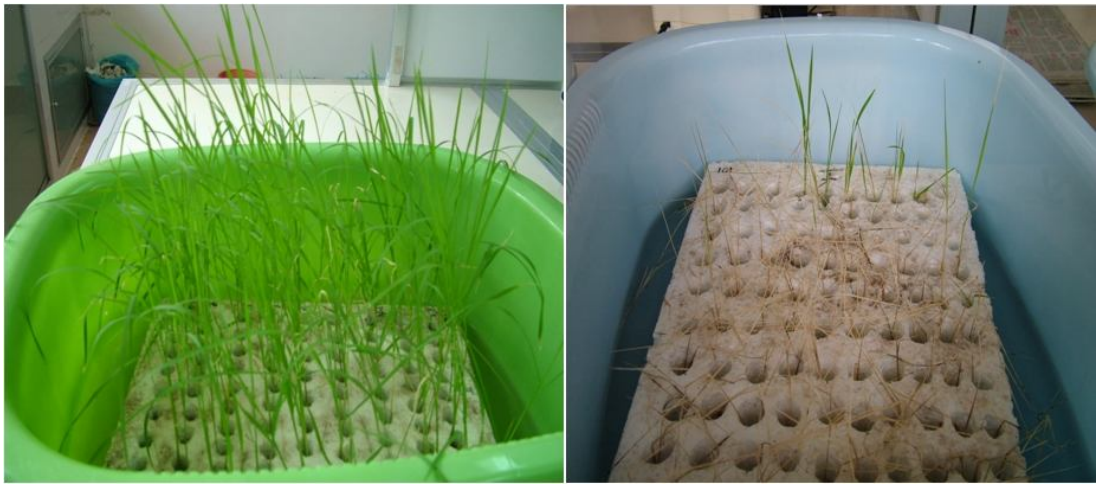

Figure 1: Screening for salt tolerance in BC_2F_2 at seedling stage with check and 12 dS/m

DNA survey

Out of 150 primer pairs tested: some markers amplified specific DNA fragments related to tolerance based on polymorphism between DNA of tolerant and susceptible plants. Of the 150 primers tested, 38 and 47 markers were polymorphic between IR64 / OMCS2000; and IR50404 / AS 996, respectively.

The overall level of polymorphism between the two parents was 23.7%. However, polymorphism between IR64 with some varieties is near genetic distance within subspecies than between subspecies. The morphology of the two parents is quite different, so the morphological differences are not necessary means that they are far in genetic distance.

Polymorphism and marker segregation

IR64 / OMCS2000

The BC_2F_2 population was evaluated by using 34 SSRs. 95% of the SSRs were polymorphic in the parents IR64 and OMCS2000. The expected segregation ratio would be 98% homozygote, 2% heterozygote.

Resulting in an allele frequency of 47.6% and 51.58% in IR64 and OMCS 2000, respectively, due to skewed allele frequencies at 34 out of 150 (22.6%) of marker loci. Skewing toward the adapted elite parent can be explained by the selection improved in the BC_1 and BC_2 generations during population developed but skewing toward OMCS2000 was not expected. This may be result of recombination due to genetic distance between the parental lines. Almost markers have detected when check with population, however, some markers did not introgress into population for example: RM283, RM286, RM339.

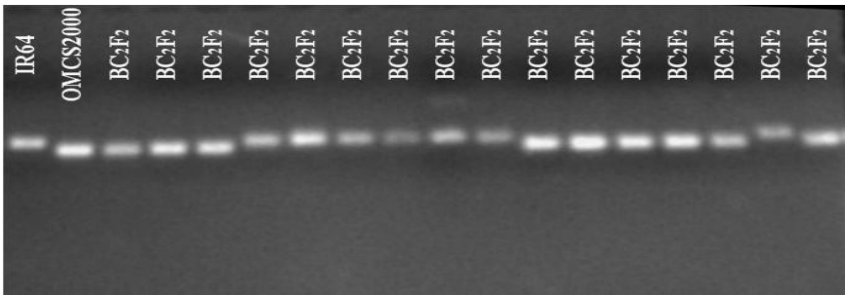

Figure 2: PCR products of BC_2F_2 population from IR64/OMCS2000 at the locus of RM315

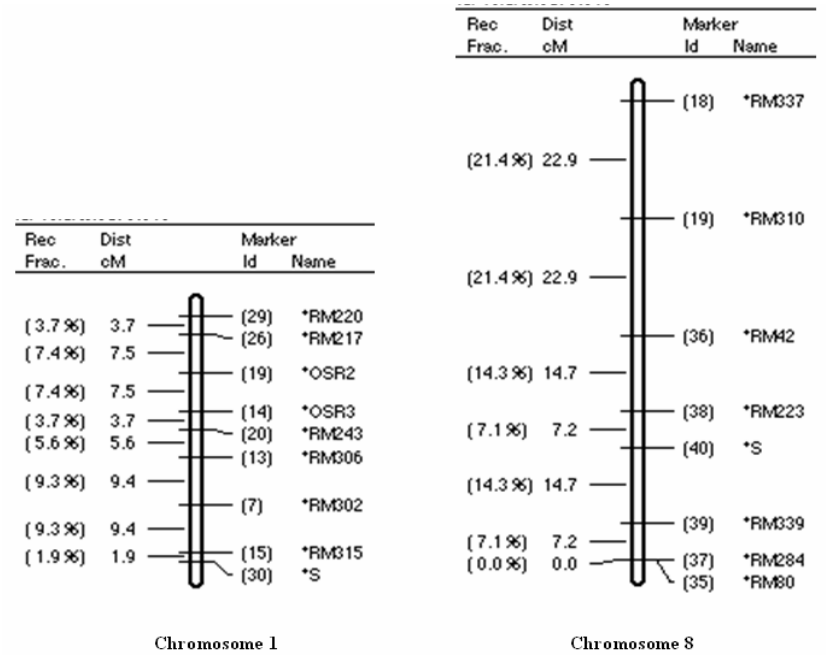

Figure 3: Microsettelite linkage map of rice chromosome 1, and 8. The makers were mapped in the indica / indica genotypes (IR64 / OMCS 2000)

IR50404/ AS996

The BC_2F_2 population was evaluated by using 44 SSRs. 92% of the SSRs were polymorphic in the parents IR50404 and AS996. The expected segregation ratio would be 97.5% homozygote, 2.5% heterozygote.

Resulting in an allele frequency of 55.5% and 37.5% in IR50404 and AS 996, respectively, due to skewed allele frequencies at 44 out of 150 (31.3%) of marker loci. Almost markers have detected target genotypes, however some markers did not introgress into population for example: RM218, RM333.

Construction of genetic linkage maps

Grouping of markers was carried out by a two-point linkage analysis with a LOD score of 4.0 and a recombination fraction D of 0.3

For the BC_2F_2 from the cross IR64 / OMCS 2000, 34 markers were used to construct a framework map with a total length of 148.6 cM, recovering two chromosomes (1 and 8). Figure 3 indicated that microsatellite linkage map of rice chromosome 1 and 8. The makers were mapped in IR64 / OMCS 2000 (both are indica rices).

Marker-assisted selection in salt tolerance

Genetic mapping of major genes and quantitative traits loci (QTLs) for many important agricultural traits is increasing the integration of biotechnology with the conventional breeding process. Exploitation of the information derived from the map position of traits with agronomical importance and of the linked molecular markers, can be achieved through marker-assisted selection (MAS) of the traits during the breeding process. However, empirical applications of this procedure have shown that the success of MAS depends upon several factors, including the genetic base of the trait, the degree of the association between the molecular marker and the target gene, the number of individuals that can be analyzed and the genetic background in which the target gene has to be transferred. MAS for simply inherited traits are gaining in breeding programs, allowing an acceleration of the breeding process. Trait related to salt tolerance in rice products are offering some

important examples of a possible routinely application of MAS. For more complex traits like abiotic stress tolerance, a number of constraints have determined severe limitations on an efficient utilization of MAS in plant breeding, even if there are a few successful applications in improving quantitative traits. Recent advances in genotyping technologies together with comparative and functional genomic approaches are providing useful tools for the selection of genotypes with superior agronomical performances (Figure 4).

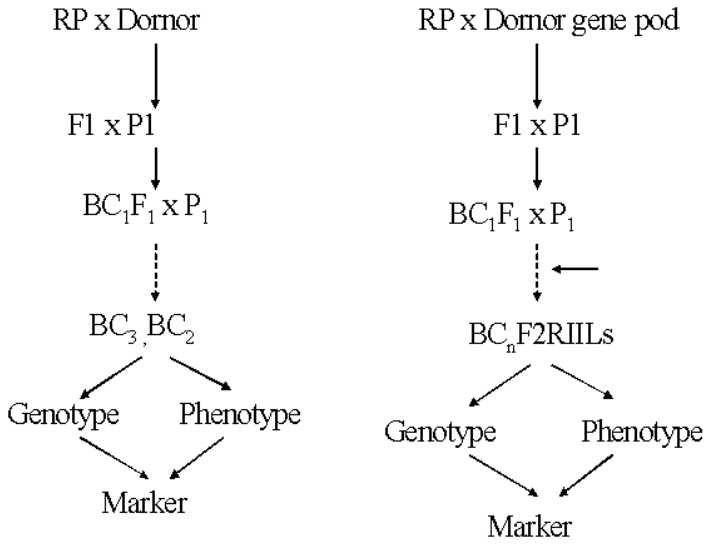

Figure 4: Protocol for MAS on salt tolerance genes in rice

217 BC_2F_2 from cross IR64 / OMCS2000 were analyzed. Regression analyses based on SSR allele class differences were performed. Highly significant associations were detected at the SSR locus RM223 on chromosome 8 in predicting the phenotype of the salt locus. The result indicated an accuracy of more than 95 % in identifying the resistant plants which was similar to that using RM223. Due to this marker, OM4498 (IR64 / OMCS2000 backcross) was successfully released in 2007.

DISCUSSION

Phenotypic measurement is very important in tagging QTL, because quantitative traits are largely affected by environment and measuring characters like salt tolerance. It is complicated because there is little agreement about how to artificially impose the stress. Phenotypic frequency distributions support the quantitative inheritance of salt tolerance gene. Some varieties are highly tolerant to salinity, while others are very sensitive. This can be interpreted to mean that under certain saline condition, plant growth, dry shoot weight, and dry root weight are more severely affected by environment. Many of the salt tolerant varieties are traditional cultivars, which tend to be tall and photoperiod sensitive. These maybe adaptive traits in many salt prone environments but modern plant breeders would like to be able to more genes controlling salt tolerance into short stature, high yielding varieties. There was no relationship between the number of shared QTL and presence/absence of phenotypic correlation. It is indicated that a large proportion of the phenotypic variance is explained by QTLs that were not detected or else by the presence of other genetic effects. Based on the salinity

tolerance excluding the major gene in chromosome 1, 8, three microsatellite loci were tightly detected minor genes for salinity tolerance, with RM223 on chr.8 and RM315 on chr.1. The ability to detect the tight linkage between markers and salt tolerance genes depends on the number of mapped markers that is available for rice. The level of polymorphism is detectable by those markers in BC_2F_2 used in tagging and the phenotyping approach. This study, which has identified salt tolerance genes associated with seedling survival under salt condition, provides a starting point for examining the effects of these genes in rice. This study has provided much more detailed informations on the relative importance of genomic segment and has increased our understanding of the genetic basis of salt tolerance. Fine maps could help identify long-term strategies for salt tolerant breeding. This gene is considered a viable approach to attaining for salt stress.With MAS, selection can be made with RM223 for salt stress.

REFERENCES

Kosambi DD. 1944. The estimation of map distances from recombination values Ann Engen 12: 172-175

Lander ES, D Botstein. 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage map. Genetics 121:185-199.

Lander ES, P Green, J Abrahamson, A Barlow, MJ Daly, SE Lincoln, L Newburg. 1987. Mapmarker: An interactive computer package for construcing primary genetic linkage maps of experimental and natural populations. Genetics 1:174-181.

Nguyen Thi Lang, S Yanagihara, Bui chi Buu. 2001. QTL analysis for salt tolerance in rice (*Oryza sativa* L.). SABRAO 33(1): 11-20

Nguyen Thi Lang. 2002. *Protocol for basic of biotechnology*. Agricultural Publishing House, Ho chi Minh City, Vietnam

Nguyen Thi Lang. 1999. QTL for salt tolerance and evaluation in rice (*Oryza sativa* L). Okinawa Subtropical Station, JIRCAS (8):145-174

Tanksley SD. 1993. Mapping polygenes. Annu Rev Genet 27:205–234

Phân tích QTL và chọn giống lúa chống chịu mặn bằng chỉ thị phân tử

Khai thác quần thể lai giữa AS996 / IR50404 và IR64 / OMCS2000, với 229 dòng cận giao tái tổ hợp (RIL) theo phương pháp SSD. Quần thể BC_2F_2 của IR64 / OMCS2000 được lập bản đồ với 34 markers phủ trên vùng mục tiêu của nhiễm sắc thể số 1 và 8, với tổng chiều dài 148.6 cM. Chọn 217 cá thể BC_2F_2 của IR64 / OMCS2000 để phân tích. Locus RM223 trên nhiễm sắc thể số 8 liên kết chặt với gen mục tiêu điều khiển tính chống chịu mặn đã được ghi nhận trong thí nghiệm này và nhiều phân tích trước đó, với mức độ chính xác khi so sánh kiểu gen và kiểu hình là 95 %. Giống OM4498 là dòng được phân lập từ RM223, đã được phóng thích trong năm 2007.