2010 International Conference on Biology, Environment and Chemistry
IPCBEE vol.1 (2011) © (2011) IACSIT Press, Singapore

Tagging and Validation of SSR markers to Salinity Tolerance QTLs in Rice (*Oryza spp*).

Aliyu R, Adamu AK, Muazu S, Alonge SO
Department of Biological Sciences,
Ahmadu Bello University,
Zaria, Nigeria.
e-mail: s.ramatu@gmail.com

Gregorio GB
International Rice Research Institute,
IRRI
Las Banos, Philippines.
e-mail: g.gregorio@cgiar.org

Abstract— **DNA markers have enormous potential to improve efficiency and precision of conventional plant breeding via MAS. QTLs mapping studies for diverse crop species have provided an abundance of DNA marker trait associations. Two F_2 populations with parental designation of IR88399-B and IR88317-B used in validation studies with 4 SALTOL markers on chromosome 1 failed to detect SALTOL QTL in the breeding populations, however, 2 QTLs for leaf diameter was obtained in the SALTOL region with Rice Markers (RM) RM493 and RM3412. These QTLs were significantly P< 0.05 associated with salinity tolerance trait. The phenotypic variation explained by the two QTLs were 4.3% and 5.6% of the total variation in F_2 population of population 1. Allelic data used to construct dendrograms clustered tolerant genotypes in population 1 but failed to cluster the tolerant genotypes in population 2. This indicates that the SALTOL QTLs could only be present in specific populations.**

To test the usefulness of microsatellite (SSR) markers associated with SALTOL QTL, a collection of 150 diverse rice genotypes were used. Result of phenotypic response of rice genotypes to salinity stress at seedling stage indicated varied genotypic response. Alleles ranged from 3 in RM493 and RM 3412 to 4 in RM 10793. Polymorphic information content value ranged from 0.6 to 0.73. RM10793 with a resolving power of 0.96 was most informative primer for genetic diversity in this study. Cluster analysis of the allelic data obtained clustered some tolerant genotypes with Pokkali. RM493 and RM3412 could discriminate tolerant genotypes based on leaf diameter. These markers could be useful in molecular mapping and marker assisted selections.

Keywords-QTLs, SALTOL, SSR markers, Salt tolerance, breeding populations.

I. INTRODUCTION

So far, conventional breeding methods for salt tolerance have been found ineffective due to the strong environmental effects on genotypic expression and the low narrow sense heritability of salt tolerance [8,9] which hinders the development of an accurate rapid and reliable screening technique under natural conditions as most of the processes found, empirically, to be important in plant tolerance to salinity exhibits quantitative inheritance. Although many component traits in salinity tolerance have now been extensively described and in some cases the underlying mechanism is at least partially understood [6], the application of this knowledge to the improvement of rice remains hampered because of the quantitative nature of the genes involved which are difficult to handle in breeding programmes as salinity affects rice growth in varying degrees at all stages due to its differential salinity sensitivity [17].

Progress in rice breeding for salt tolerance, constitutes the identification of the major locus conferring a salt tolerance gene at different growth stages. With the recent development in the field of molecular marker analysis, it is now feasible to analyze both the simple inherited traits and the quantitative traits and then identifying the individual genes controlling salinity tolerance which could facilitate selection in rice for this low heritable trait. Molecular marker could be used to tag QTL and evaluate their contributions to the phenotype by selecting for favorable allele at those loci in marker assisted selection (MAS) scheme aimed to accelerate genetic advancement in rice which is faster, more efficient and cost-efficient than conventional screening under saline field conditions [8].

II. MATERIALS AND METHODS

A. Plant Materials:

Two F_2 populations of rice (*O. sativa*) segregating for traits of interest were selected on the basis of their parental response to salinity from IRRI. Parental details and cross combinations are represented in Tab 1. Pokalli and IR29 were used as standard tolerant and susceptible checks respectively.

150 seeds of diverse rice genotypes were obtained from the International Rice Research Institute, Las Boanos and the West Africa Rice Development Agency (WARDA) Ibadan station, Nigeria and utilized in haplotypes studies.

TABLE I. TWO F2 POPULATION PARENTAGE AND PARENTAL CHARACTERISTICS

POPULATION	DESIGNATION	SOURCE
1 - IR88399-B	CHERIVIRUPPU	HB1571
	IR66946-3R-178-1-1	HB2360
	IR05A17	A1653
2 - IR88317-B	IR66946-3R-178-1-1	HB2360

IR03A477 A2163

B. *Screening for Salinity tolerance*

180 F_2 plants from population 1 and 150 F_2 plants from population 2 were screened for salt tolerance using IRRI standard protocol [9] under controlled environmental condition at the IITA screen house.

The seeds were sterilization, pregermination in a hydroponic system and salinized with NaCL to electrical conductivity EC of 12dsm^{-1} [9]. Each trays included the parental, the tolerant and sensitive checks. Plants were scored 4 weeks after salinization at the death of the sensitive check (IR29). The nutrient solution was renewed fortnightly and the pH maintained daily at 5.2±0.2.

C. *Phenotypic Evaluations and Genotyping*

Four plant phenotypic characteristics (root length, leaf width, tiller number and seedling height) and the SES score for salinity tolerance were evaluated at the end of the experiment. Four simple sequence repeats (SSR) markers (RM493, RM10793, RM3412 and RM10791) found in the SALTOL locus in chromosome 1 were used for molecular analysis.

D. *Isolation of rice DNA for PCR array:*

Genomic DNA of all 150 genotypes from diverse accessions and two f2 populations were isolated separately. DNA was extracted from a 21-day old seedling leaves collected from at least 2-3 seedling in each genotype, according to the method described by [5] with little modifications. Quantified DNA samples from each genotype were subjected to PCR amplification with SSR primers.

For each marker, allelic bands were scored based on a 1 (present) and 0 (absent) binary code and also tolerant and sensitive parental bands of the amplified products and were designated as A, B and H for homozygous tolerant, homozygous sensitive and heterozygote, respectively. Data was entered into an excel spread sheet and analyzed.

E. *Data Analysis*

For each of the defined loci, SSR allelic composition, allelic frequencies, allele per locus was determined for each genotype. The PIC value and band informativeness was determined following the formula proposed by Anderson *et al.* [1] and Prevost and Wilkinson [21].

Genetic similarities were evaluated using Jaccard similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by primers were generated using 'SMQUAL' sub-program of NTSYS-pc software [23].

F. *QTL analysis*

QTL analysis was performed using single-marker analysis using specific programme on excel work sheet which involved comparing traits for each SSR marker. The difference between the phenotypic means of tolerance and susceptible genotypes was used to estimate the phenotypic effect of the markers genotypes. The proportion of the trait phenotypic variations explained by the QTL was calculated as R^2 value which is the proportion of sum of square explained by the QTL to the total sum of squares. Additive effect was also calculated.

III. RESULT

A. *Salinity tolerance.*

Result of phenotypic score for salinity tolerance or susceptibility using the modified evaluation score (SES) of usual salt injury on the leaves of seedling of population 1 and 2 is presented in fig 1. In population one, four lines were classified as highly tolerant (HT) (visually score 1.0-2.0), 16 lines fell into the tolerant group (T) (visual scores 2.1-3.9), 61 lines were moderately tolerant (MT) (visual score 4-5.0), 77 lines were susceptible (S) (visual score 5.1-7.0), and 46 were highly susceptible (HS) (visual score 7.1-9.0). The frequency distribution of population one was continuous but skewed towards the susceptible parent (IR 05A117).

In Population two there was considerable variations among the recombinant inbred lines to salinity tolerance (Fig 1). Of the 120 lines evaluated, 8 lines were highly tolerant, 13 lines were tolerant, 40 lines moderately tolerant, 40 lines were susceptible and 19 lines were highly susceptible (Fig 1). Eight lines of the RILs were more tolerant than their parents. The frequency distribution of the population was continuous but skewed towards susceptibility.

The percentage (17.5%) of tolerant (visual score 1-3) lines and moderately tolerant (visual score 3.5-5.5) lines (33.33%) in population 2 were higher than the percentage (10.6%) of tolerant and moderately tolerant lines (28.9%) obtained in population one. However, the percentage (60.6%) susceptible (visual score 5.6-9.0) lines in population one was higher than that obtained with population 2 (49.2%). Both however recorded more susceptible than tolerant lines.

Marker analysis showed more of the tolerant parental genotype alleles which was contrary to the phenotypic scoring for visual salt injury where few of the F_2 population were tolerant to salinity in population one (19 lines) and population two (21 lines).

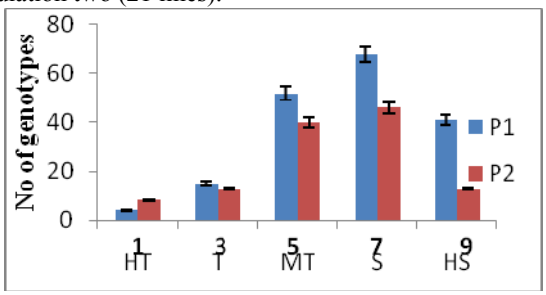

Figure 1. SES evaluation in two breeding population.

The dendrograms constructed with the marker data for population one showed five clusters (not presented). Cluster one comprised of all the tolerant genotypes and few moderately tolerant and susceptible genotypes at 67% similarity coefficient. Contrarily, at the same similarity coefficient, dendrograms obtained with population two (not

presented) revealed 5 clusters with the tolerant lines scattered around the clusters.

B. Quantitative trait loci (QTL) analysis

Single marker analysis revealed weak association with salinity tolerance (SES) in both population (Tab 2). The result obtained revealed that there was no QTL for salinity tolerance (SES) in the segment of chromosome 1 investigated. Two QTLs was however obtained at this segment for leaf diameter. RM 493 and RM 3412 was found to be associated with leaf diameter ($P<0.05$) under salt stress in population 1 (Tab 3) which revealed that there are QTLs for salinity tolerance (Ld) in this region of chromosome 1, however, these QTLs are minor. For each allele that the plant has at locus RM 493 and RM 3412, there is a 0.04cm and 0.03 cm reduction in leaf diameter respectively. The phenotypic variation explained by the two QTLs were 4.3% and 3.6% of the total variance in F_2 population of population 1. No QTL was found to be related to phenotypic traits evaluation in F_2 population of population two. The phenotypic variations obtained in this validation studies ranged between 3.6-4.4%.

TABLE II. SINGLE MARKER ANALYSIS FOR TWO F2 POPULATION.

Marker	Pop 1	Pop 2
RM493	0.39ns	0.98ns
RM3412	0.26ns	0.76ns
RM10711	0.53ns	0.16ns
RM10793	0.17ns	0.11ns

ns- not significant $P< 0.05$

TABLE III. QTL IDENTIFIED FOR SALT TOLERANT TRAIT AT 12DSM-1 IN POPULATION 1

Trait	QTL	Marker	R2	a.e.	p- value
LD	q LD-1	RM493	4.40%	-0.04	0.025*
	q LD-1	RM3412	3.60%	-0.03	0.045*

Significant at 5% level a.e – additive effect
QTL nomenclature as suggested by McCouch *et al* [18].

C. Screening for salt tolerance in diverse genotypes at SALTOL locus on chromosome 1.

1) SSR polymorphism

Three Saltol markers (RM 493, RM 3412 and RM 10793) that showed unambiguous scorable band with 150 diverse genotypes with varying response to salinity tolerance were screened. A total of 10 allele were detected with the three primers. The number of alleles ranged from 3 in RM 493 and RM 3412 to 4 in RM 10793. The allelic frequency also ranged from 90% in RM 493 to 93% in RM 3412 with no rare alleles detected. The PIC value calculated to estimate the informativeness of each primer varied from 0.64 in RM 493 to 0.73 in RM 10793 with an average of 0.69 (Tab 4) and a resolving power ranging from 0.8 in RM 493 to 0.96 in RM 10793 thus indicating that all three primers were capable of distinguishing between genotypes and highly informative. The most informative primer with the best resolving power was RM 10793 followed by RM 3412. These result revealed that markers RM 10793 would be best in screening rice germplasm for SALTOL QTL on chromosome 1 followed by RM 3412 and lastly RM 493.

TABLE IV. ALLELE, ALLELE FREQUENCY PIC VALUE AND RESOLVING POWER OF THREE SALTOL SSR MARKERS

Marker	Allele	allele freq.	PIC	RP
RM493	3	90	0.64	0.8
RM3412	3	93.3	0.69	0.89
RM10793	4	91.3	0.73	0.96

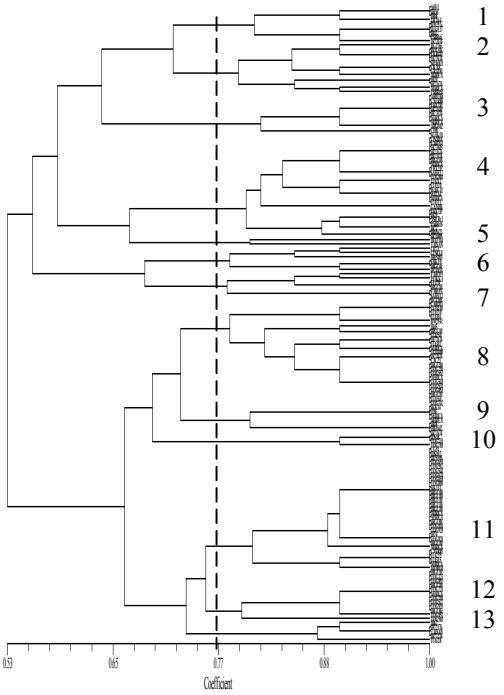

Figure 2. Dendrogram showing genetic diversity of 150 rice genotypes based on 3 Saltol polymorphic SSR markers derived from UPGMA cluster analysis using NTSYS (Jaccard coefficient).

2) Cluster analysis

The dendrogram constructed with these marker information revealed 13 clusters at a similarity coefficient of 68%. Few tolerant genotypes clustered at group 7 with Pokkali and FL 478 (fig 2).

Considering the haplotypes produced with respect to Pokkali, all primer haplotypes were linked to genotypes that were tolerant or moderately tolerant to salt stress at seedling stage. RM 493 produced 14 haplotypes of which one was same with that produced by RM 10793 (IKK 14) and of which 4 were same with RM 3412 (IAC 23, ITA 302,IR 77674-3B-21-1-1-1-2 and IR 77674-B-20-3-3-1-3-B-B). RM 3412 with 47 Pokkali haplotypes had the highest number of haplotypes with only 3 genotypes (FL 478, IRGC 101931 & IRGC 101958) associated with RM 10793.

However, some tolerant genotypes (PSB RC 50, Nerica L-41 and Nerica L-58) had no common Pokkali markers alleles for RM 493, RM 3412 and RM 10793 which suggests that alleles detected by markers tag traits independently.

IV. DISCUSSION

A. Population studies and QTL analyses.

The frequency distribution of the progenies in population 1 and 2 skewed towards susceptibility with a greater percentage of susceptible genotypes (60.8%) obtained in population 1 than population 2 (49.2%). This might be because not all progenies obtained from the crosses were screened under salt stress which might also explain their non conformation to the mendalian theory of segregation, thus the distortion observed the progenies.

Markers validation on independent populations of different genetic background is essential in determining the effectiveness and reliability of the markers to predict phenotypic [2, 3, 11, 14, 15, 24] which indicates whether or not a marker could be used in routine screening for MAS [20, 24]. Markers should also be validated by testing for the presence of the markers on a range of cultivars and other important genotypes [24, 25].

QTL analysis is based on the principle of detecting an association between phenotype and genotype of markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured [26, 27].

In rice, important traits such as salt tolerance are controlled by polygenes with additive and dominant effects that are described by quantitative trait loci (QTLs) [8] and QTL analyses of salt tolerance have been conducted by several researcher [7, 16, 29] as salt tolerance is controlled by a variety of mechanisms [6].

In the validation study, using SALTOL markers, no QTL associated with salt tolerance was detected. None of these markers were significant using single marker analysis. The failure to detect QTL related to salt tolerance might be because both parents of the two populations were salt tolerant. This revealed that the parents of these populations may not be suitable for the introgression studies of the SALTOL QTL using marker assisted selection and that parents for marker assisted breeding should be of contrasting genotypes for the traits of interest.

Therefore, marker assisted selection for salinity tolerance could be population specific. The choice of male and female parent is critical in order to improve their special combining ability in selected crosses. Some studies have also warned of the danger of assuming that marker-QTL linkage will remain in different genetic backgrounds or in different testing environments, especially for complex traits [22]. Even when a single gene controls a particular trait, there is no guarantee that DNA markers identified in one population will be useful in different populations, especially when the population originates from distinctly related germplasm [22].

Single marker analysis used for this study, though the simplest in detecting QTLs associated with single markers of the phenotypic data based on genotype at each marker position to test for the presence of one or more QTL have been reported to be adequate and efficient, giving rise to essentially similar results using marker intervals [4]. This QTL analysis method though unable to reveal QTL for salt tolerance based on SES score, revealed a QTL for leaf diameter (qSLD-1) in chromosome one with RM 493 and RM 3412 at two different locations in population one. The presence of these minor QTLs might have been responsible for clustering together the tolerant lines observed in cluster 1 in the dendrograms obtained with population 1 and their absence in population 2 could have also resulted in the non-clustering of the tolerant genotypes to a specific cluster group.

The phenotypic contribution of the major QTL (SALTOL) reported by Gregorio [9] and Niones [19] in their studies was quite bigger than as obtained in this study. However, similar results was obtained from the work of Islam [10] with entirely different breeding population which further buttresses population specificity.

The presence of moderately tolerant to highly susceptible genotypes in cluster group 1 of population 1 confirms the presence of polygene controlling salt tolerance. The failure of the Saltol markers to also cluster all salt tolerant diverse genotypes at seedling stage with varying marker response to cluster groups indicates that some markers are more suitable for use in marker assisted breeding than the other and that RM 10793 was best in marker assisted selection followed by RM 3412 then RM 493.

FL 478, PSB Rc50, Nerica L-41 and Nerica L-58 which are highly salt tolerant genotypes, were not identified by the SALTOL markers and this result suggests that there are other QTLs that control salinity tolerance in these genotypes. These genotypes could serve as potentially novel germplasm that could be exploited for the development of new breeding lines with high level of salinity tolerance.

V. ACKNOWLEDGMENT

Many thanks to IRRI for funds provided for this research, Ahmadu Bello University UBR for her monetary support, WARDA, Nigeria and IITA, Ibadan, Nigeria; my host institutions.

REFERENCES

[1] Anderson, J.A., G. A. Churchil, J.E. Aitrique, S.D. Tanksley and M. E. Sorrells (1993) Optimizing Parental Selection of genetic linkage maps. *Genome* 36: 181 – 186.

[2] Cakir, M., S. Gupta, G.J. Platz, G.A. Ablett, R. Loughman, L.C. Emebiri, D. Poulsen, C.D. Li, R.C.M. Lance, N.W. Galwey, M.G.K. Jones & R. Appels, 2003. Mapping and validation of the genes for resistance to *Pyrenophora teres* f. teres in Barley (*Hordeum vulgare* L.). *Aust J Agric Res* 54: 1369-1377.

[3] Collins, H.M., F. Panozzo, S.J. Logue, S.P. Jefferies, and A.R. Barr. (2003). Mapping and validation of chromosome regions associated with high malt extract in barley (*Hordeum vulgare* L.). Aust. J. Agric. Res. 54: 1223-1240.

[4] Davarsi A, weinreb A, Minke V, Weller J.I., and Soller M. (1993). Detecting marker QTL linkage and estimating gene effect and map location using a saturated genetics map. *Genetics* 134: 943-951.

[5] Doyle, J.J., and J.L. Doyle. (1990). Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

[6] Garcia, A., Rizzo, C.A., Ud-Din, J., Bartos, S.L. Senadhira, D., (1997). Sodium and potassium transport to the xylem are inherited independently in rice, and the mechanism of sodium: potassium selective differs between rice and wheat. *Plant cell environ*, 20: 1167-1174.

[7] Gong, J., He, P., Qian, Q., Shen, L.S., Zhu, L.H. (1999). Identification of QTL for salinity tolerance in rice (*Oryza sativa* L.). *Chinese science Bulletin*, 44(1): 68-71

[8] Gregorio G.B., Senadhira D., (1993). Genetic analysis of salinity tolerance in rice. *Theor. Appl. Genet*. 86: 333-338.

[9] Gregorio, G.B. (1997). Tagging salinity tolerance genes in rice using amplified fragment length polymorphism (AFLP). [Ph.D dissertation]. College, Laguna, Philippines: University of the Philippines Los Banõs, Laguna pp 118.

[10] Islam, M.M. (2004). Mapping salinity tolerance genes in rice (*Oryza* sativa L.) at reproductive stage. Ph.D Thesis, University of the Philippines Los Banõs, college, Laguna, Philippines, pp: 1-149.

[11] Koyama, M.L., Aurora L., Robert M.D.K., Timothy J.F. & Anthony R.Y., (2001). Quantitative Trait Loci for Component Physiological Traits Determining salt Tolerance in Rice. *Plant Physiol*. Vol. 125, pp. 406-422.

[12] Lang, N.T., S. Yanagihara and B.C. Buu. (2000). Quantitative trait loci for salt tolerance in rice via molecular markers. *OmonRice*, 8: 37-48.

[13] Langridge, P., E. Lagudah, T. Holton, R. Apples, P.Sharp & K. Chalmers, (2001). Trends in genetic and genome analyses in wheat: a review. *Aust J Agric Res* 52: 1043-1077.

[14] Li, Z., L. Jakkula, R.S. Hussey, J.P. Tamulonis & H.R. Boerma. (2001). SSR mapping and confirmation of the QTL from PI96354 conditioning soybean resistance to southern root-knot nematode. *Theor. Appl. Genet.* 103: 1167-1173.

[15] Lin, H., Yanagihara, S., Zhuang, J., (1998). Identification of QTL for salt tolerance in rice via molecular markers. *Chinese Journal of Rice Science* 12(2): 72.

[16] Manneh,B. (2004). Genetic, physiological and modeling approaches towards tolerance to salinity and low nitrogen supply in rice (*Oryza sativa* L.). Ph.D Thesis, Wageningen University, Wageningen, the Netherlands, pp:260.

[17] McCouch, S. R., Chen, X., Panaud, O., Temnykh, S., Xu, Y., Cho, Y., Huang, W., Ishi, T and Blair M (1997) Microsatellite maker development, mapping and application in rice genetics and breeding. *Plant mol. Biol.* 30: 89-99.

[18] Niones, J.M. (2004). Fine mapping of the salinity tolerance gene on chromosome 1 of rice (*Oryza sativa* L.) using near-isogenic lines. M.Sc. Thesis, University of the Philippines Los Banõs, College, Laguna, Philippines, pp: 78.

[19] Ogbonnaya, F.C., N.C. Subrahmanyam, O. Moullet, J. de Majnik, H.A. Eagles, J.S. Brown, R.F. Eastwood, J. Kollmorgen, R. Apples & E.S. Lagudah, (2001). Diagnostic DNA markers for cereal cyst nematode resistance in bread wheat. *Aust. J. Agric. Res*. 52: 1367-1374.

[20] Prevost, A., M.J. Wilkinson. (1999). A new system of comparing PCR primers applied to ISSR finger printing of Potato cultivars. *Theor. Applied. Genet.*, 98(1): 107-112. (doi:10.1007/s001220051046).

[21] Reyna, N & C.H. Sneller. (2001). Evaluation of marker assisted introgression of yield QTL alleles into adapted soybean. *Crop Sci*. 41:1317-1321.

[22] Rohlf, F.J (1993) NTSYS-PC Numerical taxonomy and multivariate analysis system. Version 2.0. Exeter Software, Applied Biostatistics Inc. Setauket, New York.

[23] Sharp, P.J., S. Johnston, G. Brown, R.A. McIntosh, M. Pallotta, M. Carter, H.S. Bariana, S. Khartkar, E.S. Lagudah, R.P. Singh, M. Khairallah, R. Potter & M.G.K. Jones, (2001). Validation of molecular markers for wheat breeding. *Aust. J. Agric. Res.* 52: 1357-1366.

[24] Speilmeyer, W., P.J. Sharp & E.S. Lagudah. (2003). Identification and validation of markers linked to broad-spectrum stem rust resistance gene Sr2 in wheat (*Triticum aestivum* L.). *Crop Sci*. 43: 333-336.

[25] Tanksley, S.D. (1993). Mapping polygenes. *Annu. Rev. Genet*. 27: 205-233.

[26] Young, N.D., (1996). QTL mapping and quantitative disease resistance in plants. *Annu. Rev. Phytopathol*. 34: 479-501.

[27] Yu J.K. Mangot J., Thompson L., Edwards K.J., Slabaugh M.B. Knapp S.J. (2000). Allelic diversity of Simple Sequence Repeats among elite inbred lines of cultivated sunflower. *Genome* 45: 652-660.

[28] Zhang, G.Y., Guo, Y., Chen, S.L., (1995). RFLP tagging of a salt tolerance gene in rice. *Plant Science* 90: 969.