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ENHANCING AND STABILIZING THE PRODUCTIVITY OF SALT- AFFECTED AREAS BY INCORPORATING GENES FOR TOLERANCE OF ABIOTIC STRESSES IN RICE

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ABSTRACT

Soil salinity is one of important stress factors limiting the growth and productivity of rice (Oryza sativa L.) in many areas of the world. Most rice varieties are moderately sensitive to salt except some traditional indica varieties such as Pokkali, Mot Bui Do and improved cultivars as IR42, AS996. Crop yields in these areas are generally low and are progressively decreasing, particularly in saline areas where farmers still use traditional varieties and practices. Effective measures are being attempted to amend these soils and use them effectively for food production. New salt tolerant rice varieties adapted to the Mekong delta region are being developed using molecular breeding with microsatellite DNA markers to accelerate progress in breeding for salt tolerance combined with submergence tolerance in rice. In the later approach, we are developing mapping populations to identify major QTLs associated with salinity tolerance, fine-mapping major QTLs and develop a marker assisted system to speed up their introgression into popular varieties and elite breeding lines. Some varieties such as OM5629, OM5891, OM4900 were developed that can yield 5-6 ton ha^{-1} under salt stress of 6.0 to 9.0 dS m^{-1}, and are being out-scaled. Results generated form on-farm trials over the last three years in these different areas and their initial impacts are discussed in this paper.

Keywords: coastal zones, problem soils, salinity, submergence,

INTRODUCTION

With the rice production at Mekong Delta, farmers expected the rice varieties with various traits as tolerance to acid sulphate soil, requirement of less fertilizer, resistance to insect and diseases (brown planthopper, stem borer, sheath borer, leaf folder, rice bugs, yellow stunt disease, grassy stunt disease, blast, sheath blight, bacterial blight, yellow leaf and grain spots), short plant, hard stems, resistance to lodging, long grain/slender grain, thin rice husk, big and long panicle, hidden panicle, synchronized flowering, big and erect leaf, high tillering capacity, high yield, sweet and soft cooked rice with aroma, well plant development after sowing, tolerance to abiotic stress. Most of the degraded soils in these coastal saline areas has poor nutrition, lacking phosphorous and potassium and are toxic with high contents of available aluminum and iron as well as excessive salts and low soil pH (Lang et al. 2009). In the Mekong Delta, there are two typical climate seasons, dry and wet. During dry season, the substances creating acid sulphate in the deeper layers of soil raises up the upper layers of soil and affects to the crop growth. The irrigation and rainfall can reduce a certain level of acid sulphate to plant crop. Thus, the rice varieties, which are tolerant to acid sulphate soil, are important for farmers' variety adoption.

Our research activities in the Mekong Delta focus on the development of proper technologies for enhancing and stabilizing farm level productivity

and for improving farmers' livelihoods (Buu et al. 2004). This is being achieved through the development of rice varieties with tolerance to prevailing abiotic stresses, adoption of proper soil, water, nutrient and crop management practices for higher.These efforts are being supported in part, by funds provided by the GTZ/BMZ Project.

MATERIALS AND METHODS

- Plant materials

Five cultivars, two landraces and one wild rice population (IR28, OM1490, AS996, IR68552-55-3-2, OM1706, Pokkali, Mot Bui Do, and *Oryza rufipogon*) were selected to represent various degree of resistance to salt (IRRI 1999).

- Screening for salt stress: The resistance cultivars were crossed to the susceptible cultivars. Subsequently F_1 were selfed and backcrossed to their respective parents to produce the F_2 and backcross generations. The parents, F_1, F_2, BC_1 and BC_2 were tested under salt stress. 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. 1,230 segregants from the BC_2F_2 were evaluated in screen for salt tolerance. Transgressive segregates were observed for salt tolerance. The BC_2F_2 family was screened using microsatellite markers. Evaluation of salt tolerance of BC_2F_2 lines, the parents were observed in the first experiment to determine the optimal electric conductivity (EC) level for the subsequent experiments. Seeds were placed at 55^{o}C for three days in order to break dormancy, and raised with 0.1% of HgCl for 1 minute, then germinated at 37^{o}C for 48 hours. Finally, the most uniform seeds were seeded in cultural solution. Seedling survival days was observed at three EC levels as 6, 12,18 dS/m. Seedlings were grown in Yoshida solution + NaCl (Yoshida et al. 1992) in green house at CLRRI in a randomized complete block (RCB) design with three replications.

For each replication, 50 seeds were maintained in cultural solution for 4 days before adding NaCl to establish the different EC levels. The pH of the cultural solution was monitored every day to keep the pH about 5. The Yoshida solution was changed every week to limit the effect of algae. When a plant was completely yellow, no more green tissue was evident, it was considered as dead. Plants survival data was score (survival day: SD). Based on the results from the first experiment, a second one was performed at the optimal EC level using BC_2F_2 population under a RCB design with three replications, each harvesting 10 plants. The level of EC was used as 12, 18 dS/m.

DNA isolation

Protocol for DNA extraction was done according to the method suggested by Zheng et al. (1995) and modified by Lang (2002). Healthy rice leaf sample (2 cm long) was collected and placed in a labeled 1.5ml centrifuge tube on ice. Cut the leaf tissue into 0.5cm long segments and ground in a well of the thick polished glass rod with a small pestle after adding 400µl of extraction buffer (50mM tris-HCl pH 8.0, 25mM EDTA, 300 mM NaCl and 1% SDS). The tissue was ground until the buffer turns dark green. Added 400µl more of DNA extraction buffer and mixed in the well by pipetting. 400µl of the lysate was transferred to the original 1.5 ml of the leaf sample. Added 400µl chloroform and mixed well by inverting. Spin the tube for 30 sec in microcentrifuge. The aqueous supernatant was transferred to a new 1.5 ml tube and DNA precipitated using absolute ethanol. Spin for 3 min at 13.000 rpm and discarded the supernatant. After drying in air, the DNA was resuspended in 50µl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). DNA samples were ready for PCR analysis. Stored DNA at –20^{o}C for later use.

RESULT AND DISCUSSION

Use of DNA markers to accelerate progress in breeding for salt tolerance

(Marker-assisted backcrossing of ***Saltol*** into Vietnam popular varieties)

There is a need to develop salt tolerant rice varieties in Mekong delta of Vietnam to solve the problem of rice production in the coastal areas affected by salinity and to adapt to more than 703,000 ha of saline soils. The primary breeding objective is to develop rice varieties adapted to saline environment. Salt tolerance genes were transferred into improved and recommended varieties through conventional crossing, with a stable yield of at least 4t / ha, early maturing with

acceptable grain quality and resistance to the major insect pests and diseases. The advent of various biotechnological techniques in recent years has offered to the plant breeder's new tools for crop improvement.

Development new varieties must be combined the *Saltol* genes with short duration, grain quality, submergence tolerance, high yielding and BPH resistance

PCR-based markers were recommended to assist the selection for salt tolerance.

Mapping population is an important factor for gene tagging and QTL analysis. The standard procedure for developing population for this research is backcross. 90 lines from the BC_1F_1 were produced that exhibited sufficient fertility for further backcrossing or selfing. Of these 30 BC_1F_1 eventually produced viable backcross to BC_2F_2.

Phenotypic variation in salinity tolerance

Performance of BC_2F_2 for salt tolerance: the first experiment was designed to find out the optimal EC level for evaluation of salt tolerance in the BC_2F_2 of IR28 / Pokkali, The basic principle in screening salinity tolerance at seedling stage was the ability of the seedlings to grow in salinized culture solution. Distribution of salt tolerance at 4 level of EC for BC_2F_2 bulk exhibited nearly normal and continuous. In case of IR28 / Mot Bui Do, there was wide variation observed for grain yield. The results were summarized in table 1. It demonstrated that the optimal EC level was 18 dS/m.

BC_2F_2 populations are nearly ideal for QTL mapping. However, RILs were also produced by continually selfing the progenies of individual members of F_2 population until homozygosity. Two major advantages are homozygosity, which allows easy propagation and experimental replication and greater of recombination. It breaks up linkage blocks and increases the analysis solution.

BC_2F_2 lines are very useful for tagging several traits in single population, because the same set of RFLP data can be used to analyze different sets of phenotypic data. BC_2F_2s are also very useful for tagging of resistance genes for complex pathogens like blast, which has many races in different geographical regions. It is worthwhile to mention that the population also segregated for other traits such as plant height, photoperiod insensitivity and disease resistance.

Selection based on genotype is not affected by environmental variation or by the complexities of interaction affecting phenotypic selection. Marker-assisted selection permits rapid identification of individuals those genes for salt tolerance. The major gene for salinity tolerance (*Saltol*) was mapped on chromosome 1 and chromosome 8 (McCouch et al. 1988; Lang 1999, Lang 2001). This reported that arraying the DNA of given varieties for the presence or absence of band of appropriate molecular weight. The banding pattern of a molecular marker at a given locus is indicative of the presence or absence of a specific chromosomal segment, which is known to carry a desired gene or allele, as previously identified. RM223 linked to salt tolerance gene at the distance of 6.3 cM on chromosome 8 at vegetative stage under EC = 10 $dS.m^{-1}$ from F_3 population of IR28 / Doc Phung (Lang et al. 1999). This was located at the distance of 7.2 cM at seedling stage under EC= 18 $dS.m^{-1}$ from BC_2F_2 of IR68552-55-3-2 / OM1706 (Lang et al. 2001). Using a selective genotyping strategy combined with the parental survey, ten varieties were found polymorphism at the locus RM223. Polymorphism were observed among varieties as Doc Phung (tolerance: T), IR28 (susceptible: S), OM2395 (T), OM1706 (T), ST5 (T), AS996 (T), OM4900 (T), OM6162 (T), Pokkali (T), Mot Bui Do (T), Doc Do (T).

Morphological characters including dry shoot weight, shoot length, survival days (SD) were noticed

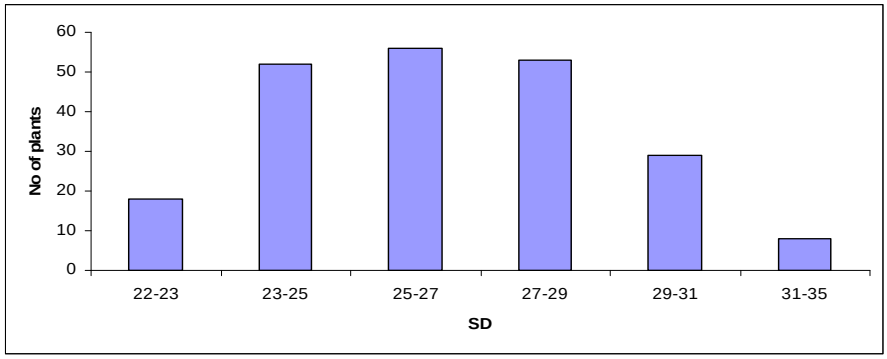

Figure 1. Survival days' distribution (SD) in BC_2F_2 of OM1490 / AS 996 // AS996

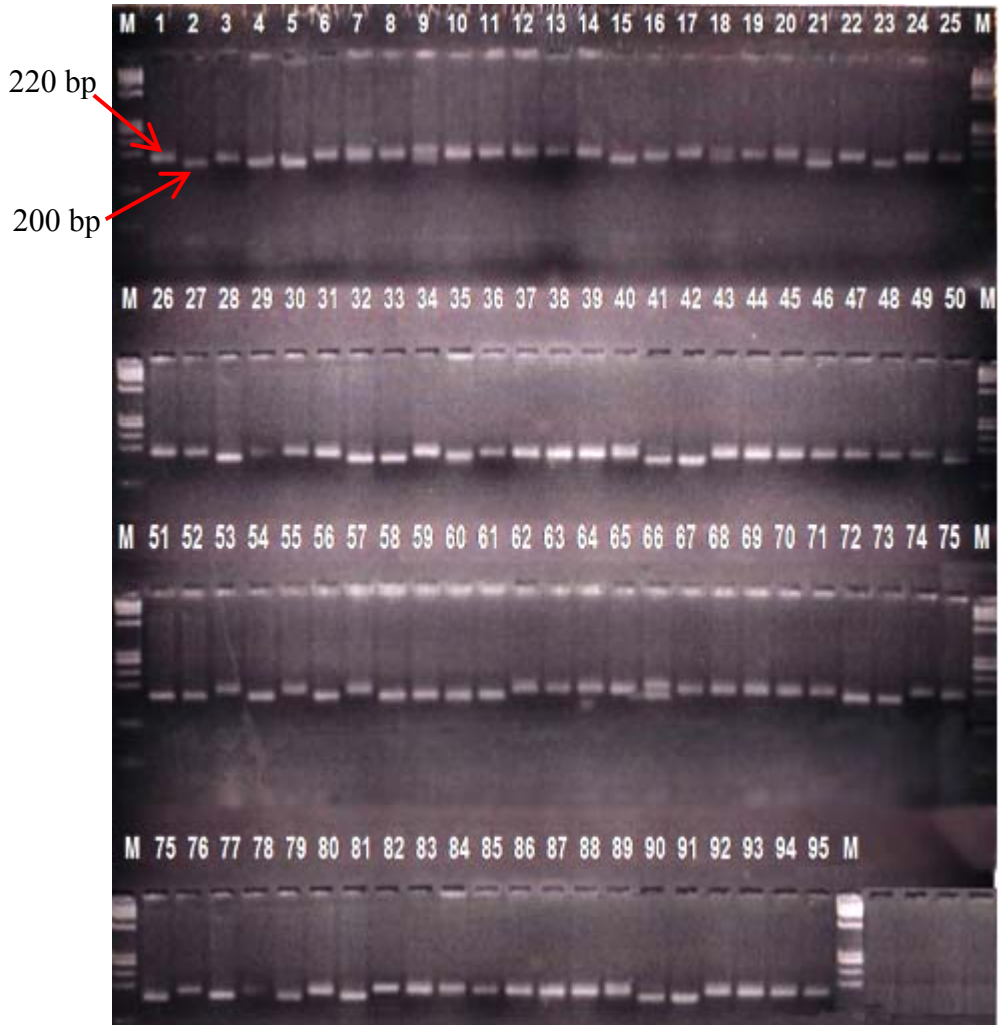

Figure 2. PCR products at the locus RM315 [motif (AT)4 (GT)10] in BC_2F_2 of OM1490 / AS996

Molecular markers linked to salt tolerance in rice

We have developed an advanced backcross population (BC_2F_2) of OM1490 / AS996. One hundred thirty BC2F2 lines were evaluated at seedling stage in CLRRI green house. Molecular markers associated to both qualitative and quantitative salt tolerance were identified by using 150 primers tested. Thirty eight markers were polymorphic. They are common markers in terms of BC_2F_2 of OM1490 / AS996 on chromosome 1's mapping.

Identification and fine-mapping of additional salinity-tolerance QTLs

OM1490 / AS996 nearly derived alleles at markers RM315. The progenies are associated to salt stress tolerance in seedling stage at the EC of 18 dS/m. Distance between RM315 and salt tolerance gene regions was 21.2, 1.9 and 0.0 cM. It means that derived alleles at the locus near salt tolerance on chromosome 1. In the cross of IR68552-55-3-2 / OM1706, the target region was located on chromosome 8 with distance between RM223 and salt tolerance gene was 7.2 cM (Lang et al. 2009). In summary, our results suggested that loci on chromosome 1, and chromosome 8 control salt stress tolerance in rice at EC = 18 dS/m. The identification provides the basis for developing genotypes more efficiently tolerant to salt stress at EC = 18 dS/m with different crosses. Generally, linked microsatellites on chromosome 1 may be used efficiently in marker-assisted selection in breeding program. Polymorphism was noticed at the locus RM 223 on chromosome 8 in case of OM1490 / AS 996.

Identification of molecular markers associated with quantitative trait loci (QTLs) linked with useful agronomic or adaptive traits will help speed the progress in breeding once developed, because these DNA markers will become effective tools for selection. Moreover, positional cloning using DNA markers will make it possible to isolate agronomically useful genes, which can also be used in breeding across species *via* transgenic approaches. Several mapping populations were developed using salt stress tolerance genotypes for QTL mapping at seedling stage (Lang et al. 2001). Two QTLs with relatively large effects were identified, one on chromosome 1 (at 18 dS/m) and the second one on chromosome 8 (at 12 dS/m). Microsatellite markers closely linked to these loci were identified such as RM223 and SL1F-SL1R on chromosome 8. These markers were further evaluated for their effectiveness in breeding using a set of 24 improved varieties, including donor (Pokkali) and sensitive genotype (IR28) as check. These cultivars were genotyped due to this marker and then phenotyped under salinity strees at 12 dS m^{-1} in culture solution (Yoshida et al. 1976) using visual SES scores (by IRRI). The results indicated an accuracy of more than 95% in identifying tolerant cultivars using this marker (Fig. 3). These results indicated the usefulness of this marker in parental surveys and in identifying tolerant lines from segregating populations. However, further tests are needed to confirm its effectiveness in different genetic backgrounds for example the polymorphism of RM223 in term of OM1490 / AS996. Some lines exhibited salt stress tolerance but not in same allele in AS996 yet (fig. 3). More efforts are needed to develop closely linked markers to these two QTLs to be used for their routine introgression into popular varieties and elite breeding lines.

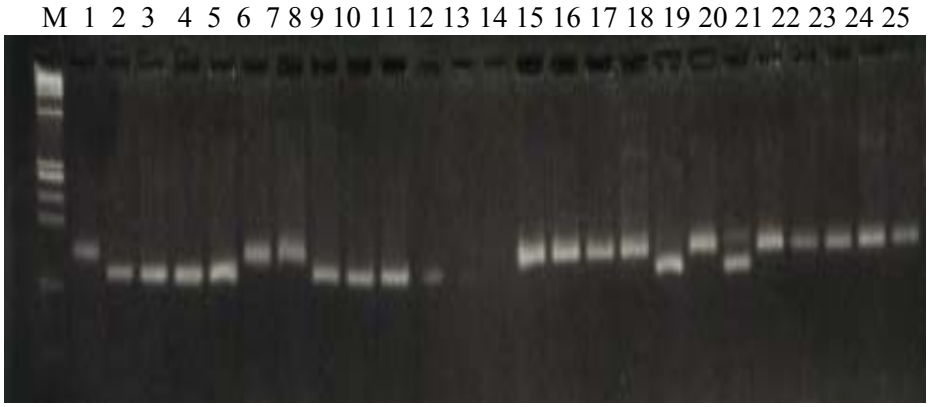

Figure 3. PCR products at the locus RM233 on chromosome 8 with BC_2F_2 of OM1490 / AS996

1: OM 1490; 2: AS 996; 3-25 lines from BC_2F_2

Testing of *Saltol* lines under different environmental condition

Performance stability is one of the most important properties of a genotype to be released as a variety to ensure wide adoption. To ensure this, we tested 8 indica rice varieties at 9 different locations during the 2010 wet and dry seasons, using a randomized block design with three replications in location. Growth duration, grain yield ($t.ha^{-1}$) and stability index were presented in Table 3. The experiment was conducted in 13 provinces as An Giang, Kien Giang, Long An, Tien Giang, Soc Trang, Vinh Long, CaMau, Can tho, Tra Vinh, Ben Tre and Bac Lieu. The highest grain yield across the 13 sites was obtained in AS996 during both seasons. The first genotype was bred by IRRI and CLRRI through wide hybridization to introgress the target genes from wild rice (*Oryza rufipogon*) into IR64 to release a derivative tolerant to acid sulfate soil. Most of the varieties showed good stability index. An understanding of environmental and genotypic causes and GxE interaction is important at all stages of plant breeding, for both selection based on specific traits or on yield (Yan and Hunt 1998; IRRI 1999).

A set of 8 rice genotypes were evaluated at 9 different locations as Tra Vinh (5 sites), Ben Tre (1), Bac lieu (2) and Can Tho (1), during the 2009 wet seasons. The multiple yield trial at 13 sites in the 2010 wet seasons was continued at the same provinces (Table 1). The purpose of these experiments were to evaluate the performance of these genotypes over years under diverse stress conditions, and also to select lines that are better adapted to one or both seasons. Overall, data showed that the performance of these selected varieties is fairly stable in these salt affected areas. Some of these lines are already been outscaled and are spreading to occupy larger areas. An example is shown for OM4900, OM6161, OM6162, OM5629 during both wet and dry seasons of 2009 and2010 (Table2).

The study showed that the released varieties were fairly stable across salt affected areas in different provinces. Several promising breeding lines were also selected and are being promoted to be released as national varieties. Another useful gene for salt tolerance was transferred to high-yielding indica varieties by backcrossing to OM1490 (as the recurrent) and with advances in cell culture and molecular markers, potentials of genes for salt tolerance more promising than before. These lines have been released as commercial varieties in Mekong delta.

Table 1. Agronomic characters and yield of some improved genotypes under salt condition of Mekong delta

Desigantion	Yield (t/ha)													Mean (t/ha)
	Hau Giang	Dong Thap	Vinh Long	An Giang	Long An	Kien Giang	Can Tho	Vien Lua	Tien Giang	Tra Vinh	Bac Lieu	Soc Trang	Ben Tre	
OM6162	5,42	6.56	6.00	6.31	6.17	6.07	6.50	6.20	5.82	6.40	6.70	6.05	6.10	6.18
OM6161	5.02	5.97	6.35	6.16	6.04	5.67	6.00	6.37	6.02	5.87	5.68	5.50	6.34	5.92
OM5629	5.13	5.78	6.25	6.16	6.07	6.60	6.53	6.24	6.00	5.37	6.40	5.80	6.31	6.05
AS996(check)	4.00	4.65	5.04	5.67	5.12	4.94	5.54	5.66	5.03	5.71	5.00	4.90	5.54	5.14
OM4900	6.54	6.34	6.78	7.00	6.46	6.81	6.50	5.76	6.35	6.66	6.41	6.17	6.71	6.50
OM7347	6.08	6.81	6.00	6.79	6.82	6.56	7.03	6.85	6.16	6.47	6.31	6.23	6.85	6.54
OM7345	6.07	6.45	6.76	7.07	5.87	6.71	6.86	6.16	6.41	6.00	6.47	6.72	7.00	6.50
OM8928	6.26	6.38	6.84	5.87	6.00	6.06	6.75	5.63	6.14	6.95	6.05	6.36	6.81	6.32
OM8927	6.15	6.36	6.76	7.00	6.48	5.67	6.76	6.45	6.17	6.00	5.78	6.04	6.57	6.32
OM7348	5.87	5.31	5.78	6.00	5.81	5.61	6.42	6.05	5.76	5.87	5.71	6.34	6.64	5.94
OMCS2009	6.34	5.46	6.41	6.08	6.34	6.51	6.75	6.57	6.61	7.00	6.86	5.67	6.72	6.41
OM7364	6.21	6.46	6.86	7.06	6.36	6.74	7.14	6.87	6.54	6.51	5.67	5.58	6.35	6.49
OM5840	5.65	6.07	6.74	6.54	6.59	6.57	6.72	6.58	5.67	6.75	5.86	6.00	6.42	6.32
OM10040	6.51	6.00	6.78	6.52	6.72	6.18	6.87	6.74	6.04	6.73	6.24	6.17	6.73	6.48
OM10041	6.00	6.76	6.87	7.00	5.92	6.83	7.25	6.80	6.18	7.00	6.52	5.76	6.78	6.59
EMS	0.177	0.147	0.145	0.110	0.125	0.185	0.173	0.138	0.198	0.099	0.245	0.213	0.200	
Mean	5.82	6.09	6.41	6.48	6.18	6.24	6.64	6.33	6.06	6.35	6.11	5.95	6.52	
t Ij	-0.43	-0.16	0.17	0.24	-0.06	-0.01	0.40	0.08	-0.19	0.11	-0.14	-0.29	0.28	

Ij: environmental index

Table 2. Agronomic characters and yield of some improved genotypes under salt condition at Mekong delta in 2010 wet season

No.	Designation	An Giang	CanTho	Hậu Giang	Kiên Giang	Vinh Long	Tra Vinh	Dong Thap	Long An	Mean Ton/ha
1	OM6677	4.1	5.2	5.2	6.2	6.2	5.2	5.3	5.7	5.4abc
2	MNR 1	5.2	5.7	4.5	6.6	6.3	5.8	5.7	5.6	5.7abc
3	MNR 5	4.6	6.7	5.7	6.4	6.4	5.6	5.5	5.9	5.9ab
4	MNR 4	4.7	6.2	6.2	6.3	5.9	5.9	6.7	5.8	6.0a
5	OM5981	6.2	5.8	5.2	6.7	5.7	5.7	6.8	5.9	6.0a
6	MNR2	4.7	4.6	5.3	6.8	5.6	3.4	4.2	6.2	5.1c
7	MNR 3	4.6	5.2	4.1	5.1	5.8	4.9	4.3	6.7	5.1c
8	AS996	4.5	4.1	4.8	6.5	5.9	4.5	4.7	6.8	5.2bc
9	OM5629	5.7	5.9	4.6	6.7	6.3	4.6	4.6	6.3	5.6abc
10	OM 4900	5.9	5.8	4.7	6.4	6.7	4.7	5.2	6.7	5.8abc
11	OM 6162	5.6	5.7	4.8	5.5	6.1	5.3	5.4	6.4	5.6abc
12	OM 6161	5.8	5.6	4.8	5.1	6.4	5.4	5.6	5.2	5.5abc
Mean		5.13c	5.54 bc	4.99c	6.19a	6.10ab	5.08c	5.33c	6.10ab	**5.56**
(Ij)		-0.43	-0.02	-0.57	0.63	0.55	-0.48	-0.23	0.54	

CONCLUSIONS AND FUTURE PROSPECTS

Salt stress affected rice yield in the Mekong delta highly serious, with a complex of abiotic stresses including salinity, acid sulfate, aluminum and iron toxicity, P deficiency. To enhance and sustain productivity of these soils, we adopt an integrated approach involving the development of adapted high yielding and salt tolerant varieties developed *via* novel breeding methods, proper management of resources. Development of salt tolerance varieties is generally considered the most effective entry point for improving productivity of salt affected soils, and it is also the cheapest option for farmers. Through the use of innovative breeding strategies involving conventional and modern tools, together with effective phenotyping techniques, good progress was made in developing salt tolerant varieties with broad adaptation to the conditions of the Mekong Delta. DNA markers tightly linked to *Saltol* as RM223 and RM315 were identified and tested in a diverse set of germplasm, where it showed >95% accuracy in identifying salt tolerant lines, suggesting that this marker is probably useful in selecting parental lines and for subsequent marker assisted selection. Some varieties such as OM4900, OM6161, OM5629 were developed. They can yield 5-6 ton ha^{-1} under EC = 6.0 to 9.0 dS m^{-1}, and are being out-scaled. The success of new varieties is assured through eventual testing and selection in target sites in partnership with farmers and under their own management to adoption. Special emphasis is placed on crop establishment because the early stages of seedling growth are extremely sensitive to salt stress. This is achieved through combined use of salt tolerant genotypes, coupled with proper nursery management and seedling handling that ensures maximum survival of transplanted seedlings. Future efforts should focus on further collection and evaluation of local germplasm to identify landraces with greater tolerance of salt stress and combine with submergence in rice, as sources of new genes or alleles for breeding. Additional resources and efforts should be directed towards identification of QTLs and genes underlying tolerance to the multiple stresses experienced in these problem soils of Mekong Delta, for their subsequent integration into modern varieties and elite breeding lines through marker aided breeding.

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Tăng cường và ổn định năng suất lúa của các vùng bị nhiễm mặn bằng cách kết hợp các gen chống chịu điều kiện bất lợi trên cây lúa

Đất nhiễm mặn là một trong những yếu tố bất lợi quan trọng làm hạn chế sự tăng trưởng và năng suất của cây lúa (*Oryza sativa* L.) tại nhiều khu vực trên thế giới. Hầu hết các giống lúa tương đối nhạy cảm với độ mặn ngoại trừ một số giống lúa mùa indica như là Pokkali, Một Bụi Đỏ và các giống cải tiến IR42, AS996. Năng suất cây trồng ở những khu vực mặn thường thấp và đang giảm dần, đặc biệt ở các vùng đất nhiễm mặn ở các vùng mà nông dân vẫn sử dụng các giống truyền thống. Các biện pháp có hiệu quả đang được cố gắng thực hiện để cải thiện các loại đất này và sử dụng chúng có hiệu quả trong sản xuất lương thực. Các giống lúa mới chịu mặn thích nghi với vùng đồng bằng sông Cửu Long đang được phát triển bằng cách sử dụng phương pháp chọn giống bằng marker phân tử với các marker DNA với chỉ thị microsatellite để đẩy nhanh quá trình chọn giống chống chịu mặn kết hợp với khả năng chống chịu ngập trên cây lúa

Phát triển quần thể lập bản đồ để xác định những QTLs chính liên quan đến tính chịu mặn, bản đồ fine-mapping các tính trạng QTLs chủ yếu và phát triển hệ thống nhờ vào marker để thúc đẩy quá trình trình chuyển gen của chúng vào các giống phổ biến và các dòng ưu tú trong chương trình chọn giống. Một số giống như OM5629, OM5891, OM4900 đã được phát triển năng suất có thể đạt từ 5-6 tấn/ha dưới điều kiện bất lợi do nhiễm mặn từ 6.0 đến 9.0 dS/m, các giống này hiện đang được phát triển và mở rộng qui mô. Kết quả đã tạo ra các dạng thử nghiệm tại nhiều địa điểm khác nhau trên cánh đồng trong 3 năm qua và những tác động ban đầu của chúng đã được thảo luận trong bài báo này.

Từ khoá: khu ven biển, các vấn đề về đất, mặn, ngập