INTROGRESSION OF SALT TOLERANCE GENE IN RICE VARIETY KARJAT-6 USING MARKER ASSISTED SELECTION

A thesis submitted to the

DR. BALASAHEB SAWANT KONKAN KRISHI VIDYAPEETH, DAPOLI
(Agricultural University)
Dist. Ratnagiri (Maharashtra State), India

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (AGRICULTURE)

In

GENETICS AND PLANT BREEDING

By

Mr. Mhatre Navadip Krishna
M.Sc. (Ag.)

2016

Approved by the Advisory Committee
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MR. MHATRE NAVADIP KRISHNA
M.Sc. (Ag.)

DEPARTMENT OF AGRICULTURAL BOTANY,
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CERTIFICATE

This is to certify that the thesis entitled **"INTROGRESSION OF SALT TOLERANCE GENE IN RICE VARIETY KARJAT-6 USING MARKER ASSISTED SELECTION"** submitted to faculty of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri, Maharashtra State in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE)** in **GENETICS AND PLANT BREEDING** embodies the results of piece of *bona fide* research work carried out by **Mr. NAVADIP KRISHNA MHATRE** (Regd. No. 139) under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma and published in other form. All the assistance and help received during the course of investigation and the sources of literature have been duly acknowledged by him.

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Chairman,
Advisory committee and
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II Shree II

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THESIS ABSTRACT

Keeping in view the different biotic and abiotic stresses caused to rice crop, salinity stress is one the serious abiotic stress being problematic and major constraint in increasing production and productivity of rice in the context of increasing human population. Therefore, the present study entitled “Introgression of Salt Tolerance Gene in Rice Variety Karjat-6 using Marker Assisted Selection” was undertaken to improve this popular high yielding, fine grained variety against salinity stress. The experiment was conducted during the period of *Rabi* 2012-13 till *Rabi* 2015-16 at Plant Biotechnology Centre, College of Agriculture, Dapoli and Khar Land Research Station, Panvel (M.S.). Rice Variety Karjat-6 was used as recurrent parent while a backcross inbred line (BIL) from the cross Pokkali x IR29 developed at IRRI, Phillipines, Manila was used as donor parent of salt resistance.

In validation of molecular markers for parental polymorphism, 8 polymorphic SSR markers linked to *saltol* QTL were screened to detect polymorphism between the parents. Out of these 8 SSR markers, RM 8094 showed maximum level polymorphism (22 bp) between the parents, Karjat-

6 and FL-478. A total of 100 SSR markers were screened to select markers in background selection for genome recovery of recipient parent. Out of these 100 SSR markers, 30 markers were found polymorphic. The level of polymorphism ranged from 4 bp (RM 3412) to 22 bp (RM 8094). SSR marker RM 8094 showed tight linkage to *saltol* QTL and hence was used in foreground selection of F1, BC_1F_1 and BC_1F_2 population. All the other polymorphic markers were used for background selection.

Several crosses between Karjat-6 and FL-478 were made during *Kharif* 2013-14 to transfer salt tolerance character from FL-478 into Karjat-6. The F_1s were evaluated during *Rabi* 2013 and 13 true F_{1s} from this population were selected by foreground selection with tightly linked SSR marker RM 8094. Backcrossing of the selected F_1s was done to the recurrent parent to get BC_1F_1 population. The BC_1F_1 population was evaluated in *Kharif* 2014 and foreground selection on these plants revealed 28 heterozygous plants. Background selection with 30 polymorphic markers showed highest recipient allele frequency (88.33 %) in plant number 34 which was selected and selfed to produce BC_1F_2 population.

The BC_1F_2 population was evaluated in *Kharif* 2015 and foreground selection on these plants with RM 8094 revealed 51 plants carrying the allele of donor plant. The BC_1F_1 and BC_1F_2 progenies were studied for segregation pattern, the proportion of heterozygous and recurrent parent type plants in BC_1F_1 population closely fitted in Chi Square Ratio 1:1 for goodness of fit. The proportion of heterozygous, donor parent and recurrent parent type plants in BC_1F_2 population closely fitted in Chi Square Ratio 3:1 for goodness of fit. The above interactions were confirmed by amplification pattern of segregating population during foreground and background selection.

In vitro screening of parents and segregating population against salinity revealed Karjat-6 to be susceptible and FL-478 to be tolerant cultivars. BC_1F_2 population showed moderate salt tolerance ability.

Field screening of parents and segregating population confirmed the findings of *in vitro* screening. Karjat-6 showed poor morphological performance as compared to FL- 478 and BC_1F_2 population. The BC_1F_2 population showed characters nearly identical to donor parent.

Plant Na and K uptake was estimated by analyzing leaf samples of Karjat-6, FL-478 and segregating population of BC_1F_2. Rice variety Karjat-6 showed more Na/K ratio (0.57) than FL-478 (0.24) indicating the higher ability of donor parent to tolerate salinity stress. Segregating population of BC_1F_2 showed variable plant Na and K uptake response and the ratio of Na/K was varied from 0.15 to 0.83. This variable response may be due to salt tolerance character being controlled by polygenes or due to the instability of the lines in the population. It may also be regarded as the presence of transgressive segregants in the population.

Findings of this study with molecular markers are useful for the improvement of high yielding rice variety Karjat-6 against salt tolerance by keeping its agronomically superior characters intact. It is possible to identify, isolate and develop NILs, RILs and BILs which could be useful breeding population. Its use at commercial level will form a solution to the problem of salinity stress, low production in saline soils of the region.

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is not only a staple food but also a way of life for millions of people around the world. It is the second largest crop in the world in terms of area and production. It supplies as much as half of the daily calories for half of the world's population. The health benefits of rice include its ability to provide fast and instant energy, stabilize blood sugar levels, and slow down the aging process, while also providing an essential source of vitamin B1 to the human body. Other benefits include its ability to boost skin health, increase the metabolism, aid in digestion, reduce high blood pressure, help weight loss efforts, improve the immune system and provide protection against dysentery, cancer, and heart disease. Rice is a fundamental food in many cultural cuisines around the world.

The various benefits can be found in more than forty thousand varieties of this cereal that is available throughout the world. The two main categories are whole grain rice and white rice. Whole grain rice is not processed very much, so it is high in nutritional value, whereas white rice is processed so that the bran or outer covering is removed, leaving it with less nutritional value. According to the International Rice Research Institute in the Philippines, the nutritional value of rice needs to be improved even more so that it benefits mankind. Rice, being the most dominant cereal crop in most of the world can improve the lives of millions of people who consume it (Anonymous, 2016b).

Rice has shaped the cultures and dietary habits of its cultivators and consumers. The combination of rice and fish in Asian countries has generated the term "rice-fish societies". The combination of rice and legumes characterizes cuisines from Cajun to Mexican to Middle Eastern to Southern European. In Columbia, "rice and beans" is acclaimed as the national food. This basic dish continues to be the sustenance of the poor in many countries.

Oryza sativa was domesticated from the wild grass *Oryza rufipogon* roughly 10,000–14,000 years ago. The two main subspecies of rice: *indica* (prevalent in tropical regions) and *japonica* (prevalent in the subtropical and temperate regions of East Asia) are not believed to have been derived from independent domestication events. Another cultivated species, *Oryza glaberrima* was domesticated much later in West Africa. Recent genetic evidence show that all forms of Asian rice, both *indica* and *japonica*, come from a single domestication event that occurred 8,200–13,500 years ago in the Pearl River valley region of China. Movement to Western India and South to Sri Lanka was also accomplished very early (Anonymous, 2016c).

Rice is estimated to be produced on 161.20 million hectares area worldwide with total milled rice production of 481.23 million tonnes and average productivity of 4.45 t/ha (Anonymous, 2016a). China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines and japan are the top rice producing countries.

In India, rice is the most important and extensively grown food crop, playing a vital role in our national food security. The Indian population has already crossed one billion and is increasing at the rate of 14- 15 million every year. For feeding the population of 1.4 billion by 2020, India will need to produce 301 Mt of food grain in addition to other commodities. India is having largest acreage under rice of 44.0 million hectare and production of 105.0 million tonnes with average productivity 3.58 t/ha (Anonymous, 2016a).

In case of Maharashtra, the area under rice cultivation is 15.13 lakh ha with annual production of 41.71 lakh tonnes rough and 28.78 lakh tonnes milled rice. The Konkan region being major rice growing tract of Maharashtra has productivity 2.75 tonnes ha^{-1} milled rice and 3.83 tonnes ha^{-1} rough rice by production of 15.26 lakh tonnes rough rice (10.53 lakh tonnes milled rice) from 3.83 lakh ha area (Anonymous, 2015).

In addition to an increasing world population, there are numerous reasons for serious concern about sufficient future global production of food from crop plants. First, the availability of arable land is decreasing because soil erosion and degradation. Second, the availability of water for agriculture will decline. Third, global climate changes will not only seriously affect crop growth but will also threaten the conservation of cultivated land. Droughts, storms, floods, heat waves and rises in sea-level are predicted to occur more frequently, and salinity and other soil toxicities are likely to be much more problematic in some areas. In semi-arid regions, reductions in yields of primary crops are predicted in the next two decades for which rice is not an exception. Lastly, demands for bio-fuels as substitutive energy sources will be increased, reducing the land available for cultivating food crops (Takeda and Mastuoka, 2008).

Rice crops are prone to various types of stresses, both biotic and abiotic. Biotic stresses include insect pests, fungus, bacteria, viruses, and herbicide toxicity. Among abiotic stresses, drought, cold, and salinity are also well studied in rice (Ansari *et al.,* 2015). According to the FAO Land and Plant Nutrition Management Service, over 6% of the world's land is affected by either salinity or sodicity. Out of the world's 227 million hectare irrigated area, 20% area is salt affected area. In India, out of 42 million hectare irrigated area, 7 million hectare (17%) area is salt affected area (Ghassemi *et al.,* 1995).

Soil salinity is the most widespread soil toxicity problem in rice growing countries. It is one of the major obstacles to increase crop production under saline soil worldwide. Rice (*Oryza sativa* L.) is a native species to swamps and freshwater marshes. Salinization is becoming an increasingly serious production constraint (Akbar and Ponnamperuma, 1980). If rice production is closely remained stagnated to the present level as increasing the human population, then an increase in salinity resistance is a necessity because a good agricultural land is a limited resource (Toenniessen, 1984). Salt stress is a major constraint across many rice-producing ecosystems worldwide, because of the high sensitivity of modern rice varieties/hybrids to salinity. This forces farmers to continue to grow their traditional landraces with low yield and low grain quality. One-third of rice growing area of the world is affected by excess salt accumulation due to irrational management. The salt tolerant breeding becomes necessary as the farmers easily and quickly respond to replace the seed of new variety. Rice breeding technique is also not cost and time consuming compared to the other techniques of salt reclamation. No new areas can be used for rice farming. Arable land is becoming more and more scarce for various reasons. To cope with these constraints, new rice varieties that combine higher yield potential with superior grain quality are needed (Padolina, 2001).

According to the inherent sensitivity of rice to salt stress, rice is referred as an especially salt-sensitive crop (Maas and Hoffman, 1977; and Reddy *et al.,* 2014). There has been a great interest in developing varieties of rice which are resistant to salinity. However, defining salt tolerance, is very difficult because of the complex nature of salt stress and the wide range of plant responses. As a consequence, the rice response to salinity depends on growth stage. Commonly in most cultivated rice, young seedling is very sensitive to salinity (Heenan *et al.,* 1988 and Lutts *et al.*, 1995). Panicle length, spikelet number per panicle and grain yield were significantly reduced after salt treatments (Heenan *et al.,* 1988 and Cui *et al.*, 1995;). In addition, salinity delayed the panicle emergence and flowering (Khatun *et al.*, 1995) and decreased seed set through reduced pollen viability (Khatun and Flowers and 1995; Khatun *et al.*, 1995). Breeding for salt tolerance has been conducted for the decade by many researchers (Akbar and Ponnamperuma, 1982; Singh *et al.,* 2011 and Huyen *et al.,* 2013). In India, a number of new varieties have been developed and released. However, these varieties have some inferior qualities. Therefore, farmers have adopted a few of them.

With the advent of marker-assisted selection (MAS), a new breeding tool is available to make more accurate and useful selections in breeding populations. MAS allows heritable traits to be linked to the DNA segments that are responsible for controlling that trait. These segments of DNA or QTLs (Quantitative Trait Loci) can be detected through specific laboratory techniques. The most commonly used method is Polymerase Chain Reaction (PCR) that amplify segments of DNA linked to heritable traits such as yield or disease resistance. This method is useful because the DNA that we amplify is different (polymorphic) between cultivars. It is this difference that we use to determine whether the plant has the desired trait or not. The process in which the differential DNA sites (or primer sites) are explored, comes from genetic mapping techniques, i.e. RAPD, microsatellites etc. PCR is an effective method for generating large quantities of a specific DNA sequence from a small amount of starting DNA. This technique is useful for a MAS breeding program because the results are reliable. To learn how MAS works, basic molecular biology principles need to be understood (Datta *et al.*, 2011).

Markers provide essentially 'yes-no' information in an individual, not probability information on a population. The markers have the potential to indicate an unequivocally genotype of a single plant. The DNA sensitivity assays means that the procedure is a non-destructive so far as an individual is concerned. Only a part of the plant sample is required since the information sought is genotypic. It is not necessary to expose the plant to stress, as is the case in assessing the physiological phenotype. The use of DNA-based marker technology is becoming routine and capable of dealing with large numbers of samples. This trend continues to be a certainty method.

To obtain salt tolerant genotypes, a large number of lines are required for selection. The selection based on morphology is laborious and time consuming, has low efficiency and slow for the development of new cultivars. The salt tolerant lines cannot be selected at an early stage of breeding. However, these problems could be overcome by indirect selection using molecular markers linked to tolerant genes by identifying salt-tolerant lines at the early seedling stage. This may lead to a considerable saving in time and space, and reducing the overall cost. Bohnert and Jensen (1996) proposed that salt tolerance might be controlled by several genes. According to this hypothesis, salt tolerance is a quantitative trait, however, molecular markers linked to salt-tolerant genes could be identified using genotypes with salt sensitive and tolerant lines (Xie *et al*., 2000).

Marker assisted selection (MAS), referring to a selection based on a genotype which is not affected by environmental variation or complexities of interaction. It involves plant selection carrying genomic regions that are involved in the expression of traits of interest through molecular markers. With the development and availability of an array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for both traits governed by major genes and quantitative trait loci (QTL) (Babu *et al*., 2004). Marker assisted backcross breeding for improvement of Karjat-6 or fine grain rice is considered as a moderately susceptible cultivar to salinity.

Karjat-6 is a rice variety developed by Dr. B. S. Konkan Krishi Vidyapeeth, Dapoli which is high yielding and has fine grain quality. It is one of the most popular rice cultivar in the Konkan region of Maharashtra. The development of a method to increase the salt tolerance level of Karjat-6 without decreasing good quality such as fineness have been becoming extremely difficult. The conventional breeding technique referring as a backcross breeding is one of the solutions to this problem. By using this method, it is possible to maintain a good characteristic of Karjat-6 while adding a new trait such as salt tolerance into the cultivar. In recent years, the Rice Genome Mapping Programme was completed. This mapping provides valuable and effective information for rice breeding program. Therefore, Karjat-6 development using marker assisted selection was emphasized on markers located on different chromosomes and the markers used in this study were selected from the chromosomal regions in previous studies. Keeping in mind all the previous studies, a research study entitled "Introgression of salt tolerance gene in rice variety Karjat-6 using marker assisted selection" was carried out with the following objectives:

1) Validation of molecular markers for parental polymorphism.
2) Introgression of *saltol* linked gene in rice cultivar Karjat-6.
3) To select the lines linked to the *saltol* gene.
4) Screening of segregating populations *in vitro* and field for salt tolerance.

CHAPTER II

REVIEW OF LITERATURE

Present research entitled "Introgression of Salt Tolerance Gene in Rice Variety Karjat-6 Using Marker Assisted Selection" was aimed to transfer salt tolerance character in fine grain variety Karjat-6. The brief review pertaining to objectives of this study is given below under respective objectives:

Since the development of molecular biology, the potential of molecular breeding (MB) contributing significantly to crop improvement has been controversial. With the identification of the first quantitative trait loci (QTL) in the 1980s, the ability of molecular markers to streamline the selection of complex traits has been oversold because scientists have largely underestimated the impact of gene networks and their interactions on plant phenotype. Some of these limitations have been overcome, due to the development of more sophisticated statistical approaches, which allow characterizing both QTL and the QTL by environment interactions (QEI), as well as the contributions made by plant models. Today, the use of markers to track transgenes or stack favorable alleles determining a significant proportion of the phenotypic variance is routine for many crops. The number of reports asserting the successes of MB in dealing with polygenic traits has been definitely increasing. In addition, it is now generally accepted that the role of MB extends beyond the manipulation of elite alleles at a few loci in bi-parental segregating populations. The modern breeding concept, which includes a combination of phenotypic and molecular selection, needs to evolve new strategies to fully exploit the massive amount of information emerging from the "-omics" technologies and various genome sequencing efforts. QTL, functional genomics, and association studies are complementary approaches, which can quantify the genetic effects of specific alleles at target loci. Once the genetic gain of favorable alleles has been validated in a suitable biological context and environment, allele-based markers can be easily developed and employed. This validation step remains a major bottleneck in the establishment of a large set of markers appropriate for deployment in plant breeding. However, considering the technological and methodological progress achieved in genomics in recent years, it is clear that the potential of MB to complement phenotypic selection and improve crop productivity is set to increase significantly in the near future (Ribaut, 2009).

2.1 Validation of molecular markers for parental polymorphism

Hossain *et al.,* (unpublished), constructed the first genetic map for reproductive stage salt tolerance in rice and demonstrated its utility for molecular mapping of QTLs controlling salinity tolerance-related traits which will be useful in marker assisted selection in the future. From their study, it was concluded that, salinity tolerance in rice varies with the growth stage with seedling and reproductive stages being most sensitive. Tolerance at reproductive stage is most crucial as it determines grain yield. An F_2 mapping population was developed from two varieties; contrasting Cheriviruppu and Pusa Basmati 1 (PB1), contrasting in salt tolerance. One hundred thirty one microsatellite markers polymorphic between the parents were used to construct a linkage map of 1458.5 cM (Kosambi) with a mean inter-marker distance of 11.1 cM. The results suggested pollen fertility, Na+ concentration and Na-K ratio in the flag leaf are the most important mechanisms controlling salt tolerance at reproductive stage in rice.

Lang *et al.* (2001), evaluated one hundred thirty BC_2F_2 lines at seedling stage in the green house at IRRI. Molecular markers associated with both qualitative and quantitative salt tolerance were identified by using 150 SSR primers. This was one of the first reports mentioning salt tolerance gene tagging based on SSR with advanced backcross populations (BC_2F_2). The suggested that, microsatellite markers on chromosome 1 may be used efficiently in rice breeding marker-assisted selection.

Araujo *et al.,* (2002), utilized RAPD technique to identify molecular markers linked to the gene Pi-ar conferring resistance to *Pyricularia grisea* race IB-45 in a somaclone derived from immature panicles of the susceptible rice (*Oryza sativa* L.) cultivar Araguaia utilizing bulked segregant analysis. Of the 240 primers tested, 203 produced amplification products. The two parental DNAs along with the resistant and susceptible bulks of F_2 population were screened using 48 primers that differentiated resistant and susceptible parents. Even though eight primers differentiated the resistant bulk from the susceptible bulk, only one primer was found to be tightly linked (1.7cM) to the resistance gene.

Baloch *et al.,* (2004), noted that, knowledge on the genetic constitution of rice plant has immense importance in plant breeding programmes. The use of molecular markers facilitated the selection process. Markers-assisted selection has provided a reliable source for identifying and selecting the desirable genotype in plant breeding programmes. Molecular marker can save time and labour. It is a laborious job to grow a large number of F_2 populations and practice selection for morphological markers in conventional plant breeding. Molecular markers will be more useful for selection when (1) the phenotype is difficult or expensive to measure directly, (2) genes of similar phenotype are being pyramided into a single line, or (3) markers are being used to select against the donor genome in a backcrossing programme.

Lang *et al.,* (2004), conducted molecular genotyping, in different rice genotypes. DNA survey from these genotypes were evaluated the polymorphism by microsatellite marker RM223 located in chromosome 8. Construction of linkage map based on BC_2F_2 population of twelve crosses was set up to analyze QTL (quantitative trait loci) for salt stress tolerance. Genotyping was done by microsatellite markers. QTL which controlled salt stress were mainly identified in chromosomes 1 and 8. Marker-assisted selection (MAS) was recommended to use microsatellite markers in chromosome 1 for rice improvement to salt tolerance. Ten SSR markers: RM202, RM223, RM231, RM235, RM237, RM214, RM218, RM201, RM232, RM206 expressed the polymorphism to facilitate the identification of tolerant and susceptible genotypes.

Rahman *et al.,* (2004), identified DNA marker for a low Na uptake trait in hexaploid wheat (*Triticum aestivum* L.). Genomic DNA from 15 tolerant and 15 sensitive F_3 plants was extracted. The bulked segregant analysis was used in the random amplified polymorphic DNA (RAPD) technique. DNA polymorphisms were observed using 148 primers. The primer OPZ-10 amplified a 680 bp polymorphic DNA fragment which linked to K:Na ratio trait. It was concluded that the primer OPZ-10 can be used for marker-assisted selection to breed for salinity tolerant wheat.

Zeng *et al.,* (2004), characterized the genetic diversity within a subset of rice germplasm with different adaptations to saline soils using microsatellite markers. A total of 123 alleles were generated at 25 microsatellite loci among the 33 genotypes. Genotypes of *japonica* rice grouped into three clusters and those of *indica* rice grouped into two clusters based on microsatellite markers. Thirty percent of the alleles detected in 20 breeding lines were not identified in the cultivars analyzed. These results indicated that the adaptation of rice to saline soils is different among genotypes with diverse genetic backgrounds. The results of the study suggested that, (1) Improving salt tolerance can be achieved by selecting parental genotypes prior to intercrossing based on microsatellite markers. (2) Phenotypic variation of ion contents in segregating populations can be increased by selecting parental genotypes prior to intercrossing based on microsatellite markers. (3) Different salt tolerance components can be combined into a cultivar by intercrossing genotypes from different microsatellite clusters with diverse salt tolerance mechanisms.

Cuartero *et al.,* (2006), studied a number of strategies to overcome the deleterious effects of salinity on plants. These strategies included using molecular markers and genetic transformation as tools to develop salinity-tolerant genotypes and some cultural techniques. They suggested that, despite innovations like better marker systems and improved genetic mapping strategies, the success of marker-assisted selection has been very limited because, in part, of inadequate experimental design. Since salinity is variable in time and space, experimental design must allow the study of genotype X environment interaction.

Afiah *et al.,* (2007), studied molecular markers as well as the genetic relationships for three hybrids of canola under the different conditions of Maryout Experimental station (Desert Research Center). Twelve polymorphic bands were detected by primer UBC-30. Only one unique band was scored in parent (L5), which was suggested to be used as marker for this genotype. The primer also gave four developed molecular markers for tolerance. Three of them were positive while the fourth molecular marker was considered as a negative marker. Only four primers out of the six primers developed ten molecular markers for salinity tolerance, four of them were positive markers, while the other six were negative ones. It was suggested that, these negative markers could be used as *indica*tors to discard the salt sensitive canola genotypes in breeding programs.

Bertrand *et al.,* (2007), presented an overview of the advantages of MAS and its most widely used applications in plant breeding, providing examples from cereal crops. They also discussed the reasons of why the greater adoption of MAS in the future is inevitable. Achieving a substantial impact on crop improvement by MAS represents the great challenge for agricultural scientists in the next few decades.

Bhowmik *et al.,* (2007), concluded that, microsatellite markers could be efficiently used for identification of salt tolerant rice varieties in marker- assisted breeding and quantitative trait loci analysis. In their study, genotypic evaluation of eleven genotypes of rice including the salt tolerant cultivar Pokkali as check for salinity tolerance was conducted. Three selected SSR primers *viz.,* RM7075, RM336 and RM253 were used to evaluate rice genotypes for salt tolerance. The genotypes having similar banding pattern with Pokkali were considered as tolerant. Phenotypically, three genotypes Pokkali, THDB and TNDB-100 and five genotypes RD-2586, TNDB-1 00, Dhol Kochuri, PNR-519 and Pokkali were identified as salt tolerant at the seedling and reproductive stages, respectively. These genotypes were also identified as salt tolerant genotypically (with markers).

Lang *et al.,* (2008), conducted the mapping and marker-assisted selection for salt tolerance genes in rice. SSR technique combined with selective genotyping was used to salt tolerance in rice. 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). 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, analysis of the genotypes of 93 improved varieties at RM223 locus was done. The results indicated an accuracy of more than 95% in identifying the resistant plants which was similar to that using RM223. It was concluded that, 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.

Sabouri and Sabouri (2008), constructed the linkage map by 74 simple sequence repeat (SSR) molecular markers which covered a total of about 1231.50 cM distance rice genome. An $F_{2:3}$ population derived from the cross between Tarommahalli (*indica*) and Khazar (*indica*) was used to map salt tolerance in rice. Plant stand, chlorophyll content, root and shoot length, fresh weight of root and shoot, dry weight of root and shoot, Na+ uptake, K+ uptake, Na+/K+ ratio related to uptake ions and green leaf area were mapped. Four QTLs for root length under salt stress were detected on chromosomes 1, 4, 7 and 9. Also, two QTLs (on chromosome 9) for dry weight root and three QTLs for ion exchanges (on chromosome 3 and 10) were identified. Tarommahalli alleles in these loci increased salt tolerance. Of these QTLs, the five major QTLs with the very large effect, qRL-7 for root length, qDWRO-9a and qDWRO-9b for dry weight root, qBI-1a and qBI-1b for biomass explained 16.21, 27.43, 25.50, 22.24 and 26.83% of the total phenotypic variance, respectively. All these results reinforced the idea that, new QTLs of this study play an important role in the growth of rice at seedling in Iranian local population under salinity condition.

Bhowmik *et al.* (2009), studied the usefulness of three selected SSR markers; RM7075, RM336 and RM253 to evaluate rice genotypes for salt tolerance. Phenotypic and genotypic evaluation for salinity tolerance was done at the seedling stage.

Suchismita Raha (2009), studied the genetic variation for submergence tolerance in rice and biochemical basis of improved submergence tolerance exhibited by FR13A. Attempts were also made towards marker assisted introgression of *Sub1* locus controlling submergence tolerance in FR13A into a mega variety of TN namely, CO 43. True F_1 hybrids between CO 43 and FR13A were selected through SSR genotyping using the SSR marker RM421 on chromosome 5. Out of 232 SSR markers, 76 showed polymorphism between FR13A and CO 43 which can be used in foreground selection, recombinant selection and background selection.

Titov *et al.,* (2009), stated that, recent advent of molecular markers, microsatellites or simple sequence repeats (SSRs), have been useful in finding salt tolerant rice genotypes. Three selected SSR markers already known to be polymorphic, *viz*., RM7075, RM336 and RM253, were used to evaluate rice genotypes for salt tolerance.

Mohammadi-Nejad *et al.,* (2010), used thirty three polymorphic SSR markers located on chromosome 1 to determine the impact of these markers associated with salt tolerance in rice. In their study, assessment of role of *Saltol* QTL in regards to effects of salinity on plant growth and yield components of different genotypes of rice at different growth stages was done. Cluster analysis of the rice genotypes based on SSR data divided the genotypes into three groups each of which having 12, 8 and 16 genotypes including highly salt-tolerant IRRI elite lines, salt tolerant and moderate tolerant genotypes as well as Pokkali and FL478 sensitive and highly sensitive genotypes, respectively. The impact of chromosome 1 for tolerance to salinity at the seedling stage in rice was emphasized by the results. The SSR marker, RM8094, was found to be superior for analysis of genetic diversity in this study. RM8094 and RM10745 microsatelite markers found to be the most effective markers for discriminating the salinity tolerant genotypes.

Senguttuvel *et al.,* (2010), studied the genotyping through microsatellite markers for salinity tolerance in a set of twenty five genetically divergent genotypes. The polymorphic SSR markers already reported for major *saltol* QTLs were utilized in their studies found highly reproducible. The association of SSR markers *viz;* RM 23, 493 and 8053 for the trait linked to Na+/K+ ratio was regarded as the most reliable marker for marker-assisted selection to identify salinity tolerance in rice. The study revealed that the selection of genetically diverse and resistant genotypes based on association of Na^+/K^+ ratio with molecular markers is reliable.

Ahmadi and Fotokian (2011), used a total of 114, out of 235 simple sequence repeats (SSRs) markers that showed polymorphism in the parents for genotyping of the BILs. A linkage map was constructed with an average interval of 15.3 centiMorgan (cM) between the markers, spanning 1747.3 cM across all 12 rice chromosomes. Using the composite interval mapping (CIM) and a minimum logarithm of the odds (LOD), a total of 14 QTLs were detected as: on chromosome 1 (5 QTLs), 3 (1QTL), 4 (3 QTLs), 5 (2 QTLs), 6 (1 QTL), and 8 (2 QTLs) for all six traits except, Sodium (Na+) in the shoot. A QTL (*QKr1.2*) for K+ content in the root was identified with the highest LOD score (7.8) on chromosome 1. This QTL explicated 30% of the total variation and was identified as a major QTL conferring salt tolerance in rice.

Aliyu *et al.*, (2011), studied tagging and validation of SSR markers to salinity tolerance QTLs in rice. Two F_2 populations the validation studies with 4 *SALTOL* markers on chromosome 1 which failed to detect *SALTOL* QTL in the breeding populations, however, 2 QTLs for leaf diameter were obtained in the *SALTOL* region with Rice Markers (RM)

RM493 and RM3412. These QTLs were significantly associated with salinity tolerance trait. To test the usefulness of microsatellite (SSR) markers associated with *SALTOL* QTL, a collection of 150 diverse rice genotypes were used. 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. It was concluded that, RM493 and RM3412 could discriminate tolerant genotypes based on leaf diameter. These markers could be useful in molecular mapping and marker assisted selections.

Islam *et al.,* (2011), in their study reported the detection of QTLs for salinity tolerance at the seedling stage in a F_2 breeding population derived from the cross between BRRI dhan40 and IR61920-3B-22-2-1 (NSIC Rc106). A total of 260 SSR and two EST markers evenly spread throughout the whole rice genome at 5 Mb intervals were used for parental polymorphism survey. The 90 polymorphic makers were used for QTL mapping for salinity tolerance at seedling stage. QTL analysis using single marker, interval mapping and composite interval mapping detected three major QTLs on chromosome 1, 8 and 10. The position of QTL on chromosome 1 was flanked by RM8094 and RM3412 marker which is in the same region as a previously identified major QTL designated as *Saltol*. However, two other QTLs with relatively large effects were flanked by RM25 and RM210 on chromosome 8, and RM25092 and RM25519 on chromosome 10, and appeared to be novel QTLs. It was concluded that, the markers flanking these QTLs could be useful for molecular marker assisted breeding for salinity tolerance.

Singh *et al.,* (2011), screened a total of 63 rice varieties of different salinity tolerance with seven *saltol* linked SSR (simple sequence repeat) markers. The number of SSR alleles ranged from 3 to 5 and a total of 27 alleles were detected, of which the primer markers AP 3206 and RM 10793 produced the highest number of 5 alleles while RM 493 and RM 562 showed 4 alleles. The RM 3412 marker showed the highest allele frequency. The gene diversity ranged from 0.3427 to 0.7770 with RM 10793 as the highest and RM 3412 as the lowest and with a mean of 0.5786.

Baker (2012), performed Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and inter simple sequence repeats (ISSRs) markers analysis to detect the genetic diversity among 6 new rice lines and 4 cultivars with different responses to drought tolerance and establish specific DNA markers associated with drought tolerance. Among 16 RAPD primers tested, only 5 produced bands polymorphic between lines with an average of 5.2 bands per primer (ranging from approximately 252 to 1232 bp) and 73.02 % were polymorphic. Among the tested ISSR primers, only five amplified polymorphic ISSR loci with an average number of 4.4 bands per primer (ranging from approximately 80 to 813 bp) and the mean percentage of ISSR polymorphism was 90.91.

Vu *et al.,* (2012), i+n their study, applied a MABC (marker-assisted backcrossing) system on foreground selection, recombinant selection followed by background selection for development of Vietnamese rice variety that can tolerate salinity of rised sea water. FL478 was used as a donor to transfer *Saltol* QTL into Bacthom 7 recipient rice cultivar. A total of 368 SSR markers were used to identify 8 markers in *Saltol* locus and 81 markers in other loci with total of 89 (24%) polymorphic markers between the parents, out of which 88 markers were then applied to analyze genotyping of each backross generation with three steps of selection.

Deepti Davla *et al.,* (2013), conducted the mapping and marker-assisted selection for salt tolerance genes in rice with an objective to evaluate genetic diversity among 19 rice genotypes, representing highly tolerant as well as susceptible rice cultivars using SSR markers. Among 39 SSR markers used, 26 SSR marker loci generated polymorphic patterns and a total of 185 alleles were detected. From these 26 SSR markers, 16 SSR markers were located on the *Saltol* region on chromosome 1 of rice. The number of alleles per locus ranged from 3-11 with a mean of 7.1 alleles per locus. The PIC values for 26 SSR markers varied from 0.50 (RM6737) to 0.89 (RM3412) with an average PIC of 6.7. It was concluded that, the SSR markers can detect high polymorphism and are very useful in studying variation among different genotypes.

Diwan *et al.,* (2013), studied identification of Quantitative Trait Loci (QTLs) for early vigour related traits using IR64/Azucena as a reference population. The simple sequence repeat (SSR) markers flanking QTLs in IR64/Azucena and other populations were used for validation in the new populations (BPT5204/A67 and BPT5204/Dodiga). Twenty-two QTLs distributed on chromosomes 1 to 6 were identified for different vigour related traits. The QTLs controlling germination, rate of germination, seedling dry weight and vigour index, shoot length, root length were identified.

Huyen *et al.,* (2013), in their study, focused on developing new rice lines with salinity tolerance and high yield by applying markers assisted selection (MAS). Total of 21 primers in the *Saltol* QTL region were checked with the two parents varieties to identify polymorphic primers for screening the *Saltol* QTL region of the breeding populations.

Lang *et al.,* (2013), used SSR genotyping with selective genotyping to map quantitative trait loci (QTLs) associated with drought tolerance in rice in their study. A total of 229lines (BC_2F_2) were evaluated for drought at flowering (DRF), root dry weight (RDW), and root length (RL). A microsatellite map of this population was constructed with 232 markers to detect the linkage to the target traits. The map covered 2,553.7 cM with an average interval of 10.97 cM between marker loci. Markers associated with drought tolerance were located on chromosomes 2, 3, 4, 8, 9, 10 and 12. QTL mapping was used to determine effects of loci associated with drought tolerance traits. QTLs for morphological attributes related to drought tolerance were also mapped. Chisquare tests ($\chi2$), single maker analysis (SMA), interval mapping (IM) were combined in the QTL analysis. All approaches used for QTL detection obtained similar results. QTLs were identified for drought tolerance with the emphasis on 2 QTLs for RL, and 2 QTLs for RDW. This study provided detailed information on the potential of using marker-assisted selection for drought tolerance.

Khatab and Samah (2013), studied development of molecular marker (s) associated with salt tolerance in barley using Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) markers and usefulness of both markers to detect possible specific markers to be utilized in the barley future breeding programs for salt tolerance. The 10 ISSR primers showed low resolution to distinguish the two barley groups. However, SSR primer HVM09 exhibited a band with molecular size of 125 bp which could be considered a positive molecular marker associated with salt tolerance.

Mahmood *et al.,* (2013), determined genetic diversity of thirteen salt tolerant plant species namely *Pergularia tomentosa, Salvadora oleoides, Malcolmia africana, Peganum hermala, Capparis spinosa, Tamarix aphylla, Prosopis juliflora, Rhazya stricta, Cynodon dactylon, Aerva persica, Cymbopogon jwarancusa, Calotropis procera* and *Salavadora persica* by using RAPD markers.

In total, ten primers from the OPC series were used, out of which nine primers gave reproducible amplification profiles. Out of the nine all except OPC-1, produced polymorphic bands and Numerical Taxonomy and Multivariate Analysis System (NTSYS) revealed 56% similarity among the selected species. Monomorphic bands produced by OPC- 1 were related to some genetic elements involved in managing the life under high salt conditions.

Nisha Kottearachchi (2013), reviewed applied uses of DNA markers in rice breeding and provided the updated information on different types of DNA markers with sequence information. It was concluded that, markers such as SSR, InDel and Perfect markers or functional markers are the main choices of rice breeders nowadays as they are convenient to use and easily discriminable between parents. The limitations of conventional breeding such as linkage drag and lengthy time consumption can be overcome by utilizing DNA markers in breeding.

Ali *et al.,* (2014), studied the genetic diversity and relationships among the salt-tolerant rice landraces cultivated in the coastal districts of Bangladesh using random amplified polymorphic DNA (RAPD) analysis. Polymorphic bands generated with five primers were scored and used for determining polymorphic information contents (PIC) and in deriving a dendrogram using the Jaccard similarity coefficient-based unweighted pair-group method with arithmetic means (UPGMA). The five primers generated 84 reproducible bands of the size range 0.24-1.90 kbp and 73% of the bands were polymorphic. It was concluded that the RAPD markers identified could be useful in developing high-yield salt-tolerant rice strains with improved grain quality.

Anh *et al.,* (2014), identified QTLs controlling the salinity tolerance of rice by using F_2/F_3 population derived from the cross combination between the Chanhtrui (high salinity tolerance) and Khangdan18 (susceptible) rice cultivars. The molecular map constructed covered 192 polymophic SSR markers which distributed on the 12 chromosomes with the total distance 1.797cM, and the average distance between two markers was 9.4cM. 10 QTLs were detected on the chromosomes 1, 3, 4, 6, 7 and 9. Six QTLs (*qSFW-1b-CK, qRK-1-CK, qSN-1-CK, qSTR-4-CK, qSNK-6-CK, qSDW- 7-CK*) had the nagetive AE (*Additive effect*) values which explained Khangdan18 variety contributed to increasing salinity tolerance.

Wang *et al.,* (2014), conducted a study to develop simple sequence repeat (SSR) markers based on cotton salt-tolerant expressed sequence tags. To test the efficacy of these SSR markers, their polymorphism and cross-species transferability were evaluated, and their value was further investigated on the basis of genetic diversity and evolution analysis.

2.2 Introgression of *saltol* linked gene in rice

Hossain *et al.,* (unpublished), developed an F_2 mapping population from two *indica* varieties contrasting in tolerance; Cheriviruppu and Pusa Basmati 1 (PB1). Cheriviruppu is highly tolerant at reproductive stage, while PB1 is sensitive at both seedling and reproductive stages. From their study, it was concluded that, salinity tolerance in rice varies with the growth stage, with seedling and reproductive stages being most sensitive.

Lang *et al.* (2001), developed an advanced backcross population (BC_2F_2) with the parents including OM1706, ChengHui448, FR13A, Type3, Almol3 and Madhukar as the donors of salt tolerance and IR64, IR68552-55-3-2, Tequing as the recurrent parents with good quality traits.

Nguyen *et al.,* (2002), in their study derived a doubled-haploid population from the rice (*Oryza sativa* L.) breeding lines CT9993 and IR62266 to map genes controlling Al tolerance. A genetic linkage map consisting of 280 DNA markers (RFLP, AFLP and SSR) was constructed to determine the position and nature of quantitative trait loci (QTLs) affecting Al tolerance.

Yao *et al.,* (2005), derived an F_2 population from the cross between Jiucaiqing (*japonica*) and IR36 (*indica*) to analyze the inheritance of salt tolerance in rice by genetic model of major-genes plus polygenes, and to map the corresponding QTLs by SSR molecular markers.

Haq *et al.,* (2008), used a mapping population of 120 recombinant inbred lines (RILs) derived from the cross between Co39 (lowland, *Indica*) and Moroberekan (upland, *Japonica*) rice (*Oryza sativa* L.) cultivars to map QTLs associated with shoot growth traits under salinity on all chromosomes of rice.

Lang *et al.,* (2008), conducted the mapping and marker-assisted selection for salt tolerance genes in rice as the salinity stress is the major constraint in rice production. SSR technique combined with selective genotyping was used to salt tolerance in rice. Salt tolerant cultivar AS996 was crossed to IR50404 and 229 recombinant inbred lines (RILs) were produced by single seed descent.

Mohammadi-Nejad *et al.* (2008), developed F_8 recombinant inbred lines (RILs) of Pokkali/IR29 cross, to map a major quantitative trait locus (QTL) for salt tolerance named *Saltol* on chromosome 1 which is responsible for low Na+, high K+ uptake and maintaining Na+/K+ homeostasis in the rice shoots.

Sabouri and Sabouri (2008), developed an $F_{2:3}$ population from the cross between Tarommahalli (*indica*) and Khazar (*indica*) to map salt tolerance in rice.

Zang *et al.*, (2008), developed 99 BC_2F_8 introgression lines (IL) from a cross between IR64 (*indica*) as a recurrent parent and Bi-nam (*japonica*) from Iran as the donor parent and identified QTLs for salt-tolerance (ST) related traits at the seedling and tillering stage.

Janaki Ramayya P. *et al.,* (2009), generated an F_2 and F_3 population from a cross between an alkaline tolerant variety, CSR27 and a sensitive variety, IR28 and ealso valuated a set of 194 F_3 segregants at high (pH 9.7) in sodic microplots at Central Soil Salinity Research Institute, Karnal, Haryana.

Suchismita Raha (2009), Attempted a marker assisted introgression of *Sub1* locus controlling submergence tolerance in variety FR13A into a mega variety of TN namely, CO 43. Screening for submergence tolerance revealed the superiority of FR13A over CO 43 for its ability to withstand 14 days submergence. True F_1 hybrids between CO 43 and FR13A were selected through SSR genotyping using the SSR marker RM421 on chromosome 5. F_2 plants harboring the *Sub1* locus from FR13A were identified by genotyping the population using RM219 which was tightly linked to *Sub1* locus. Foreground selection revealed that 61 F_2 plants were carrying CO 43 allele of RM219, 125 F_2 plants carrying both the alleles (heterozygotes) and 64 F_2s carrying FR13A allele of RM219.

Absa Jaw (2010), developed several near isogenic lines with *rymv1-2* resistant allele and evaluated in the field for their resistance to RYMV in the Republics of Mali and Guinea. The study examined 100 near isogenic lines from BC_2F_7 population, 7 parental lines and 3 checks were screened for RYMV resistance.

Ahmadi and Fotokian (2011), developed 62 advanced backcross-inbred lines (BILs), at the BC_2F_5 generation, from the cross of

Tarome-Molaei (salt tolerant) and Tiqing (Salt sensitive to identify the QTLs involved in salinity stress tolerance, using SSR markers.

Islam *et al.,* (2011), derived an F_2 breeding population from the cross between BRRI dhan40, a moderately tolerant female parent with IR61920-3B-22-2-1 (NSIC Rc106), a highly tolerant male parent to detect QTLs for salinity tolerance at the seedling stage.

Lang *et al.,* (2011), attempted to develop new salt tolerant rice varieties adapted to the Mekong delta region 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, they 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.

Singh *et al.,* (2011), concluded that, marker assisted backcross breeding (MABB) provides a great opportunity for precise transfer of desirable donor segment by minimizing the linkage drag into a recurrent parent. In their lab, MABB was used for incorporating bacterial blight (BB) resistance genes (*xa13* and *Xa21*) into the genetic background of Pusa Basmati1, which led to development of Improved Pusa Basmati 1 (Pusa 1460) as one of the first products of molecular breeding. Further, the parental lines of superfine grain aromatic rice hybrid Pusa RH 10 namely, Pusa 6A, Pusa 6B and PRR78 were improved for resistance to BB and blast by transferring genes *xa13* + *Xa21* and *Pi54* + *Piz5*, respectively. In addition, a major QTL for salt tolerance (*Saltol*) was also being transferred to Pusa Basmati 1121, which is widely grown in Haryana, the state having problem of salinity owing to underground brackish water. In order to develop genetically enhanced donor sources for resistance to biotic (BB, blast and BPH) and abiotic (salt tolerance, and phosphorus uptake) stresses in Basmati background, isogenic lines were being developed for major resistance genes/QTLs for respective stresses in the background of Pusa Basmati 1.

Alam *et al.,* (2012), used Marker Assisted Selection (MAS) technique to develop salt tolerant rice genotypes using molecular markers during June 2009 to November 2010 in the experimental field and Biotechnology Laboratory of Plant Breeding Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. FL-378 was identified as donor or male parent for *saltol* QTL and Binadhan-7 as recurrent or recipient parent which had high yield with short life cycle. Crossing was done between them and 10 F_1 seeds were produced. PCR bands from all the 10 F_1 plants were scored as "H" represented heterozygous alleles for donor and recipient parent. Backcrossing was done to produce 105 BC_1F_1 seedlings.

Cuc *et al.,* (2012), developed a new rice variety from KDDB into the one containing *Sub1* QTL that can tolerate with submergence while maintaining its original characteristics preferred by farmers and consumers. The results proved that MABC aids in the transfer of target segments and may improve the recovery of the recipient genome KDDB if background selection is employed.

Huyen (2012), found that Marker Assisted Selection (MAS) is a tool for enhancing the efficiency of Rice Molecular Breeding. Introgression of Bph3 and BphZ genes in the line IS1.2 into the elite cultivar SL12 was confirmed using MAS combine with conventional breeding. After generations, the most promising rice line KR1(DTE2-3) was selected. The rice line KR1 was shown high resistance level with most of brown planthopper biotypes in Vietnam.

Linh *et al.,* (2012), attempted to improve rice salt tolerance in Bac Thom7 cultivar by using FL478 as a donor parent to introgress the *saltol* QTL conferring salt tolerance into Bac Thom7. Three backcrosses were conducted to transfer positive alleles of *saltol* from FL478 into Bac Thom7.

Rasheed *et al.,* (2012), reported the gene stacking of *Lr10*, *Lr17a* and *Lr27+31* as a resistant combination for spring wheat cultivated in rain fed areas based on the field observations and marker assisted screening. To demonstrate this, a rain fed leaf rust resistant variety *'Chakwal86'*, was genetically characterized following its cross with a susceptible variety '*Inqilab91'*. The parents, NILs and F_2 population were studied in the greenhouse through inoculation and in the field under natural conditions revealed a 3:1 resistance to susceptible ratio, while the $F_{2:3}$ populations revealed a 1:2:1 ratio suggesting the dominant mode of resistance. The individual lines carrying gene combinations as found in *Chakwal86* remained resistant in the field while all those not carrying *Lr17a* in the stack were susceptible. Hence, the gene stack *Lr10*, *Lr17a* and *Lr27+31* was found an effective resistant combination for spring wheat in rain fed areas.

Vu *et al.,* (2012), applied a MABC (marker-assisted backcrossing) system on foreground selection, recombinant selection followed by background selection for development of Vietnamese rice variety that tolerates salinity of rised sea water. The highly salt tolerant FL478 was used as a donor to transfer *Saltol* QTL into Bacthom 7 recipient rice cultivar. The results showed that, the best plants of BC_3F_1 generation carried the segments of the donor (11.16 - 12.6 Mb), which had 96.8% - 100 % of the recipient genome.

Lang *et al.,* (2013), in their study combined SSR genotyping with selective genotyping to map quantitative trait loci (QTLs) associated with drought tolerance in rice. A total of 229lines (BC_2F_2) were derived from the cross of OM1490/WAB880-1-38-18-20-P1- HB and evaluated for drought at flowering (DRF), root dry weight (RDW), and root length (RL).

Huyen *et al.,* (2013), in their study, focused on developing new rice lines with salinity tolerance and high yield by applying markers assisted selection (MAS). For this, crosses between Q5DB x FL478 were made. The individual plants in BC_1, BC_2 and BC_3 generations were analyzed to evaluate the introgression of *Saltol* fragment into Q5DB cultivar. After screening of 3 Back-Cross generations, the best individual plants of BC_3F_1 of the plant numbers QF-3-1, QF-3-2, QF4-3-3, QF6-3 with almost the recipient alleles were selected. Conventional breeding was developed on BC_3F_2 for selection of the new salt tolerance rice lines with high yield, resistance to the biotic stress. The salinity tolerance of the new lines was also performed using the standard system of screening.

Mohammadi *et al.,* (2013), developed an F_2 mapping population from a Sadri/FL478 cross which was exposed to saline field conditions (6–8 dS m−1*)* after the active tillering stage to identify reproductive stage specific QTLs for salinity tolerance.

Anh *et al.,* (2014), in their study attempted to identify QTLs controlling the salinity tolerance of rice by using F_2/F_3 population derived from the cross combination between the Chanhtrui (high salinity tolerance) and Khangdan18 (susceptible) rice cultivars.

2.3 To select the lines linked to the *saltol* gene.

Yao *et al.,* (2005), derived an F_2 population from the cross between Jiucaiqing (*japonica*) and IR36 (*indica*) to analyze the inheritance of salt tolerance in rice by genetic model of major-genes plus polygenes, and to map the corresponding QTLs by SSR molecular markers.

Lang *et al.,* (2008), conducted the mapping and marker-assisted selection for salt tolerance genes in rice as the salinity stress is the major constraint in rice production. 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 produced. With the help of identified SSR primer RM223, they 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. It was concluded that, 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.

Zang *et al.,* (2008), identified QTLs for salt-tolerance (ST) related traits at the seedling and tillering stages using 99 BC_2F_8 introgression lines (IL) derived from a cross between IR64 (*indica*) as a recurrent parent and Bi-nam (*japonica*) from Iran as the donor parent.. It was concluded that, to develop salt tolerant rice variety for both stages by pyramiding of salt tolerance QTLs of different stages or selection against the overlapping QTLs between the two stages via marker-assisted selection (MAS) is effective.

Suchismita Raha (2009), studied the genetic variation for submergence tolerance in rice and biochemical basis of improved submergence tolerance exhibited by FR13A. Attempts were also made towards marker assisted introgression of *Sub1* locus controlling submergence tolerance in FR13A into a mega variety of TN namely, CO 43. True F_1 hybrids between CO 43 and FR13A were selected through SSR genotyping using the SSR marker RM421 on chromosome 5. The F_2 plants harboring the *Sub1* locus from FR13A were identified by genotyping the population using RM219 which was tightly linked to *Sub1* locus. Foreground selection revealed that 61 F_2 plants were found carrying CO 43 allele of RM219, 125 F_2 plants carrying both the alleles (heterozygotes) and 64 F_2s carrying FR13A allele of RM219. Phenotyping of selected F_2 lines confirmed the effect of *Sub1* locus on tolerance against submergence and recovery after desubmergence.

Absa Jaw (2010), in a study conducted at the Africa Rice Center, Cotonou, Benin, developed Several near isogenic lines with *rymv1-2* resistant allele by the Biotechnology Unit of Africa Rice Center and evaluated in the field for their resistance to RYMV in the Republics of Mali and Guinea. The study examined 100 near isogenic lines from BC_2F_7 population, 7 parental lines and 3 checks were screened for RYMV resistance. Results from phenotypic screening identified 20 NILs to be resistance to RYMV B27 isolate. Foreground selection using the gene marker RM252 revealed 22 of the lines showed introgression of *rymv1-2* allele and the rest do not show the resistant gene.

Islam *et al.,* (2011), in their study reported the detection of QTLs for salinity tolerance at the seedling stage identified in a F_2 breeding population derived from the cross between BRRI dhan40, a moderately tolerant female parent with IR61920-3B-22-2-1 (NSIC Rc106), a highly tolerant male parent. Out of total 300 F_2 segregating plants, 93 plants with extreme phenotype for salinity stress response, i.e. tolerant and sensitive, were used for selective genotyping based on of visual seedling stage salt tolerance symptom.

Alam *et al.,* (2012), used Marker Assisted Selection (MAS) technique to develop salt tolerant rice genotypes using molecular markers during June 2009 to November 2010 in the experimental field and Biotechnology Laboratory of Plant Breeding Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. FL-378 was identified as donor or male parent for *saltol* QTL and Binadhan-7 as recurrent or recipient parent which had high yield with short life cycle. Crossing was done between them and 10 F_1 were identifed. Backcrossing was done to produce 105 BC_1F_1 seedlings. Foreground selection on 72 BC_1F_1 plants revealed 33 plants carrying heterozygous allele and were selected for RM21. The selected segregants were subjected to further recombinant and background selections at BC_1F_1 generation. These selected genotypess could be used for further foreground, recombinant and background selections with appropriate markers upto BC_3 generation for the development of salt tolerant rice genotypes.

Huyen (2012), found the efficiency of Marker Assisted Selection (MAS) as a tool for enhancing the Rice Molecular Breeding. Introgression of Bph3 and BphZ genes in the line IS1.2 into the elite cultivar SL12 was confirmed using MAS combine with conventional breeding. After generations, the most promising rice line KR1 (DTE2-3) was selected. The rice line KR1 was shown high resistance level with most of brown planthopper biotypes in Vietnam.

Vu *et al.,* (2012), in their study, applied a MABC (marker-assisted backcrossing) system on foreground selection, recombinant selection followed by background selection for development of Vietnamese rice variety that can tolerate salinity of rised sea water. The highly salt tolerant FL478 was used as a donor to transfer *Saltol* QTL into Bacthom 7 recipient rice cultivar.

Huyen *et al.,* (2013), In their study, focused on developing new rice lines with salinity tolerance and high yield by applying markers assisted selection (MAS). Total of 21 primers in the *Saltol* QTL region were checked with the two parents varieties to identify polymorphic primers for screening the *Saltol* QTL region of the breeding populations. The individual plants in BC_1, BC_2 and BC_3 generations of the Q5DB/FL478 were analyzed to evaluate the introgression of *Saltol* fragment into Q5DB cultivar. After screening of 3 Back-Cross generations, the best individual plants of BC_3F_1 of the plant numbers QF-3-1, QF-3-2, QF4-3-3, QF6-3 with almost the recipient alleles were selected. Conventional breeding was developed on BC_3F_2 for selection of the new salt tolerance rice lines with high yield, resistance to the biotic stress.

Linh *et al.,* (2012), stated that use of marker assisted backcrossing (MABC) to develop a new salt tolerance rice variety is one of the feasible methods to cope with climate change and fast rising sea levels. In their study, to improve rice salt tolerance in Bac Thom 7 cultivar, FL478 was used as a donor parent to introgress the *saltol* QTL conferring salt tolerance into Bac Thom 7. Three backcrosses were conducted to transfer positive alleles of *saltol* from FL478 into Bac Thom 7. The plants number IL-30 and IL-32 in BC_3F_1 population expected recurrent genome recovery of up to 99.2%, 100%, respectively. These selected lines that carried the *saltol* alleles were screened in field for their agronomic traits. All improved lines had *saltol* allele similar to the donor parent FL478, whereas their agronomic performances were the same as the original Bac Thom 7. It showed the success of improving rice salt tolerance by MABC and the high efficiency of selection in early generations. It was concluded that MABC accelerated the development of superior qualities in the genetic background of Bac Thom 7.

2.4 Screening of segregating population *in vitro* and field for salt tolerance

Hollington (1998), described recently-developed screening techniques for use in the development of salt-tolerant and waterlogging-tolerant wheat genotypes and recent advances in breeding for tolerance. Developments in screening under controlled field conditions using sprinkler and drip irrigation systems were described and their benefits and drawbacks noted. The potential for the development of tolerant wheat through hybridization with wild relatives which show enhanced tolerance, including the development of *Tritypyrum* was discussed and progress in this field noted. A screening technique for testing for combined salinity and waterlogging tolerance was also proposed. It was concluded that, although progress has been slow, the availability of the new techniques will lead to much more rapid progress in the future.

Munns and James (2003), tested fast an effective glasshouse screening techniques that could identify genetic variation in salinity tolerance. The objective was to produce screening techniques for selecting salt-tolerant progeny in breeding programs in which genes for salinity tolerance have been introduced by either conventional breeding or genetic engineering. A set of previously unexplored tetraploid wheat genotypes, from five subspecies of *Triticum turgidum*, were used in a case study for developing and validating glasshouse screening techniques for selecting for physiologically based traits that confer salinity tolerance. Specific traits were assessed.

Ali *et al.*, (2004), screened ten advanced rice lines for salinity tolerance at seedling stage using a rapid screening technique. Out of the lines tested, five were found tolerant, four were graded as moderately tolerant and one as susceptible. It appeared that tolerant lines had higher root and shoot ratio at seedling stage thus providing a clue about salt tolerance potential of a genotype. Further comparative studies for salt tolerance in these rice genotypes in artificially saline conditions showed that salinity in general caused a marked reduction in yield and yield components in all the genotypes.

Lang *et al.,* (2004), phenotyping in different rice genotypes during the period of 2000-2003. The genotypes were screened in field, green house and in hydroponics to assess their response to salt stress. The genotypes were evaluated for seedling survival in culture solution (EC= 4 - 8 – 12 dS/m). Phenotyping was done in Yoshida solution with added NaCl under EC of 6 and 12 dS/m at seedling stage.

Lang *et al* (2004), determined the genetic variability of salt tolerance in rice. Yield trials were conducted at nine sites in Bac Lieu and Tien Giang to understand GxE interaction. Salt stress treatment was done in Yoshida solution under the EC of 6 and 12 dS/m at seedling stage. The results showed high yields and stability in some varieties such as: AS 996 and OM 2395.

Rahman *et al.,* (2004), identified DNA marker for a low Na uptake trait in hexaploid wheat (*Triticum aestivum* L.). The individual plants from F_3 population segregating for salinity tolerance and the parents (LU-26S & Rohtas-90) were grown in polyethylene tubes under saline conditions (EC 25 dS m-1) and screened for K:Na ratio, chloride ions and net photosynthesis at the fourth leaf stage. The plants were then transplanted into pots filled with 7 Kg of fertile soil and supplied with optimum water and nutrients until maturity. Correlations of K:Na and net photosynthesis with yield components were calculated.

Zeng *et al.,* (2004), studied phenotyping and characterization of the genetic diversity within a subset of rice germplasm with different adaptations to saline soils using microsatellite markers. Plants of 33 genotypes were grown in sand tanks under greenhouse condition and irrigated with Yoshida nutrient solution. Two salt treatments were imposed with electrical conductivities of 0.9 dSm−1 (control) and 6.5 dSm−1 (6:1 molar ratio of NaCl and CaCl2). Results indicated that the adaptation of rice to saline soils is different among genotypes with diverse genetic backgrounds.

Duangjai (2005), conducted screening of sixteen rice lines and cultivars for salinity tolerance in a screenhouse of Ubon Ratchathani Rice Research Center, Ubon Ratchathani, Thailand during April - June 2001. A modified standard evaluation system (SES) was adopted to record the salt injury scoring of the experimental material.

Natarajan *et al.,* (2005), conducted a field experiment in the saline field of Annamalai University, experimental farm, Annamalai Nagar during *Navarai* season of 2000-2001 to study the grouping of rice accessions for salinity tolerance and yield under coastal environment. Fifteen rice accessions from the International Rice Soil Stress Tolerance Screening Nursery Trial (IRSSTN) of IRRI were used for the study. Based on the grain yield the rice accessions were grouped in to High Yielding Tolerant (HYT), High Yielding Susceptible (HYS), Low Yielding Tolerant (LYT) and Low Yielding Susceptible (LYS) for salinity tolerance. The rice accessions from the high yielding and tolerant group recorded a lower value for the Na:K ratio higher value of grain yield.

Yao *et al.,* (2005), treated rice plants of P_1, P_2, F_1 and F_2 at 5 to 6 leaf stage under 140 mmol/L NaCl for 10 days. Three indices representing the ability of salt tolerance of rice seedlings were measured, including salt tolerance rating (STR), Na+/K+ ratio in roots and dry matter weight of shoots (DWS). In this study reported that, STR, Na+/K+ and DWS were all controlled by two major genes with modification by polygenes.

Bhowmik *et al.,* (2007), phenotypically and genotypically evaluated eleven genotypes of rice including the salt tolerant cultivar Pokkali as check for salinity tolerance. Two setups were maintained for this study *viz.,* the seedling and reproductive stages. Phenotyping at the seedling stage was done in hydroponic system using salinized (EC 12 dS^{-1}) nutrient solution and at the reproductive stage using salinized tap water (EC 6 dS m-^{1}). IRRI standard protocol was followed to evaluate salinity tolerance in rice. Phenotypically, three genotypes Pokkali, THDB and TNDB-1 00 and five genotypes RD-2586, TNDB-1 00, Dhol Kochuri, PNR-519 and Pokkali were identified as salt tolerant at the seedling and reproductive stages, respectively.

Haq *et al.,* (2008), studied phenotyping in mapping population of 120 recombinant inbred lines (RILs) derived from the cross between Co39 (lowland, *Indica*) and Moroberekan (upland, *Japonica*) rice (*Oryza sativa* L.) cultivars to map QTLs associated with shoot growth traits under salinity on all chromosomes of rice. The dilution of salt concentration in shoot tissues by higher vegetative growth is an established mechanism of salt tolerance in plants. The fresh weight of shoots was recorded after 42 days of growth at 100 mol m–3 NaCl + 5.0 mol m–3 CaCl2 in nutrient solution in a flood bench system. They also determined shoot dry weight and shoot fresh/dry weight ratio traits from the above data.

Mohammadi-Nejad *et al.,* (2008), assessed phenotypic response of the collection of 36 diverse rice genotypes to salt stress with EC=12 was under controlled environmental conditions at seedling stage using a visual score of 1 to 9 scale. The results of phenotypic response of rice genotypes to salinity stress at the seedling stage indicated the varied genotypic responses. The genotypes were classified into five groups from highly tolerant (score 1) to highly sensitive (score 9).

Bhowmik *et al.* (2009), studied Phenotyping of 11 genotypes in hydroponic system using salinized (EC 12 dS/m) nutrient solution. IRRI standard protocol was followed to evaluate salinity tolerance. Large variation in salinity tolerance among the rice germplasms was detected. Plant height and total dry matter of tolerant lines were reduced by 19.0 and 40.6%, respectively under salt stress (EC 12 dS/m), whereas those of susceptible lines were reduced by 46.0 and 73.5%, respectively.

Mahmood *et al.,* (2009), suggested that, selection for salinity tolerance in rice can be carried out at an early stage of growth. Salt tolerance of 4 commercial varieties and 17 breeding lines of Basmati rice (*Oryza sativa* L.) was assessed at early growth stage and at maturity in field plots artificially salinzed with NaCl and CaCl2 (1:1 by weight). The average electrical conductivity (EC) of soil was 1.2, 5.2 and 10.5 dS m-1. Forty-five days after sowing (20 days in saline or control conditions), shoot dry weights and sodium (Na) and potassium (K) contents of shoot were determined. At maturity, plant height, number of tillers per plant, panicle length, number of grains per panicle, 1000-grain weight, grain sterility, shoot dry weight, grain straw ratio and grain yield per plant were measured. There was significant variation between genotypes for all the characters studied.

Janaki Ramayya P. *et al.,* (2009), evaluated an F_2 and F_3 population from a cross between an alkaline tolerant variety, CSR27 and a sensitive variety, IR28. Evaluation was done at high pH 9.7 in sodic microplots at Central Soil Salinity Research Institute, Karnal, Haryana. An effort was made to study genetic nature and association between genes/genomic regions conferring alkaline tolerance and DNA based simple sequence repeats (SSRs). Observations recorded included physiological parameters *viz.,* sodium uptake, potassium uptake and alkalinity injury scores during seedling stage. The normal distribution of the physiological traits in the F_3 segregants indicated the polygenic control. In this study, it was also observed that the occurrence of transgressive segregants demonstrated the feasibility of finding more tolerant progenies than existing tolerant parent by employing proper selection from tolerant extremes of population distribution.

Suchismita Raha (2009), studied the genetic variation for submergence tolerance in rice and biochemical basis of improved submergence tolerance exhibited by FR13A. Screening for submergence tolerance revealed the superiority of FR13A over CO 43 for its ability to withstand 14 days submergence. FR13A was found to exhibit greater degree of recovery ability after 14 days of submergence than CO 43. FR13A was found to accumulate significantly higher levels of total carbohydrates than the susceptible CO 43. The quantitative traits *viz.,* days to flowering, plant height, number of tillers/hill, number of panicles/plant, panicle length, number of grains per panicle, 100 grain weight and grain yield per plant were found to show continuous variation within the population.

Titov *et al.,* (2009), conducted evaluation study in rice with the help of Phenotyping and genotyping for salinity tolerance at the seedling stage. Phenotyping was done in hydroponic system using salinized (EC 12 dS/m) nutrient solution following IRRI standard protocol. Large variation in salinity tolerance among the rice germplasms was detected. Salt stress (EC 12 dS/m) reduced seedling height by 19.0% and total dry matter of tolerant lines by 40.6%, whereas, total dry matter of susceptible lines were reduced by 46.0-73.5%. Through phenotypic and genotypic study, three genotypes *viz*., Pokkali, TNDB-100 and THDB were identified as salt tolerant rice genotypes.

Absa Jaw (2010), examined screening 100 near isogenic lines from BC_2F_7 population, 7 parental lines and 3 checks for RYMV resistance in a study conducted at the Africa Rice Center, Cotonou, Benin, Biotechnology Unit of Africa Rice Center. Results from phenotypic screening identified 20 NILs to be resistance to RYMV B27 isolate. It was concluded that, the integration of screen house experiments together with marker-assisted selection would be more efficient and durable for the poor resource farmer.

Cha-um *et al.,* (2010), in their study, photoautotrophically grown seedlings of thirteen genotypes of rice on MS medium and subsequently exposed to 0 (control) or 200 mM NaCl (salt stress) for 14 days. Chlorophyll a (Chla), chlorophyll b (Chlb) and total carotenoids (Cx+c), in the salt stressed leaves of all genotypes decreased significantly, but the extent of the decrease varied among different genotypes. Moreover, growth parameters including shoot height, root length, fresh weight, dry weight and leaf area in salt stressed plantlets of all genotypes were significantly inhibited. The pigment degradation, photosynthetic abilities and growth inhibition in saline regimes were subjected to hierarchical cluster analysis, which lead to the classification of Kumuangluang (KML), Khao Dawk Mali (KDML), Pokkali (POK), HJ, DPY, Chewmaejan 1 (CMJ1), CMJ2, UR1 and Chowho (CH) as salt tolerant and R258, Pathumthani 1 (PT1), IR29 and upland rice 2 (UR2) as salt sensitive.

Genc *et al.,* (2010), in their study, grown a doubled-haploid bread wheat population (Berkut/KrichauV) in supported hydroponics to identify quantitative trait loci (QTL) associated with salinity tolerance traits commonly reported in the literature (leaf symptoms, tiller number, seedling biomass, chlorophyll content, and shoot Na+ and K+ concentrations), to understand the relationships amongst these traits, and determine their genetic value for marker assisted selection.

Mohammadi-Nejad (2010), assessed the role of *Saltol* QTL in regards to effects of salinity on plant growth and yield components of different genotypes of rice at different growth stages. For this, a greenhouse study was conducted to evaluate the response of 30 rice genotypes to three levels of salt stresses (0, 60, 100 mM NaCl) at reproductive stage. The seedling stage response of these genotypes to salinity with electrical conductivity at 12 dSm-1 was also investigated. Pollen viability, number of unfilled and filled grain and grain yield per plant were evaluated. The rice genotypes differed significantly for salt tolerance at seedling stage. The genotypes were also significantly varied for the traits measured at the reproductive stage. The interactions of genotypes × salinity treatments were significant for pollen viability, number of unfilled grain and grain yield. Grain yield reduction due to salinity was more sever for control to 60mM than for 60mM to 100mM. Pollen viability was found to be a robust criterion to screen the genotypes for salt tolerance at the reproductive stage. Thirty rice genotypes divided into 16 different haplotypes based on *Saltol* QTL.

Senguttuvel *et al.,* (2010), studied the genotyping and phenotyping through microsatellite markers for salinity tolerance in a set of twenty five genetically divergent genotypes for Na+/K+ ratio grown under Yoshida solution with 60 and 120 mM NaCl. The study revealed that the selection of genetically diverse and resistant genotypes based on association of Na+/K+ ratio with molecular markers is reliable. These markers can also be used to screen large set of Germplasm collection to identify and discriminate more salt tolerant rice genotypes from susceptible based on sequence homology with already identified salt tolerant rice genotypes, which can be utilized as donors in the breeding programme for generating salt tolerant varieties.

Ahmadi and Fotokian (2011), evaluated 62 advanced backcross-inbred lines (BILs of BC_2F_5 generation derived from the cross of Tarome-Molaei (salt tolerant) and Tiqing (Salt sensitive along with their parents for six parameters *viz.* Sodium (Na+) and Potassium (K+) in roots and shoots and the Na+/K+ ratio, using the modified Yoshida's nutrient solution at an electrical conductivity of 6 and 12 dS/m.

Cattarin Theerawitaya *et al* (2011), investigated Genetic variations associated with salt tolerance in Khao Dawk Mali105 (KDML105), a well-known Thai rice that has high cooking quality and aroma. The M_2 seedlings were screened for salt tolerance in Hoagland solutions containing 171 mM NaCl or 342 mM NaCl. Salt tolerance (ST) lines could withstand for 15 days under salt stress up to 342 mM NaCl, whereas those of sensitive (SS) lines are drastically susceptible to condition of 171 mM NaCl after 5 days of screening.

Singh *et al.,* (2011), screened a total of 63 rice varieties of different salinity tolerance at salinity (electrical conductivity of water: EC~12.0) in hydroponics and with seven *saltol* linked SSR (simple sequence repeat) markers.

Cuc *et al.,* (2012), developed a new rice variety from KDDB into the one containing *Sub1* QTL that can tolerate with submergence while maintaining its original characteristics preferred by farmers and consumers. The results proved that MABC aids in the transfer of target segments and may improve the recovery of the recipient genome KDDB if background selection is employed. The function of QTL *Sub1* was affirmed via submergence tolerant stress in green house and on the field conditions.

Linh *et al.,* (2012), conducted a screening of BC_3F_1 population of a cross between BT7 x FL478 in field for their agronomic traits. Their agronomic performances were the same as the original BT7. The study showed the success of improving rice salt tolerance by MABC and the high efficiency of selection in early generations.

Mansuri *et al.,* (2012), screened 40 rice genotypes in saline soil of electrical conductivity (EC) of 4, 8 and 12 ds/m in vegetative growth stage in 2009. Tolerant genotypes were tested in young seedling stage in hydroponic system and then reproductive stage in 2010. Results shown that vegetative growth was less affected by salt stress in comparison to reproductive stage. Na and Na:K ratio in tolerant genotypes were lower than susceptible genotypes in salt condition in young seedling stage.

Sultana Raziya (2012), used ten rice genotypes to evaluate salt tolerance phenotypically and genotypically at the germination, seedling and reproductive stages under different salinized conditions (50, 100, 150 and 200 mM). At germination stage, under different salinized conditions rice genotypes FL478, BRRI Dhan40 and BRRI Dhan47 were identified as salt tolerant in comparison with Pokkali. Rice genotypes showed wide variations in salinity tolerance phenotypically. The effect of prolonged salinity stress on yield and yield components of rice genotypes at reproductive stage were evaluated. Large variations in salinity tolerance among rice genotypes were detected.

Huyen *et al.,* (2013), in their study, focused on developing new rice lines with salinity tolerance and high yield by applying markers assisted selection (MAS). The individual plants in BC_1, BC_2 and BC_3 generations of the Q5DB/FL478 were analyzed to evaluate the introgression of *Saltol* fragment into Q5DB cultivar. Conventional breeding was developed on BC_3F_2 for selection of the new salt tolerance rice lines with high yield, resistance to the biotic stress. The salinity tolerance of the new lines was also performed using the standard system of screening.

Maryam Foroozanfar (2013), conducted an experiments in order to study the genetic control of salt stress in the model legume, *Medicago truncatula*. The experiment was conducted to study the effect of salt stress on some morpho-physiological parameters in *M. truncatula* genotypes and to determine the eventual use of some traits as tolerance criteria. Genotypes were studied under 6 salinity treatments (0, 30, 60, 90,120 and 150 mM NaCl) in a factorial experiment based on randomized complete blocks with three replications.

Mohammadi *et al.,* (2013), stated that, salinity tolerance in rice is critical at reproductive stage because it ultimately determines grain yield. An F_2 mapping population derived from a Sadri x FL478 cross was exposed to saline field conditions (6–8 dS m−1*)* after the active tillering stage to identify reproductive stage specific QTLs for salinity tolerance.

Anh *et al.,* (2014), identified QTLs controlling the salinity tolerance of rice by using F_2/F_3 population derived from the crossed combination between the Chanhtrui (high salinity tolerance) and Khangdan18 (susceptible) rice cultivars. The results shown that: salinity tolerance was controlled by multiple genes. Fresh and dried shoot weights of F3 rice lines ranged from 0.13g to 0.40g and from 0.05g to 0.12g respectively; Na^+ ion concentration in dry roots of the susceptible lines was higher than those of tolerance lines; K^+ ion concentration in dried shoots and roots of the susceptible lines showed lower than those of tolerance lines; Na^+/K^+ ion ratio in dried shoots and roots of tolerance lines were lower than those of susceptible lines.

CHAPTER III

MATERIAL AND METHODS

Present study entitled "Introgression of Salt Tolerance Gene in Rice Variety Karjat-6 Using Marker Assisted Selection" was undertaken to transfer salt tolerance gene in the popular fine grain cultivar of rice Karjat-6 at Department of Agril. Botany and Plant Biotechnology Centre, Dr. B. S. Konkan Krishi Vidyapeeth, Dapoli during 2012- 2015. In this chapter, the details of different material used and methodologies followed for the study have been presented.

3.1 Material

The experimental material consisted of two rice genotypes (Plate 1). List of genotypes and their description is as below:

Sr. No.	Name of genotype	Agronomic characters	Source
1.	Karjat-6	High yielding, dwarf, fine grain, white kernel colour and susceptible to salinity	Regional Agricultural Research Station, Karjat, Maharashtra.
2.	FL-478	Tolerant to salinity and red kernel colour	IRRI, Manila, Phillipines.

3.2 Programme of research work

Present investigation was conducted at Plant Biotechnology Center, College of Agriculture, Dapoli. The field screening experiment was conducted at Khar Land Research Station, Panvel, Dist- Raigad, Maharashtra. The brief schematic representation of research is as follows:

Details	Period
Selection of Parents	*Rabi*, 2012-13
Validation of Molecular markers	2013-14
Hybridization	*Kharif*, 2013-14
Raising of F_1s, Foreground selection and backcrossing	*Rabi*, 2013-14
Raising of BC_1F_1 population, Foreground Selection, Background selection and generation of BC_1F_2 population	*Kharif*, 2014-15
Raising of BC_1F_2 population, Foreground selection, screening of BC_1F_2 population.	*Kharif*, 2015-16

Conventional hybridization was adopted coupled with Marker Assisted Selection and the programme was conducted in following manner:

KJT-6 X FL-478
⇩
F_1 ⇨ Foreground selection (using markers)
⇩
Selection of heterozygous plants
⇩
BC_1: F_1 X Recurrent Parent (Back cross)
⇩
BC_1F_1 ⇨ Background and Foreground Selection
⇩
Selfing
⇩
BC_1F_2 (Foreground selection)
⇩
Screening for salt tolerance *in vitro* and field

3.2.1 Selection of parental genotypes

Karjat-6 is a popular high yielding variety developed by DBSKKV, Dapoli and was released in the year 2005 which has good agronomic base but it is susceptible to salinity stress. It was used as recipient parent while, FL-478 (an inbred line developed by crossing IR-29 X Pokkali) was used as a donor parent of salinity tolerance.

3.2.2 Validation of molecular markers for parental polymorphism

The DNA was extracted from 15 days old seedling of both the selected parents by using standard rapid method (Doyle and Doyle, 1990). Total of 100 SSR markers (BioResource Biotech Pvt Ltd., Pune) were used for detection of parental polymorphism. The molecular markers which were found polymorphic were used for foreground and background selection. The list of markers used and their sequences are given in following table:

Sr. No.	Primer	Sequence Forward primer	Sequence Reverse Primer	Chr. No.
1	RM 10793	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC	1
2	RM 140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG	1
3	RM 1287	CCATTTGCAGTATGAACCATGC	ATCATGCAATAGCCGGTAGAGG	1
4	RM 562	GGAAAGGAAGAATCAGACACAGAGC	GTACCGTTCCTTTCGTCACTTCC	1
5	RM 7075	GCGTTGCAGCGGAATTTGTAGG	CCCTGCTTCTCTCGTGCAGTCG	1
6	RM-8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA	1
7	RM-3412	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC	1
8	RM-493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG	1
9	RM 212	AAGGTCAAGGAAACAGGGACTGG	AGCCACGAATTCCACTTTCAGC	1
10	RM 302	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG	1
11	RM 3825	CCACTAGCAGATGATCACAGACG	GAGCACCTCATAAGGGTTTCAGC	1
12	RM 323	CAACGAGCAAATCAGGTCAG	GTTTTGATCCTAAGGCTGCTG	1
13	RM 283	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG	1
14	RM 104	GGAAGAGGAGAGAAAGATGTGTGTCG	TCAACAGACACACCGCCACCGC	1
15	RM 315	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	1
16	RM 272	AATTGGTAGAGAGGGGAGAG	ACATGCCATTAGAGTCAGGC	1
17	RM 102	AACTTTCCCACCACCACCGCGG	AGCAGCAGCAAGCCAGCAAGCG	1
18	RM 14	CCGAGGAGAGGAGTTCGAC	GTGCCAATTTCCTCGAAAAA	1
19	RM 154	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	2
20	RM 166	GGTCCTGGGTCAATAATTGGGTTACC	TTGCTGCATGATCCTAAACCGG	2
21	RM 112	GGGAGGAGAGGCAAGCGGAGAG	AGCCGGTGCAGTGGACGGTGAC	2
22	RM 53	ACGTCTCGACGCATCAATGG	CACAAGAACTTCCTCGGTAC	2
23	RM 154	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	2
24	RM 318	GTACGGAAAACATGGTAGGAAG	TCGAGGGAAGGATCTGGTC	2
25	RM 174	AGCGACGCCAAGACAAGTCGGG	TCCACGTCGATCGACACGACGG	2
26	RM 48	TGTCCCACTGCTTTCAAGC	CGAGAATGAGGGACAAATAACC	2
27	RM 231	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG	3
28	RM 85	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC	3
29	RM 130	TGTTGCTTGCCCTCACGCGAAG	GGTCGCGTGCTTGGTTTGGTTC	3
30	RM 338	CACAGGAGCAGGAGAAGAGC	GGCAAACCGATCACTCAGTC	3
31	RM 60	AGTCCCATGTTCCACTTCCG	ATGGCTACTGCCTGTACTAC	3
32	RM 251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC	3

33	RM 22	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTCACTCGTC	3
34	RM 218	TGGTCAAACCAAGGTCCTTC	GACATACATTCTACCCCCGG	3
35	RM 335	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG	4
36	RM 119	CATCCCCCTGCTGCTGCTGCTG	CGCCGGATGTGTGGGACTAGCG	4
37	RM 185	AGTTGTTGGGAGGGAGAAAGGCC	AGGAGGCGACGGCGATGTCCTC	4
38	RM 261	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	4
39	RM 142	CTCGCTATCGCCATCGCCATCG	TCGAGCCATCGCTGGATGGAGG	4
40	RM 303	GCATGGCCAAATATTAAAGG	GGTTGGAAATAGAAGTTCGGT	4
41	RM 280	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAGG	4
42	RM 122	CTTCTTCCGCTTCCTCCCTTCC	TGTACCAGTGCACCGAGAGTTGG	5
43	RM 146	CTATTATTCCCTAACCCCCATACCCTCC	AGAGCCACTGCCTGCAAGGCCC	5
44	RM 178	TCGCGTGAAAGATAAGCGGCGC	GATCACCGTTCCCTCCGCCTG	5
45	RM 413	CCAATCTTGTCTTCCGGATCTTGC	AGATAGCCATGGGCGATTCTTGG	5
46	RM 26	GAGTCGACGAGCGGCAGA	CTGCGAGCGACGGTAACA	5
47	RM 289	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG	5
48	RM 6775	AATTGATGCAGGTTCAGCAAGC	GGAAATGTGGTTGAGAGTTGAGAGC	6
49	RM 6273	CTTCGCACTCCAGTCGCTCTCC	GTTGAGGAGGTGTATGGGCTTGG	6
50	RM 276	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA	6
51	RM 343	CCACGAACCCTTTGCATC	GTGATGATGCGTCGGTTG	6
52	RM 8101	GTGTAGTTACGACCAATGATACGC	TATAATGAGTTCGAGCCGATCC	6
53	RM-225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC	6
54	RM 8225	GCGTGTTCAGAAATTAGGATACGG	GATCTCGCCACGTAATTGTTGC	6
55	RM 253	TCCTTCAAGAGTGCAAAACC	GCATTGTCATGTCGAAGCC	6
56	RM 234	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	7
57	RM 02	ACGTGTCACCGCTTCCTC	ATGTCCGGGATCTCATCG	7
58	RM 70	GTGGACTTCATTTCAACTCG	GATGTATAAGATAGTCCC	7
59	RM 118	CCAATCGGAGCCACCGGAGAGC	CACATCCTCCAGCGACGCCGAG	7
60	RM 325	GACGATGAATCAGGAGAACG	GGCATGCATCTGAGTAATGG	7
61	RM 336	CTTACAGAGAAACGGCATCG	GCTGGTTTGTTTCAGGTTCG	7
62	RM 22709	CGCGTGGGCGAGACTAATCG	CCTTGACTCCGAGGATTCATTGTCC	8
63	RM 547	TTGTCAAGATCATCCTCGTAGC	GTCATTCTGCAACCTGAGATCC	8
64	RM 38	ACGAGCTCTCGATCAGCCTA	TCGGTCTCCATGTCCCAC	8
65	RM 223	GAGTGAGCTTGGGCTGAAAC	GAAGGCAAGTCTTGGCACTG	8
66	RM 310	CCAAAACATTTAAAATATCATG	GCTTGTTGGTCATTACCATTC	8
67	RM 149	GCTGACCAACGAACCTAGGCCG	GTTGGAAGCCTTTCCTCGTAACACG	8
68	RM 152	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG	8
69	RM 337	GTAGGAAAGGAAGGGCAGAG	CGATAGATAGCTAGATGTGGCC	8
70	RM 215	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	9
71	RM 160	AGCTAGCAGCTATAGCTTGCTGGAGATC	TCTCATCGCCATGCGAGGCCTC	9
72	RM 108	TCTCTTGCGCGCACACTGGCAC	CGTGCACCACCACCACCACCAC	9
73	RM 219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	9
74	RM 245	ATGCCGCCAGTGAATAGC	CTGAGAATCCAATTATCTGGGG	9
75	RM-201	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	9
76	RM 484	TCTCCCTCCTCACCATTGTC	TGCTGCCCTCTCTCTCTCTC	10
77	RM 147	TACGGCTTCGGCGGCTGATTCC	CCCCCGAATCCCATCGAAACCC	10
78	RM 333	GTACGACTACGAGTGTCACCAA	GTCTTCGCGATCACTCGC	10

79	RM 171	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG	10
80	RM 311	TGGTAGTATAGGTACTAAACAT	TCCTATACACATACAAACATAC	10
81	RM 271	TCAGATCTACAATTCCATCC	TCGGTGAGACCTAGAGAGCC	10
82	RM-5926	ATATACTGTAGGTCCATCCA	AGATAGTATAGCGTAGCAGC	11
83	RM 5961	GATCAGCAGTGGACGATTCACC	TCTCCTGTATGCTCCTCCTCACC	11
84	RM 1233	ATGGGCACGTGTAATTCATTCG	ATCCTCGAAAGTAGGAGTAGGAAAGC	11
85	RM 332	GCGAAGGCGAAGGTGAAG	CATGAGTGATCTCACTCACCC	11
86	RM 206	ATCGATCCGTATGGGTTCTAGC	GTCCATGTAGCCAATCTTATGTGG	11
87	RM 181	ACGGGAGCTTCTCCGACAGCGC	TATGCTTTTGCCGTGTGCCGCG	11
88	pTA248	AGACGCGGAAGGGTGGTTCCCGGA	AGACCGGGTAATCGAAAGATGAAA	11
89	RM 144	CATGTTGTGCTTGTCCTACTGC	AGCTAGAGGAGATCAGATGGTAGTGC	11
90	RM 167	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTTGTC	11
91	RM 224	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	11
92	RM 309	CACGCACCTTTCTGGCTTTCAGC	AGCAACCTCCGACGGGAGAAGG	12
93	RM 5479	CTCACCATAGCAATCTCCTGTGC	ACTTCGTTCACTTGCATCATGG	12
94	RM 270	GGCCGTTGGTTCTAAAATC	TGCGCAGTATCATCGGCGAG	12
95	RM 101	GTGAATGGTCAAGTGACTTAGGTGGC	ACACAACATGTTCCCTCCCATGC	12
96	RM 20	ATCTTGTCCCTGCAGGTCAT	GAAACAGAGGCACATTTCATTG	12
97	RM 6217	CGCAGATGGAGATTCTTGAAGG	ACAGCAGCAAGAGCAAGAAATCC	12
98	RM 277	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG	12
99	RM 19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	12
100	RM 248	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG	12

3.2.3 Hybridization

After detection of parental polymorphism, staggered sowing and transplanting of the selected diverse parents was done to obtain synchronized flowering. Hybridization was carried out by hand emasculation and pollination. Dusting of pollens was continued for 2-3 days. The pollinated panicles were wrapped by butter paper bag and grain setting was recorded 3-4 days after the last pollination. The fully matured filled seeds were harvested from each panicle and bulked.

3.2.4 Raising of F_1s, Foreground selection and backcrossing

The F_1 hybrids between susceptible and tolerant parent were grown in the field. The true F_1 plants were identified among the hybrid population by foreground selection. The true F_1 plants were used for back crossing to the recipient parent (high yielding susceptible variety). The spikelets of the hybrids (F_1) were emasculated and pollinated using the pollen grains produced from recurrent parent to get BC_1F_1 population.

3.2.5 Raising of BC_1F_1 population, Foreground Selection, Background selection and generation of BC_1F_2 population

The BC_1F_1 population was grown in pots, DNA was extracted and foreground selection was performed using tightly linked marker. Heterozygous plant population was selected. Background selection was performed using other polymorphic markers. Plants showing maximum allelic frequency of background genome were selfed to generate BC_1F_2 population.

3.2.6 Raising of BC_1F_2 population, Foreground selection, screening of BC_1F_2 population

The BC_1F_2 population was sown in pots. DNA was extracted and foreground selection was performed. Plants showing allele of donor parents were selected and used for *in vitro* and field screening against salinity stress. *In vitro* screening was performed using standard protocol of IRRI, Philippines. Visual salt injury level was determined by using modified standard evaluation score (SES) of visual salt injury given by Gregorio *et al.*, 1997. For field screening natural salinity stress conditions were utilized at Khar Land Research Station, Panvel, Dist- Raigad, Maharashtra. Biochemical analysis was performed by estimating Na:K ratio in leaves at Department of Soil Science and Agricultural Chemistry, College of Agriculture, Dapoli.

Screening method:

1. Seeds of Karjat-6, FL-478, F_1, F_2, F_3, $BC1F_2$ and Panvel-3 were surface sterilized with 10% clorox (5.25% w/w sodium hypochlorite) for 30

minutes, then rinsed with distilled water to prevent the infection from fungi or other types of disease and insect.

2. Sterilized seeds from step 1 were incubated in petridishes on germinated paper sheath at room temperature (25-32°C) for 5-7 days.

3. The germinated seeds of each genotype were placed individually in small holds of a thermocol plates with a nylon net supported at the bottom (10 seeds for one genotype). The thermocol plates were floated on rectangular plastic trays half filled with nutrient solution recommended by Yoshida *et al*. (1976) (Appendix II). They were placed in a screenhouse under the natural light of a day/night temperatures at 30-32/25-27 °C.

4. After 14 days of growth in normal nutrient solution, the seedling were subjected to salinization at electrical conductivity value (EC) of 4 dS/ m by added NaCl to the nutrient solution, and to 6 dS/ m two days later. The nutrient solution was renewed once a week, and its pH was maintained daily at 5.8 (adjusted by adding either 1 N NaOH or HCl). The EC measurements were taken daily.

5. Sixteen days after salinization in nutrient solution with 6 dS/ m, salinity stress symptoms were scored according to the standard evaluation system (1- 3: tolerant, 4-5: moderate and 6-9: susceptible) developed at IRRI.

The experiment was carried out at of Plant Biotechnology Center, Dapoli. Aaverage temperature of day/night were approximately 30/25°C. The treatment consisted of 7 rice genotypes and 2 salinity levels; 0 and 6 dS/m.

Modified standard evaluation score (SES) of visual salt injury (Gregorio *et al.*, 1997)

Score	Observation	Tolerance Level
1	Normal growth	Highly tolerant
3	Nearly normal growth; leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately Tolerant
7	Complete cessation of growth ; most leaves dry; some plants dying	Susceptible
9	Almost all plant dead or dying	Highly Susceptible

3.2.7 Estimation of K and Na content in plants

The most commonly used method for K estimation is by flame photometry (Tandon, 2004). It is based on the principle that atoms of some specific element take energy from flame and get excited to the higher orbit. Such atoms release energy of wavelength which is specific to that element and is proportional to the concentration of the atoms of that element.

The procedure for Na is similar to that of Potassium. However a different fitterment for Na has to be used as the radiation emitted by excitation of Na atoms is of a different wavelength.

3.2.7.1 Standard stock solution for Potassium

To prepare a stock solution, 1.9069 gms of analytical grade KCl was dissolved in deionized water and volume was made upto 1 liter. This solution contained 1000 ppm K. From this, 100 ppm K solution was prepared by diluting the 1000 ppm K solution 10 times (10 ml in 100 ml final volume). Final standard solutions of 5, 10, 20, 40, 80 ppm were prepared from 100 ppm stock solution.

3.2.7.2 Estimation of potassium

The plant samples for K estimation were digested by diacid (Nitric acid: Perchloric acid) through wet ashing. The digest was diluted to suitable concentration range between 0 to 100 ppm. The samples were then read in flame photometer at 548 nm wavelength or using filter for K.

3.2.7.3 Standard stock solution for Sodium

To prepare stock solution, 2.541 gms NaCl was dissolved in 1000 ml deonised distilled water to get 1000 ppm Na solution. 10 ml of this stock solution was diluted to 100 ml water to get 100 ppm solution. From this 100 ppm stock solution, a final stock solution of 10, 20, 40, 80 ppm were prepared from 100 ppm stock solution.

3.2.7.4 Estimation of Sodium:

The concentration of Na in plants was determined by wet ashing of samples by diacid (Nitirc acid : Perchloric acid) digestion. The digest was diluted to suitable concentration range between 0 to 100 ppm. The samples were then read in flame photometer at 548 nm wavelength.

3.3 Molecular Analysis:

3.3.1 Plant materials used for DNA extraction

Sr. No	Parents	Generations
1.	Karjat-6	F_1 (Karjat-6 x FL-478)
2.	FL- 478	BC_1F_1
3.		BC_1F_2

3.3.2 Chemicals: List of chemicals used:

Sr. No	DNA isolation	PCR reaction	Gel electrophoresis	Screening
1	Extraction buffer	Taq buffer	Agarose gel	Yoshida nutrient solution
2	CIA solution	Primers	1x TAE	$HgCl_2$
3	0.5M Glucose	$MgCl_2$	EtBr	$NaCl_2$
4	PVP.	dNTPs	Bromophenol blue	HCl
5	Sodium bisulphate	Taq polymerase		NaOH
6	Sodium lauryl sulphate	Double distilled water		
7	Lauryl Sarcosine	DNA		
8	Isopropanol			
9	Alcohol (70%)			
10	TE.			

3.4 Plant DNA extraction: DNA was isolated by the protocol of Doyle and Doyle (1990) with slight modification in buffer composition and concentration.

3.5 DNA Purification: Purification of DNA was done to remove RNA, proteins and polysaccharides which were the major contaminants. RNA was removed by RNase treatment. RNase was added to the DNA sample @100 g ml^{-1} and incubated at 37°C for 1 hour.

3.6 DNA quantification: Concentration of DNA in the sample was determined after electrophoresis with standard DNA ladder.

3.7 DNA Amplification: DNA amplification was carried out by master thermal cycler (Eppendorf PCR Machine). For PCR analysis, SSR markers were used.

3.7.1 Standard reaction mixture for PCR (Appendix III):

Components	Stock concentration	Vol. for one reaction/20 µl
Taq buffer	10X	2.5 l
$MgCl_2$	25 mM	0.5 l
dNTP mix	10 mM	1.0 l
Forward Primer	25nmole	1.0 l
Reverse Primer	25nmole	1.0 l
Taq DNA polymerase	3 U/ l	0.5 l
Template DNA	30-50 ng	1.0 l
Sterile Distilled water	-	12.5 l

3.7.2 Thermal Profile for PCR:

Particular	Temp. (°C)	Period (min.)
Initial Denaturation	94°C	5 min.
Denaturation	94°C	20 sec.
Annealing	53°C - 63°C	45 sec.
Extension	72°C	1 min.
Final extension	72°C	7 min.
Hold	4°C	-

3.8 Agarose gel electrophoresis: The amplified products in PCR reaction were separated by electrophoresis in 2% Agarose gel containing ethidium bromide in 1X TAE buffer and constant voltage of 60 V for 70-80 min.

3.9 Gel Documentation: The images of gels were documented for further analysis using Uvitec Fire Reader Software.

3.10 Data analysis: Based on the banding pattern obtained, the polymorphism for each marker was detected. For foreground selection and background selection, the amplified products for each primer were scored as H for presence of hybrids alleles, R for recurrent parent allele and D for presence of the donor parent allele. The allele frequency (%) was calculated by using the Hardy-Weinburg equilibrium formula:

$$\text{Donor allele frequency (\%)} = \frac{D + 1/2\,H}{N} \times 100$$

OR

$$\text{Recipient allele frequency (\%)} = \frac{R + 1/2\,H}{N} \times 100$$

Where,

D= No. of donor alleles

H= No. of Heterozygous alleles

R= No. of recipient alleles

N= Total number of locus

3.11 Observations recorded:

3.11.1 Validation of molecular markers for parental polymorphism

a. Amplification pattern

b. Size of amplicon

c. No. of alleles

d. Polymorphism

3.11.2 Introgression of *saltol* linked gene

a. No. of crosses made

b. No. of successful crosses

3.11.3 Marker based selection of lines linked to the *saltol* gene

a. No. of true hybrids

b. No. of parental types

c. Amplicon size of fragments

d. Amplification pattern

3.11.4 Screening of segregating population *in vitro* and field for salt tolerance

a. Salt toxicity scoring

b. Survival %

c. Plant height

d. No. of tillers/plant

3.12 Statistical analysis

The results obtained from present experiment were analyzed by chi-square statistics.

$$X^2 = \Sigma \frac{(O-E)^2}{E}$$

Where,

X^2= Chi-square

O= Observed Frequency

E= Expected Frequency

CHAPTER IV

EXPERIMENTAL RESULTS

Experiments were carried out to introgress salt tolerance gene in rice fine garin variety Karjat-6 through marker assisted selection. The results obtained from different experiments pertaining to the respective objectives are presented in this chapter in the following order.

4.1 Validation of molecular markers for parental polymorphism

4.2 Introgression of *saltol* gene in rice variety Karjat-6

4.3 Foreground selection of lines linked to *saltol* gene

4.4 *In vitro* and field screening of segregating population for salt tolerance

4.1 Validation of molecular markers for parental polymorphism

4.1.1 Validation of *saltol* linked markers

The results of validation of molecular markers for parental polymorphism are presented in Table 1. Out of 8 known polymorphic SSR markers, RM-8094 showed maximum polymorphism between the parents and was tightly linked to *saltol* gene which was further used for foreground and background selection (Plate 2).

4.1.2 Validation of markers for background selection for the genome recovery of recipient parent

Out of 100 SSR markers used, 30 SSR markers were found to be polymorphic between Karjat-6 and FL-478 (Table 1) (Plate 2, 2a, 2b, 2c). Among these 30 polymorphic markers, RM 8094 was tightly linked to *saltol* gene which was further used for foreground and background selection. Remaining 29 markers were used for background selection in BC_1F_1 population.

Table 1. List of polymorphic SSR markers

Sr. No.	Marker	Amplicon size		Level of polymorphism
		Karjat-6	FL- 478	
1	RM 8094	197	175	22 bp
2	RM 1233	230	220	10 bp

3	RM 3412	206	210	4 bp
4	RM 493	260	248	12 bp
5	RM 215	190	198	8 bp
6	RM 231	210	222	12 bp
7	RM 253	134	146	12 bp
8	RM 102	450	440	10 bp
9	RM 122	270	279	9 bp
10	RM 302	290	302	12 bp
11	RM 3825	110	100	10 bp
12	RM 303	220	208	12 bp
13	RM 140	196	182	14 bp
14	RM 562	150	159	9 bp
15	RM 5926	100	88	12 bp
16	RM 8225	270	281	11 bp
17	RM 181	150	140	10 bp
18	RM 333	190	200	10 bp
19	RM 248	139	150	11 bp
20	RM 223	185	175	10 bp
21	RM 335	150	140	10 bp
22	RM 283	171	165	14 bp
23	RM 206	191	197	6 bp
24	RM 276	103	94	9 bp
25	RM 154	62	56	6 bp
26	RM 149	290	282	12 bp
27	RM 14	198	190	8 bp
28	RM 1928	240	248	8 bp
29	RM 10793	50	44	6 bp
30	RM 5961	200	210	10 bp

4.2 Introgression of *saltol* gene in rice variety Karjat-6

For the introgression of *saltol* gene in rice variety Karjat-6, panicles of Karjat-6 were hand emasculated and pollinated with the pollens of FL-478. A total of 25 crosses were made which produced 20 F_1 seeds (Table 2). Out of these, only 17 seeds were germinated when sown in pots. DNA from individual plant were isolated and foreground selection with SSR marker RM-8094 was done. It identified 13 true hybrids and 4 Karjat-6 type individuals (Plate 3). For the generation of BC_1F_1 seeds, 13 hybrids were backcrossed to Karjat-6. Total 13 crosses were made which produced 70 BC_1F_1 seeds (Table 3). Out of these 70 BC_1F_1 seeds, 58 plant were germinated when sown in pots. DNA from individual plant were isolated and foreground selection was performed. Out of the 58 BC_1F_1 plants 28 plants were carrying heterozygous alleles and 30 plants showed Karjat-6 type alleles (Plate 4). Background selection on these 28 plants was performed using polymorphic SSR markers. Recipient allele frequency of these 28 plants was calculated using the Hardy-Weighnburg formula (Table 4). Plant no. 34 had the highest recipient allele frequency which was selfed to generate 228 BC_1F_2 seeds. Out of these 228 BC_1F_2 plants, 204 plants germinated when sown in pots. DNA from individual plants were isolated and foreground selection was done with the help of RM 8094. Foreground selection revealed 96 heterozygous plants, 57 recipient parent type plants and 51 donor parent type plants (Table 2) (Plate 5,6,7). Segregation pattern of BC_1F_1 and BC_1F_2 plant population was studied based on the banding pattern obtained in foreground selection with the help of chi-square test (Table 5). In BC_1F_1 population, 1:1 (Heterozygous : recessive) frequency was observed indicating salt tolerance was controlled by dominant resistant gene. Whereas, in BC_1F_2 population, 3:1 (Heterozygous + dominant : Recessive) ratio was observed. Thus, chi-square test confirmed the results of expected frequency in BC_1F_1 and BC_1F_2 generation.

Table 2. Number of crossed seed obtained after hybridization.

Cross	Total no. of crosses made/ panicles emasculated	Total no. of crossed seed obtained	Total no. of selfed seed obtained	No. of Seeds used for evaluation	Seeds germinated	Heterozygous types	Recurrent parental types (Karjat-6)	Donor Parental types (FL-478)
Karjat-6 X FL-478	25	20	--	20	17	13	4	--
F_1 X Karjat-6	13	70	--	70	58	28	30	--
BC_1F_1 (selfing)	1	--	228	228	204	96	57	51

4.3 Selection of lines linked to *saltol* gene

For the identification and selection of hybrids and heterozygous population in BC_1F_1 generation, RM 8094, a tightly linked SSR marker to *saltol* gene was used. Out of the 20 F_1 seeds obtained, 17 seeds were germinated and foreground selection with SSR marker 8094 identified 13 true hybrids which were selected and used for backcrossing with recurrent parent Karjat-6 (Table 2). Backcrossing of selected 13 hybrids to Karjat-6 produced 70 BC_1F_1 seeds, from which 58 plant were germinated. Foreground selection on these 58 BC_1F_1 plants showed 28 plants carrying heterozygous alleles which were selected for further background selection and those with parental type were discarded (Table 2). Background selection on these 28 plants was performed using polymorphic SSR markers. Recipient allele frequency of these 28 plants was calculated using the formula. Plant no. 34 had the highest recipient allele frequency (Table 4) which produced 228 BC_1F_2 seeds upon selfing. From these 228 BC_1F_2 seeds, 204 plants were germinated which revealed 96 heterozygous plants, 57 recipient parent type plants and 51 donor parent type plants (Plate 5,6,7) on the basis of foreground selection with tightly linked SSR marker, RM 8094. Those plants having donor parent type alleles were selected and were used to study further screening and stable integration of *saltol* gene.

Table 3. List of BC_1F_1 seeds produced

Sr. No.	**F_1**	**Recurrent parent**	**BC_1F_1 seeds**
1	1	Karjat-6	4
2	2	Karjat-6	7
3	3	Karjat-6	3
4	4	Karjat-6	8
5	5	Karjat-6	4
6	6	Karjat-6	2
7	7	Karjat-6	7
8	8	Karjat-6	2
9	9	Karjat-6	5
10	10	Karjat-6	10
11	11	Karjat-6	9
12	12	Karjat-6	7
13	13	Karjat-6	2
		Total	70

Table 4. Recipient allele frequency (%) of BC$_1$F$_1$ population with 30 SSR markers observed polymorphic in background selection.

Sr. No.	Plant No.	R	H	D	Recipient Allele%	Rank background
1	1	18	12	0	80.00	10
2	2	20	10	0	83.33	6
3	3	15	15	0	75.00	16
4	4	11	19	0	68.33	22
5	6	17	13	0	78.33	12
6	9	22	8	0	86.67	2
7	13	10	20	0	66.67	23
8	15	9	21	0	65.00	25
9	16	14	16	0	73.33	17
10	17	13	17	0	71.67	19
11	19	12	18	0	70.00	20
12	20	6	24	0	60.00	28
13	22	20	10	0	83.33	5
14	23	13	17	0	71.67	18
15	24	19	11	0	81.67	8
16	32	16	14	0	76.67	15
17	33	20	10	0	83.33	4
18	**34**	**23**	**7**	**0**	**88.33**	**1**
19	41	11	19	0	68.33	21
20	42	21	9	0	85.00	3
21	44	18	12	0	80.00	9
22	46	16	14	0	76.67	14
23	48	17	13	0	78.33	13
24	49	8	22	0	63.33	26
25	50	7	23	0	61.67	27
26	51	9	21	0	65.00	24
27	53	19	11	0	81.67	7
28	56	17	13	0	78.33	11

Table 5. Seggregation pattern of BC$_1$F$_1$ and BC$_1$F$_2$ population based on banding pattern in foreground selection

Cross	Name of cross	BC$_1$F$_1$ observations	X^2	X^2	P

no.		Heterozygotes	Karjat-6 type		ratio	value	value
I	Karjat-6/ FL-478 // Karjat-6	28	30		1 : 1	0.06	0.20-0.10
		BC$_1$F$_2$ observations					
		FL-478 type	**Heterozygotes**	**Karjat-6 type**			
II	BC$_1$F$_1$ / BC$_1$F$_1$ (Selfing)	51	96	57	3 : 1	0.95	0.20-0.10

Table value at 1 d.f. and 0.05% level of significance = 3.84

4.4 *In vitro* and field Screening of segregating population for salt tolerance

In vitro screening of segregating population was conducted at Plant Biotechnology Center, College of Agriculture, Dapoli. Both the parents *viz;* Karjat-6, FL-478 and segregating generations *viz;* F$_1$, F$_2$, F$_3$, BC$_1$F$_2$ plants were used for screening along with Panvel-3 (Plate 8). Panvel-3 variety was used as a control. Results of this study are presented in Table 6 in which the salt injury scoring recorded after the screening is presented. The results revealed that, FL- 478 had the lowest salt injury scoring (2) as 8 out of 10 plants survived after 16 days of salinization (Plate 9). Hence, it was categorized as tolerant cultivar. Genotype Karjat-6 had the highest salt injury score (8) and categorized as susceptible genotype. All the other genotypes showed salt injury score in between 5 to 6 and were categorized as moderately tolerant genotypes.

Table 6. Salt injury scoring of genotypes after *in vitro* screening.

Sr. No.	Genotypes	Tolerance Scoring	Tolerant Grouping
1.	Karjat-6	8	Susceptible
2.	FL-478	2	Tolerant
3.	F$_1$	5	Moderately Tolerant

4.	F_2	6	Moderately Tolerant
5.	F_3	5.5	Moderately Tolerant
6.	BC_1F_2	5	Moderately Tolerant
7.	Panvel-3	5.5	Moderately Tolerant

Field screening for salinity was conducted at Khar Land Research Station, Panvel. Both the parents *viz;* Karjat-6, FL-478 and segregating generations of F_2, F_3 and BC_1F_2 population were screened at natural conditions of Khar Land Research Station, Panvel (Plate 10). Salinity level was in between 4 to 5 ds/m during the growing period and 6 to 7 ds/m during seedling stage. Observations were recorded on plant survival, plant height and number of tillers per plant and the data is presented in Table 7. Results revealed that, FL-478 had highest survival percentage (80%) while, Karjat-6 showed lowest survival percentage (40%). Maximum height was recorded in BC_1F_2 (86 cms) and FL-478 (85 cms) genotypes. Maximum number of tillers per plant were recorded in F_3 genotype (13.10) followed by BC_1F_2 (11.88), F_2 (11.50) and FL-478 (11.0) genotype. Lowest number of tillers per plant were recorded in Karjat-6 (9.0) genotype. It clearly indicates FL- 478 is tolerant and Karjat-6 is susceptible to salinity stress. It also showed that segregating population is having the salt tolerance ability of donor parent.

Table 7. Response of genotypes to salinity in Field Screening.

Sr. No.	Genotypes	Survival %	Height (cms)	No. of tillers/plant
1	Karjat-6	40%	75.0	9.00
2	FL-478	80%	85.0	11.00
3	F_2	53%	81.0	11.50
4	F_3	60%	74.0	13.10
5	BC_1F_2	60%	86.0	11.88

- Figures in table are average values for characters.

4.4.1 Estimation of sodium (Na) and potassium (K) uptake from plants

During field screening, leaf samples from parents, F_2, F_3 and BC_1F_2 population were studied to estimate sodium and potassium uptake in plants. Leaf samples from individual plants were taken and oven dried for 2 days. These leaf samples were then finely ground and 0.5 g of sample was used for estimation of Na and K uptake using flame photometry at Department of Soil Science and Agricultural Chemistry, College of Agriculture, Dapoli. Results of these analysis are presented as follows

4.4.1.1 Estimation of Na and K content in parental plants

The data regarding Na, K content and Na/K ratio in parental plants is presented in Table 8. It was observed that, the donor parent had low Na uptake (12.2 ppm) as compared to recipient parent (28.8 ppm). The K content was also high in donor parent (51.6 ppm) as compared to recipient parent (50.6). The Na/K ratio in donor parent was low (0.24) as compared to recipient parent (0.57) indicating high Na exclusion capacity of donor parent.

Table 8. Estimation of Na and K content in parental plants

Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio	Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio
FL-478	12.2	51.6	**0.24**	Karjat-6	28.8	50.9	0.57

4.4.1.2 Estimation of Na and K content in F_2 plants

The data with regards to estimation of Na, K content and Na/K ratio of F_2 plants is given in Table 9. It was observed that, Na content varied in between 15.4 ppm (Plant No. 11) to 56.4 ppm (Plant No. 14). The highest Na content was recorded in Plant No. 33 (56.4 ppm) followed by Plant No. 24 (43.3 ppm). Whereas, K content in plants ranged between 31.9 ppm (Plant No. 6) to 81.0 ppm (Plant No. 13). The highest K content (81.0 ppm) was showed by Plant No. 32 followed by Plant No. 21 (80.9 ppm). The Na/K ratio was observed in between 0.20 (Plant No. 23) to 1.10 (Plant No. 6). The minimum Na/K ratio was observed in Plant No. 23 (0.20) followed by Plant No. 11 and 19 (0.25). Only single plant (Plant No. 6) showed more than 1.0 NA/K ratio. Whereas, only single plant (Plant No. 23) showed Na/K ratio of 0.20 which was less than even donor parent- FL-478 (0.24). There were total 22 plants which showed the Na/K ratio in between the parents range (0.24 – 0.57).

Table 9. Estimation of Na and K content in F_2 plants

Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio	Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio
1	38.2	51.5	0.74	17	29.7	71.5	0.42
2	18.7	44.6	0.42	18	19.2	63.8	0.30
3	16	47.9	0.33	19	15.7	63.1	**0.25**
4	34	48.8	0.70	20	17.4	51.2	0.34
5	32.6	43.1	0.76	21	34.0	**80.9**	0.42
6	35.0	**31.9**	**1.10**	22	27.6	54.1	0.51
7	15.6	59.4	0.26	23	13.0	66.3	**0.20**
8	37.8	74.7	0.51	24	**43.3**	69.9	0.62
9	23.2	46.6	0.50	25	35.0	64.8	0.54
10	27.6	49.1	0.56	26	25.9	58.4	0.44
11	15.4	61.3	**0.25**	27	21.4	56.2	0.38
12	23.5	47.6	0.49	31	39.7	60.9	0.65
13	25.7	51.2	0.50	32	25.6	**81.0**	0.32
14	33.8	42.5	0.80	33	**56.4**	63.0	0.90
15	20.75	38.75	0.54	34	28.6	59.7	0.48
16	37.92	66.2	0.57				

4.4.1.3 Estimation of Na and K content in F_3 plants

The data with regards to estimation of Na, K content and Na/K ration of F_3 plants is given in Table 10. It was observed that, Na content varied in between 9.4 ppm (Plant No. 16) to 134.7 ppm (Plant No. 30). The highest Na content was recorded in Plant No. 30 (134.7 ppm) followed by Plant No. 14 (64.3 ppm). Whereas, K content in plants ranged between 35.0 ppm (Plant No. 12) to 97.5 ppm (Plant No. 8). The highest K content (97.5 ppm) was showed by Plant No. 8 followed by Plant No. 14 (76.0 ppm). The Na/K ratio was observed in between 0.15 (Plant No. 16) to 3.44 (Plant No. 30). The minimum Na/K ratio was observed in Plant No. 16 (0.15) followed by Plant No. 8 (0.21). Three plants showed more than 1.0 NA/K ratio (Plant No. 30, 12 and 11). Whereas, two plants (Plant No. 16 and 8) showed Na/K ratio lesser than donor parent- FL-478 (0.24). There were total 16 plants which showed the Na/K ratio in between the parents range (0.24 – 0.57).

Table 10. Estimation of Na and K content in F_3 plants

Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio	Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio
1	25.5	63.2	0.40	17	39.2	42.7	0.92
2	19.7	46.5	0.42	18	28.9	46.3	0.62
3	22.8	50.9	0.45	21	35.1	44.8	0.78
4	19.3	35.6	0.54	22	34.6	42.5	0.81
5	25.9	43.9	0.59	25	12.6	41.6	0.30
6	40.5	49.6	0.82	26	19.9	43.7	0.46
7	12.5	37.3	0.34	27	28.5	59.9	0.48
8	20.3	**97.5**	**0.21**	28	25.5	49.3	0.52
9	34.4	41.4	0.83	29	32.2	51.6	0.62
10	11.1	47.2	0.24	30	**134.7**	39.2	**3.44**
11	60.9	42.4	1.44	32	32.3	60.1	0.54
12	61.1	**35.0**	**1.75**	35	14.5	59.7	0.24
13	19.3	44.5	0.43	40	13.8	45.9	0.30
14	**64.3**	**76.0**	0.85	41	19.1	59.9	0.32
15	27.4	65.1	0.42	47	32.8	43.8	0.75
16	**9.4**	64.6	**0.15**	48	28.3	41	0.69

4.4.1.4 Estimation of Na and K content in BC_1F_2 plants

The data with regards to estimation of Na, K content and Na/K ration of BC_1F_2 plants is given in Table 11. It was observed that, Na content varied in between 7.5 ppm (Plant No. 29) to 43.9 ppm (Plant No. 24). The highest Na content was recorded in Plant No. 24 (43.9 ppm) followed by Plant No. 3 (41.5 ppm). Whereas, K content in plants ranged between 22.2 ppm (Plant No. 42) to 72.9 ppm (Plant No. 17). The highest K content (72.9 ppm) was showed by Plant No. 17 followed by Plant No. 4 (66.5 ppm). The Na/K ratio was observed in between 0.15 (Plant No. 41) to 0.83 (Plant No. 27). The minimum Na/K ratio was observed in Plant No. 41 (0.15) followed by Plant No. 29 (0.17). No any plant showed more than 1.0 NA/K ratio. Whereas, 6 plants (Plant No. 5, 25, 28, 29, 37 and 41) showed Na/K ratio lesser than donor parent- FL-478 (0.24). There were total 36 plants which showed the Na/K ratio in between the parents range (0.24 – 0.57).

Table 11. Estimation of Na and K content in BC_1F_2 plants

Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio	Plant No.	Na content (ppm)	K content (ppm)	Na/ K ratio
1	25.3	63.3	0.40	27	15.6	18.9	**0.83**
2	25.7	64.7	0.40	28	8.3	43.6	0.19
3	**41.5**	64.5	0.64	29	**7.5**	43.0	**0.17**
4	28.2	**66.5**	0.42	30	13.0	43.5	0.30
5	10.1	49.4	0.20	31	21.3	**29.6**	0.72
6	23.3	51.0	0.46	32	15.1	46.7	0.32
7	26.5	47.9	0.55	33	22.6	37.3	0.61
8	20.8	43.8	0.47	34	28.2	34.3	**0.82**
9	13.8	35.1	0.39	35	12.2	49.7	0.25
10	28.0	55.1	0.51	36	15.1	51.2	0.29
11	17.1	40.3	0.42	37	**8.4**	46.9	0.18
12	28.0	55.5	0.50	38	19.8	39.4	0.50
13	15.3	60.2	0.25	39	17.5	35.1	0.50
14	23.3	55.2	0.42	40	23.3	46.2	0.50
15	23.1	49.4	0.47	41	8.7	56.7	**0.15**
16	13.2	48.0	0.28	42	8.6	**22.2**	0.39
17	23.4	**72.9**	0.32	43	17.5	46.9	0.37
18	23.4	55.0	0.43	44	36.3	56.1	0.65
19	16.3	49.1	0.33	45	26.2	49.6	0.53
20	13.1	41.7	0.31	46	16.2	53.4	0.30
21	23.9	43.1	0.55	47	25.3	61.5	0.41
22	34.4	57.6	0.60	48	10.0	36.9	0.27
23	20.6	47.3	0.44	49	26.1	33.5	0.78
24	**43.9**	66.2	0.66	50	13.2	46.6	0.28
25	12.0	59.3	0.20	51	19.6	43.1	0.45
26	12.2	40.2	0.30				

CHAPTER V

DISCUSSION

Rice (*Oryza sativa* L.) is not only a staple food but also a way of life for millions of people around the world. It is the second largest crop in the world in terms of area and production. It supplies as much as half of the daily calories for half of the world's population. China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines and japan are the top rice producing countries.

Konkan region of Maharashtra consists of Thane, Mumbai, Raigad, Ratnagiri and Sindhudurg districts. The Konkan region is divided into two meteorological sub-divisions i.e. North Konkan and South Konkan. In North Konkan, the climate is warm and humid with more than 2500 mm of rain. The maximum and minimum temperatures are 30^0 to 31^0 C and 22^0 to 24^0 C respectively. This sub-division consists of Thane, Raigad, Mumbai, Navi-Mumbai districts. The general topography is hilly to undulating but mostly less than 300 m in altitude. On the other hand, South Konkan, This sub-division consists of Sindhudurg and Ratnagiri districts. This region receives very high rainfall ranging between 3,000 to 4,000 mm, 90% of it during June to October (100-110 days). The relative humidity varies from 90-95% in *Kharif* and 80-85% in *Rabi* season.

As the Konkan region lies in the coastal region, the soils of this region are lateritic and saline alkaline soils. The saline alkaline soils, Depending upon the causes of their formation, these are also of two types *viz.*, a) coastal saline soils, and b) saline-alkaline soils of the inland region. The coastal saline soils all along the west coast in the districts of Sindhudurg, Ratnagiri, Thane and Raigad. In the immediate vicinity of the coast or creeks, the soil impregnated with salts (NaCl) from sea to the extent of 0.2 per cent. In spite of the high rainfall to the extent of about 3000 mm and above in these areas, they are suitable only for growing saline resistant rice varieties which is the common crop of the region. These soils are locally known as *khar* or *khajan* lands. The saline-alkaline soils of the inland areas are developed as a result of various causes, such as 1) rise of subsoil water level due to indiscriminate irrigation over a long period of time, 2) use of subsoil water level due to indiscriminate irrigation over a long period of time, 3) Occurrence of impervious subsoil 4) salt bearing subsoil strata (Thaware *et al.*, 2009).

Soil salinity is the most widespread soil toxicity problem in rice growing countries. It is one of the major obstacles to increase crop production worldwide. Rice is a native species to swamps and freshwater marshes. If rice production is closely remained to the present level as increasing the human population, then an increase in salinity resistance is a necessary because a good agricultural land is a limited resource (Toenniessen, 1984).

Conventional breeding methods of variety development such as selection, back cross breeding etc. are time consuming, laborious and requires more years for the development of improved population. 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 (Aliyu *et al.* 2011).

Marker Assisted Selection (MAS) is the indirect selection not based on the trait itself but based on the markers linked to it which is not affected by the environmental variations or complexities. With the advent of Marker Assisted Selection technology, a new breeding tool is available to make more accurate and useful selections in breeding populations.

There are two major groups of genetic markers: morphological markers and molecular markers. Morphological markers are usually visually characterized phenotypic traits such as flower colour, seed shape, and plant pigmentation. The major disadvantage of morphological markers is that they may be less in number and are influenced by environment or developmental stage of the plant, whereas molecular markers are the most widely used type of markers as they are unlimited in number. The efficiency of conventional plant breeding is greatly improved by the advent of molecular markers. In this case, selection is made directly on molecular markers linked to the trait rather than the trait itself; however the molecular marker should be tightly linked to the trait of interest. After identification of tightly linked molecular markers, they can be used to select a large germplasm at the early growth stage. Polygenic or quantitative traits are those traits which are controlled by more than one gene. The regions within genomes which are associated with the quantitative traits and have more effect on the expression of a trait as compared to other regions are called quantitative trait loci (QTLs). In Molecular breeding, DNA markers are mainly used for the development of linkage maps which have been very helpful for the identification of genomic regions (QTLs) involved in the control of simple as well as quantitative traits through QTL analysis. QTL mapping is the process of constructing linkage maps and performing QTL analysis for the detection of chromosomal regions associated with quantitative traits. This approach has wide scope for the genetic dissection of complex traits such as salt and drought tolerance that ensured better crop performance. In MAS, phenotypic selection is made on the basis of a presence of a DNA marker which is independent of the environmental effects and the developmental stage of the plant. It is more efficient, effective, reliable and cost effective compared to the conventional plant breeding methodologies. The use of DNA markers is becoming very common in the improvement of rice (Lang *et al*., 2001; Lang *et al*., 2011; Linh *et al*., 2012), wheat (Rahman *et al*., 2004), maize (Monsento, 2007), barley (Khatab and Samah, 2013), pulses (Datta *et al*., 2011), oilseeds (Afiah *et al*., 2007; Holbrook *et al.,* 2011) and horticultural crop species (Cuartero *et al*., 2006).

Therefore, the present study entitled "Introgression of Salt Tolerance Gene in Rice Variety Karjat-6 using Marker Assisted Selection" was undertaken with a view to transfer salt tolerance character from a backcross inbred line, FL-478 into a fine grain variety Karjat-6.

Validation of molecular markers for parental polymorphism

The primary resource of plant breeding programs is the genetic variability available within germplasm closely related to the crop of interest. However, the success of crop improvement programs is highly reliant on the power and efficiency with which this genetic variability can be manipulated. DNA marker technologies offer plant breeders the potential of making genetic progress more precisely and more rapidly than phenotypic selection. Genetic markers also offer the possibility of addressing previously unattainable goals. This is now equally true for both temperate and tropical crops. In particular, progress in model systems offers the possibility of supporting both substantial and rapid developments in tropical crop improvement, which would not be conceivable through traditional methods. There is now a wide array of DNA marker assays, each having a different set of advantages in any particular application. However, with the development of the polymerase chain reaction (PCR) there is now a range of assays which have great potential for molecular breeding.

Simple sequence repeat (SSR), also known as microsatellite, variable number of tandem repeats (VNTR) or sequence tagged microsatellite site (STMS) markers. This technique provides high quality, highly consistent results and remains the assay of choice for marker assisted selection.

In the present investigation, popular rice variety, Karjat-6 was used as a recurrent parent which is susceptible to salinity stress and a back cross inbred line (BIL), FL-478 derived from the cross of Pokkali X IR29 was used as a donor of salinity tolerance. The aim of the study was to improve the high yielding, fine grain variety Karjat-6 for salt tolerance. Before starting any molecular breeding programme, a degree of genetic diversity between the two parents needs to be estimated for the success of breeding programme. Molecular markers help to serve this purpose by detecting the level of polymorphism between the parents for a particular trait or trait of interest.

In order to check the polymorphism between the selected parents, 8 polymorphic SSR markers linked to *saltol* QTL were validated. The results showed RM 8094 to be more polymorphic (22 bp) and hence was used as a marker for foreground selection in F_1, BC_1F_1 and BC_2F_2 generations. A total of 100 SSR markers were selected from the different chromosomes of rice to test the polymorphism and background selection for recovery of recurrent parent genome. Genomic DNA was extracted from both the parents and Polymerase Chain Reaction was performed at the specific annealing temperature of individual marker. Out of 100 SSR markers, 30 markers showed polymorphism between the parents. The polymorphism level ranged from 4bp (RM 3412) to 22 bp (RM 8094) between the parents. Hence, RM 8094 showed tight linkage for the *saltol* gene in FL-478. Similar results for parental polymorphism in rice genotypes for salt tolerance with SSR marker RM 8094 have been reported (Mohammadi-Nejad *et al.,* 2008; Islam *et al.,* 2011; Singh *et al.,* 2011; Anh *et al.,* 2014 and Reddy *et al.,* 2014). Many workers have used SSR markers for determining polymorphism to facilitate MAS in salt tolerance breeding of rice (Lang *et al.,* 2001, 2008; Bhowmik *et al.,* 2007; Ahmadi and Fotokian, 2011; Aliyu *et al.,* 2011; Alam *et al.,* 2012). SSR markers are now the most widely used markers in major cereals as they are highly reliable, reproducible, co-dominant in inheritance, relatively simple and cheap to use and also highly polymorphic (Bertrand *et al.,* 2008). SSR marker analysis is promising to identify major gene locus for salt tolerance that can be helpful to plant breeders to develop new cultivars or to improve existing cultivars in their few characteristics. SSR markers are now being predominantly used to map and introgress agronomically important Quantitative Trait Loci (QTLs) into popular varieties using marker assisted backcrossing (MABC).

Introgression of saltol linked gene in rice variety Karjat-6

Introgression means the transfer of one or few genes from a one genotype to another to improve it in one or few characters. This can be achieved by conventional plant breeding. One agronomically superior genotype lacking in characters like tolerance to biotic or abiotic stresses is crossed with other agronomically inferior genotype having biotic and abiotic stress resistance ability or sometimes with wild relative. To transfer resistance or simply resistance breeding, involves backcross breeding method. Backcross breeding method requires several backcrosses to be done to introgress the desired resistant gene in the existing variety which is expensive, time consuming and labourious.

Marker Assisted Selection (MAS) or Marker assisted backcrossing (MABC) is an indirect selection of traits which are difficult to score (technically or due to environmental-specific expression), expressed late in the growth season and/or traits which are a primary selection criterion but occur infrequently in breeding populations. The benefits of this approach are compounded when multiple traits can be simultaneously selected. When introgressing traits from exotic germplasm, DNA markers can be used for indirect selection of that trait plus simultaneous selection of offspring with the least amount of other genomic material from the exotic parent.

Phenotypic evaluation of salinity tolerance in rice is time consuming and expensive. First plants must be evaluated in controlled environment chambers using culture solution systems. Results are then confirmed under glasshouse pot experiments using saline irrigation. Finally, the selected advanced generation lines are then evaluated in replicated field trials costing around $30 per genotype. Most significantly, the success of field evaluation is highly unpredictable, particularly in terms of the level of salinity stress applied. PCR-based markers have been identified for genes underlying salinity tolerance in rice. Using these markers for indirect selection of this trait costs less than 10% of phenotypic evaluation and allows a magnitude more plants to be screened in a season. In the IRRI breeding program, the use of this approach is reducing the breeding cycle by many years (Crouch, 2000).

In the present study, FL-478 was used as donor parent to introgress salt tolerance gene in a popular rice variety Karjat-6 which is susceptible to salinity stress. Several crosses were made by transferring pollens from FL-478 on to stigma of emasculated flowers of Karjat-6. The true hybrids (F_1s) were selected (Foreground selection) by using tightly linked SSR marker RM 8094. A total of 13 true hybrids were detected in foreground selection. These hybrids were then backcrossed to recurrent parent produce BC_1F_1 population. Foreground selection in this population detected 28 heterozygous and 30 recurrent parent type plants. Background selection on the selected 28 heterozygous plants was performed with the help of 29 polymorphic markers which showed recipient allele frequency ranging from 61.67% to 88.33%. Plant No. 34 showed highest recipient allele frequency (88.33%). Similar result were observed by Singh *et al.,* 2011 in improvement of Basmati rice for different biotic and abiotic stresses. This plant was then selfed to produce BC_1F_2 population.

Backcrossed lines could be developed rapidly in which only a small amount of foreign DNA is linked to the gene/genes of interest. After the first backcross all lines with the gene/genes of interest could be screened with markers linked with interested traits. Those lines in which a crossover occurred at this marker would be selected. Thus, the amount of wild DNA could be reduced. The key to this procedure is to have markers that are polymorphic between the two parents that are closely linked to the gene of interest. Similar results to introgress resistance genes and foreground selection using SSR markers in rice are reported by Lang *et al.,* (2011); Singh *et al.,* (2011); Alam *et al.,* (2012); Cuc *et al.,* (2012); Huyen, (2012); Linh *et al.,* (2012); Reddy *et al.,* (2014).

Selection of Lines linked to *saltol* gene

With the recent development in the field of molecular markers analysis, it is now feasible to analyze both the simply inherited traits as well as the quantitative traits and identify the individual genes controlling the traits of interest. Molecular markers are now used to tag Quantitative Trait Loci (QTLs) and evaluate their contributions to the phenotype by selecting for favourable alleles at these loci in a Marker-Assisted Selection (MAS) scheme that aim to accelerate genetic advancement in rice. Individual genotypes with target gene in a segregating population can be identified with the assistance of DNA markers. Identifying molecular markers that are linked to genes controlling salinity tolerance could facilitate selection in rice for this low heritable trait. MAS in rice is faster, more efficient and cost-effective than conventional screening under saline field conditions. Progress in rice breeding for salt tolerance constituted the identification of the major locus conferring a salt tolerance gene at different growth stages (Bhowmik *et al.,* 2007).

The most effective and environment friendly management strategy of combating different biotic and abiotic stresses is exploitation of host plant resistance. Under such situations, marker assisted backcross breeding (MABB) offers a great opportunity for transferring desirable genes from unadapted donors to otherwise agronomically superior cultivars having specific weakness. With the availability of molecular markers and saturated molecular genetic map of rice, MAS has now become feasible both for traits controlled by major genes as well as QTLs. Given the information available, molecular markers can be successfully deployed for foreground as well as background selection in order to confirm the presence of resistance gene(s) and speedy recovery of recurrent parent genome (RPG) and phenome.

In our study, a backcross inbred population (BC_1F_2) was produced by selfing the selected BC_1F_1 plant which showed highest allelic frequency in background selection. A total of 204 plants were screened with the help of SSR marker RM 8094 to select lines linked to *saltol* gene. Foreground selection on the BC_1F_2 population revealed 51 plants carrying *saltol* allele in these plants. It indicates that, the segregation of *saltol* linked gene carries in the successive generations which are expected at Mendelian fashion. These results were in accordance with the results obtained by Lang *et al.,* (2008); Singh *et al.,* (2011); Alam *et al.,* (2012) and Linh *et al.,* (2012).

In vitro and field screening of segregating population for salt tolerance

Selection of highly salt tolerant genotypes within a species can be expected to provide useful material for experimental comparisons with the salt sensitive ones. It is intrinsic to a screening procedure that the phenotype (which is evaluated) should adequately reflect the potential of the genotype; and salinity resistance has been treated as if it were a single factor (which could include a genetically linked group of factors). If this were not true, i.e. if salt tolerance in non-halophytes were the product of several independent factors, there follow two important conclusions. Firstly, there would be cause to believe that salt resistance in rice can be increased beyond the present phenotypic range because there is no reason to expect that, in the absence of selection pressure, current varieties have evolved the optimal combination of characters for salt resistance. Secondly, such characters will commonly be cryptic, i.e. the genotype for one may not on its own influence the phenotype sufficiently for that phenotype to be selected in a screening process (Natarajan *et al.,* 2005).

In this study, phenotyping of parents *viz;* Karjat-6 and FL-478, F_1 hybrids and segregating population of F_2, F_3 and BC_1F_2 was done *in vitro* to check the response of these genotypes to salt stress along with the local check Panvel-3. A modified Standard Evaluation System (SES) suggested by Gregorio *et al.,* 1997 was followed. The results of *in vitro* screening revealed visual salt injury scoring in the range of 1 to 9. The lowest salt injury score (2) was recorded by donor parent FL-478 while, highest salt injury score (8) was recorded in recurrent parent, Karjat-6. Local check and all the other genotypes showed salt injury score in the range of 5 to 6. The result indicated that, the donor parent FL-478 to be tolerant genotype while recurrent parent Karjat-6 to be susceptible genotype to the salinity. Local check Panvel-3 and segregating population showed moderate salt tolerance ability. These results were in close conformity with the results obtained by Ali *et al.,* 2004; Mohammadi-Nejad *et al.,* 2008; Bhowmik *et al.,* 2009; Aliyu *et al.,* 2011; Anh *et al.,* 2014) in rice.

Field screening for salinity tolerance was conducted in natural field salinity conditions at Khar Land Research Station, Panvel using both the parents *viz;* Karjat-6 and FL-478 and segregating population of F_2, F_3 and BC_1F_2. Observations were recorded on survival %, plant height and number of tillers at reproductive stage. Leaf samples of these plants were analysed for Na^+ and K^+ uptake (ppm).

In case of survival %, highest survival % (80%) was observed in FL-478 followed by F_3 and BC_1F_2 (60%) plants. Lowest survival % (40%) was recorded in Karjat-6 plants.

In case of plant height, highest plant height (86 cm) was observed in BC_1F_2 plants followed by FL-478 (85 cm). Lowest plant height was observed in F_3 plants (74 cm).

In case of number of tillers per plant, highest number of tillers were observed in F_3 plants (13.10) followed by BC_1F_2 plants (11.88). Lowest number of tillers were observed in Karjat-6 plants (9.0).

Plant leaf Na^+ and K^+ estimation was done and Na^+/K^+ ratio was calculated. In case of parents, FL-478 showed low Na^+/K^+ ratio (0.24), while Karjat-6 showed high Na^+/K^+ ratio (0.57).

In case of F_2 plants, Na^+/K^+ ratio ranged from 0.20 to 1.10. Highest Na^+/K^+ ratio was observed in Plant No. $F_{2:6}$, while lowest was in Plant No. $F_{2:23}$.

In case of F_3 plants, Na^+/K^+ ratio ranged from 0.21 to 3.44. Highest Na^+/K^+ ratio was observed in Plant No. $F_{3:30}$, while lowest was in Plant No. $F_{3:8}$.

In case of BC_1F_2 plants, Na^+/K^+ ratio ranged from 0.15 to 0.83. Highest Na^+/K^+ ratio was observed in Plant No. $BC_1F_{2:27}$, while lowest was in Plant No. $BC_1F_{2:41}$.

Field screening gave the results in which donor parent showed good performance for survival %, plant height and number of tillers per plant. On the other hand, recipient parent Karjat-6 showed poor response for survival %, plant height and number of tillers per plant. It indicated that, FL-478 is resistant to salinity and Karjat-6 is susceptible to salinity. Segregating population of F_2, F_3 and BC_1F_2 showed variable response for different characters studied under salinity stress. In some parameters, segregating population was superior to resistant parent. It may be due the presence of transgressive segregants in the population or instability of lines under study.

Both the parents and segregating population of F_2, F_3 and BC_1F_2 was evaluated for Na^+ and K^+ uptake in plants as it is an indicator of salt tolerance mechanism in plants. The result of Na^+/K^+ ratio in parents showed 0.24 Na^+/K^+ ratio in donor parent while in Karjat-6 the Na^+/K^+ ratio was 0.57. It may be due to the FL-478 being more capable of taking K+ ions during the growing phase and excluding more Na^+ ions. On the contrary Karjat-6 had lesser K^+ ion uptake. The ratio of Na^+/K^+ in segregating population was in range of 0.15 to as high as 3.44. This may be due to the presence of transgressive segregants in the population or instability of lines in the study. To avoid these kind of results in advanced generations of breeding population, more of number of SSR markers needed to be screened and backcrossing of selected lines with the recurrent parent is necessary to recover maximum genome of recipient parent. There is also a chance that the salt tolerance mechanism is governed by polygenes and due to this these genes may have accumulated these variable results for Na^+/K^+ ratio. Similar results were also reported by Duangjai (2005); Mahmood *et al.,* (2009) and Janaki Ramayya P. *et al.,* (2009).

CHAPTER VI

SUMMARY AND CONCLUSION

The present investigation entitled "Introgression of salt tolerance gene in rice variety Karjat-6 using marker assisted selection" was conducted at Plant Biotechnology Center, College of Agriculture, Dapoli and Experimental Farm of Khar Land Research Station, Panvel (M.S.) during the period from *Rabi*, 2012-13 to *Rabi*, 2015-16 in respect of the fulfillment of following objectives:

1. Validation of molecular markers for parental polymorphism.
2. Introgression of *saltol* linked gene in rice cultivar KJT-6.
3. To select the lines linked to the *saltol* gene.
4. Screening of segregating population *in vitro* and field for salt tolerance.

Validation of molecular markers for parental polymorphism:

- The investigation comprised of popular high yielding, fine grained variety, Karjat-6 which is salt susceptible as a recurrent parent while a backcross inbred line (BIL), FL-478 developed by cross of Pokkali x IR29 as a donor parent.
- Among the 8 different *saltol* linked polymorphic SSR markers used, RM 8094 showed maximum polymorphism (22 bp) which was selected as a marker for foreground selection in F_1, BC_1F_1 and BC_1F_2 population.
- Out of 100 SSR markers used, 30 SSR markers were found polymorphic between the parents.
- The polymorphic markers showed the polymorphism in the range of 4 bp (RM 3412) to 22 bp (RM 8094).

- SSR marker RM 8094 was used in foreground selection while all the other polymorphic SSR markers were used for background selection.

Introgression of *saltol* linked gene in Karjat-6:

- Successful transfer of *saltol* gene was achieved in Karjat-6 as 13 true hybrid plants were produced after hybridization.
- With the help of backcross in selected hybrids, 58 BC_1F_1 plants were produced.
- Maximum parental allele frequency was found in Plant no. 34 (88.33%) in background selection.
- Selfing in selected plants was affected to obtain 204 BC_1F_2 plants.

Selection of lines linked to *saltol* gene:

- In F_1 population, 13 true hybrids were detected and selected with the help of foreground selection.
- Foreground selection on BC_1F_1 plants detected 28 heterozygous plants at *saltol* locus.
- Foreground selection in BC_1F_2 plants detected 51 plants carrying *saltol* QTL.

***In vitro* and field screening of segregating population for salt tolerance:**

- *In vitro* screening of parents and segregating population showed Karjat-6 to be susceptible parent, FL-478 to be tolerant parent while BC_1F_2 and local check showed moderate salt tolerance abiity.
- Field screening of parents and segregating population showed Karjat-6 to be susceptible parent and FL-478 to be tolerant parent. While, BC_1F_2 showed salt tolerance ability nearly similar to tolerant parent FL-478.
- Na and K uptake in plants was determined and it showed high Na^+/K^+ ratio in Karjat-6 while FL-478 showed low Na^+/K^+ ratio indicating Karjat-6 to be salt susceptible and FL-478 to be salt

tolerant. BC_1F_2 plants showed variable response regarding Na^+/K^+ ratio which may be due to association of polygenes for saltol QTL or presence of transgressive segregants in the population.

Conclusion:

The present study revealed that, marker assisted selection (MAS) for the transfer of salt tolerance in rice is very effective. It could be concluded that, Marker Assisted Selection for introgression of salt tolerance gene in rice variety Karjat-6 is useful tool for improving its ability against salinity stress. The SSR marker RM 8094 found more suitable for foreground selection of all populations carrying *saltol* linked allele. For background selection of similar population, 30 markers were polymorphic and these markers showed desired level of allelic frequency for recovery of recurrent parent genome. An *in vitro* and field screening data showed reliability of marker assisted selection (MAS) for introgression. But, at the same time more robust and stringent screening strategy needs to be adopted and more number backcrosses in selected lines are necessary to recover more recipient parent genome in selected lines. Hence for specific breeding programme, marker assisted selection is very effective, quick and reliable method for improvement of salt tolerance level in desired genotypes of rice.

Findings of this study with the help of molecular analysis will be helpful for the improvement of high yielding Karjat-6 variety against salt tolerance by keeping its fine grain character intact. It is possible to identify, isolate and develop NILs, RILs and BILs for salt tolerance of the variety Karjat-6. Their use at commercial level will form a solution to the problem of salinity stress, low production in saline soils of the region.

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*Originals not seen