"लवण सहनशीलता लिया पूसा बासमती ११२१ का चिह्नक सहायता से सधुार"

"Marker assisted improvement of Pusa Basmati 1121 for salinity tolerance"

Naresh Babu N.

DIVISION OF GENETICS
INDIAN AGRICULTURAL RESEARCH INSTITUTE
NEW DELHI - 110012

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Marker assisted improvement of Pusa Basmati 1121 for salinity tolerance

By

Naresh Babu N.

A thesis

Submitted to the Faculty of Post Graduate School,

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in

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Approved by:

Chairman :	**Dr. A.K. Singh**	_____
Co-Chairman:	**Dr. K.V. Prabhu**	_____
Members :	**Dr. N.K. Singh**	_____
	Dr. Madan Pal	_____

Division of Genetics
Indian Agricultural Research Institute
New Delhi – 110 012, India

Dr. A. K. Singh
Professor

CERTIFICATE

This is to certify that the thesis entitled **"Marker assisted improvement of Pusa Basmati 1121 for salinity tolerance"** submitted to the Faculty of Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of requirement of degree of **Doctor of Philosophy in GENETICS,** embodies the results of a bona fide research work carried out by **Mr. Naresh Babu N** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information, as has been availed of in this work, has been duly acknowledged.

Date: 02.07.2014
Place: New Delhi

(**Dr. A.K. Singh**)
Chairman, Advisory Committee

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Any research for that matter is not one man's show, it is a congregated performance done collectively. It would certainly be wrong on my part if I claim I have carried entire research on my own without any body's help. Hence it is my sincere duty to acknowledge the persons who directly or indirectly involved or gave supporting hands while doing the research or preparation of this manuscript. Without my supporters this thesis would not have taken its present shape. I thank one and all.

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CONTENTS

Sl. No.	Title	Page No.
1	Introduction	01-03
2	Background	04-25
3	Material and Methods	26-45
4.1	Research Paper I	46-57
4.2	Research Paper II	58-78
4.3	Research paper III	79-94
4.4	Research Paper IV	95-100
5	Discussion	101-108
6	Summary and Conclusion	109-111
	Abstract	112-117
	Bibliography	I - XXVII
	Appendix	I - VI

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4.4	Research Paper IV	95-100
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6	Summary and Conclusion	109-111
	Abstract	112-117
	Bibliography	I - XXVII
	Appendix	I - VI

Dedicated to my
beloved Parents

1 Introduction

Salinity is one of the major factors limiting productivity of crops including rice around the world. Soil salinity is often accompanied by osmotic imbalance, mineral deficiency and toxicity that have adverse effects on crop growth (Asch et al. 1999; Ahmad and Prasad 2012). Rice is cultivated in more than 115 countries across the world, of which Asia's share is more than 91%. Majority of the Asian rice production zone is confined to South and Southeast Asia wherein salinity stress during various stages of crop growth affects 47 M ha (20%) of the rice area (Abbas et al. 1994), which consists of warm humid coastal regions and marshy inlands (Vinod et al. 2013).

Basmati, the unique quality rice, is a nature's gift to Indian sub-continent notable for its grain quality and delicate nuanced flavour. A superior Basmati variety Pusa Basmati 1121 (PB1121), developed by the Indian Agricultural Research Institute. New Delhi, occupies more than 60 per cent of the total Basmati rice area in India and almost 80 per cent of the Basmati rice area in the state of Haryana, which has sizable proportion of rice lands affected by salinity. PB1121 commands a premium in the market over other Basmati rice varieties because of its excellent grain and cooking quality traits. However, this variety is sensitive to soil salinity, a major constraint to sustainable production of Basmati rice in Haryana and the neighbouring areas of Punjab.

Rice is sensitive to salinity, particularly during the seedling stage (Maas and Hoffman 1977).The earlier set benchmarks (Maas and Grattan 1999; Hanson et al. 1999) indicate that rice yield decreases 12% for every unit (dS m^{-1}) increase in ECe above the threshold tolerance of 3.0 dS m^{-1} (Maas 1990). Generally, rice plant is very sensitive to salt accumulation at seedling stage and less sensitive at reproductive stages (Lutts et al., 1995). However, Zeng et al., (2001) reported that the early reproductive stages *i.e.*, panicle initiation or pollination (Khatun and Flowers 1995) are the most salt sensitive growth stages affecting the formation of grains and grain quality. In contrast, Khan et al., (1997) reported that rice is more tolerant to salt at germination than other stages. Salt tolerance is a complex trait to measure (Munns 2002) because phenological screens demand huge labour, space and laborious experiments coupled with ambiguity created by high environmental influence, which make them impractical for screening large number of genotypes (Flower and Yeo 1997; Gregorio et al., 2002; Yamaguchi and Blumwald 2005; Ismail et al., 2007; Thomson et al., 2010).

In the Basmati rice germplasm, only one variety namely, CSR30 (Yamini) is reported to have some degree of salinity tolerance (Singh et al., 2005), however the mechanism and genomic region involved in conferring salinity tolerance in CSR30 is not yet well characterized. Conversely, in non-Basmati rices, remarkable variation exists for salt tolerance, which provides great opportunities to improve salt stress tolerance through genetic means (Akbar et al., 1987; Flowers and Yeo 1981). Although initial progress in breeding for salt tolerance in rice through conventional means was slow, accelerated development of molecular marker technologies in recent times and their rapid application in construction of genetic linkage map, molecular dissection of the important complex traits has aided in identifying several genomic regions associated with salt tolerance (Vinod et al., 2013). Recent advances in molecular marker assisted breeding in several crops have unequivocally proved the advantage in enhancing the efficiency and accuracy of breeding process through introgressing genes/QTLs governing target traits into high yielding varieties (Alpuerto et al., 2008).

A number of Genes/QTLs have been mapped for several salt tolerance parameters like Na^+ and K^+ uptake, Na^+ and K^+ concentration and Na^+/K^+ ratio in shoot on different chromosomes of rice (Koyama et al., 2001; Lin et al., 2004; Singh et al., 2005; Ammar et al., 2009; Pandit et al., 2010; Haq et al., 2010). Lin et al., (2004) mapped two major QTLs namely shoot K^+ concentration on chromosome 1 (*qSKC-1*) and shoot Na^+ concentration on chromosome 7 (*qSNC-7*) in $F_{2:3}$ population derived from the tolerant *indica* landrace Nona Bokra with the susceptible *japonica* variety Koshihikari. The *SKC1* gene was subsequently cloned and found to encode a sodium transporter that helps control K^+ homeostasis under salt stress (Ren et al., 2005). A major QTL associated with the Na–K ratio and seedling-stage salt tolerance, named *Saltol* (Gregorio et al., 1997) was identified on chromosome 1 using recombinant inbred line population developed by crossing a salt sensitive variety IR29 with a salt tolerant land race Pokkali, explaining 43 to 70 per cent phenotypic variance for salt tolerance (Bonilla et al., 2002). Developed from this cross, IR 66946-3R-178-1-1 (also named FL478), a semi dwarf, early and photoperiod insensitive RIL with high level of seedling stage salinity tolerance has been extensively used as a donor for salinity tolerance in rice breeding. Further analysis of single feature polymorphisms in the *Saltol* region of FL478 revealed that it contained a <1 Mb DNA fragment from Pokkali at 10.6–11.5 Mb on chromosome 1, flanked by IR29 alleles (Kim et al., 2009). The region also contains many other Pokkali QTLs including that of *SKC1* (Thomson et al., 2010).

The candidate markers within *Saltol* QTL region namely, AP 3206, RM 8094 and RM 3412 for use in marker assisted breeding for increased salt tolerance have been identified (Thomson et al., 2010; Aliyu et al., 2011). Further SSR marker based fine mapping of *Saltol* locus using RILs of Pokkali/ IR29 saturated this region with more than 20 microsatellite markers spanning on a 5Mb region [Niones 2004; Thomson et al. 2007, 2010; Alam et al. 2011]. Group of researchers at IRRI validated all the reported SSR markers present within *Saltol* in three independent studies. However there is no consistency in recommendation robust foreground marker for *Saltol* selection in MABB. Therefore, there is need to identify useful Microsatellite marker for efficient selection of *Saltol* in MABB strategy.

IR 66946-3R-178-1-1 (FL478) was identified as a novel source of seedling stage salt tolerance (Walia et al., 2005) and has been widely used in transfer of *Saltol* though marker assisted backcross (MABC) breeding into elite varietal backgrounds. Although, work on QTL introgression of *Saltol* in major non-basmati rice varieties like BR11, BRRI Dhan 28, IR64, AS996 and Swarna has been initiated (Rahman et al., 2008; Ho et al., 2010; Linh et al. 2012;STRASA 2011), there is no report of similar attempt in Basmati rice varieties,

Keeping in view the above points the present study entitled 'Marker assisted improvement of Pusa Basmati 1121 for salinity tolerance' will be undertaken with the following objectives:

1. Molecular and physiological characterization of rice germplasm lines for salinity tolerance
2. Marker assisted introgression of *Saltol* locus into Pusa Basmati 1121.
3. Characterization of backcross derived lines for salinity tolerance, yield, grain and cooking quality traits.
4. Expression profiling *SKC1* gene in parental lines and *Saltol* derived NILs

2. Background

Soil Salinity in Rice Growing Regions of the World

More than 6.2% of the total world area amounting to 837 million hectares (M. Ha.) is salt affected (Fisher et al. 2002), of which roughly about 48% is saline and 52% is sodic (Bot et al. 2000) with saline soils predominating major rice areas of the world (Fig. 10.1). Area under salt stress is on the rise with an estimated 10–40 M ha becoming salinized every year due to secondary salinization that renders vast areas of land underutilized (Pessarakli and Szabolcs 1999; Ahmed and Qamar 2004; Ansari et al. 2007). Published estimates show that more than 76 M ha of land world- wide has become salt-affected due to secondary salinization of which about 45 M ha is irrigated (Dregne et al. 1991; Oldeman et al. 1991).

Rice is cultivated in more than 115 countries, of which Asia's share is more than 91% of the world total. Majority of the Asian rice production zone is confined to South and South east Asia wherein 47 M ha (20%) area suffers from severe salinity related problems (Abbas et al. 1994), this area consis of warm humid coastal regions and marshy inlands (Fig. 10.2). India and Pakistan have the largest share of area under salinity among the South Asian nations.

About 7% of India's land area amounting to 21 M ha is salt affected. In India, salinity coupled with water logging is seriously threatening agricultural economy in Indo-Gangetic plains covering the states of Haryana, Punjab and Uttar Pradesh.

Salinity is a common problem along the coastal belts of rice growing countries, especially in India. Such areas are characterized by occasional or frequent sea water ingression during tides resulting in submergence that builds up salt accumulation. Rice is the only viable crop in these areas because it can withstand submergence and shows wide variability for salt tolerance. However, under salt prone conditions, yield is reduced from 10% to 80%, and coupled with erratic rainfall; loss can reach up to 100%. Further, low productivity can also be attributed to occasional flooding, rainfed rice cultivation, frequent water-deficit stress and continued cultivation of traditional low-yielding rice varieties and landraces (Singh et al. 2009). In near future, Asia requires more rice to feed its burgeoning population, since rice is the staple food for more than 90% of Asians. Ironically, millions of hectares of rice cultivable areas are either being rendered uncultivable or are grown with very low yields because of growing soil salinization. Two options to enhance the productivity out of such problem areas are reclaiming soil off the salinity and by cultivating salt

tolerant varieties. The second option seems more feasible and sustainable, as there is enough variability in the rice germplasm for salt tolerance which could be utilized for development of rice varieties with salinity tolerance.

Salt Stress in Rice

Rice is sensitive to salinity, particularly during the seedling stage (Maas and Hoffman 1977) and the earlier set benchmarks (Maas and Grattan 1999; Hanson et al. 1999) indicate that rice yield decreases 12% for every unit (dS/m) increase in ECe above the threshold tolerance of 3.0 dS m^{-1}(Maas 1990). Salt sensitivity in rice is now revised to a much lower threshold tolerance of 1.8 dS m^{-1} with yield decline slope of 9.1% (Grattan et al. 2002). Salinity affects yield components such as panicle length, spikelet number per panicle, and grain yield (Zeng and Shannon 2000), besides delayed panicle emergence and flowering, and reduced seed set percentage due to lower pollen viability (Khatun and Flowers 1995). As against the earlier belief that the salt induced damage in rice is caused by osmotic imbalance and accumulation of chloride (Cl^-) ions (Tagawa and Ishizaka 1963), it is now known that injury is primarily caused by sodium ion (Na^+) toxicity due to cellular ion imbalance (Mandhania et al. 2006). In contrast to adverse effect on root growth, presence of excess amounts of Na^+ results in greater reduction in shoot growth and yield (Esechie et al. 2002). When compared to other cereals, Cl^- ions are relatively well tolerated by rice at varying concentrations pre-empting them being toxic (Clarkson and Hanson 1980).

Rice plants uptake excess levels of Na^+ under salt rich conditions, interfering with the uptake of potassium (K^+) and calcium (Ca^{2+}) inciting deficiency symptoms. Low K^+ uptake result in high Na^+/K^+ ratio within plants, which together with low Ca^{2+} uptake causes impairment of mineral transport resulting in reduced shoot growth. The rate of conversion of soluble sugars into starch is reduced concomitantly with the reduced uptake of K^+, as K^+ is needed for the catalytic activities of starch biosynthesis enzymes (Zhang et al. 2012). Further, decrease in carbohydrate accumulation under severe salt stress may also occur due to reduced carbon assimilation (Moradi and Ismail 2007; Pattanagul and Thitisaksakul 2008) resulting from the damage of photosynthetic machinery (Moradi and Ismail 2007). Photoinhibition along with salt stress can cause serious damage to photosynthesis, nutrient uptake, water absorption, root growth and cellular metabolism leading to yield loss (Hasegawa et al. 2000; Zeng and Shannon 2000; Zhu 2001). Besides, salt stress rapidly

activates several lipid responses in rice leaves; however, whether these responses do have any role in salt tolerance is not clear (Darwish et al. 2009). Further, on long term exposure to salinity, especially during development, morphological modifications may be seen occurring in leaves by development of smaller and densely packed cells with thickened cell walls (Qiu et al. 2007)

Rice cultivars show variable sensitivity towards salinity at different phenological stages, with better tolerance during germination and tillering (Khan et al. 1997). Tolerance drops significantly at young seedling stage and especially during early reproductive stage becoming very sensitive during panicle initiation and fertilization, directly affecting the crop yield (Heenan et al. 1988; Zeng et al. 2001). A possible reason for this variability is the ability of rice plants to grow in standing water that can dilute and leach away excess salts in the soil (Bhumbla and Abrol 1978). Particularly due to the early seedling susceptibility, older seedlings are generally recommended for transplanting into saline soils. Young rice plants of susceptible varieties die after germination, while those of the adapted survive showing reduced growth, together with osmotic adjustments to avoid dehydration. Seedling biomass is now recognized as an important parameter for the survival of transplanted seedlings in saline soils (Summart et al. 2010). However, there are reports in which rice varieties show poor correlation of seedling and adult plant salt tolerance (Moradi et al. 2003), which can pose a major challenge because combinatorial expression of tolerance sustaining throughout the crop lifespan is essential for breeding tolerant varieties (Ismail et al. 2007). Therefore, most of these reports are only suggestive because the earlier investigations on salt tolerance are done on seedlings grown under hydroponics, tissue culture environment, and under artificial salinization of the culture media. Although artificial screening may simulate near perfect situations of salt sufficiency, it may fail to duplicate natural situations that are practically relevant for breeding.

Physiological Basis of Salt Tolerance in Rice

Salt tolerance in rice, is an integrated phenomenon contributed by several traits relating to water and mineral uptake, transpiration, osmotic balance, tissue tolerance, oxidant scavenging and growth vigor (Moradi et al. 2003). Physiological basis of salt tolerance in rice plants is primarily manifested by Na^+ exclusion from young tissues and flag leaves and developing panicles (Asch et al. 2000; Haq et al. 2010). High Na^+ concentration in the apoplastic solution results in increased accumulation of proline in the cytoplasm especially in tolerant rice genotypes, which helps in restoring the osmotic potential between the cytosol and apoplastic solution (Demiral and Turkan 2005). Other mechanisms operating in rice include confining of toxic ions to older leaves and vacuoles, secondary responses such as scavenging reactive oxygen species (ROS), enhanced growth response to dilute salts and increased stomatal response. Salt stress was reported to increase chlorophyll concentration in leaves of tolerant and moderately tolerant rice genotypes, with significant levels of chlorophyll *a* concentration and high chlorophyll *a*/*b* ratio. Being the major photosynthetic pigment, reduction in chlorophyll *a* may be associated with reduced photosynthetic activity under salt stress (Moradi and Ismail 2007). Salt induced inhibition of conversion of soluble sugars into starch was less in salt tolerant genotypes (Zhang et al. 2012). Additionally, long-term reduction of mesophyll conductance under salt stress results in anatomical modifications in leaves (Chaves et al. 2009). A comprehensive review of mechanisms of salt tolerance (Munns and Tester 2008) with major focus on rice has been published by Ismail et al. 2007 and Negrão et al. 2011.

In a recent investigation to identify biochemical markers for salt tolerance, significantly reduced level of H_2O_2 activity was observed in the tolerant variety Pokkali, suggesting the existence of an efficient antioxidant defense system to cope up with salt-induced oxidative stress. Supporting this hypothesis, higher activities of antioxidant enzymes and isozyme patterns that are either directly or indirectly involved in the detoxification of reactive oxygen species (ROS), were observed in Pokkali. Further, Pokkali exhibited a higher reduced versus oxidized glutathione ratio together with a higher ratio of reduced versus oxidized ascorbate ratio and higher activity of methylglyoxal detoxification system (glyoxalase I and II). As reduced glutathione is involved in the ascorbate–glutathione pathway as well as in the methylglyoxal detoxification pathway, the results suggest that both ascorbate and glutathione homeostasis, which is also modulated via glyoxalase enzymes, can be considered as biomarkers for salt tolerance in rice (el-Shabrawi et al. 2010).

In salt tolerant genotypes of rice, activity of enzymes such as ascorbate peroxidase, catalase and peroxide dismutase, that are known to be involved in ROS scavenging was found to be either constitutively expressed or induced by salt stress (Moradi and Ismail 2007; Nagamiya et al. 2007; Ahmad et al. 2008).

Genetics of Salt Tolerance in Rice

Rice genetic diversity harbours natural variability for salt tolerance. In a large scale screening of rice genotypes conducted at the International Rice Research Institute(IRRI), about 17% of the total 1,38,000 genotypes have been found to possess acceptable levels of salt tolerance (EC 10 dsm^{-1}) at seedling stage (Datta et al.1993). A basic understanding on the genetics and relationships between varietal groups and phenotypic variation for salt tolerance is vital for bioprospecting of genes and mining useful alleles. Till date, there have been no systematic studies for comparing within and between group variability for salt tolerance in rice. Several independent studies have reported many landraces and varieties tolerant to salt accumulation by one mechanism or another (Gregorio et al. 2002; Ismail et al. 2007; Mohammadi-Nejad et al. 2010). However, most of the traditional salt tolerant varieties from coastal regions of India such as *pokkali* rice types (Pokkali, Cheruvirippu, Bali, Orkayama, Eravapandy, Orpandy, Oorumundakan, Chettivirippu Kuruka Anakodan chottupokkali etc.) from Kerala and Nona Bokra, Getu, Kalarata 1–24, SR26B, Damodar, Dasal, Patnai and Nona Sail from West Bengal possess undesirable agronomic and grain quality characters. Other salt tolerant *indica* cultivars grown traditionally in the coastal areas of other countries include Kalimekri, Bhirpala, Kajalsail (Bangladesh), Ketumbar, Kuatik Putih (Indonesia), Khao Seetha (Thailand) and Soc Nau (Vietnam).

Several breeding lines have been developed at the IRRI with specific characteristic features for salt tolerance namely, IR4630-22-2-5-1-3 (a donor for leaf compartmentation), IR60167-129-3-4 (a donor for tissue tolerance), and IR66946-3R-178-1-1 (also known as FL478, a donor for seedling stage salinity tolerance). Similarly, popular salt tolerant varieties such as CSR10, CSR13, CSR27 and CSR30 have been developed and released from the Central Soil Salinity Research Institute (CSSRI) at Karnal in India using traditional salt tolerant varieties as parents (Negrao et al 2011). Hardly little was known about inheritance of salt tolerance in rice until 1970s, when Akbar and Yabuno (1977) reported that panicle sterility under salt stress was a dominant trait controlled by a small number of genes. Later, overdominance of salt tolerance was demonstrated in crosses

between tolerant and susceptible varieties, accompanied by dominance and a sizeable degree of fixable additive variance (Moeljopawiro and Ikehashi 1981). Subsequent genetic studies indicated that salt tolerance in rice was a complex trait under polygenic control (Flowers 2004) with large environmental effects and low heritability (Gregorio and Senadhira 1993). However, in crosses with moderately tolerant and susceptible parents, duplicate type of epistasis was also reported (Ray and Islam 2008). It is rather difficult to consolidate and quantify genetic effects of tolerant traits since screening methods for tolerance were different at different growth stages and hardly any relation exists between phenological tolerance expressions. Nevertheless, considerable variation for difference in survival traits and components of salt injury are reported coupled with significant genotype × environment interactions (Zhou et al. 2010; Ali et al.2006).

Many workers have attempted genetic component analysis on various traits, especially on Na^+/K^+ compensation, and reported both additive and dominant gene effects and overdominance (Gregorio and Senadhira 1993; Ray and Islam 2008). Agronomic performance under salinity showed preponderance of dominant gene action for yield components and additive gene action for morphological traits (Kalaiyarasi et al. 2002; Sankar et al. 2008). Recent mixed model genetic analysis (Wang et al. 2010) on seed germination under salinity, reported polygenic control of the component traits with preponderance of two to three major genes showing high heritability and accounting for 12.5–99.0% of the total phenotypic variation. A specific genetic model was fitted for each trait, that showed control of two major genes on imbibition rate, two major genes plus polygene on germination and vigor indices, three major genes plus polygene on germination rate and two major genes or two major genes plus polygene on shoot and root length. Significant dominant effects and absence of epistasis for salt tolerant traits have also been reported suggesting the possibility of hybrid rice development under saline situations (Ray and Islam 2008) and using modified bulk and single seed descent methods with later generation selections using larger population as breeding strategies (Gregorio and Senadhira 1993).

Molecular Basis of salinity tolerance

Na^+-K^+ homeostasis governs the principal mechanisms of salt tolerance in plants such as ion uptake and transport, sequestration and extrusion. The major genes involved in Na^+ uptake under saline conditions are governed by high affinity potassium transporters

(HKTs), while vacuolar Na^+/H^+ antiporters regulate Na^+ sequestration in vacuoles and membrane Na^+/ H^+ antiporters regulate Na^+ extrusion as seen in halophytes (Yamamoto and Yan 2008). Plant HKTs represent a class of xylem–parenchyma-expressed Na^+-permeable genes that govern primary mechanism mediating salt tolerance and Na^+ exclusion from leaves. HKT genes are the most widely studied genetic system for salt tolerance in *Arabidopsis*, and in rice they constitute a large gene family of nine genes consisting of two distinctly grouped sub-families of *OsHKT1* with five genes (Garciadeblás et al. 2003; Platten et al. 2006; Huang et al. 2008) and *OsHKT2* containing four genes(Table 2.1). Although named after their relation with bacterial high affinity K^+ transport genes, HKT genes are primarily Na^+ transporters and regulates a variety of cellular mechanisms such as Na^+ sequestration, extrusion and exclusion (Hauser and Horie 2010) and play a key role in regulation of Na^+ homeostasis (Rodríguez-Navarro and Rubio 2006). Evidences show that *OsHKT1* genes distinctly act as Na^+ uniporters and *OsHKT2* as Na^+-K^+ symporters or uniporters depending on the ionic conditions (Huang et al. 2008b; Pardo 2010). A detailed review of HKT transporter-mediated salt tolerance mechanisms can be found at Horie et al. (2009) and Hauser and Horie (2010).

HKT transporters fulfill distinctive roles at the whole plant level in rice, each system playing decisive roles in different cell types. *OsHKT1;5* (*OsHKT8*), was the first among the HKT genes to be mapped, identified initially as a quantitative trait locus (QTL), *shoot* K^+ *content 1* (*SKC1*; Lin et al. 2004). Functional analysis later identified *SKC1* to code for a transporter that unloads Na^+ from the root xylem and preferentially expressed in the parenchyma cells surrounding xylem vessels. It was postulated that relative salt tolerance of rice landraces Pokkali and Nona Bokra is due to the presence of *OsHKT1;5* (Ren et al. 2005). Recently an isoform of *OsHKT2;2* (No-*OsHKT2;2/1*) that is likely to have originated from a deletion in chromosome 6, producing a chimeric gene is identified in Nona Bokra, a highly salt-tolerant cultivar (Oomen et al. 2012). It has a 5¢ region corresponding to that of *OsHKT2;2*, as found in Pokkali but with a 3¢ region corresponding to that of *OsHKT2;1*. In contrast to *OsHKT2;1*, No-*OsHKT2;2/1* is essentially expressed in roots and displays a significant level of permeability to Na^+ and K^+ even at high external Na^+ concentrations. No-*OsHKT2;2/1* perhaps contributes to the salt tolerance of Nona Bokra by enabling high root K^+ uptake under saline conditions.

Other genes of the sub-family 2, *OsHKT2;3* and *OsHKT2;4* are structurally 93% similar at the amino acid sequence level and traceable on the reference rice genome of cv. Nipponbare (Horie et al. 2011). They retain the four selectivity filter Gly residues typical of class II HKT transporters (Horie et al. 2009; Hauser and Horie 2010). Recently, *OsHKT2;4* was shown to possess a typical Na^+ transport properties and show dominant selectivity for K^+ under competition over Mg^{2+} and Ca^{2+} ions, however, *OsHKT2;3* failed to complement a high-affinity K^+ uptake-deficient mutant of yeast strain (Horie et al. 2011).

Quantitative Trait Loci and Markers

Extending the DNA based molecular technology to classical linkage analysis, mapping of QTLs is the simplest way of identifying trait-related genomic regions that are otherwise difficult to identify due to several interfering factors such as poly- genes, linkage, and low heritability. To date, several QTLs have been mapped for salt tolerance related traits in rice (Table 2.2), especially for the vegetative stage tolerance. Significant QTLs for reproductive stage tolerance are yet to be identified in rice (Jena and Mackill 2008). However, excepting few significant ones (Table 2. 3) most of the QTLs identified so far are small effect QTLs and many of those reported from populations of early generations probably may remain in reports.

Earliest attempt to map QTL for salt tolerance in rice was reported by Zhang et al. (1995), in which a QTL was mapped on chromosome 7 in an F_2 population derived of the cross M-20 × 77–170. M-20 was a stable mutant of 77–170 derived by *in vitro* selection. Later a large effect QTL was mapped on chromosome 1 in an IR29/Pokkali derived recombinant inbred line(RIL) population significantly influencing three salt tolerant traits viz., high K^+ uptake, low Na^+ uptake and low Na^+/K^+ ratio (Gregorio 1997). Named *Saltol*, this remains as the most prominent QTL mapped so far for salt tolerance in rice.

Subsequently, two large effect QTLs were mapped for shoot concentration of Na^+ and K^+, *qSNC-7* on chromosome 7 and *qSKC-1* on chromosome 1, respectively in an F_{2-3} population between Nona Bokra, a salt tolerant *indica* landrace and Koshihikari (Lin et al. 2004), with *qSNC-7* explaining 49% and *qSKC-1* explaining 40% of the phenotypic variation. Later, *qSKC-1* was cloned and found to encode a member of HKT-type transporters, *OsHKT1;5*, that is preferentially expressed in the parenchyma cells surrounding

the xylem vessels. *SKC1* protein functions as a Na^+-selective transporter, involved in regulating K^+/Na^+ homeostasis under salt stress (Ren et al. 2005).

Saltol, a major QTL for salinity tolerance

The *Saltol* was mapped on the short arm of rice chromosome 1 derived from the tolerant parent Pokkali by AFLP genotyping (Gregorio 1997). The QTL had a LOD score of 14.5 and explained up to 81% of the phenotypic variation. Subsequently Bonilla et al. (2002) integrated RFLP and SSR markers to the *Saltol* map, and in a hydroponic screen at the seedling stage using 54 RILs remapped this QTL that explained 43% of the phenotypic variation for shoot Na–K ratio. Further, many workers remapped and fine mapped *Saltol* in other mapping populations (Niones 2004; Lin et al. 2004; Elahi et al. 2004; Thomson et al. 2010). This QTL region is now confined within a 1.2 Mb region (Niones 2004). Recent confirmative mapping of *Saltol* locus (Fig. 10.3), shows that *Saltol* contributes to Na^+/K^+ homeostasis with an LOD of 7.6 and R^2 of 27% across the 140 RILs and a 30% decrease in the shoot Na–K ratio (Thomson et al. 2010). *Saltol* is flanked between microsatellite markers, RM1287 and RM7075 at physical position between 10.8 and 15.3 Mb on chromo- some 1 (Alam et al. 2011).

SalT, another important gene, co-localized with *Saltol* on chromosome 1 wasfirst isolated and characterized from the roots of salt-treated rice plants (Claes et al. 1990). Its expression is correlated with osmoprotectants, such as trehalose and proline. The treatment of rice with trehalose improved salt tolerance but suppressed *SalT* upregulation, while proline treatment increased growth inhibition of salt treated rice plants and upregulated *SalT* (Garcia et al. 1997). *SalT* transcripts were found to accumulate with wounding and heat treatment and the gene is also induced by fungal elicitors, jasmonic acid and abscisic acid (de Souza et al. 2003; Kim et al. 2004; Moons et al. 1997), which suggests that the role of *SalT* protein may be involved in a broader response/ sensor mechanism to the imposed stress (de Souza et al. 2003).

FL478 (IR 66946-3R-178-1-1), a highly salt tolerant recombinant inbred line otherwise similar to IR29 (Bonilla et al. 2002), was subsequently used as donor for salt tolerance breeding worldwide. Contrary to the expectations, investigations revealed that *Saltol* region of FL478 was indeed contributed by the sensitive parent IR29, but activated to trigger high salt tolerance in presence of other positive alleles form Pokkali (Walia et al. 2005). *Saltol* region of FL478 is very complex, and now poised to contain many

Pokkali QTLs including that of *SKC1* (Thomson et al. 2010) and a <1 Mb Pokkali DNA fragment at 10.6–11.5 Mb flanked by IR29 alleles (Kim et al. 2009). The fact that *Saltol* affected the Na–K ratio predominantly, the causal gene underlying this effect could be the sodium transporter *SKC1* (*OsHKT1;5*) (Thomson et al. 2010).

Comparative genomic investigations reveal that *Saltol* region seemed to contain an array of homologous sequences of known genes, viz., transcription factors, signal transduction components, cell wall components, and membrane transporters (Walia et al. 2005, 2007). The membrane transporter genes included those coding for carriers and channels involved in transporting cations, anions and organic substrates such as sugar transporters (Senadheera et al. 2009). Specific genes identified so far are root tissue and membrane transporters such as cation- proton exchanger (*OsCHX11*) and Cyclic nucleotide-gated ion channel (*OsCNGC1*) (Senadheera et al. 2009), HKT1, ABC1 (ATP-binding cassette transporter) genes (Walia et al. 2005). Various other genes identified near *Saltol* are, salt stress-induced protein (eF576533) and tetra- copeptide repeat domain containing protein (eF575991) showing salt induced activation (Kumari et al. 2009).

Improving Salt Tolerance in Rice

Cultivation of salt tolerant rice in India perhaps had begun much earlier than any-where else in the world. Although there is no historical record available of its beginning, a traditional organic rice-shrimp farming system known as *Pokkali* still exists in the coastal saline areas of Kerala that is characterized by daily ingression of tidal waves causing partial flooding of rice fields and seasonal shrimp farming in the rice fallows during high saline phase (Pillai 1999; Shylaraj and Sasidharan 2005). In fact, there were many *Pokkali* rice varieties in use, all of which were salt tolerant. In India, however, organized research on breeding salt tolerant rice was begun *circa* 1940, especially in the states of Maharashtra and Madras. In 1943, two salt tolerant varieties Kala Rata 1–24 and Bhura Rata 4–10 were released in Maharashtra (Shendge et al. 1959). Around this time, in 1939, a salt-tolerant landrace called 'Pokkali' was introduced to Sri Lanka, which was later recommended for cultivation in saline areas in 1945 (Fernando 1949).

However, varietal development program for salt affected areas met with low success due to many reasons such as (a) lack of understanding of the complex nature of inheritance of salt tolerance, (b) lack of sufficient sources of resistance, (c) complexity and diversity of salt affected areas, (d) lack of precise and reliable screening techniques, and (e) lack of sufficient

research backing. Conventional breeding methods such as introduction and selection of landraces, pedigree method, modified bulk pedigree method, mutation and shuttle breeding were used for development of new varieties in India (Singh et al. 2009). Shuttle breeding under an IRRI- India collaborative project had resulted in development of two salt-tolerant rice varieties, CSR23 and CSR27 (Mishra 1994).

Despite decade long breeding efforts, potential yield gap between the salt affected areas and normal regions remains wide, and there is an immediate need to harness resources to develop target specific, locally adapted high-yielding varieties. Thanks to the modern approaches such as improved screening technique for phenotyping, in vitro and marker assisted selection, gap between potential and actual yield within these coastal areas is narrowing because of the development of salt-tolerant, fertilizer responsive and intermediate stature high-yielding varieties.

Screening for Salt Tolerance

Success of a target specific varietal development programme such as salt tolerance depends on reliable screening techniques that translate the results to reality in the field. Based on the target traits, methods of screening can be either phenological or physiological. While phenological screens included germination, survival, injury, morphology, yield and index such as mean tolerance index, physiological screening was done for Na^+, K^+, Cl^- concentrations and their derived ratios. Several screening techniques have been developed in rice such as hydroponics, pot culture, microplots and field evaluation, besides specialized solution culture screening methods such as bread boxes with perforated lids, seedling float technique and adult plant screening system (Singh et al. 2010). Among all, field screening is the best because it is the only method that could accommodate salt tolerance in its holistic form with entire temporal and spatial variability. Notwithstanding, field screening is the most cumbersome of all the methods and hence limits the number of genotypes/ progenies to be handled per screening. However, augmented designs allow screening of large number of varieties than conventional complete designs. Artificially created soil plots that resembles mini *in situ* fields but devoid of soil heterogeneity and maintains gradient levels of salinity in each designated plots are used for microplot screening. Such microplots are utilized for screening early generation materials. For precise individual plant studies pot culture experiments are employed, which facilitate closer observations. Hydroponics screens have been very popular because of its simplicity in setting up, precise control over salt concentration and it helps in creating a water-plant interface to which rice is adapted.

Furthermore, specialized laboratory screening such as prolonged soaking in high salt concentration for 9 days prior to germination test was proven to be effective in delineating salt tolerant varieties (Abeysiriwardena 2004). Salt tolerance at juvenile screening are classified based on a modified standard evaluation score (Gregorio et al. 1997) of visual salt injury at seedling level.

Most of these methods except field screening are limited to seedling stage, and therefore could not account for adult plant salt tolerance. Notwithstanding, artificial screens remain different from natural soil-water-plant interface limiting their direct application in crop improvement research. Therefore, an integrated approach is desirable that uses different screens so that the selected variety performs well under all stages of growth and therefore can be feasible for commercial cultivation.

In Vitro Techniques and Transgenesis

Since 1980s, cell and tissue culture techniques, have been recognized as powerful tools augmenting conventional breeding for the development of plants with increased tolerance to stresses such as salinity. Later in vitro technology found their applications in molecular linkage mapping through Doubled Haploid (DH) lines and as an integral part of genetic engineering for the development of transgenic plants. In vitro techniques such as anther and pollen culture, somaclonal variation and protoplast fusion were used to develop salt tolerant lines in rice (Ram and Nabors 1985; Lynch et al. 1991). Among these, anther culture was used extensively in deriving salt tolerant lines, because of its advantage of faster development and efficiency in handling large number of progeny lines.

Anther Culture

The success of anther culture derived salt tolerant lines was established by the release of PSBRc50 (Bicol) targeted for saline-prone areas. Developed at IRRI, from the *indica-indica* cross IR5657-33-2/IR4630-22-2-5-1-3 (Zapata et al. 1991) this variety, originally known as IR51500-AC11-1, was the first ever anther culture derived variety to be released in the Philippines (Senadhira et al. 2002) and also the first cultivar recommended for adverse environments (Datta et al. 2009). Two other anther culture derived lines from IRRI, IR51500-AC17 and IR51485-AC6534-4 were released as commercial cultivars CSR21 and CSR28, respectively, for cultivation in saline-alkaline soils of India. Several anther derived DH lines were developed at IRRI, most of which had been used as a donor parents in breeding programs in various rice growing nations (Datta et al. 2009).

Although anther culture has limitations of reduced success, anther derived lines are still being developed for salt tolerance. Recently in Bangladesh, Rahman et al. (2010) generated 25 salt tolerant DH lines from a cross IR52724/ BR36 with line AC 1 showing excellent seedling stress survival coupled with moderately low Na/K ratio close to that of the tolerant control Pokkali, besides producing good yield in field trials conducted in a saline zone. Similarly many anther derived lines were tested in Vietnam (Tam and Lang 2004) and Thailand (Cha-um et al. 2008).

Somaclonal Variation

Successful exploitation of somaclonal variants, the mutant cell lines that survive in salt rich selective media and regeneration of whole plants from such variants, stimulated many attempts for the development of salt-tolerant plants (Reddy and Vaidyanath 1986; Kavi-Kishor 1988). Although several salt tolerant lines have been reported, in many cases regenerants either failed to inherit the trait effectively, or showed developmental defects or in extreme cases showed complete reversal of tolerance. Such failure are now attributed to lack of distinction between mutant and adapted cell lines, distinct driving mechanisms for cellular and whole plant tolerance, multigenicity of salt tolerance and loss of regeneration capacity during selection (Tal 1993; Oono 1984). In an earlier reported attempt from IRRI, Pokkali cell lines were subjected to *in vitro* induction of somaclonal variants, with the objective of improving agronomic traits. A variant, TCCP 266-2-49-B-B-3 had improved agronomic performance coupled with good salt tolerance (Senadhira et al. 1994), showing vigorous growth, semi-dwarf nature, white pericarp and better cooked rice quality, features that were distinctly different from the original Pokkali line, which was tall with red pericarp and poor cooking quality. TCCP266-2-49-B-B-3 has later become a popular donor for producing new high-yielding salt-tolerant lines, some of which were released as varieties (Datta et al. 2009).

Transgenesis

Development of transgenic plants, by introducing new genes from external sources is an ideal tool to test the expression of orthologous genes. With the advancement in molecular mapping of QTLs together with transcriptome and whole genome expression profiles, map-based cloning has been successfully used to isolate and clone candidate genes and QTLs of biological and/or agricultural importance (Senadheera et al. 2009). However, before putting them into use it is essential to functionally validate the genes for trait expression.

This helps to target the gene precisely and develop markers for marker assisted selection (MAS) programmes. In the modern biology, information on useful genes accumulate from different directions such as whole-genome information of both eukaryotic and prokaryotic model organisms, expressed sequence tag (EST) libraries, QTL mapping, microarrays etc. and transgenic system is the most handy tool in testing the expression of target genes under given environments such as high salinity. The testing can either be on the same or different organisms.

Several rice genes have already been positively tested in plant systems such as Arabidopsis, maize, tobacco and within rice itself, besides testing of foreign genes in rice for their role in imparting salt tolerance through transgenic approaches (Tables 2.4). In many cases *Agrobacterium* mediated gene transfer has been used to generate transgenic plants, proving the usefulness of transgenic approach in gene validation.

Molecular Breeding

With the availability of several 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. Molecular breeding, a generic term now includes different MAS approaches, such as marker assisted backcrossing (MABC), marker assisted recurrent selection (MARS), genomic selection and MAS based diallel selective mating system (MAS- DSMS). MAS has two distinct advantages translating to significant monetary and time benefits, namely, (a) it reduces the product delivery time considerably and (b) it reduces the genetic load or linkage drag associated commonly with backcross breeding programmes (Alpuerto et al. 2008; Gopalakrishnan et al. 2008; Singh et al. 2012). Furthermore, it is now possible to defer early generation phenotyping of segregating populations, by foreground selecting for stable QTLs and genes that have already been validated to confer stable salt tolerance under varying situations. This can accelerate breeding cycle as well as helps in handling large number of individuals per population, channelling to a successful salt tolerant variety. MAS as a tool to augment breeding programme, is widely used in targeted transfer of specific genes/ QTLs into popular cultivars through indirect selection based on gene or QTL linked based markers for foreground selection (Singh et al. 2011).

Marker Assisted Backcrossing (MABC)

MABC is of great practical interest in applied breeding programmes, because it is done almost in the conventional way, but without or minimized phenotype testing in the early

generations, rapidly advancing to the target genotype by following the inheritance of simple molecular tags that segregate in classical Mendelian fashion. 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 (Singh et al. 2011). Detailed reviews on usefulness of MABC in rice are available (Collard and Mackill 2008; Singh et al. 2011).

MABC for transferring salt tolerance QTL, *Saltol* is currently practiced in many rice growing nations, viz., Philippines, India, Thailand, Vietnam and Bangladesh (Elahi et al. 2004; Singh et al. 2011; Lang et al. 2011). The procedure involves crossing of the *Saltol* donor (preferably FL478) with the recipient with 2-3 repeated backcrosses, selecting for the markers flanking the *Saltol* (foreground selection) and selecting against the donor markers for other regions (background selection) within each backcross generations. At the end of the programme recombinants are selected in which *Saltol* alleles are fixed to expresssalt tolerance as against the original recipient. Various MABC procedures are in practice that combines phenotype selection for faster background recovery (Singh et al. 2011), together with stepwise transfer or simultaneous transfer or simultaneous but stepwise transfer for QTL pyramiding (Joshi and Nayak 2010).

In India, FL478 is being used as a donor to transfer *Saltol* into the recurrent parents Pusa Basmati 1121 and Pusa Basmati 6 through MABC in two independent backcross programs at the Indian Agricultural Research Institute, New Delhi. Three *Saltol* linked markers RM8094, RM3412 and RM493 that are polymorphic between the recurrent and donor parents are used for foreground selection in each back cross generation, coupled with stringent phenotypic selection for rapid recovery of RPG and phenome with salt tolerance (Singh et al. 2011; Babu et al. 2012). Furthermore, various institutions across India are working on transferring *Saltol* in the backgrounds of popular rice varieties such as Sarjoo 52, Pusa 44, PR114, Gayatri, Savithri, MTU 1010, White Ponni and ADT45 under DBT, GOI funded project “QTL to Variety”. IRRI in collaboration with national institutes under Stress-Tolerant Rice for Africa and South Asia (STRASA) project is taken up a massive MABC for transferring *Saltol* into popular varieties such as BRRI dhan 28, IR64, BR11 and Swarna (STRASA 2011).

Table 2.1: **High affinity potassium transporters (HKT) on rice reference genome cv. Nipponbare**

Gene	Chromosome	Genome location	Other names	Length (bp)	No. of transcripts
OsHKT1;1	4	LOC_Os04g51820	*OsHKT4*	2,443, 2,224, 2,097	3
OsHKT1;2	4	–	*OsHKT5*	Pseudo gene	–
OsHKT1;3	2	LOC_Os02g07830	*OsHKT6*	1,733	1
OsHKT1;4	4	LOC_Os04g51830	*OsHKT7*	2,269	1
OsHKT1;5	1	LOC_Os01g20160	*OsHKT8, SKC1*	2,164	1
OsHKT2;1	6	LOC_Os06g48810	*OsHKT1*	1,881	1
OsHKT2;2	?	–	*OsHKT2*		–
OsHKT2;3	1	LOC_Os01g34850	*OsHKT3*	1,628	1
OsHKT2;4	6	LOC_Os06g48800	*OsHKT9*	1,557	1

Table 2.2: Quantitative trait loci (QTLs) mapped for salt tolerance in rice

Cross	Population	No. of QTL	Method	Marker system	References
M20/77–170	F_2	1		RFLP	Zhang et al. (1995)
IR29/Pokkali	RIL	10	IM	AFLP	Gregorio (1997)
Tesanai 2/CB	RIL	1	SF-ANOVA	RFLP	Lin et al. (1998)
M20/77–170	F_2	1	SMA	RAPD	Ding et al. (1998)
Zhaiyeqing 8/Jingxi l7	DHL	8	SIM, CIM	RFLP	Gong et al. (1999)
IR64/Azucena	DHL	7	SIM	RFLP	Prasad et al. (2000)
IR 59462[a]	F_7	16	–	AFLP	Flowers et al. (2000)
IR4630/IR15324	RIL	25	SMA	AFLP, RFLP, SSR	Koyama et al. (2001)
Zhaiyeqing 8/Jingxi l7	DHL	24	SIM	RFLP	Gong et al. (2001)
IR29/Pokkali	RIL	1	–	AFLP, SSLP	Bonilla et al. (2002)
Tesanai 2/CB	F_2	31	SMA	RFLP	Masood et al. (2004)
Nipponbare/Kasalath	BIL	28	IM	RFLP	Takehisa et al. (2004)
Nona Bokra/Koshihikari	F_2	8	SIM	RFLP	Lin et al. (2004)
IR64/ Tarom Molaii	BIL	??	CIM	SSR	Fotokian et al. (2005)

[a]IR 59462= Nona Bokra/Pokkali//IR 4630-22-2-5-1-3/IR 10167-129-3-4

Table 2.2 : continued...

Cross	Population	No of QTLs	Method	Marker system	References
Milyang 23/Gihobyeo	RIL	3	IM	RFLP	Lee et al. (2007)
CSR 27/MI 48	RIL	6	CIM	STMS	Ammar et al. (2007)
IR64/Binam	BIL	13–22	SIM	SSR	Zang et al. (2008)
AS996/IR50404	RIL	1	Regression	SSR	Lang et al. (2008)
Tarommahali/Khazar	F3	32	CIM	SSR	Sabouri and Sabouri (2008)
Tarommahali/Khazar	F2	14	CIM	SSR	Sabouri et al. (2009)
Tarommahali/Khazar	F2	12	CIM	SSR	Sabouri and Biabani 2009
Ilpumbyeo/Moroberekan	BIL	8	CIM	SSR	Kim et al. (2009)
CSR 27/MI 48	RIL	18	CIM	SSR, SNP	Pandit et al. (2010)
IR29/Pokkali	RIL	17	CIM	SSR	Thomson et al. (2010)
Co39/Moroberekan	RIL	Many	IM	RFLP	Ul Haq et al. (2010)
IR26/Jiucaiqing	F9	16	MIM	SSR	Wang et al. (2011)
Tarome-Molaei/Tiqing	BIL	14	CIM	SSR	Ahmadi and Fotokian (2011)
Pokkali/IR29	BIL	13	SMA, Regression	SSR	Alam et al (2011)

Table 2.2 : continued...

Pokkali/Shaheen Basmati	F3	22	SMA	SSR	Javed et al (2011)
BRRI Dhan40/IR61920-3B-22-2-1	F2	3	SMA, CIM	SSR	Islam et al. (2011)
Teqing/Oryza rufipogon	IL	15	SMA	SSR	Tian et al.(2011)

Table 2.3: Prominent quantitative trait loci mapped under salt tolerance screening

Trait	QTL	Chromosome	Flanking markers	R2 (%)	References
Salt tolerance	*Saltol*	1	P3/M9-8 – P1/M-9-3	81	Gregorio (1997)
Seedling survival days	–	1	RG612 – C131	14.3	Gong et al. (1999)
Seedling root length	*qSRTL-6*	6	RG162 – RG653	18.9	Prasad et al. (2000)
Salt tolerance	*Saltol*	1	C52903S – C1733S	39.2	Bonilla et al. (2002)
Salt tolerance	*Saltol*	1	RM23 – RM140	43.2	Bonilla et al. (2002)
Salt tolerance	*Saltol*	1	CP03970 – CP06224	–	Niones (2004)
Shoot K+ concentration	*qSKC-1*	1	C1211-S2139	48.5	Lin et al. (2004)
Shoot Na+ concentration	*qSNC-7*	7	C1057-R2401	40.1	Lin et al. (2004)
Seedling salt tolerance	*qST-1*	1	Est12 – RZ569A	27.8	Lee et al. (2007)
Shoot Na–K ratio	*qSNK1(Saltol*	1	RM1287 – RM10825	20	Thomson et al. (2010)
Imbibition rate	*qIR-6*	6	RM3687 – RM3306	33.6	Wang et al. (2011)
Imbibition rate	*qIR-9*	9	RM276 – RM5531	33.7	Wang et al. (2011)
Germination percentage	*qGP-2*	2	RM8254 – RM5804	36.5	Wang et al. (2011)
Germination percentage	*qGP-9*	9	RM219 – RM7048	43.7	Wang et al. (2011)
Relative root dry weight	*qRDW-10*	10	RM273	22.7	Tian et al. (2011)
Relative shoot dry weight	*qRSW-10*	10	RM273	17.3	Tian et al. (2011)
Relative total dry weight	*qRTW-10*	10	RM273	18.5	Tian et al. (2011)

[a]Salt tolerance = high K^+ uptake + low Na^+ uptake + low Na^+/K^+ ratio

Table 2.4 Rice genes that are functionally tested for improved salt tolerance through transgenesis

Gene	Encoded protein	Transgene candidate	Promotor	Associated trait	References
OsCDPK7	Calcium-dependent protein kinase	*Oryza sativa*	CaMV 35S	Cytosolic Ca^{2+} influx	Saijo et al. (2000)
GS2	Chloroplastic glutamine synthetase	*Oryza sativa*	CaMV35s	Photorespiration	Hoshida et al. (2000)
OsMAPK5a	Mitogen-activated protein kinase	*Oryza sativa*	Ubiquitin	Cytosolic Ca^{2+} influx	Xiong and Yang (2003)
OsDREB1A	DREB Transcription factor	*Arabidopsis*	CaMV35s	Dehydration response	Dubouzet et al. (2003)
OsNHX1	Na^+/H^+ antiporter	*Oryza sativa*	CaMV 35S	Na^+ homeostasis	Fukuda et al. (2004)
OsMAPK44	Mitogen-activated protein kinase	*Oryza sativa*	–	Cytosolic Ca^{2+} influx	Jeong et al. (2006)
OsDREB1A,OsDREB1B	Dehydration responsive element binding (DREB)	*Oryza sativa*	Ubiquitin	Dehydration response	Ito et al. (2006)
SNAC1	Stress-responsive NAC 1	*Oryza sativa*	CaMV 35S	Stress response	Hu et al. (2006)
OsSOS1	Salt overlay sensitive	*Arabidopsis thaliana*	CaMV 35S	Na+ homeostasis	Martinez-Atienza et al. (2007)
Rab16A	Responsive to ABA dehydrins	*Nicotiana tabacum*	Rab16A	Dehydration response	RoyChoudhury et al.2007
OsNHX1	Vacuolar type Na^+/H^+ antiporter	*Oryza sativa*	CaMV 35S	Na^+ homeostasis	Chen et al. (2007)
OsCIPK12	Calcineurin B-like protein-interacting protein kinase	*Oryza sativa*	Ubiquitin	Cytosolic Ca^{2+} influx	Xiang et al. (2007)
OsKAT1	Shaker family K^+ channel	*Oryza sativa*	CaMV 35S	K^+ uptake	Obata et al. (2007)
OsAPXa, OsAPXb	Ascorbate peroxidase	*Oryza sativa*	CaMV 35S	Antioxidant activity	Lu et al. (2007)

Table 2.4 : **continued**

Gene	Encoded protein	Transgene candidate	Promotor	Associated trait	References
OsNAC6	NAC transcription factor	*Oryza sativa*	Ubiquitin	Stress response	Nakashima et al. (2007)
glyII Glyoxalase II Oryza sativa	Glyoxalase II	*Oryza sativa*	CaMV 35S	Salt tolerance	Singla-Pareek et al. 2008
OsTOP6A1	Meiotic recombination protein	*Arabidopsis thaliana*	CaMV 35S	Multiple stress response	Jain et al. (2008)
OsiSAP8	Stress associated protein	*Nicotiana benthamiana*	CaMV 35S	Multiple stress response	Kanneganti and Gupta(2008)
OsiSAP8	Stress associated protein	*Oryza sativa*	CaMV 35S	Multiple stress response	Kanneganti and Gupta (2008)
OsHsfA2e	Heat shock transcription factor TFIIIA-type zinc finger protein	*Arabidopsis thaliana*	CaMV 35S	Stress response	Yokotani et al. (2008)
ZFP252	Trehalose-6-phosphate	*Oryza sativa*	aMV 35S	Proline accumulation	Xu et al. (2008)
OsTPP1	phosphatase Stress-responsive	*Oryza sativa*	CaMV 35S	Trehalose	Ge et al. (2008)
SNAC2	NAC	*Oryza sativa*	Ubiquitin *Ubi1*	accumulation Stress response	Hu et al. (2008)
ONAC063	NAC transcription factor	*Arabidopsis thaliana*	CaMV 35S		Yokotani et al. (2008)
ONAC045	Stress-responsive NAC	*Oryza sativa*	CaMV 35S	LEA gene expression	Zheng et al. (2009)
AP37	APETALA 2 transcription factor	*Oryza sativa*	*OsCC1*	Stress regulatory	Oh et al. (2009)
DST	ethylene zinc finger protein	*Oryza sativa*	CaMV 35S	Stomatal control	Huang et al. (2009)

Table 2.4 : continued

Gene	Encoded protein	Transgene candidate	Promotor	Associated trait	References
OsSIK1	Receptor-like kinase	*Oryza sativa*	*OsSIK1*	Antioxidant activity	Ouyang et al. (2010)
OsNAC10	Stress-responsive NAC	*Oryza sativa*	*GOS2*, *RCc3*	Stress response	Jeong et al. (2010)
OsNAC5	Stress-responsive NAC	*Oryza sativa*	Ubiquitin	LEA gene expression	Takasaki et al. (2010)
ZFP179	Cys2/His2-type zinc finger protein	*Oryza sativa*	CaMV 35S	Proline accumulation	Sun et al. (2010)
OsNHX1	Na^+/H^+ exchanger	*Oryza sativa*	CaMV 35S	Osmoregulation system	Liu et al. (2010b)
OsVP1	H^+-pyrophosphatase in tonoplasts	*Oryza sativa*	CaMV 35S	Na^+ homeostasis	Liu et al. (2010b)
OsTPKb	Two pore K^+ channel	*Oryza sativa*	CaMV 35S	K^+ homeostasis	Mian (2010)
OsAKT1	K^+ inward rectifying channel	*Oryza sativa*	CaMV 35S	K^+ uptake	Mian (2010)
OsTPS1	Trehalose-6-phosphate synthase	*Oryza sativa*	*Actin1*	Trehalose accumulation	Li et al. (2011)
OsHAK5	Sodium-insensitive potassium transporter	*Nicotiana tabacum*	CaMV 35S	K^+ transport	Horie et al. (2011b)
OsNAC5	Stress-responsive NAC	*Oryza sativa*	CaMV 35S	Proline accumualtion	Song et al. (2011)
OsHsfC1b	Heat shock factors	*Oryza sativa*	Ubiquitin	Stress response	Schmidt et al. (2012)

3. Material and Methods

The details of the plant materials used and methods followed in the study entitled 'Marker assisted improvement of Pusa Basmati 1121 for salinity tolerance in rice (*Oryza sativa* L.) are presented objective wise separately. Information on protocols and statistical tools used for analysis is also presented in respective objective.

Objective 3.1: Molecular and physiological characterization of rice germplasm lines for salinity tolerance.

First objective has been divided into two parts and conducted as separate experiments

3.1.1. Association analysis of *Saltol* linked and random markers in diverse germplasm set

3.1.1.1. Plant materials:

A set of 85 diverse genotypes including accessions of *Oryza rufipogon* and *Kaipad* land races, Basmati genotypes, induced mutant lines and improved varieties were screened for seedling stage salinity tolerance through association analysis using *Saltol* linked and random markers. The characteristic features and origin of genotypes is presented in Table 3.1.

3.1.1.2. Molecular markers:

19 SSR markers spanning 5Mb region of *Saltol* locus and 22 genome wide random SSR markers were used for association analysis. The product size and sequence of SSR markers is presented in Appendix 1.

3.1.1.3. Isolation of plant genomic DNA:

The extraction of plant DNA was carried out by CTAB (Cetyl- Tri Methyl Ammonium Bromide) method as described by Murray and Thompson (1980). Reagents prepared for DNA isolation are given in Appendix I.

The genomic DNA was extracted from all 85 varieties. Fresh healthy and young leaf tissue (1g) of 15 day old seedlings were harvested and stored in minus 80^{o}C until used for DNA extraction.

1. The leaf samples were ground to fine powder in liquid nitrogen using sterile, pre-cooled mortars and pestles.

2. The powder was transferred to a 50 ml polypropylene centrifuge tube containing 10ml of pre-warmed (65^{o}C) DNA extraction buffer.

3. Suspension was incubated at 65^{o}C for 1 hr. with intermittent shaking at 15 minutes interval.

4. The tubes were mounted on stands and left to cool down at room temperature, after which equal volume of chloroform: isoamyl alcohol (24:1) was added using a micro-pipette. Solution was mixed by gentle inversion of tubes for 5 minutes.

5. An equal volume of chloroform: isoamyl alcohol (24:1) was added using a micro-pippette in order to remove the phenols and other non-aqueous substances like lipids and proteins.

6. The content was then centrifuged at 10000 rpm for 10 minutes at 4^{o}C. The supernatant was carefully transferred to a fresh centrifuge tube with wide bore tip to avoid DNA shearing.

7. Again equal volume of chloroform: isoamyl alcohol (24:1) was added to perform second wash. The previous step was repeated.

8. Afterwards, pre-chilled iso-propanol 0.6 volume of the content in tube was added followed by quick and gentle inversion until fibrous mass was visible through naked eye. Tubes were incubated at –20 ^{0}C for 1 hour.

9. Tubes were then put to centrifugation at 5000 rpm for 10 minutes at 4^{o}C to obtain a precipitate. The supernatant was drained by gently inverting the tubes. The tubes were left inverted with lids open on blotting paper to drain the residual iso-propanol.

Table 3.1. List of rice germplasm lines used for association analysis

SI No	Genotype	Type	SI No	Genotype	Type
1	Longphou	*Japonica*	44	Pusa44	Released Variety
2	TaiPei	*Japonica*	45	IR8	Released Variety
3	Khao-Khao	*Japonica*	46	Pusa1342	Released Variety
4	China988	*Japonica*	47	ADT43	Released Variety
5	Leimpore	*Japonica*	48	IR36	Released Variety
6	Heibao	*Japonica*	49	DU85	Released Variety
7	TomphaKhau	*Japonica*	50	Swarna	Released Variety
8	Nipponbare	*Japonica*	51	IR72	Released Variety
9	Bhoi	*Japonica*	52	ADT38	Released Variety
10	Zang Bhuman	*Japonica*	53	DhanaPrasad	Land race
11	Khao Daen Krai	*Japonica*	54	Tilkchandan	Land race
12	Dom Siah	*Japonica*	55	Jeerasambha	Land race
13	Type3	Basmati	56	SahPasand	Land race
14	SuperBasmati	Basmati	57	Bhadrakali	Land race
15	Indira Sugandhit Dhan1	Basmati	58	Danteshwari	Land race
16	Basmati564	Basmati	59	RedTrinaini	Land race
17	Pusa Sugandh 5	Basmati	60	SalamPikit	Land race
18	Haryana Basmati 1	Basmati	61	Khandagiri	Land race
19	Pusa Sugandh 3	Basmati	62	Mahanadi	Land race
20	Pant Dhan 11	Hill variety	63	Badami	Land race
21	PusaSugandh 2	Basmati	64	Bindli	Land race
22	Pusa1401	Basmati	65	Erramallalu	Land race
23	Improved Pusa Basmati 1	Basmati	66	Pokkali	Land race
24	Rajendra Basmati	Basmati	67	Nilagiri	Land race
25	Ranbir Basmati	Basmati	68	Tripura Medicinal Rice	Land race
26	Pant Sugandh Dhan17	Basmati	69	PediBadam	Land race
27	Chembalati Basmati	Basmati	70	Acharmati	Land race
28	Pusa Basmati1	Basmati	71	N22	Drought tolerant
29	Pusa Basmati 1121	Basmati	72	SN36	Mutants
30	Sanwal Basmati	Basmati	73	SN26	Mutants
31	Seond Basmati	Basmati	74	SN22	Mutants
32	Basmati386	Basmati	75	SN7	Mutants
33	Basmati370	Basmati	76	SN11	Mutants
34	CSR30	Basmati	77	SN9	Mutants
35	CSR23	Salt tolerant variety	78	SN3	Mutants
36	CSR27	Salt tolerant variety	79	SN4	Mutants
37	CSR36	Salt tolerant variety	80	NKSWR2	Wild rice
38	FL478	Salt tolerant variety	81	NKSWR5	Wild rice
39	IR64	Released Variety	82	NKSWR9	Wild rice
40	MI48	Released Variety	83	NKSWR6	Wild rice
41	Pusa1301	Released Variety	84	NKSWR1	Wild rice
42	Jaya	Released Variety	85	NKSWR17	Wild rice
43	MTU3626	Released Variety			

11. After a while, the DNA pellet was washed twice with 70% ethanol, and kept overnight at room temperature for drying the pellet.

12. 100-200 µl of TE buffer (pH 8.0) was added to dissolve the pellet.

13. After 6-8 hrs. RNase (10mg/ml) was added to the DNA voil @ of 1 µl/100 ul of crude DNA. Mixture was incubated in a water-bath for 1 hour at 37^{o}C with intermittent mixing.

14. Purification of DNA was performed by adding an equal volume of Chloroform: isoamyl alcohol 24:1 to aqueous phase mixed gently for 5 minutes and again centrifuged at 10000 rpm.

15. Aqueous phase was collected in a fresh tube to which 1/10th volume of 3M Sodium-Acetate (pH 5.2) was added, mixed well and then 2 volumes of chilled ethanol was added to the mixture, mixed gently and incubated at –20^{o}C for 2 h.

16. The tubes were centrifuged for 5 minutes at 10000 rpm. The supernatant was discarded and pellet washed twice with chilled 70% ethanol. Pellets was dried properly (overnight air drying) and dissolved in 100µl TE buffer (pH 8.0).

17. DNA was quantified using gel quantification method. The 2 µl samples were loaded on 0.8% agarose gel in 0.5 x TAE buffer and using diluted λ uncut genomic DNA as standard in parallel well. The intensity of individual samples was compared with a range of known amount of λ DNA (25, 50, 75 and 100 ng). According to the concentration of DNA, samples were diluted, and again loaded on 0.8% gels till all of the samples finally reached at ~25 ng /µl in a uniform manner.

3. 1.1.3. Polymerase Chain Reaction

PCR assay was performed for foreground selection using gene linked markers and background selection by using genome wide polymorphic markers. For each sample, DNA quantity was adjusted to ~25 ng/µl. PCR reaction mix contained 25 ng of DNA, 10x PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM $MgCl_2$), 0.05 mM dNTPs (MBI, Fermentas, Lithuania, USA), 5 pmol each of forward and reverse primer and 0.5 U of Taq DNA

polymerase (Bangalore Genei Pvt. Ltd., Bangaluru, India) in a reaction volume of 10 µl (Table 3.1. 3.2).

Table 3.2. The PCR reaction mixture

Reagent	**Stock conc.**	**Aliquot**	**Final conc.**
DNA	25 ng/µl	1 µl	25 ng
PCR buffer	10x	1 µl	1x
dNTP mix	25mM	1 µl	2.5mM
Forward Primer	30 ng/µl	0.5 µl	15 ng
Reverse Primer	30 ng/µl	0.5 µl	15 ng
Taq DNA polymerase:	3 U/µl	0.2 µl	0.6 U
Milli-Q water	-	5.8 µl	-
Total		10 µl	-

Polymerase chain reaction (PCR) was performed in a thermal cycler (G-Storm, Somerset, UK) with following thermal regimes:

Table 3.3. PCR thermal regimes and conditions

Step	**Reaction**	**Temperature**	**Time**	**Cycle**
I	Initial denaturation	94^{o}C	4	1
II	Denaturation	94^{o}C	1	35
	Annealing	55^{o}C	1	
	Extension	72^{o}C	2	
III	Final extension	72^{o}C	7	1

3.1.1.4 Resolution of amplified PCR products:

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Here we used the technique for size separation of amplified DNA. A concentration of 3.5% (w/v) agarose gel was prepared by dissolving 17.5 g of weighed Agarose powder in 500 ml of 1xTAE [490 ml double distilled water + 10 ml of 50x TAE buffer (242.2g Tris base: Mwt. 121.14; 100 ml of 0.5M EDTA: PH 8.0; 57.1ml Glacial acetic acid: Mwt. 61.83; make vol. to 1000 ml using de-ionized Milli-Q water)] in a conical flask. The suspension was heated in microwave oven for 10 minutes at 900 watt till clear solution was obtained. The solution

was allowed to cool down and to this was added 24 µl (0.05 µl/ml of 1x TAE) of Ethidium Bromide stock solution (10mg/ml of double distilled water). After gentle shaking, the gel was poured onto gel casting tray. After 15-20 min., the gel was immersed inside the gel tank filled with 1x TAE (PH 8.0). To each PCR product of 10 µl was added 1 µl 6x loading dye (0.25% bromophenol blue; 0.25% xylene cyanol FF; 40% sucrose). With the help of 10 µl pipettes samples were loaded in individual wells. In parallel, was also loaded 50 bp size reference ladder (Fermentas, Lithuania, USA). The power pack was adjusted at 5 Volts/cm of run and the total duration of electrophoresis varied from 1.5 to 2.5 hours. After optimum run of samples the gel slabs were visualized under UV trans-illuminator and documented in gel documentation system (Bio-Rad Laboratories Inc., USA). This was followed by scoring of bands, where in our study remark of A, B and H will be followed in description of results for recurrent type, donor type and heterozygous individuals in various backcross generations.

3.1.1.5 Screening for seedling stage salinity tolerance

Twenty three rice genotypes (Table 3.1) were screened for seedling stage salt tolerance under controlled environment in National Phytotron Facility at IARI, New Delhi during *kharif* 2012. Pre-germinated (3 days after germination) seeds were sown in punch holes made on extruded polystyrene foam floats fitted with a nylon wire mesh on the bottom side and suspended on trays filled with Yoshida nutrient solution (Yoshida et al. 1976). As shown in the Figure 1 Each tray carried 12 entries and controls, Pokkali (salt tolerant) and IR29 (salt sensitive) and these trays formed fundamental unit of replication. Two replications were used for each set of genotypes, with nine individual plants per line evaluated for each replication. In order to avoid border effect, one of the controls, FL478 was sown along the border on all sides to normalize competition for light and space for next rows. Salt stress was imposed 14 days after germination by adding 60mM NaCl (ECe of 6 $dS.m^{-1}$) and salt concentration was increased to 120mM (ECe of 12 $dS.m^{-1}$) after 3 days in Yoshida nutrient solution and was maintained until final phenotypic scoring. The pH of the nutrient solution was adjusted daily to 5.0, and the culture solution was replaced every 7 days. 16 days after salt stress, entries were scored based on visual symptoms using the modified score scale, (Gregario et al. 1997) with scores ranging from 1 (highly tolerant) to 9 (highly sensitive) Table 3.

3.1.1.6 Estimation of salt ion concentrations in shoot and root

In the controlled hydroponic conditions, sampling of plant tissues for Na^+ and K^+ ion concentration in shoot and root was done immediately after phenotypic scoring. Whole plant was washed first for 1 min. in tap water followed by 1 min. in distilled water twice, blotted dry and recorded observations of length and fresh weight of shoot and root. Further root and shoot samples were kept in oven at 60°C for 3 days until completely dry and the dry weight was recorded. Dry samples (known weight) were mixed with 20 ml of 100 mM glacial acetic acid in 30ml test tube. The samples were kept in water bath for 2 h at 90°C which was followed by cooling, and the debris was removed by filtration. An aliquot of the digested material was taken and diluted ten times with 100mM glacial acetic acid to determine Na^+, and K^+ concentration by Flame photometer (ELICO CL360) by the equation:

$$Na^+ \text{or } K^+ \text{ concentration} = \frac{CdV}{1000DW}$$

Where, C is flame photometer reading, *d* is dilution factor, V is extraction volume (ml) and DW is oven dry weight (g). A standard curve of KCl and NaCl solutions of different concentrations, ranging from 10 to 100 $mg.l^{-1}$ was prepared and used for calibration. Na^+ and K^+ content of leaves and stem were expressed on $mg.kg^{-1}$ basis.

3.1.1.5. Statistical Analysis:

3.1.1.5. 1 Diversity and population structure

The SSR allele segregation data were used to construct dissimilarity matrix between genotypes using simple matching coefficient (Sokal and Michener 1958). The dissimilarity matrix was used for clustering of genotypes, based on unweighted neighbour-joining method (NJ). Analysis was performed using DARWin 5.0 (Perrier *et al.* 2003). To check the goodness of fit of the clustering, a cophenetic correlation was computed between similarity and the cophenetic matrices (Rohlf and Sokal 1981). Confidence limits of different clades were tested by bootstrapping 10,000 times to assess the repetitiveness of genotype clustering (Felsenstein 1985).

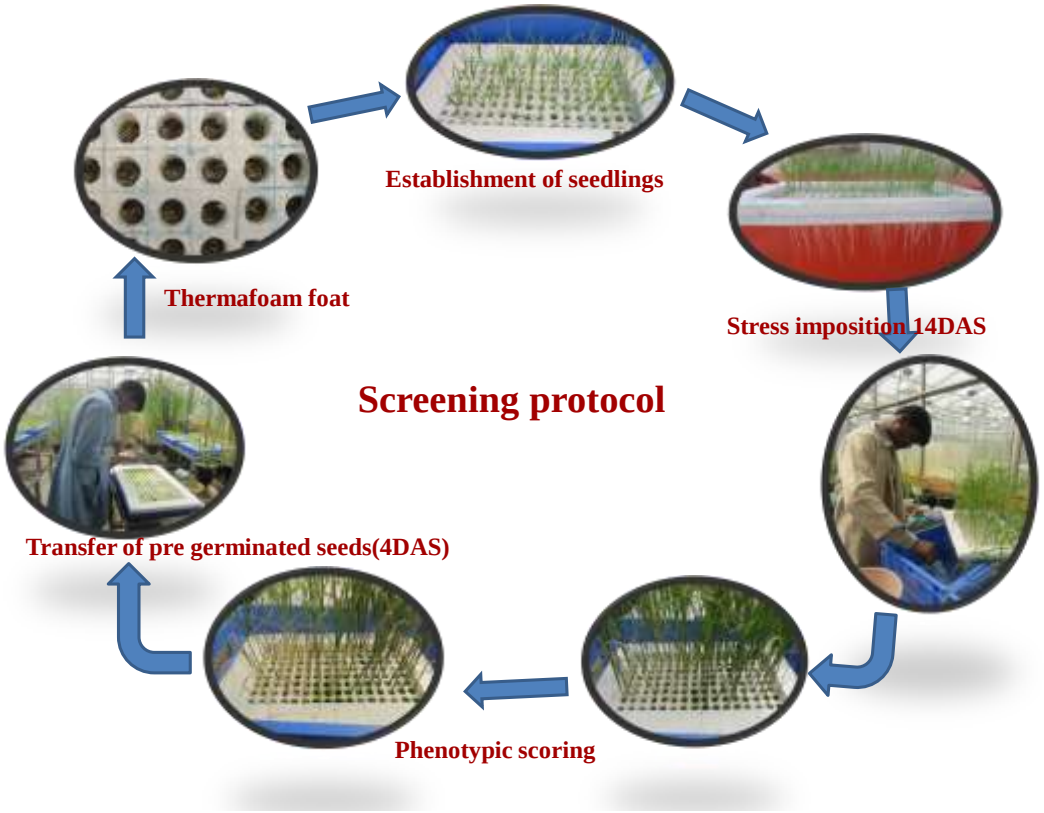

Figure 3.1: Screening of rice genotypes for seedling stage salinity tolerance

For SSR markers, polymorphism information content (PIC), was calculated as the measure of in formativeness of markers (Botstein *et al.* 1980). As an alternative to graphical clustering methods, a model-based Bayesian approach implemented in the software package Structure 2.3.3 (Pritchard *et al.* 2010) was used to analyse the population structure of rice accessions. Optimum number of populations was inferred by running an admixture ancestry model with correlated allele frequencies starting from two populations $K = 1$ to $K = 10$, with three runs at each K. For each run, 500,000 burn-ins followed by 500,000 Markov Chain Monte Carlo (MCMC) simulations were performed. The ideal value of K was determined from the uppermost hierarchical level of population structure, detected using an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values (Evanno *et al.* 2005). In addition, the ideal number according to Pritchard *et al.* (2000) was used as the criterion for defining the number of groups (*k).* The most trustworthy value was estimated based on the lowest negative number of Ln (the log-likelihood of the data) and the lowest standard deviation found during statistical analysis. Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected subpopulation (Pritchard *et al.* 2000).

3.1.1.5.2 Linkage disequilibrium

LD was estimated for each pair of SSR loci using Tassel 2.1 software (Bradbury et al. 2007), both in overall population and in subpopulations. LD was measured using D' and r2 estimates modified for multiple loci (Hedrick 1987). Significance (P values) of D' for each SSR pair was determined by 100,000 permutations.

3.1.1.5.3 Phenotype diversity and LD mapping

Agronomic data were analysed for phenotypic diversity using principal component approach. Principal component scores obtained for each genotype were used for computing squared Euclidean distances, and grouping of genotypes were performed using unweighted NJ method (Gascuel 1997) with bootstrapping of 10,000 iterations. LD mapping (association mapping) was performed to analyse marker–trait association by structured association approach using ancestry coefficient (Q values) estimates as covariate in a general linear model (GLM) function using TASSEL 2.1. For each marker–trait combination, GLM estimated the ordinary least squares solution (Searle 1987). Multiple testing corrections were performed by adjusting maker probability values for multiple test runs, by a permutation test

derived using a step-down MinP procedure (Ge *et al.* 2003). The significant association for a marker and trait was selected when adjusted *P* value (false discovery rate) was below.

3.1.2 Marker based haplotype diversity of *Saltol* QTL in relation to seedling stage salinity tolerance in selected genotypes of rice.

3.1.2.1 Plant material

A set of 23 diverse genotypes including accessions of *Oryza rufipogon* and *Kaipad* land races Basmati genotypes and induced mutant lines and other improved varieties were screened for seedling stage salt tolerance and haplotypes diversity in *Saltol* region. The characters and origin of genotypes is presented in Table 3.4.

3.1.2.2 Screening for seedling stage salinity tolerance

Twenty three rice genotypes (Table 4) were screened for seedling stage salt tolerance using similar procedure fallowed in section **3.1.1.4**

3.1.2.3 Molecular Marker

Twenty polymorphic SSR markers in the *Saltol* region on chromosomes 1 were used to study the diversity in the *Saltol* haplotypes in rice genotypes Fig 3.2).

3.1.2.4 DNA extraction and polymerase chain reaction amplification

Same procedure as followed in section **3.1.1.2 and 3.1.1.3**

3.1.2.5 Statistical analysis

3.1.2.5.1 Polymorphism information content (PIC)

SSR allelic composition for each genotype at every marker locus was determined by counting the number of alleles per locus and the allele frequencies and polymorphism information content (PIC) was determined using the formula,

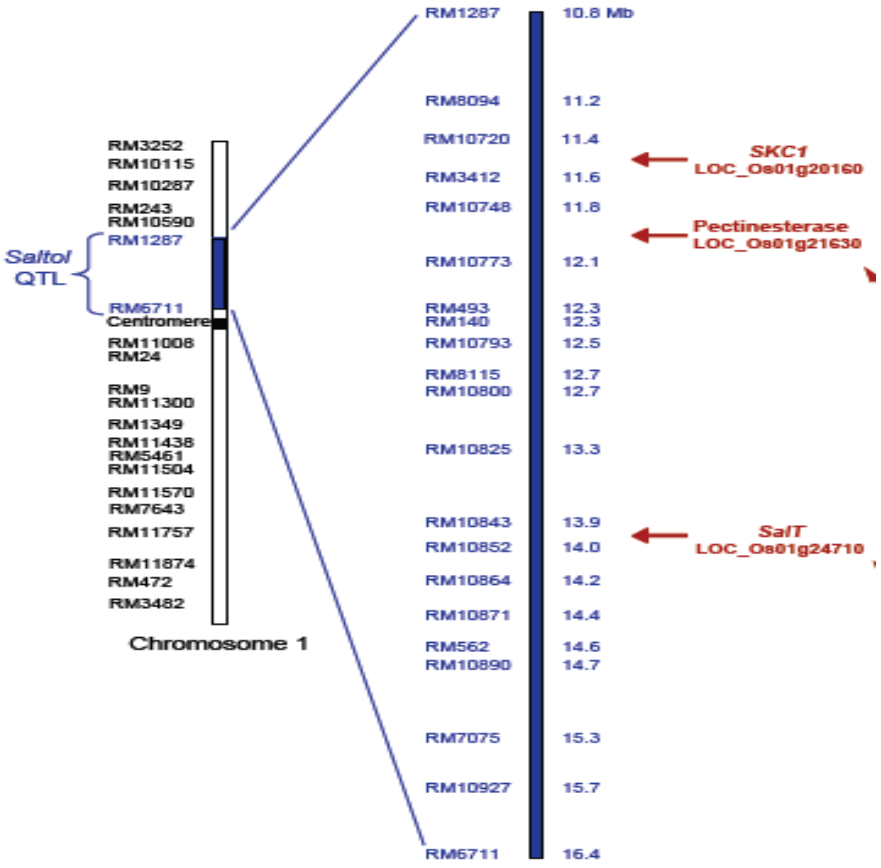

Fig 3.2. The *Saltol* region on chromosome 1 markers showing 21 polymorphic SSR (Thomson et al 2010)

$$PIC = \left(1 - \sum_{i=1}^{k} \hat{p}_i^2\right) \frac{2n}{2n-1} - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2\hat{p}_i^2 \hat{p}_j^2$$

where $\hat{p}_i$ was the estimated allele frequencies of k alleles (i=1 to k) and n was the number of individuals sampled (Botstein et al. 1980).

Table 3.4. Origin, characteristics and salt tolerance reaction of 23 rice genotypes

No	Genotype	Source origin	Characteristics
1	FL478	IRRI	Salt tolerant RIL of IR29
2	IR 29	IRRI	Salt susceptible
3	Pokkali	South Kerala, India	Salt tolerant landrace (*Pokkali*)
4	Kuthiru	North Kerala, India	Salt tolerant landrace (*Kaipad*)
5	Okayama	North Kerala, India	Salt tolerant landrace (*Kaipad*)
6	Jaiphula	Orissa	Landrace
7	Nipponbare	Japan	Landrace (*japonica*)
8	PB1121	IARI, India	Basmati variety
9	PB1	IARI, India	Basmati variety
10	Pusa1734-4	IARI, India	Salt tolerant NIL of PB1121
11	Pusa1734-24	IARI, India	Salt tolerant NIL of PB1121
12	Taraori Basmati	India	Basmati landrace
13	N22	India	Drought tolerant genotype
14	N292	India	Mutant line of N22
15	N295	India	Mutant line of N22
16	CSR10	CSSRI, India	Salt tolerant variety
17	CSR 30	CSSRI, India	Salt tolerant variety
18	Ezhome 1	North Kerala, India	Salt tolerant variety (*Kaipad*)
19	Ezhome 2	North Kerala, India	Salt tolerant variety (*Kaipad*)
20	NKSWR19	Kevali, UP, India	wild rice (*Oryza rufipogon*)
21	NKSWR20	Rawak, UP, India	wild rice (*O. rufipogon*)
22	NKRWR32	Lokmanpur, UP, India	wild rice (*O. rufipogon*)
23	NKSWR35	Bariin, UP, India	wild rice (*O. rufipogon*)

3.1.2.5.2 Cluster analysis

Cluster analysis was performed on a dissimilarity matrix of simple matching coefficients (Sokal and Michener 1958) using unweighted neighbour joining algorithm using DARwin version 5.0.158 (Perrier et al 2003) with 30000 permutations.

3.1.2.5.3 Haplotype analysis

Haplotype analysis was conducted according to Bai et al. (2003) and Liu and Anderson (2003) using tightly linked best six SSR markers, i.e., RM1287, RM8094, RM10720, RM3412, RM10748 and RM493 (Islam et al. 2005, Mohammadi-Nejad et al. 2008, Aliyu et al. 2010; Islam et al. 2012) to compare with *Pokkali* as a reference.

Objective 2 & 3: **Marker assisted introgression of *Saltol* locus into Pusa Basmati 1121 and Characterization of backcross derived lines for salinity tolerance, yield, grain and cooking quality traits.**

Since objective 2 mainly related to intogression of *Saltol*, while objective 3 related to continuation of objective 2 mainly on characterisation of introgressed lines. Therefore, both objectives are presented together in section 3.2

3.2.1 Plant materials and development of improved lines

Plant materials used in the study include PB1121, a salt susceptible popular Basmati rice variety, as the recurrent parent and FL478 (IR 66946-3R-178-1-1), a *Saltol* QTL carrying non-Basmati RIL derived from IR29/Pokkali cross, as donor parent. FL478 has seedling-stage salt tolerance upto 18 $dS.m^{-1}$ (Thomson et al., 2010). A single F_1 plant from cross PB1121 X FL478 (designated as Pusa1734) was backcrossed with PB 1121 to generate the BC_1F_1s seeds (Fig 3.3). Marker-assisted foreground selection was employed using QTL linked markers to identify plants heterozygous for *Saltol* linked marker. Further, the selected plants were subjected to analysis of background recovery using polymorphic markers augmented with stringent phenotypic selection for agro-morphological, grain and cooking quality traits. A single BC_1F_1 plant with maximum recovery of the recurrent parent genome (RPG) as well as phenotypic similarity to recurrent parent was backcrossed to develop the BC_2F_1 generation. The BC_2F_1 plants were also subjected to foreground and background selection followed by phenotypic selection to identify one best plant heterozygous for *Saltol* and with maximum recovery of RPG, which was backcrossed to develop BC_3F_1s. The foreground, background and phenotypic selection cycle was repeated in BC_3F_1 as well to identify plants heterozygous for *Saltol* and with maximum recovery of RPG. These plants were then selfed to generate BC_3F_2 populations. In the BC_3F_2 generation, plants homozygous for *Saltol* were identified and then advanced to the BC_3F_4 generation with further selection

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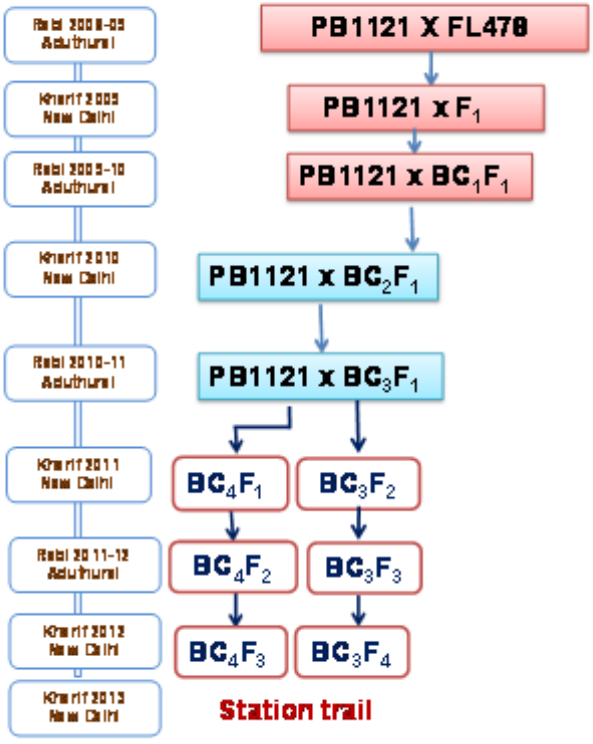


Fig. 3.3. Marker Assisted Backcross Breeding (MAB) scheme followed for incorporation of *Saltol*, major QTL for seedling stage salinity tolerance in to genetic background of Pusa Basmati 1121.

for morphological traits, grain and cooking quality in the subsequent generation. RPG recovery was quantified at every step using STMS markers polymorphic between parents.

3.2.2 DNA extraction and polymerase chain reaction amplification

Similar procedure fallowed as described in section **3.1.1.2 and 3.1.1.3**

3.2.3 Molecular marker analysis

3.2.3.1Foreground selection

Foreground selection was carried out using RM3412, which is the peak marker for *Saltol* QTL. Additionally, the markers RM35, RM1287, RM8094, RM10720, RM10748 and RM493 present on carrier chromosome (Chr 1) flanking the *Saltol* QTL were used for parental polymorphism between PB1121 and FL478 and the nearest flanking polymorphic markers were used for recombinant selection to minimize donor segment

3.2.3.2 Background selection

A set of 600 SSR primer pairs distributed uniformly across the rice genome was used for parental polymorphism survey between the donor FL478 and the recurrent parent PB1121. The polymorphic SSR markers were then used to identify plants with maximum recovery of recurrent parent genome (RPG) in each backcross generation. The PCR products were amplified and resolved in 3.5 % Metaphor^{TM} gel. The extent of Recurrent Parent Genome (RPG) recovery was calculated as per the formula:

$$\text{RPG (\%)} = \frac{(A+\frac{1}{2}H)}{(A+B+H)} \text{ X } 100,$$

Where, A is the number of recurrent parent alleles at genome wide marker loci; B is the number of donor fragments and H represents markers showing heterozygosity.

The genomic contribution of the parents in the elite selections in different backcross generation was estimated using the software Graphical GenoTypes (GGT) Version 2.0 (Van Berloo 1999).

3.2.4 Screening for seedling stage salinity tolerance

Saltol introgressed lines were screened for seedling stage salt tolerance using procedure fallowed in section **3.1.1.5**

3.2.5 Estimation of salt ion concentrations in shoot and root

Salt ion concentrations in shoot and root of some improved lines was estimated using procedure fallowed in section **3.1.1.6**

3.2.6 Evaluation of agronomic performance

The observation was recorded on five plants selected at random for each genotype. Data recorded for the five plants was averaged and used for statistical analysis. The details of the observation recorded on various traits are presented.

3.2.6.1 Days to 50 per cent flowering

The total number of days from sowing to the emergence of panicle in 50 per cent of the total plants in each plot was recorded.

3.2.6.2 Plant height (cm)

Height of the plant at maturity from the base of the plant to the tip of the main panicle was measured and expressed in centimetres.

3.2.6.3 Number of panicles per plant (Productive Tillers)

The total number of panicles in each plant was counted at the time of maturity.

3.2.6.4 Panicle length (cm)

The length of the main panicle measured from the base to tip and is expressed in centimetres.

3.2.6.5 Spikelet fertility (%)

This was calculated by number of filled spikelets in a panicle divided by total number of spikelets in a panicle multiplied by 100 and expressed in percentage.

3.2.6.6 Days to maturity

Total number of days taken by each genotype from sowing to physiological maturity of plant was taken as days to maturity.

3.2.6.7 Yield per plant (g)

The weight of the grains per plant was recorded in grams after threshing, cleaning and drying.

3.2.6.8 1000 grain weight (g)

Weight of thousand randomly selected filled grains was measured and recorded in grams.

3.2.7 Evaluation of Grain and cooking quality

The analysis of grain and cooking quality traits such as

3.2.7.1 Kernel length before cooking (mm) (KLBC), Kernel breadth (mm) (KBBC) and Length/ breadth ratio (LBR): Approximately, 5 grams of paddy from single plant selections at each backcross generation was de-hulled with palm huller. The brown rice was milled with rice polisher (Kett Rice Polisher, AI43590, Ota-ku, Tokyo). Twenty to thirty well milled whole kernels were scanned at 300 dpi in the eVision System (CDAC, Kolkata, India) for recording KLBC, KBBC and LBR.

3.2.7.2 Kernel length after cooking (mm) (KLAC), Kernel breadth after cooking (KBAC): Cooking was done as per the standard practice. 10 milled whole kernels for each sample were placed in 15 cm test tubes. Each test tube was filled with 10 ml of RO water and left standing for half an hour. The test tubes mounted on stands were placed in water bath at 100^{o}C for 7 minutes. Then the cooked kernels along with water were poured in 2.5" petri-plates. Five Whole cooked grains were scanned at 300 dpi in the eVision System (CDAC,

Kolkata, India) for recording KLAC and KBAC.

3.2.7.3 Elongation ratio(ER): Elongation ratio was calculated as:

$$\text{Elongation ratio} = \frac{\text{Average length of cooked rice (mm)}}{\text{Average length of raw rice (mm)}}$$

3.2.7.4 Aroma (ARO): Aroma was determined after cooking by a panel of experts through sensory evaluation and given score 0(no aroma) to 2(highly aromatic).

3.2.8 Statistical analysis:

The statistical analysis of the data on the individual character was carried out on the mean value of 5 randomly selected plants on each genotype using Augmented design.

Field Layout for Augmented Design

The experimental plot was divided into six blocks and each block contains only a subset of entries and common checks. Five check varieties were assigned at random to plots within the blocks and replicated in each block. Experimental error was estimated by treating the checks as if they were treatments in a RBD. MSE was then used to construct standard errors for comparisons. A two way table of check variety x block means was constructed and the grand mean ($\bar{\bar{X}}$) and the mean of the checks ($\overline{Xi}$) in each block was computed.

The block adjustment (a_i) was computed as:

$a_i = \overline{Xi} - \bar{\bar{X}}$

Adjust yields of new selections as

$$\bar{Y}ij = Yij - ai$$

$\bar{Y}ij$= adjusted yield

Yij = Actual yield

Table 3.5: Standard ANOVA for augmented design using check yields

Source	d.f.	MS
Block	b-1	
Checks	c-1	
Treatments	t-1	
Checks Vs Treatments	1	
Error	(c-1) (b-1)	MSE
Total Entries	n-1	

Difference between two check varieties

$$Sc = \sqrt{2MSE}/b$$

Difference between adjusted means of two selections in the same block

$$Sd = \sqrt{2MSE}$$

Difference between adjusted means of two selections in different blocks

$$Sv = \sqrt{2(c+1)MSE}/C$$

Difference between adjusted selection and check mean

$$Svc = \sqrt{(b+1)(c+1)MSE}/bc$$

Where b= number of block, c=number of checks

Objective 4. Expression profiling *SKC1* gene in *Saltol* derived NILs, donor and recipient parents

3.3.1 Plant Material

The recipient parent PB 1121, the donor parent FL478 (IR 66946-3R-178-1-1) and Two BC_3F_5NILs namely Pusa1734-8-3-3(NIL1) and Pusa1734-8-3-26 (NIL2) derived from

PB1121 X FL478 cross carrying *Saltol* from Donor parent with more than 98 per cent of recurrent parent genome and phenotype. These two NILs had also shown consistent salinity tolerance reaction at seedling stage both in controlled and field condition. All four genotypes were used for stress imposition and *SKC1* gene expression

3.3.2 Experimental set up and stress imposition

The experiment was conducted under controlled condition in National Phytotron Facility at IARI, New Delhi during July 2013. Pre-germinated (3 days after germination) seeds were sown in punch holes made on extruded polystyrene foam floats fitted with a nylon wire mesh on the bottom side and suspended on trays filled with Yoshida nutrient solution (Yoshida et al. 1976). Air temperature ranged from30- 35^0C during the day and 20–24^0C during night. Relative humidity ranged from 70% to 80%. The pH of the nutrient solution was adjusted daily to 5.0, and the culture solution was replaced every 7 days. Each tray contain 16 rows and 10 column where each genotypes was planted in three rows and two border two rows were planted with parental lines to eliminate border effect. All four genotypes are planted in single tray with three rows each. Each tray will form one fundamental unit of replication. Salinity stress was imposed at 21 days after germination with 50mM and 100 mM of NaCl in three replications each. One separate control set was grown using normal nutrient solution.

3.3.3 Sampling for RNA extraction

Shoot sample for RNA extraction was collected at 0hr, 3hr, 6hr, 12hr, 24hr by selecting 3 plants randomly from each genotype in each replication of 50mM, 100mM and control. Samples were homogenised and wrapped in aluminium foil and defreeze in liquid nitrogen and store in -80^0C.

3.3.4 General guidelines

In order to avoid any contamination from bacteria and RNase potentially existing in the working place the bench and routinely used equipments, such as the pipettes, were regularly cleaned with 70 % (v/v) ethanol and RNase Zap(Ambion). Milli-Q water (Millipore was used in all reagents and procedures were required for the following molecular biology protocol. Additional Milli-Q water (Millipore) autoclaved with 0.1% diethylpyrocarbonate

(DEPC) (Sigma) was also prepared for those works associated with RNA. All glass and plastic wares were either autoclaved or manufacturer-certified sterile, prior to use.

3.3.5 RNA Preparation and cDNA synthesis

To examine the expression levels of SKC1 transcript in shoot, the total RNA was isolated from the shoots collected at 0 hour, 3 hr, 6 hr, 12 hr, 24 hr 21 days after germination. The

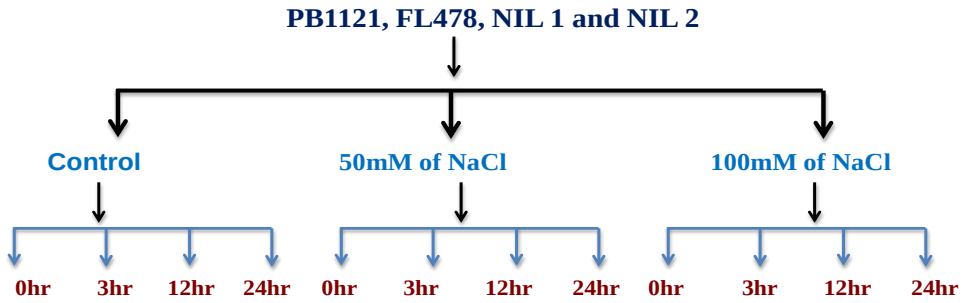


Fig 3.5. Experimental set up *SKC 1* gene expression analysis in parents and NILs

isolation of total RNA from all 48 (4 genotypes X 3 levels of stress X 4 time intervals) samples carried out using RNeasy plant mini kit (Qiagen,Germany) according to manufacturers protocol. The concentration of RNA samples was determined using NanoDrop^{TM} 1000 spectrophotometer (Thermo Fisher Scientific,USA).

3.3.6 Quantification of RNA

Using a pipette, 1µl of undiluted RNA was placed directly on to the lower measurement pedestal of the Nano Drop 1000. The upper optical pedestal was lower and the measurement was taken. After each reading, sample was wiped from both the upper and lower pedestals using a clean kimwipe. The solvent were not necessary to clean the optical surfaces of the instrument. The each RNA sample was quantified in this manner. The concentration of the total RNA varied with different samples and thus the total RNA from each sample was

diluted with nucleus free water to normalize the RNA concentration in all samples prior to cDNA synthesis.

3.3.7 Assessment of Quality of total RNA

2 µl of each sample was incubated at 850c for 3 mins to denature RNA before running on a 1% RNase free agarose gel with an RNA size marker (MBI fermentas) to identify if any degradation of RNA occurred. The clear integrity of 18 and 28S ribosome RNA bands was used to indicate that the total RNA was not degraded.

3.3.8 cDNA preparation

The Affinity Script QPCR cDNA Synthesis Kit is designed for the highest efficiency conversion of RNA to cDNA and is fully optimized for two-step quantitative reverse transcription-PCR (QRT-PCR) applications. A two-step RT-PCR format is useful for amplifying multiple targets from a single cDNA source, for maintaining archival cDNA, and for providing maximum flexibility in selecting a downstream QPCR reagent system. The AffinityScript QPCR cDNA synthesis kit provides a streamlined master mix format and fast protocol, with a 15-minute cDNA synthesis step and an overall protocol time of just 25 minutes. The kit is formatted for high-yield production of cDNA up to 12 kb and each reaction accommodates a range of RNA amounts from pg to µg.

3.3.9 Quantitative Real time qPCR

Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was performed using Agilent technologies stratagene Mx3005P (Agilent technology,USA). The reaction mixture consisted of 1µl cDNA, 1µl forward and reverse primer mix(µM each), and 25 µl iQSYBR green supermix. No-templetes controls were included in each PCR run to ensure purity of reagents and minimal carryover contamination. *SKC1* and 18S rRNA standard primer sequence (Table 3.6) were used in real time quantitative PCR.

To confirm that the SYBR green fluorescence is a direct measure of the product of interest subsequent to the PCR reaction cycles, dissociation curve analysis was performed. The amplification results were analysed using Stratagene Mx3005P software (Agilent technology,USA). The log fold change(dRn) (Goss *et al* 2001) was used to quantify the

results obtained by real time PCR and 18S rRNA was used as endogenous control as the levels of the molecule were found to be fairly stable throughout the stages of plant development. In all experiments, appropriate negative controls containing no template cDNA were subjected to the same procedure to exclude or detect any possible contamination, e.g. by genomic DNA. All reactions were set up in hexaplate. All reaction mixture were analysed by agarose gel electrophoresis to confirm that only one PCR product was synthesised. The thermal cycling condition were composed of 50°C for 10 min followed by an initial denaturation step at 95°C for 10 min,40 cycle at 95°C for 30s,then 60°C for 40 min and 72°C for 1 min.

Table 3.6: Sequence information of the primers used for expression analysis

SI No	**Gene**	**Sequence**	**Reference**
1	*SKC1*	F-TTCATGGCGGTCAACTCGA R-TTTGCTGGTGTTTGTCTTGGA	Walia *et al* 2005
2	*18S rRNA*	F-ATGATAACTCGACGGATCGC R-CTTGGATGTGGTAGCCGTTT	Kim *et al* 2003

4.1 Research Paper I

Marker based Haplotype diversity of Saltol QTL in relation to seedling stage salinity tolerance in selected genotypes of rice.

Abstract

Rice is sensitive to salinity especially during early seedling and reproductive stages. A major quantitative trait locus (QTL) for seedling stage salt tolerance in rice, *Saltol* has been mapped on chromosome 1. The present study was carried out with the objective of characterising the haplotypes diversity in the Saltol region and it association with seedling stage salinity tolerance in 23 diverse germplasm including land races, wild germplasm and improved varieties. Twenty polymorphic simple sequence repeat (SSR) markers linked with *Saltol* were used to study marker haplotypes diversity in Saltol region. The genotypes were phenotyped at seedling stage for salt stress response using a visual score scale of 1 to 9 by exposing them to NaCl concentration of ECe of 12 $dS.m^{-1}$ under controlled environmental conditions. Seedling stage salt response of the test genotypes varied significantly. Genotypes such as Kuthiru, Orkayama and Ezhome 1 from Kaipad region of Kerala showed salinity tolerance level and haplotypes of Saltol locus similar to Pokkali. Whereas salt tolerant wild rice genotypes NKSWR19 and NKSWR20, were found to possess a different haplotypes and therefore may be novel sources for salt tolerance. Allele diversity within the *Saltol* markers used for haplotypes analysis, showed variation ranging from 2 to 6 alleles (RM7075). The polymorphism information content (PIC) value varied from 0.08 (RM8115) to 0.73 (RM7075) with an average of 0.51. Two markers, RM493 and RM7075 exhibited high PIC value and were found to be superior for genetic diversity analysis. Cluster analysis based on SSR marker data divided the rice genotypes into six groups with each group comprising of genotypes with varying level of salt tolerance. Altogether, 23 rice genotypes were found to possess 14 different haplotypes based critical markers linked to *Saltol* QTL using Pokkali as the reference. The haplotypes possessing both of RM8094 and RM3412 markers could discriminate the salt tolerant genotypes from the susceptible genotypes and hence could be useful for marker-assisted selection of *Saltol* QTL.

Key wards: Saltol, Salinity tolerance, Haplotype diversity,

Introduction

Salinity is one of the major factors limiting productivity of crops including rice around the world. Soil salinity is often accompanied by osmotic imbalance, mineral deficiency and toxicity that has adverse effects on crop growth (Asch et al. 1997, 1999; Ahmad and Prasad 2012). Rice is cultivated in more than 115 countries, of which Asia's share is more than 91% of the world total. Majority of the Asian rice production zone is confined to South and Southeast Asia wherein severe salinity related problems are rampant in 20% of the rice area amounting to 47 M ha (Abbas et al. 1994), which consists of warm humid coastal regions and marshy inlands (Vinod et al. 2013).

Rice is sensitive to salinity, particularly during the seedling stage (Maas and Hoffman 1977) and the earlier set benchmarks (Maas and Grattan 1999; Hanson et al. 1999) indicate that rice yield decreases 12% for every unit (dS m^{-1}) increase in ECe above the threshold tolerance of 3.0 dS m^{-1} (Maas 1990). In rice, tremendous variation exists for salt tolerance within species, which provides great opportunities to improve salt stress tolerance through genetic means. Breeding for salt tolerant rice varieties has been difficult task owing to the complexity in the inheritance pattern of salinity tolerance, strong GE interaction and difficulties experienced in the conventional screening techniques (Gregorio and Senadhira 1993; Gregorio 1997; Mohammadi-Nejad et al. 2008; Islam et al. 2012; Vinod et al. 2013).

Rapid advancements in the molecular marker technologies and their application in practical plant breeding during last two decades (Mackill et al. 1999; McCouch and Doerge 1995) have helped in mapping several rice genes/QTLs for salt tolerance parameters like Na^+ and K^+ uptake, Na^+ and K^+ concentration and Na^+/K^+ ratio in shoot (Gregorio 1997; Koyama et al. 2001; Lin et al. 2004; Ammar et al. 2009; Pandit et al. 2010). A major QTL among these was *Saltol* (for *salt tolerance*) mapped on the short arm of chromosome 1 by using an F_8 recombinant inbred lines (RIL) developed from the cross of a salt tolerant land race Pokkali from kerala and IR29, salt sensitive rice variety(Gregorio 1997). Later studies identified that *Saltol* controlled Na-K absorption (Bonilla et al., 2002) and accounted for substantial phenotypic variation for Na^+, K^+ and Na-K absorption ratio amounting to 39.2, 43.9 and 43.2% respectively. Further SSR marker based fine mapping of *Saltol* locus using RILs of Pokkali/ IR29 saturated this region with more than 20 microsatellite markers spanning on a 5Mb region (Niones 2004; Thomson et al. 2007, 2010; Alam et al. 2011). Since the original *Saltol* donor Pokkali possessed several undesirable characters, a highly tolerant RIL from the IR29/ Pokkali cross, IR 66946-3R-178-1-1 (FL478) was identified as a novel source of seedling stage salt tolerance (Walia et al. 2005). Since then, FL478 has been

widely used in transfer of *Saltol* though marker assisted backcross (MABC) breeding into elite varietal backgrounds such as BR11, BRRI dhan 28, IR64, AS996 and Swarna (Rahman et al. 2008; Ho et al. 2010; Linh et al. 2012, Huyen etal. 2012; STRASA 2011). Although FL478 has been widely used as donor for *Saltol* in breeding programmes, it possess red pericarp colour of endosperm, which is an undesirable trait. Therefore, there is a need to identify new donors with improved grain and cooking quality traits and normal pericarp colour.

In addition to Pokkali tract of south Kerala, *Kaipad* is an unique coastal organic wetland rice ecosystem from north Kerala,which is naturally saline prone and unfamiliar to the scientific world(Chandramohanan and Mohanan 2012; Vanaja 2012). *Kaipad* ecosystem is charcterised with marshy lands rich in biodiversity of flora and fauna embedded with a traditional rice production system. However, *Kaipad* soils are coarser than *Pokkali* soils (Vanaja 2013). Cultivation of traditional low yielding saline tolerant rice landraces is the most common practise of the *Kaipad* region. The most popular varieties are Kuthiru and Orkayama, alongwith other landraces such as Mundon, Kandorkutty, Orpandy, Odiyan, Orissa, Punchakayama and Kuttadan (Chandramohanan and Mohanan 2012; Vanaja 2012). Recently, high yielding rice varieties such as Ezhome 1 and Ezhome 2 were developed for *kaipad* tracts by Kerala Agricultural University utilizing the traditional land races (Vanaja et al 2010). Although there are few reported studies of SSR based survey of *Saltol* region on diverse germplasm sets (Mohammadi-Nejad et al. 2008, Aliyu et al 2010; Islam et al 2012), to our knowledge, there is no such attempt so far on *kaipad* landraces and improved lines and wild germplasm lines. In this study, we used SSR based haplotyping of *Saltol* region in a set 23 diverse genotypes including accessions of *Oryza rufipogon* and *Kaipad* land races and improved varieties with special focus (i) to evaluate the haplotype diversity of *Saltol* region in rice genotypes, (ii) Identification of most discriminating SSR markers for salt tolerance and (iii) to identify putative novel genotypes that possess distinct *Saltol* haplotypes, as new sources of salt tolerance in rice.

Material and Methods

Plant material

A set of 23 diverse genotypes including accessions of *Oryza rufipogon* and *Kaipad* land races Basmati genotypes and induced mutant lines and other improved varieties were screened for

seedling stage salt tolerance and haplotypes diversity in *Saltol* region. The characters and origin of genotypes was presented in Table 4.1.1.

Screening for seedling stage salinity tolerance

Twenty three rice genotypes (Table 4.1.1) were screened for seedling stage salt tolerance under controlled environment in National Phytotron Facility at IARI, New Delhi during *kharif* 2012. Pre-germinated (3 days after germination) seeds were sown in punch holes made on extruded polystyrene foam floats fitted with a nylon wire mesh on the bottom side and suspended on trays filled with Yoshida nutrient solution (Yoshida et al. 1976). As shown in the Figure 4.1.1 Each tray carried 12 entries and controls, Pokkali (salt tolerant) and IR29 (salt sensitive) and these trays formed fundamental unit of replication. Two replications were used for each set of genotypes, with nine individual plants per line evaluated for each replication. In order to avoid border effect, one of the controls, FL478 was sown along the border on all sides to normalize competition for light and space for next rows. Salt stress was imposed 14 days after germination by adding 60mM NaCl (ECe of 6 $dS.m^{-1}$) and salt concentration was increased to 120mM (ECe of 12 $dS.m^{-1}$) after 3 days in Yoshida nutrient solution and was maintained until final phenotypic scoring. The pH of the nutrient solution was adjusted daily to 5.0, and the culture solution was replaced every 7 days. 16 days after salt stress, entries were scored based on visual symptoms using the modified score scale, (Gregario et al. 1997) with scores ranging from 1 (highly tolerant) to 9 (highly sensitive).

Molecular Marker Analysis

Twenty polymorphic SSR markers in the *Saltol* region on chromosomes 1 were used to study the diversity in the *Saltol* haplotypes in rice genotypes. Total genomic DNA from 23 genotypes was extracted by the micro-extraction protocol of Prabhu et al. (1998). Polymerase chain reaction (PCR) was performed in a thermal cycler (G-Storm, Somerset, UK) using a 10 µl total reaction volume as described previously (Basavaraj et al. 2010). This contained 30 ng/ µl of template DNA, 5 pmol of each primer (synthesized from Sigma Inc., St. Louis, MO, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (MBI, Fermentas, Vilnius, Lithuania) and 0.5 U of Taq polymerase (Bangalore Genei, Bangalore, Karnataka, India). Polymerase chain reaction comprised one cycle of denaturation at 95 °C for 5 min, followed by 35 cycles each at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 7 min. The amplified products were resolved on 3.5% Metaphor^{TM} gel (Lonza, Rockland, ME, USA) containing 0.1 mg/ml of ethidium bromide (Amresco, Solon, OH, USA) along with a

DNA size standard 50bp ladder (MBI, Fermentas) and documented in a gel documentation system (BioRad, Hercules, CA, USA).

SSR allelic composition for each genotype at every marker locus was determined by counting the number of alleles per locus and the allele frequencies and polymorphism information content (PIC) was determined using the formula,

$$\mathrm{PIC}=\left(1-\sum_{i=1}^{k}\hat{p}_i^2\right)\frac{2n}{2n-1}-\sum_{i=1}^{k-1}\sum_{j=i+1}^{k}2\hat{p}_i^2\hat{p}_j^2$$

where $\hat{p}_i$ was the estimated allele frequencies of k alleles (i=1 to k) and n was the number of individuals sampled (Botstein et al. 1980). Cluster analysis was performed on a dissimilarity matrix of simple matching coefficients (Sokal and Michener 1958) using unweighted neighbour joining algorithm using DARwin version 5.0.158 (Perrier et al 2003) with 30000 permutations. Haplotype analysis was conducted according to Bai et al. (2003) and Liu and Anderson (2003) using tightly linked best six SSR markers, i.e., RM1287, RM8094, RM10720, RM3412, RM10748 and RM493 (Islam et al. 2005, Mohammadi-Nejad et al. 2008, Aliyu et al. 2010; Islam et al. 2012) to compare with *Pokkali* as a reference.

Results and Discussion

Rice is sensitive to salinity especially during early seedling and flowering stages. Early seedling stage salinity is one of the major constraints in rice production, where it would affect the establishment of rice crop and finally yield. Original *Saltol* donor *Pokkali* and improved donor FL478 derived from *Pokkali* possess some undesirable traits which pose difficulties in using them in marker assisted selection. Therefore, there is a need to identify improved germplasm lines possessing seedling stage salinity tolerance with similar Saltol haplotypes of *Pokkali* and it would help to accelerate breeding for salinity tolerance.

Phenotypic response to salinity

Seedling stage salinity tolerance of the twenty three rice genotypes under evaluation indicated varied responses to salt stress (Table 4.1.1). The genotypes were classified into five groups from highly tolerant (score 1) to highly sensitive (score 9). Further truncation of the results identified 11 genotypes as tolerant to highly tolerant (scores 1 and 3), 6 were moderately tolerant (score 5) and 6 susceptible (scores 7 and 9). Kuthiru and Orkayama land races from *Kaipad* region were found to be as tolerant of *Pokkali* but possessed white endosperm and lustrous grains in contrast to the red endosperm of *Pokkali* and FL478 and

thus can be a good alternate source for seedling stage salinity tolerance. Two improved *Kaipad* lines showed tolerant (Ezhome 1) to sensitive (Ezhome 2) reaction to salt exposure at seedling stage. Among the *O rufipogon* (wild) accessions, NKSWR19 showed highly tolerant resposne while NKSWR20 and NKSWR32 were found tolerant. The basmati cultivars Pusa Basmati 1121(PB1121), Taraori Basmati and Pusa Basmati1(PB1) were salt sensitive reaction while two *Saltol* introgressed PB1121 NILs (Pusa1734-4, Pusa1734-24) exhibited tolerance to salt stress. The EMS (ethylmethane sulphonate) induced mutant lines of N22 (N292, N295) did not show any significant variation for salt tolerance from that of N22.

Genotype grouping based on molecular diversity

Genotypic cluster analysis based on the allele pattern of twenty SSR markers divided the genotypes into six clusters (Fig 4.1.2). First cluster comprised of four salt tolerant genotypes with a bootstrap value of 82%. All the *kaipad* landraces and improved lines except Ezhome 2 were grouped with Pokkali indicating the common allelic profile of markers within the *Saltol* region. Jaiphula, a highly salt sensitive genotype shared common allelic profile for 13 markers and showed distinct alleles for seven markers, namely RM1287, RM8094, RM10720, RM10843, RM10852, RM7075 and RM10927 (Appx 1). This indicate indirectly that these markers may form the basis of identifying key markers associated with for salt tolerance. Highly salt sensitive genotypes IR29 and PB1 formed the second cluster with a high bootstrap value of 96% along with FL478 and W32, both salt tolerant. IR29 is the parent of FL478. Looking back to the pedigree of PB1, its lineage can be easily trace back to IR22 (IR579-160-2) as the female parent of Pusa 150, from which PB1 was derived by crossing to Karnal Local. IR579-160-2 falls in the lineage of IR29 as one of its immediate grandparents; hence this grouping has lines that are identical by descent (IBD). However, the genetic proximity of NKSWR32 with the group members could not be explained. Interestingly, looking at the allele pattern with respect to salt tolerance within this group, an apparent departure from similarity can be found for two markers RM8094 and RM10793, relative to other marker loci that are predominantly similar across the members. The fourth cluster constituted three wild *O. rufipogon* lines NKSWR19, NKSWR20 and NKSWR35 indicating that they have different allelic configuration at the *Saltol* region. This is well substantiated by the unique allele pattern observed for at least eight markers. Six genotypes of cluster 5 had two robust groupings, N22 and its mutant lines forming the first with a bootstrap percentage of 100 and the Near Isogenic lines(NIL) of PB1121 (Pusa1734-3 and Pusa1734-26) forming the second with a bootstrap value of 99% along with PB1121. Both the NILs of PB1121

carrying *Saltol* introgression showed high level of salinity tolerance while PB1121 was highly sensitive. This provided another opportunity of comparing the *Saltol* haplotypes between near isogenic salt tolerant and susceptible lines. Five genotypes falling in cluster 6 also showed varied level salt tolerance, with CSR10 being highly tolerant. CSR10 differed from other members of this cluster with for two markers *viz,* RM8094 and RM3412, for which it possessed *Pokkali* type alleles. Deducing marker allele pattern associated with salt tolerance among the clusters, we could not find any consistent association for four markers (RM10843, RM10852, RM7075 and RM10927) that were suspected to be associated with salt tolerance in the first group in comparison with Jaiphula. Therefore, we could narrow down to six key SSR markers, namely RM1287, RM8094, RM10720, RM3412, RM10748 and RM493 by genotype grouping based on haplotype heterogeneity of the *Saltol* region *vis-à-vis* salt tolerance response.

Molecular diversity and haplotype analysis of *Saltol*

The *Saltol* region is 5.6 Mbp long and is relatively very large with respect to haplotype conservation among the rice gene pool. All of the 20 markers spanning 5.6Mbp in the *Saltol* region on chromosome 1 used in this study were reported to be associated with salt tolerance in several previous investigations (Niones 2004; Thomson et al 2010; Alam et al 2011). Interestingly, but not unexpected, these markers were found to be highly polymorphic in 23 rice genotypes screened (Table 4.1.2, Fig 4.1.4) indicating that *Saltol* haplotype is poorly conserved across rice gene pool. The results of SSR polymorphism shows that shortest amplicon was produced by RM10825 with size of 80bp while RM10864 produced the longest amplicon size (330bp). Haplotype allele diversity of the SSR markers ranged from one hexaallelic (RM7075) to seven biallelic markers. Additionally, there were four triallelic, three quadriallelic and five pentaallelic markers. The polymorphic information content (PIC) varied from 0.08 (RM8115) to 0.73 (RM7075) with an average of 0.51. Since PIC is a function of allelic diversity, this result implies that some loci within *Saltol* region had relatively more frequent recombinations and evolutionary reorganisations resulting in more number of alleles and high PIC values. The results indicated that two SSR markers RM493 (PIC=0.72) and RM7075 (PIC=0.73) found to be better indicators of genetic diversity within *Saltol* region. It is desirable to have markers with low PIC value with positive association with salt tolerance for use in *Saltol* introgreesion by marker assisted backcross breeding(Singh et al 2011).

To confirm the association of six key markers (RM1287, RM8094, RM10720, RM3412, RM10748 and RM493) to salt tolerance, a graphical comparison of *Saltol* haplotypes of *Pokkali*, IR29 and FL478 was made along with a PIC heatmap of the marker loci obtained for all 23 genotypes (Fig 4.1.4). The allelic distribution clearly indicated that haplotype variability existed only within the key SSR markers, which was further confirmed by the uniform pattern of PIC values for this region ranging from 0.40 to 0.69. These six key SSR markers spanned between 10.8Mb to 12.3Mb in the reference rice genome (www.gramene.org).

By deciphering the haplotype pattern within these linked SSR markers using Pokkali as reference, fourteen haplotypes were identified among 23 genotypes (Figure 4.1.4). Kuthiru, a popular *Kaipad* landrace had haplotypes similar to that of *Pokkali*, indicating that a complete *Saltol* region was conserved across landraces of two different salt tolerant rice farming systems (*Kaipad* and *Pokkali*). However, both these systems being geographically very close to each other, possibility of common ancestry between this landraces could not be ruled out. Eighteen genotypes had different combination of *Pokkali* alleles at different loci, while three genotypes did not share any allele (Haplotype 14) with *Pokkali* haplotype. From the comparison of haplotypes with high frequency of *Pokkali* alleles, it can be deduced that the marker, RM8094 showed association with high salt tolerance response in the present study. However, this marker did not discriminate tolerance found in PB1121 and its NILs Pusa1734-4 and Pusa1734-24. Pokkali allele at marker RM3412 was present in salt tolerant genotypes such as Kuthiru, FL478, Orkayama, Ezhome 1, Pusa1734-4 and Pusa1734-24. However some of highly sensitive lines IR29, PB1 and Jaiphula also possess *Pokkali* allele at this locus. Further, some of the genotypes, which carried *Pokkali* allele at marker loci RM1287, RM10720, RM10843 and RM493 showed differential reaction to salinity stress. The results of haplotypes analysis demonstrated that no individual marker showed strong positive association with salt tolerance. The combination of RM8094 and RM3412 showed strong positive association with salt tolerance and consistently discriminated the salt tolerant genotypes from sensitive ones with some exceptions such as PB1 which is a salt sensitive genotype but possessed *Pokkali* allele at both these loci. Therefore identification of additional polymorphic markers between donor and recurrent parent is being necessary for marker assisted backcross breeding. Highly tolerant wild rice line NKSWR19 and tolerant NKSWR20 did not possess any *Pokklai* allele that could be associated with salt tolerance in

this study, implying that they may possess novel QTLs/alleles for salt tolerance and can be used for mapping.

In the present study, significant level of diversity for *Saltol* was observed in the large genomic region it encompasses. This is the maiden attempt to analyse the salt tolerance of landraces of the *Kaipad* ecosystem along with few salt tolerant wild rice lines. Results are indicative that *Kaipad* genotypes also possess *Saltol* locus similar to *Pokkali*. The genotypes such as Kuthiru and Ezhome 1 which have normal pericarp colour and high degree of salinity tolerance can be used as new *Saltol* donors to improve high yielding varieties. The salt tolerant wild rice genotypes like NKSWR19, NKSWR20 and NKSWR32 did not seem to possess *Saltol* locus. They can become sources for mapping novel QTLs for seedling stage salinity tolerance. The results further suggest that the combination of RM8094 and RM3412 would be useful for marker assisted transfer for *Saltol in*to popular rice varieties sensitive to seedling stage salinity.

Table4.1. 1. Origin, characteristics and salt tolerance reaction of 23 rice genotypes

No	Genotype	Source origin	Characteristics	Score	Reaction to salinity*
1	FL478	IRRI	Salt tolerant NIL of IR29	3	T
2	IR 29	IRRI	Salt susceptible	9	HS
3	Pokkali	South Kerala, India	Salt tolerant landrace (*Pokkali*)	1	HT
4	Kuthiru	North Kerala, India	Salt tolerant landrace (*Kaipad*)	1	HT
5	Okayama	North Kerala, India	Salt tolerant landrace (*Kaipad*)	1	HT
6	Jaiphula	Orissa	Landrace	9	HS
7	Nipponbare	Japan	Landrace (*japonica*)	5	MT
8	PB1121	IARI, India	Basmati variety	7	S
9	PB1	IARI, India	Basmati variety	9	HS
10	Pusa1734-4	IARI, India	Salt tolerant NIL of PB1121	3	T
11	Pusa1734-24	IARI, India	Salt tolerant NIL of PB1121	3	T
12	Taraori Basmati	India	Basmati landrace	9	HS
13	N22	India	Drought tolerant genotype	5	MT
14	N292	India	Mutant line of N22	5	MT
15	N295	India	Mutant line of N22	5	MT
16	CSR10	CSSRI, India	Salt tolerant variety	3	T
17	CSR 30	CSSRI, India	Salt tolerant variety	5	MT
18	Ezhome 1	North Kerala, India	Salt tolerant variety (*Kaipad*)	3	T
19	Ezhome 2	North Kerala, India	Salt tolerant variety (*Kaipad*)	7	S
20	NKSWR19	Kevali, UP, India	wild rice (*Oryza rufipogon*)	1	HT
21	NKSWR20	Rawak, UP, India	wild rice (*O. rufipogon*)	3	T
22	NKRWR32	Lokmanpur, UP, India	wild rice (*O. rufipogon*)	5	MT
23	NKSWR35	Bariin, UP, India	wild rice (*O. rufipogon*)	3	T

*HT, highly tolerant; T, tolerant; MT, moderately tolerant; S, sensitive; HS, highly sensitive.

Table 4.1.2. Number of alleles and polymorphism information content (PIC) value of SSR markers for 23 rice genotypes

Marker	No of alleles	PIC value	Amplicon size rage (bp)
RM1287	3	0.47	160-190
RM8094	5	0.59	80-220
RM10720	5	0.64	190-260
RM3412	4	0.62	220-250
RM10748	4	0.52	70-110
RM493	5	0.72	220 - 260
RM140	4	0.55	250-280
RM8115	2	0.08	120 -130
RM10793	5	0.49	130-230
RM10800	2	0.23	140-150
RM10825	3	0.52	80-90
RM10843	2	0.34	160-170
RM10852	2	0.2	170-190
RM10864	2	0.42	210-330
RM10871	5	0.81	160-220
RM562	4	0.48	230-260
RM10890	3	0.47	230-260
RM7075	6	0.73	120-180
RM10927	2	0.48	150-160
RM6711	3	0.64	130-150

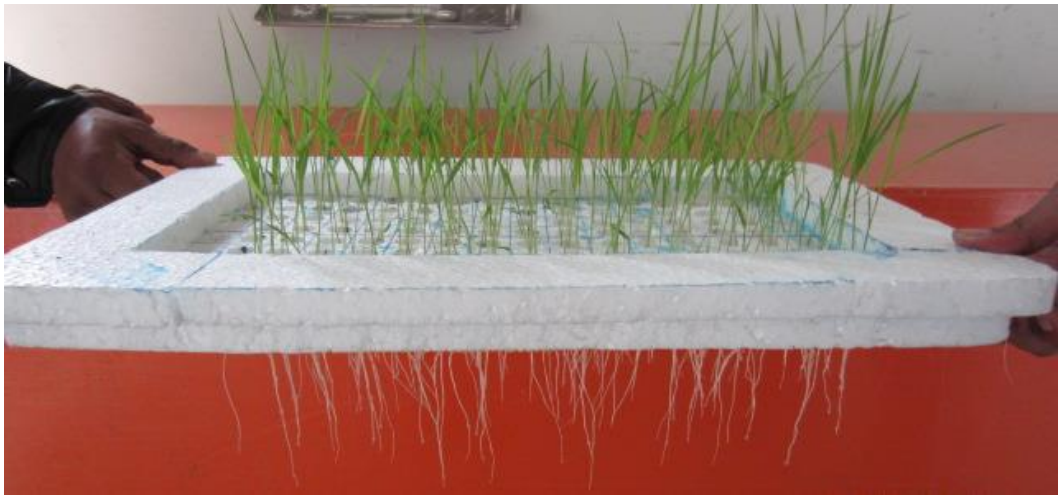


Fig 4.1.1: Screening of rice genotypes for seedling stage salinity tolerance

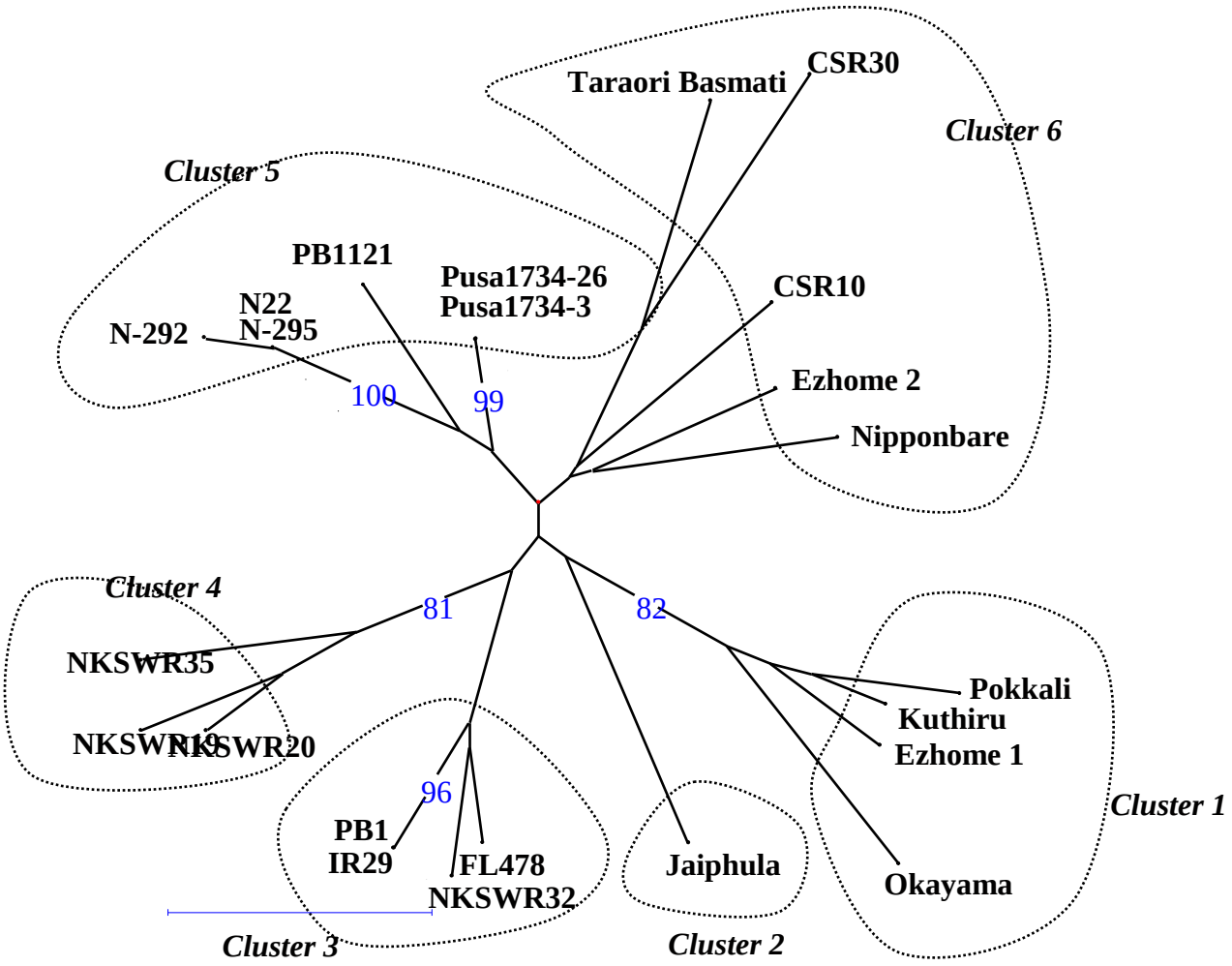


Fig 4.1.2. Radial Dendrogram of 23 rice genotypes based on 20 polymorphic SSR markers on *Saltol* region of chromosome 1 according to the un-weighted neighbour joining using dissimilarity matrix of simple matching coefficients

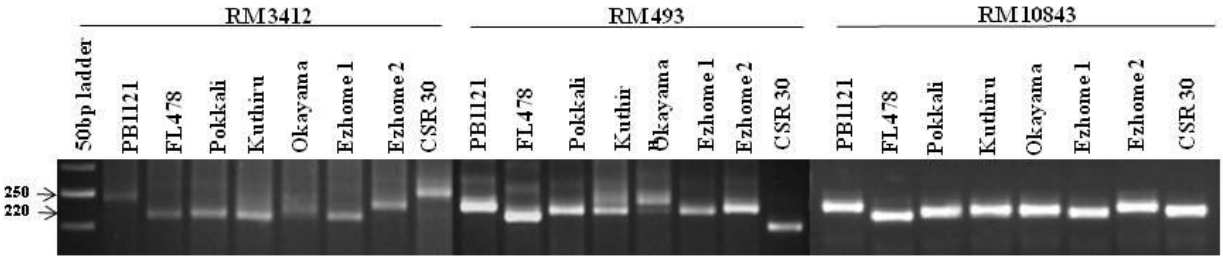


Fig 4.1.3. Gel picture of three SSR markers of four *kaipad* salt tolerant lines, PB1121, Pokkali, FL478 and CSR30

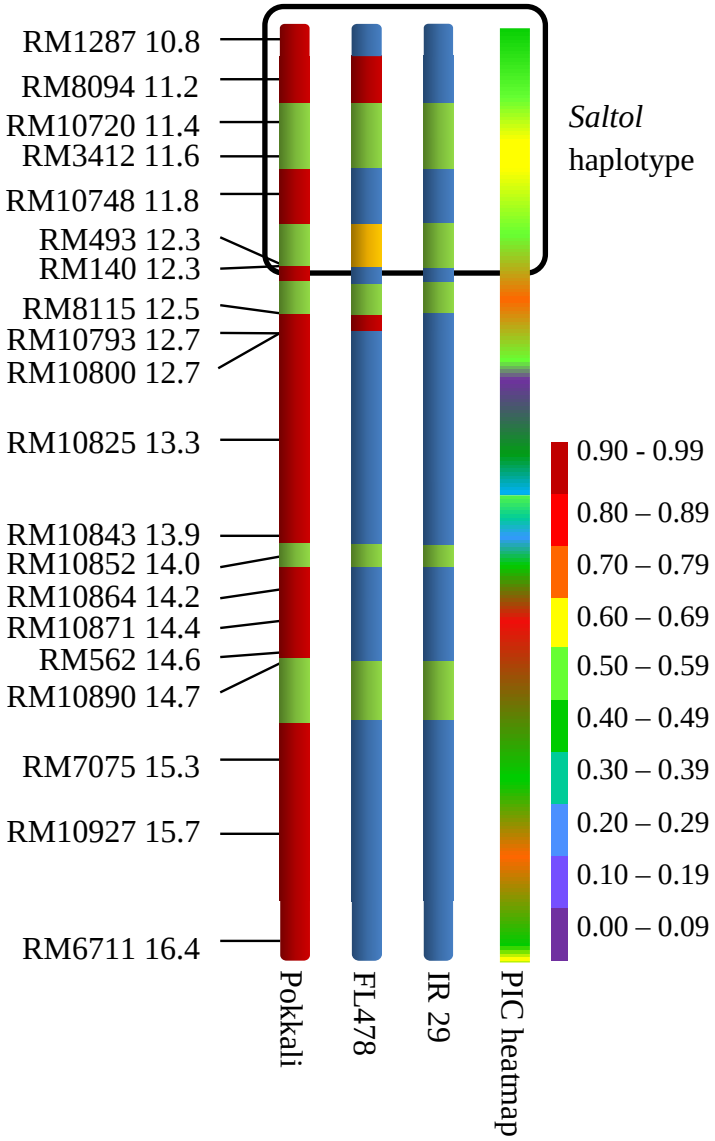


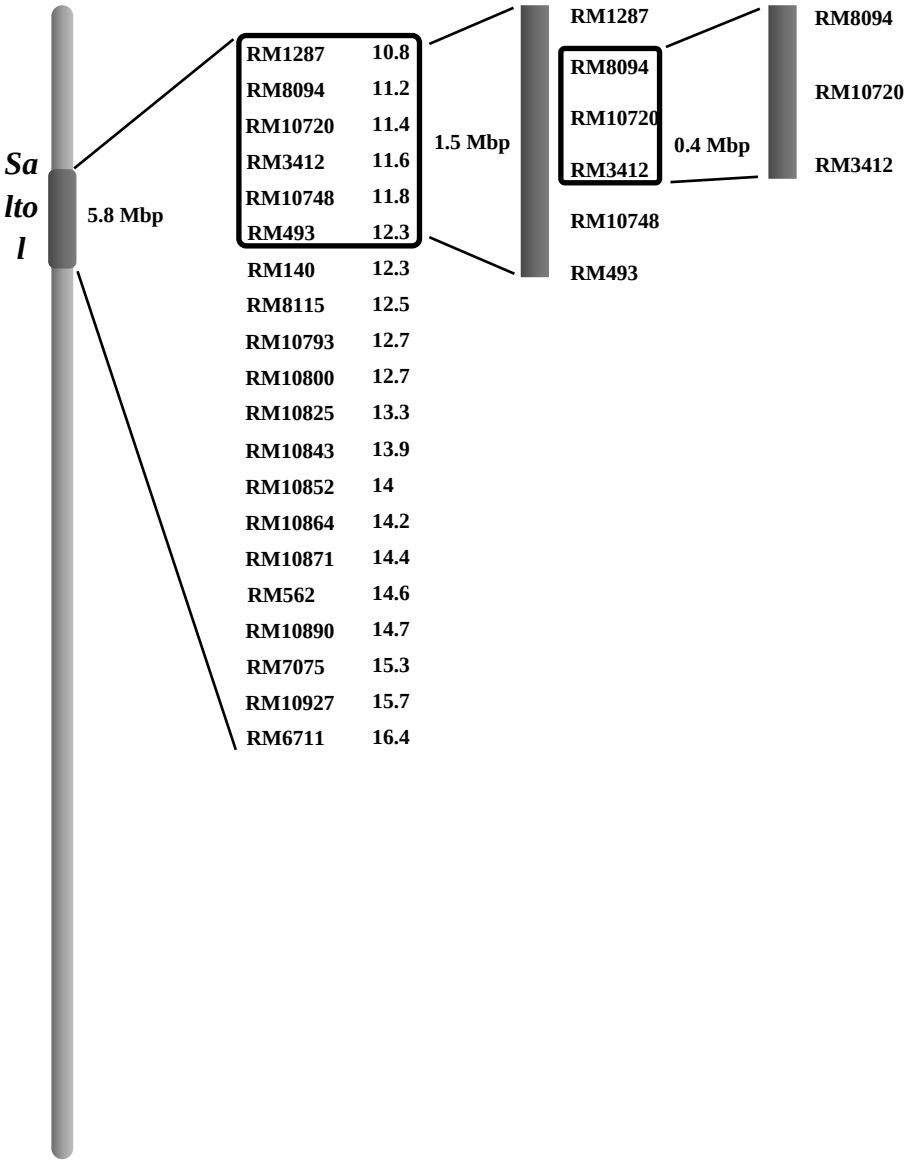
Fig 4.1.4: Graphical haplotypes of Pokkali, IR29 and FL478 showing high level of recombination within the *Saltol* region. The PIC heatmap showing high level of conserved diversity (depicted by similar PIC values) at the Saltol haplotype.

	Haplotypes													
Markers	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
RM1287	■											■		
RM8094	■	■	■	■		■								
RM10720	■	■			■		■				■			
RM3412	■	■	■	■	■	■			■					
RM10748	■		■					■					■	
RM493	■			■	■		■	■	■	■				

1. Pokkali, Kuthiru; 2. FL478; 3.Okayama; 4. Ezhome 1; 5. IR29, PB1, Pusa1734-4, Pusa1734-24; 6. NKSWR32, CSR10; 7. PB1121; 8. Ezhome 2; 9. Jaiphula; 10. Nipponbare; 11. NKSWR19, N22, N292, N295; 12. NKSWR35, 13. CSR 30; 14. NKSWR20,Taraori Basmati

Figure 4.1.5. Fourteen rice haplotypes produced by key SSR markers located in *SalTol* QTL region on chromosome 1 with reference to Pokkali.

Figure 4.1. 6. *Saltol* segment on chromosome 1 of rice A. Bonila et al., 2002; Niones, 2004 and Thomson et al 2007 with new arrangement of used SSR markers



Appendix 1. Haplotypes of the *Saltol* region in 23 genotypes.

Genotype	Cluster	Salt Tolerance	RM1287	RM8094	RM10720	RM3412	RM10748	RM493	RM140	RM8115	RM10793	RM10800	RM10825	RM10843	RM10852	RM10864	RM10871	RM562	RM10890	RM7075	RM10927	RM6711
Pokkali	1	1																				
Kuthiru	1	1																				
Okayama	1	1																				
Ezhome 1	1	3																				
Jaiphula	2	9																				
FL478	3	3																				
NKSWR32	3	3																				
PB1	3	7																				
IR 29	3	9																				
NKSWR19	4	1																				
NKSWR20	4	3																				
NKSWR35	4	5																				
Pusa1734-26	5	3																				
Pusa1734-3	5	3																				
N22	5	5																				
N-292	5	5																				
N-295	5	5																				
PB 1121	5	9																				
CSR10	6	3																				
CSR 30	6	5																				
Nipponbare	6	5																				
Ezhome 2	6	7																				
Taraori Basmati	6	9																				
PIC values			0.47	0.59	0.64	0.62	0.52	0.72	0.55	0.08	0.49	0.23	0.52	0.34	0.2	0.42	0.81	0.48	0.47	0.73	0.48	0.64

4.2 Research Paper II

QTL linked and random microsatellite marker based association mapping for seedling stage salt tolerance in rice (*Oryza sativa* L.)

Abstract

In the present study, we tested 85 diverse set of rice genotypes to ascertain their seedling stage salt tolerance and genetic diversity structure. A set of 19 polymorphic SSR markers linked to *Saltol* QTL region and 22 random markers spread across the rice genome were used for generating the marker segregating data. Based on the phenotypic screening for salt stress tolerance under hydroponics, one basmati rice variety (Seond Basmati), two japonica lines Tomphakhu and Nipponbare, three indica landraces viz., Badami, Shah Pasandand Pedi Badam and two *Oryza rufipogon* accessions, NKSWR2 and NKSWR17 were identified to be highly salt tolerant genotypes, as comparable to Pokkali and FL478. Phenotypic data collected under salt stress screen showed consistent negative association of morpho-physiological data with salt tolerance score. However, no such relation was found for biochemical parameters pertaining to Na^+-K^+ loading into plants. The genotypic clustering and population structure divided the total germplasm into two sub-specific populations. The structured association mapping using co-ancestry coefficients and genotypic PC scoresrevealed six robust marker trait associations. The stable marker trait associations RM1287 (10.8Mb) associated with shoot Na^+/K^+ ratio and RM140 (12.3Mb) associated with phenotypic salt tolerance score were detected. These results clearly indicated that the critical region of *Saltol* is located within 10.8mb to 12.3mb. RM10927 was found linked to root and shoot Na^+ content. The results reveal that there are genomic regions other than *Saltol* that govern salt tolerance in rice. In order to identify novel QTL regions for salt tolerance, more markers are to be used in screens. The locus around RM140 may be targeted for marker assisted improvement any variety for salt tolerance, that lacks *Saltol* locus.

Introduction

Rice (*Oryza sativa* L.) is staple food for more than half of the world population. In rice producing countries, soil salinity is a major production constraint after drought, adversely affecting quantity and quality of crop produce (Gepstein et al. 2006;Blumwald and Grover2006). Rice is particularly sensitive to salinity, during the seedling (Maas and Hoffman 1977) and reproductive stages. Under the threat of salinity, rice yield is estimated to

decreases 12% for every unit (dS m^{-1}) increase in ECe above the threshold tolerance of 3.0 dS m^{-1}(Maas 1990; Maas and Grattan 1999; Hanson et al. 1999).

Tremendous variation is reported in rice with respect to salt tolerance that provides great opportunities to improve salt stress tolerance through genetic means. A major QTL associated with the Na–K ratio and seedling-stage salt tolerance has been reported in recent genetic studies for mapping quantitative trait loci (QTL) associated with salt tolerance in rice (Bonilla et al.2002).Designated as *Saltol* this QTL was identified on the short arm of chromosome 1 by linkage mapping using a recombinant inbred line population developed by crossing a salt sensitive variety IR29 with a salt tolerant landrace Pokkali, originally sourced from coastal saline belt of Kerala in India. *Saltol* explained 43 percent phenotypic variance for salt tolerance, and was mapped between RFLP markers C52903S and C1733S (5 cM flanking interval) and SSR markers RM23 and RM140, with an interval length of 6.3 cM (Bonilla et al. 2002). Subsequently by SSR marker based fine mapping *Saltol* region was saturated with more than 20 microsatellite markers spanning on a 5Mb region (Niones 2004; Thomson et al. 2010; Alam et al. 2011). A highly tolerant RIL from the IR29/ Pokkali cross, IR 66946-3R-178-1-1 (FL478) was identified as a novel source of seedling stage salt tolerance because Pokkali possessed several undesirable characters (Walia et al. 2005). Since then, FL478 has been used for marker assisted backcross (MABC) transfer of *Saltol* into elite varietal backgrounds such as BR11, BRRI dhan 28, IR64 and AS996 (Rahman et al. 2008; Ho et al. 2010; Gregario et al. 2013; Huyen etal. 2012). However, FL478 possesses red colour of endosperm pericarp, which is an undesirable trait. Further, salt tolerant rice genotypes available in India and elsewhere are adapted to different ecological zones and are largely unrelated, it is unlikely that all of them carry same *Saltol* locus for salt tolerance. Therefore, there is a need to identify new donors with new sources for salt tolerance together with traits of better grain and cooking quality and normal pericarp colour.

Although there are few reported studies of SSR based survey on diverse germplasm sets to identify candidate markers linked to *Saltol* gene (Mohammadi-Nejad et al. 2008;Aliyu et al.2011; Islam et al. 2012; Babu et al.2014),neither of these studies were conclusive nor consistent, inciting confusion in selection of such candidate markers. Almost allof these studies either had smaller set of genotypes (Mohammadi-Nejad et al. 2008) or few markers within *Saltol* region(Aliyu et al.2011; Islam et al. 2012).

There are several physiological and biochemical traits associated with seedling stage salttolerance in rice. We have observed that none of the previous studies on validation of

Saltol QTL and associated markers have included biochemical and physiological parameters associated with seedling stage salt tolerance in rice. In several occasions marker validation was confined to haplotype analysis of the *Saltol* region as done in the case of qualitative traits like disease resistance, where target trait is tested for association with a biallelic marker. However salt tolerance itself is a quantitative trait, and there is every reason to suspect for polyallelic nature of candidate loci. Under such circumstances, haplotype analysis may not bring out all necessary information especially regarding novel and rare alleles.

Therefore in the present study, we have tested a large and diverse germplasm set of 85 genotypes consisting of Basmati lines, land races, released varieties, *japonica* lines, EMS induced mutants and wild accessions of *Oryza rufipogon* for variation in salt tolerance and association of markers linked to *Saltol* region together with several random microsatellite (SSR) markers spanning across the genome. The study has special focus on(i) to identify putative new salt tolerant sources in rice, (ii) to decipher genetic diversity structure of the germplasm set, (iii) to assess the linkage disequilibrium (LD) structure within germplasm set and (iv) identification of putative markers associated with different components of salt tolerance using LD mapping.

Material and Methods

Plant material

A set of 85 diverse genotypes including basmati genotypes that are adapted to inland salinity of the Indo-Gangetic plains, stress tolerant cultivars, induced mutant lines, high yielding rice cultivars that are partially salt tolerant or intolerant and *Oryza rufipogon* lines were used in this study. The characters and origin of genotypes was presented in Appdx 2.

Screening for seedling stage salt tolerance

Genotypes were screened for seedling stage salt tolerance under controlled environment in the National Phytotron Facility at IARI, New Delhi during *kharif* 2012. Pre-germinated (3 days after germination) seeds were sown in punch holes made on extruded polystyrene foam floats fitted with a nylon wire mesh on the bottom side and suspended on trays filled with Yoshida nutrient solution(Yoshida et al. 1976). As shown in the Figure 4.2.1, each tray carried 12 entries and three controls, Pokkali and FL478 (salt tolerant) and IR29 (salt sensitive). The screen trays formed fundamental unit of replication and two replications were maintained for each set of genotypes. Nine individual plants per genotype were evaluated in each replication. In order to avoid border effect, one of the controls, FL478 was sown along

the border on all sides to normalize competition for light and space for next rows. Salt stress was imposed 14 days after germination by adding 60mM NaCl (ECe of 6 $dS.m^{-1}$) and salt concentration was increased to 120mM (ECe of 12 $dS.m^{-1}$) after 3 days in Yoshida nutrient solution and was maintained until final phenotypic scoring. The pH of the nutrient solution was adjusted daily to 5.0, and the culture solution was replaced every 7 days. 16 days after salt stress, entries were scored based on visual symptoms using the modified score scale (Gregario et al. 1997) with scores ranging from 1 (highly tolerant) to 9 (highly sensitive).

Phenotype data and estimation of salt ion concentrations

In the controlled hydroponic conditions, sampling of plant tissues for Na^+ and K^+ ion concentration in shoot and root was done immediately after phenotypic scoring. Whole plant was extracted from the screening floats and washed first for 1 min in tap water followed by 1 min in distilled water twice, blotted dry and recorded observations of length and fresh weight of shoot and root. Further root and shoot samples were kept in oven at 60°C for 3 days until completely dry and the dry weight was recorded. Dry samples of known weight were mixed with 20 ml of 100 mM glacial acetic acid in a 30ml test tube. The samples were kept in water bath for 2 h at 90°C followed by cooling, and the debris was removed by filtration. An aliquot of the digested material was taken and diluted ten times with 100mM glacial acetic acid to determine Na^+, and K^+ concentration by Flame photometer (ELICO CL360) by the equation:

$$Na^+ \text{or} K^+ \text{ concentration} = \frac{CdV}{1000DW}$$

Where, C is flame photometer reading, d is dilution factor, V is extraction volume (ml) and DW is oven dry weight (g). A standard curve of KCl and NaCl solutions of different concentrations, ranging from 10 to 100 $mg.l^{-1}$ was prepared and used for calibration. Na^+ and K^+ content of leaves and stem were expressed on $mg.kg^{-1}$ basis.

Molecular markers

A total of 41 SSR markers that contained random and *Saltol* linked markers were used for analysis in this study. An initial set of 65 random SSR markers distributed on 12 rice chromosomes was used for a polymorphism survey that was reduced to 22 markers based on polymorphism. To this, 19 polymorphic SSR markers out of 21 markers spanning 5Mb region of *Saltol* locus in chromosome 1 were added.

DNA extraction and polymerase chain reaction amplification

Total genomic DNA was extracted from 15 days old seedlings using CTAB method developed by Doyle and Doyle (1990). Polymerase chain reaction (PCR) was performed in a thermal cycler (G-Storm, UK) using a 10 µl total reaction volume as described previously (Basavaraj et al. 2010). This contained 30 ngµl^{-1}of template DNA, 5 pmol of each primer (Sigma Inc., USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (MBI, Fermentas) and 0.5 U of Taq polymerase (Bangalore Genei, India). PCR comprised one cycle of denaturation at 95°c for 5 min, followed by 35 cycles at 95°c for 30 s, 55 °c for 30 s and 72 °c for 1 min, with a final extension at 72°c for 7 min. The amplified products were resolved on 3.5% Metaphor^{TM}agarose gel (Lonza, USA) containing 0.1 mg/ml of ethidium bromide (Amresco, USA) along with a DNA size standard 50bp ladder (MBI, Fermentas) and documented in a gel documentation system (BioRad, USA).

Statistical analyses

Phenotype variability and correlations

The phenotype data comprising of root and shoot morphology and weight along with biochemical data were subjected to standard statistical analyses. Pearson's correlations were worked out among all the traits as well as in relation to phenotypic salt tolerance score.

Diversity and population structure

The SSR allele segregation data were used to construct dissimilarity matrix between genotypes using simple matching coefficient (Sokal and Michener 1958). The dissimilarity matrix was used for clustering of genotypes, based on unweighted neighbour-joining method. Analysis was performed using DARWin 5.0 (Perrier et al. 2003). To check the goodness of fit of the clustering, a cophenetic correlation was computed between similarity and the cophenetic matrices (Rohlf and Sokal 1981). Confidence limits of different clades were tested by bootstrapping 10,000 times to assess the repetitiveness of genotype clustering (Felsenstein 1985).

For SSR markers, polymorphism information content (PIC), was calculated as the measure of in formativeness of markers (Botstein et al. 1980). As an alternative to graphical clustering methods, a model-based Bayesian approach implemented in the software package Structure 2.3.4(Pritchard et al.2010) was used to analyse the population structure of rice accessions. Optimum number of populations was inferred by running an admixture ancestry model with correlated allele frequencies starting from two populations $K = 1$ to $K = 10$, with three runs at

each *K*. For each run, 500,000 burn-ins followed by 500,000 Markov Chain Monte Carlo (MCMC) simulations were performed. The ideal value of *K* was determined from the uppermost hierarchical level of population structure, detected using an ad hoc statistic Δ*K* based on the rate of change in the log probability of data between successive *K* values (Evanno et al.2005). In addition, the ideal number according to Pritchard et al. (2000) was used as the criterion for defining the number of groups (*k)*. The most trustworthy value was estimated based on the lowest negative number of Ln (the log-likelihood of the data) and the lowest standard deviation found during statistical analysis. Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected subpopulation (Pritchard *et al.* 2000).

Linkage disequilibrium

LD was estimated for each pair of SSR loci using Tassel 2.1 software (Bradbury et al. 2007), both in overall population and in subpopulations. LD was measured using D' and r^2 estimates modified for multiple loci (Hedrick 1987). Significance (P values) of D' for each SSR pair was determined by 100,000 permutations.

Phenotype diversity and LD mapping

Agronomic data were analysed for phenotypic diversity using principal component approach using simple correlations. Principal component scores obtained for each genotype were used for computing squared Euclidean distances, and grouping of genotypes were performed using unweighted NJ method (Gascuel 1997) with bootstrapping of 10,000 iterations. LD mapping (association mapping) was performed to analyse marker–trait association by structured association approach separately using ancestry coefficient (Q values) estimates and principal component scores as covariates in a general linear model (GLM) function using TASSEL 2.1. For each marker–trait combination, GLM estimated the ordinary least squares solution (Searle 1987). Multiple testing corrections were performed by adjusting maker probability values for multiple test runs, by a permutation test derived using a step-down MinP procedure (Ge et al.2003). The significant association for a marker and trait was selected when adjusted *P* value (false discovery rate) was below. From the results obtained, most common marker-trait association were extracted as the most valid ones.

Results

Variability in salt tolerance

Large variation was observed for seedling stage salt tolerance among the 85 genotypes screened (Table 4.2.1, Appedx 2). On exposure for 16 days to a maximum salt concentration

of 12dSm^{-1}, none of the genotypes showed absolute tolerance to salt stress. Twelve lines out of the total 85 (14.1%) showed salt tolerance score of 3.0. All of them were having normal growth with occasional leaf tip whitening and/or leaf rolling. All the known salt tolerant lines (FL 478, CSR 23, CSR 27 and Pokkali) showed considerable level of tolerance. The remaining tolerant lines included one basmati cultivar (Seond Basamti), two *japonica* lines (TomphaKhau and Nipponbare), three landraces (Shah Pasand, Badami and Pedi Badam) and two *O. rufipogon* accessions (NKSWR2 and NKSWR17).

Of the remaining lines, 16.5% (14 lines) showed moderate tolerance to salt exposure, by exhibiting severe growth retardation, extensive leaf rolling with occasional elongation of few leaves (score 5). Among these there were four basmati lines (Pusa Sugandh 2, Pusa Sugandh 3, Pusa 1401 and Pusa 1460), two japonicas, six landraces, one *O. rufipogon* accession and one known salt tolerant cultivar, CSR36. The rest of the genotypes tested were showing susceptible to highly susceptible reaction with no growth and were almost dead or dying.

Phenotypic variability and correlation among different component traits

Under salinity screening, the test genotypes showed wide phenotypic variation (Table 4.2.2)Shoot length of the seedlings ranged from 12.1 cm (Rajendra Basmati) to 54.75cm (Khandagiri) with average of 31.75 cm.Root length varied between 3.15 cm (Rajendra basmati) to 19.75 cm (NKSWR1) with a CV of 26.4%. Significant variations were recorded for shoot and root weight among the genotypes. Highest shoot weight (both fresh and dry) was recorded for FL 478 among all the genotypes tested. Similarly root dry weight was highest in FL478, while Pant Sugndh Dhan had highest root fresh weight. Seedling weight was highest in FL 478 (6.24g) and least in Rajendra Basmati (0.11g). Most of the tolerant lines had high seedling weight that ranged between 2.46g (Seond Basamati) and 6.24g in FL 478.

Among the biochemical traits, root Na^+ content varied between 0.08 nmol g^{-1} (Pusa 1342) to 2.77 nmol g^{-1} (Indira Sugandh Dhan). Simlarly Shoot Na^+ content was also highest for Indira Sugandh Dhan (3.81 mmol g^{-1}) and lowest for Pusa 1342 (0.15 mmol g^{-1}). Root K^+content varied between 0.10 mmol g^{-1} (Danteswari) and 1.52 mmol.g^{-1} (ADT43) with an average of 0.54 mmol g^{-1}. However, shoot K^+ content was highest in Pokkali (1.88 mmolg^{-1}) and lowest was in Erramallalu (0.15 mmol.g^{-1}). The Na^+/K^+ ratio in roots varied from 0.09 to 10.19 while shoot Na^+/K^+ ratio ranged between 0.18 and 7.77.

Average performance of rice genotypes falling under different classes of salt tolerance was given in Table 4.2.3. Mean values for shoot and root length, shoot and seedling weight decreased with increasing susceptibility among the different classes. Root fresh weight was high in salt tolerant classes when compared to susceptible classes. Among the biochemical parameters, no consistent pattern for root as well shoot Na^+-K^+ content could be deciphered. For Na+/K+ ratio in both roots and shoots the tolerant class had low values when compared to susceptible class.

Phenotypic association between different parameters are presented in Table 4.2.4. All the morpho-physiological traits such as length and weight (fresh and dry) of roots and shoots, showed significant positive correlation among themselves. Further, the association between morphological traits and salt tolerance scores (PS) were found to be significant and negative. However there were no significant association between biochemical traits and morphological traits, except for a positive association of root length and shoot Na^+/K^+ ratio. Among the biochemical traits, Na^+ content of roots showed positive relations with shoot Na^+ content and Na^+/K^+ ratios in both roots and shoots. Similarly root K^+ content was found positively correlated to shoot K^+ content, but its associations were negative with Na^+/K^+ ratios in both shoots and the roots. Root Na^+/K^+ ratio was found positively associated to shoot Na^+/K^+ ratio as well as shoot Na^+ content, and negatively associated to shoot K^+ content. Similar trend was observed for Na^+/K^+ ratio in shoots with shoot Na^+ (positive) and shoot K^+ (negative) contents. In overall, no association was found between biochemical traits and phenotypic salt tolerance score.

SSR marker segregation

SSR marker allele distribution among 85 test genotypes is presented in Table 4.2.5. 41 SSR marker generated 188 alleles with an average of 4.6 alleles per marker. The number of alleles per marker varied from two to ten. The overall frequency of tri-allelic markers was the highest (24.4%) followed by hexa-allelic (19.5%). Tetra and penta-allelic markers were equal in frequency accounting for more than 34% of the markers. Octa- and deca-allelic markers shared a common frequency of 2.4% each. The frequency of bi-allelic marker was 9.6%. The PIC values of markers range from 0.23 (RM10748) to 0.88 (RM10871).

Genotypic diversity

Assessment of genotypic diversity among 85 genotypes revealed two major groups, I and II from the dendrogram constructed from the matrix of simple matching coefficients (Fig 4.2.1).

Having grouped genotypes from different component sets like japonicas, landraces, mutants, improved and high yielding varieties, the grouping did not show distinct grouping based on salt tolerance. In overall, there were three distinct sub-clusters each with bootstrap value of more than 90%, present among the test genotypes. The first distinct sub-cluster consisted of N22 and its mutant lines with a bootstrap value of 98%, followed by another cluster of high yielding varieties, Pusa44, MTU3626, Jaya and MI48. Both of these were constituent of the first group. Pusa 1460 and Pusa 1401 formed another distinct cluster in the group II.

Empirically grouping genotypes in group I can be further divided into three subclusters named I-A to I-C, that included the sub-cluster containing N22 and its mutants. Sub cluster I-B contained 6 *Oryza rufipogon* accessions, while the largest sub-cluster I-C contained several landraces, basmati lines, japonicas, varieties and salt tolerant lines including Pokkali and FL478. In the group II, sub-cluster II-A had several of the popular basmati varieties, few landraces and varieties such as Swarna and ADT43.

Population structure

Analysis of population structure distinguished two sub-populations *POP1* and *POP2* with a highest ΔK value of 295.8 (Fig 4.2.2a). *POP2*was the largest with a membership proportion of 77.3% (Table 4.2.6) consisting most of the Basmati lines, wild rice accessions and Japonicas. The bar plot (Fig 4.2.2b) shows the distribution pattern of genotypes within and between subpopulation based on the inferred ancestry coefficients. The fixation index (F_{ST}) values of two sub populations ranged from 0.528 (*POP1*) to 0.081(*POP2*). The allele frequency divergence between subpopulation *POP1* and *POP2* was 0.2504.

Principal component analysis

Fifteen principle components that had Eigen values more than 1derived from the correlation coefficients of genotype data explained 76% of total genotype variance (Table 4.2.7). The first component explained 12.6% of the variation followed by 9.4% by second component.

Linkage Disequilibrium mapping using coefficient of co-ancestry

GLM analysis of marker trait association using coefficient of co-ancestry as covariates (Table 4.2.8) identified marker RM140 associated with phenotypic salt tolerance score with a PVE of 13.76 %. Seven SSR markers were putatively found associated to root length, amongst which three markers were located on chromosome 1(*Saltol* region) and one each on chromosome 2,7,11 and 12. RM1234 located on chromosome 2 had the highest value (33.4%) for the phenotypic variation explained (PVE), followed by RM336 with a PVE of

28.7%. One of the *Saltol* peak markers, RM1287 explained 19.94% of phenotypic variance for root length. The two important salt tolerance parameters such as Na^+ concentration in root and shoot showed significant association with *Saltol* marker RM10927, while the marker RM1287 (26.5% PVE) found significant associated with Na^+/K^+ ratio in the shoot.

Linkage disequilibrium mapping using PCA score as covariates

Association analysis using principal components as covariates in the GLM identified a total of fifteen significant marker-trait association with different salt tolerant component traits (Table 4.2.8).Two markers (RM10871 and RM140) were found associated to salt tolerance score with PVE values 38.6 and 16.6% respectively. Among the morphological traits, root length was linked to two markers RM7003 (PVE 14.5%) and RM1234 (PVE 21.4%) while root dry weight was found associated to RM84 with a PVE of 28.9%. RM84 was also found associated to shoot dry weight with a PVE of 32.2%. Among the biochemical traits, both the Na^+ contentof root and shoot was associated to marker RM10927 explaining phenotypic variations of 25.9% and 18.1% respectively. RM493 also showed significant association with shoot Na^+ content. RM493 and RM10825 exhibited significant association with root Na^+/K^+ ratio with PVE of 39.0% and 27.8% respectively. Four markers (RM10890, RM16, RM1287 and RM493) were found associated to shoot Na^+/K^+ ratio, with PVE values ranging from 22.5 to 31.1%.

To identify robust association of markers and traits, six QTLs were found common in both the approaches of LD mapping in the present study. Among the *Saltol* linked markers, RM140 was found linked to phenotypic salt tolerance score. Two markers, one from chromosome2 (RM1234) and another from chromosome 12 (RM7003) showed consistent association with root length. The key salt tolerant traits like Na^+ content of roots and shoots was found linked to RM10927, while RM1287 was found consistently associated to shoot Na^+/K^+ ratio in both the approaches.

Discussion

Rice genome is a storehouse of vast genetic variability for different traits of commercial importance. There are several genotypes that show differential tolerance to salinity, a major constraint in contemporary rice production worldwide (Vinod et al. 2013).The major QTL *Saltol* mapped on the short arm of chromosome 1 governing seedling stage salt tolerance with the phenotypic variability of 40-70% was the first identified QTL conferring salt tolerance in rice (Gregario et al. 1997). Mapped in the RIL population

generated from the cross IR29/Pokkali, the putative *Saltol* region spans more than 5 Mb of physical chromosome length (Linh et al. 2012). Using haplotype analysis, several researchers have reported alternate markers (*viz.*, RM3412, RM493, RM8094 etc.) spanning in the putative *Saltol* region (Islam et al. 2012). Recently, Babu et al. (2014) identified RM8094 and RM3412 as the most reliable marker from the *Saltol* region that could discriminate the salt tolerant genotypes from the susceptible genotypes. However, these two markers could not discriminate salt sensitivity of Pusa Basmati 1, a highly sensitive line to reproductive stage salt injury, which is currently being used to map QTLs for reproductive stage salt tolerance in rice by combining with resistant variety, Cheruvirippu (Hossain et al. 2010).Currently, there is a need to identify most reliable marker linked to component traits of the salt tolerance associated with the *Saltol* locus which would aid in MAS programs for efficient transfer of *Saltol* locus, as well as to identify novel QTLs for salt tolerance in rice.

Phenotype screening of 85 genotypes for seedling stage salt tolerance under hydroponic conditions revealed ample diversity of 14% tolerance. Taken together with moderately tolerant lines, the total useful proportion of genotypes was well above 30% of the germplasm tested. Salt tolerance is a quantitative trait, and therefore, it would be ideal to search both tolerant and moderately tolerant germplasm for QTLs conferring tolerance. Such QTLs when put together may make remarkable difference in salt tolerance. In the present screening, we could delineate one basmati rice variety (Seond Basmati), two *japonica* rice varieties(Tompha Khau and Nipponbare), three *indica* landraces viz., Badami, Shah Pasandand Pedi Badam and two *O. rufipogon* accessions NKSWR2 and NKSWR17 as salt tolerant that performed as good as already known salt tolerant lines such as Pokkali and its RIL, FL478, which are being currently used in marker assisted breeding for introgression of *Saltol*. Since, the currently identified lines are genetically and geographically distant to Pokkali which comes from Kerala, these genotypes could be used to study mechanisms of salt tolerance and can be potential sources for mapping of novel QTLs for salt tolerance.

Most common morpho-physiological traits that are affected by salt injury in rice are length and weight of roots and shoots and seedling weight (Misra et al. 1997). Seedling weight was one of the important component traits, as it directly determines the biomass accumulation under salt stress. Most of the salt tolerant genotypes had shown higher seedling weight indicating that, genotype which maintained good osmotic balance under salt stress could accumulate higher biomass that lead to higher seedling weight. However in susceptible genotypes, biomass yield was very less due to ionic toxicity. A common feature of salt stress

in plants is loss of cell turgor, causing leaf rolling and necrosis. Cellular turgor depression causes stomatal closure resulting in decreased photosynthesis (Gale and Zeroni1984) and decreased biomass production. Also under salt stress, growth inhibition occurs due to reduced uptake of essential mineral nutrients, due to imbalances in ion uptake incited by the Na^+ competition (Hu and Schmidhalter 2005). Similar findings were reported earlier in rice (Koyoma et al. 2001; Munns 2002). Salt sensitivity of susceptible genotypes were also perceptible from their poor growth and establishment that was reflected in all the morphological traits studied. Morphological traits had shown consistent phenotypic correlation among themselves and strong negative association to salt tolerance score. However, biochemical parameters that are associated to Na^+ and K^+ loading into plants as the mechanism of salt tolerance did not show any significant pattern in the set of germplasm analysed in this study. Differential Na^+ and K^+ loading was reported as the major mechanism of *Saltol* locus(Gregario 1997; Bonilla et al. 2002), however, in the present study only few of the germplasm lines such as Pokkali and FL 478 were known to have *Saltol* locus, while mechanism of salt tolerance shown by other genotypes remained largely unknown. However, observations showed that there may be additional mechanisms other than Na^+-K^+ loading operating in some of these genotypes. In support of this, we have observed similar range of variation for the biochemical parameters in both the salt susceptible and salt tolerant lines in this study, indicating the Na^+-K^+ loading was not only the criteria of salt sensitivity in many of the genotypes. Also, these observations were slightly in contrast to what was observed in case of *Saltol* introgressed Pusa Basmati 1121 lines in a separate marker assisted back cross program in our lab where in root Na^+ and K^+ were found to be negatively associated indicating preferential competition for K^+ over Na^+, and root K^+ content and Na^+/K^+ ratio was significantly related to salt tolerance score in *Saltol* carriers (Babu, unpublished data).

Marker segregation pattern revealed that the proportion of tri-allelic and hexa-allelic markers was more compared to tri-allelic, tetra-allelic and bi-allelic markers. Among the two classes of markers used, *Saltol* linked markers had shown high allelic variation when compared to random markers. This indicated that *Saltol* region in rice gene pool remains highly diversified and not all markers linked to *Saltol* region of Pokkali are associated to salt tolerance. Similar observations on non-ubiquity of *Saltol* was reported in different germplasm sets recently (Aliyu et al. 2011; Babu et al. 2014; Chattopadhyay et al. 2014)

Genotype clustering using simple matching coefficients and determination of population structure based on co-ancestry coefficients produced two large groupings of germplasm.

Although almost 50% of the markers were linked to *Saltol* locus, the genotype grouping did not stratify salt tolerant genotypes into definite clusters. Even after empirical grouping of clusters, there was no definite pattern in relation to salt tolerance. This emphasises our earlier observation that variation in *Saltol* region alone was not the contributing factors for observed tolerance in many of the genotypes under study. Further, the genotype based PCA identified 15 principal components explaining 76% of the genotype variation. Inconsistency in genotype grouping based on different methods indicated that markers used in this study were highly random vis-à-vis salt tolerance, and could only reveal a loose diversity pattern of the genotypes.

Nevertheless, the grouping of sub-populations in the model based approach was the best grouping possible (Goldstein1991), wherein the sub-population (*POP1*) containing wild rice accessions, landraces and Basmati lines had maximum *Fst* value suggesting a distinct population structure (Vanniarajan et al. 2012). One of the Basmati lines, Pusa Basmati 1 that was phenotypically susceptible to salt stress was found clustered together with tolerant CSR varieties. Pusa Basmati 1 is now known to have a different *Saltol* haplotype compared to CSR varieties (Babu et al. 2014). This again proved that *Saltol* and its haplotype variants were less informative in describing salt tolerance of germplasm in this study. The unique clustering pattern of *O. rufipogon* accession revealed their distinct genetic structure from cultivatedlines.

The present observations revealed that salt tolerance in rice a complex trait(Chinnusamy et al. 2005); therefore phenotyping of salt tolerance in different growth stages may bring in different tolerance pattern among genotypes (Khatun and Flowers 1995). However, with respect to seedling stage salt tolerance, in contrast to the earlier belief, measurement of Na^+ and K^+ loadings in shoot and root and their ratio may only supplement to phenotyping for salt tolerance in a germplasm where mechanisms other than *Saltol* is operating. It is always better to augment data on morphological traits such as root, shoot or seedling parameters that gives better indication of salt tolerance. Further, our observations showed that although Na^+/K^+ ratio was low in *Saltol* carriers (Pokkali and FL478), other tolerant genotypes had wide variation ranging from low to high Na^+/K^+ ratio either in roots or shoots.

LD mapping (association mapping) is an approach that exploits marker-gene linkage in the naturally occurring haplotype blocks that are conserved in the germplasm (Malysheva-Otto et al. 2006; Rostocks et al. 2006). These haplotype blocks are in LD and their sizes vary

depending on the pollination behaviour, geographical isolation, evolutionary time gaps, mutation, selection and genetic drift (Gupta et al. 2005). In the present study, the random combinations of landraces, *japonica* lines, cultivars and wild accessions which had limited LD blocks conserved among themselves, have brought together the LD blocks to a sufficient level that could be utilized for genome-wide association mapping. In this approach, the subpopulation structure derived ancestry coefficients were used as covariates in the model that predicted marker–trait association to avoid spurious associations (Pritchard and Rosenberg 1999; Pritchard et al.2000).To suppress ambiguity of spurious association, an additional association mapping using the genotypic PC scores as covariates was done parallelly and the common marker-trait association were picked as unambiguous ones. Amongst the total twenty six marker-trait associations identified, six were picked as the most robust ones. Two markers, RM1234 on chromosome 2 and RM7003 on chromosome12 were found associated to root length, an important morphological parameter for related to salt tolerance in this study. However, these markers were not found associated to salt tolerant score *per se*, indicating that there may be other augmenting mechanisms that confer salt tolerance while improving the root growth. Interestingly, four out of six marker-trait associations identified were related to *Saltol* linked markers. This is probably because of the predominance of *Saltol* linked markers in the analysis. RM140 was linked to salt tolerance score, while RM10927 was linked to root and shoot Na^+ content. Since the study had only measured phenotypes associated to salt tolerance, the results indicated that *Saltol* linked markers remained major loci associated with Na^+-K^+ loading in rice plants, irrespective of the phenotype. This reiterates their proximity to *shoot* K^+ *content 1* (*SKC1*) locus on chromosome 1 (Lin et al. 2004).*SKC* is a transporter gene located almost at the same location of *Saltol* that codes for a protein which unloads Na^+ from the root xylem and preferentially expressed in the parenchyma cells surrounding xylem vessels. It was reported that relative salt tolerance of rice landraces such as Pokkali and Nona Bokra is due to the presence of *SKC1* (Ren et al. 2005). The identification of RM140, a flanking marker to *Saltol* region also indicated that among the markers studied most probable association could only reveal with this marker in respect of salt tolerance score but with a relatively low PVE values of 13.8 to 16.6% in both the analyses. This implied that the markers used in the study other than the *Saltol* linked ones were insufficient in number to reveal novel marker-trait association in salt tolerance among the lines other than *Saltol* carriers.

In the present study, we could observe that the significant marker-trait association of *Saltol* linked markers ranged within a physical region from 10.8Mb to 15.7Mb. The most significant region associated to salt tolerance score was falling in between this region at the physical position of 12.3Mb where the marker RM140 was localised. Therefore this region was identified as the hotspot *Saltol* region in the present investigation. Therefore, for MABC of *Saltol*,this region can be recommended to the plant breeders for foreground selection. The marker region around RM140 has been widely studied in respect of *Saltol* in several studies (Al-Amin et al. 2013; Vu et al. 2012; Islam et al. 2012; Elahi et al. 2004; Niones 2004; Bonilla et al. 2002). The present results open up avenues for further investigations for identification of novel QTLs and candidate genes within the salt tolerant lines identified.

Table 4.2.1. Spectrum of phenotypic response among germplasm lines for seedling stage salt tolerance

Group	HT	T	MT	S	HS	Total
Basmati	0	1	4	10	8	23
Japonica	0	2	2	6	2	12
Landraces‡	0	3	6	8	1	18
O. rufipogon accessions	0	2	1	3	0	6
Salt tolerant lines*	0	4	1	0	0	5
Cultivars and breeding lines	0	0	0	7	6	13
Mutants of N22	0	0	0	5	3	8
Membership (%)	0.00	14.12	16.47	45.88	23.53	100.00

* Salt tolerant lines includes cultivars, landraces and breeding lines; ‡ includes Nagina 22

Score scale: HT - Highly tolerant, 0; T - tolerant, 3; MT - moderately tolerant, 5; S - Susceptible, 7; HS - highly susceptible, 9 (Gregario et al. 1997)

Table 4.2.2 Mean and range of physiological and biochemical traits in rice germplasm

Traits	Minimum	Maximum	Mean	CV%
Shoot length (cm)	12.10	54.75	31.75	30.10
Root length (cm)	3.15	19.75	10.98	26.38
Shoot fresh weight (g)	0.07	5.78	1.24	77.49
Root fresh weight (g)	0.04	0.59	0.11	83.10
Seedling weight (g)	0.11	6.24	1.35	75.92
Shoot dry weight (g)	0.01	0.34	0.08	67.33
Root dry weight (g)	0.01	0.05	0.02	44.31
Root Na^+content ($mmol.g^{-1}$)	0.08	2.77	0.61	73.74
Root K^+ content ($mmol.g^{-1}$)	0.10	1.52	0.54	58.97
Root Na^+/K^+ratio	0.09	10.19	1.67	106.96
Shoot Na^+ content ($mmol.g^{-1}$)	0.15	3.81	1.07	62.63
Shoot K^+ content ($mmol.g^{-1}$)	0.15	1.88	0.86	45.79
Shoot Na^+/K^+ratio	0.18	7.77	1.57	83.30

Table 4.2.3. Mean phenotype performance of genotypes falling into distinct salt tolerance classes

Salt tolerance class	PS	SL	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS
Highly tolerant	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Tolerant	3	39.80	13.40	2.59	0.19	2.78	0.15	0.02	0.54	0.58	1.14	1.03	1.00	1.22
Moderately tolerant	5	34.16	12.02	1.61	0.15	1.75	0.09	0.02	0.62	0.60	1.74	1.32	0.85	1.96
Susceptible	7	30.29	10.36	0.94	0.08	1.02	0.06	0.01	0.49	0.48	1.56	0.84	0.86	1.28
Highly susceptible	9	28.07	10.04	0.75	0.10	0.85	0.06	0.01	0.88	0.58	2.16	1.36	0.78	2.08

PS, phenotypic salt tolerance score;SL, shoot length (cm); RL, root length (cm); SFW, shoot fresh weight (g); RFW, root fresh weight (g); SEW, seedling weight (g); SDW, shoot dry weight (g); RDW, root dry weight (g); NR, root Na^+ content (mmol.g-1); KR, root K^+ content (mmol.g-1); NKR, root Na^+/K^+ ratio; NS, shoot Na^+ content (mmol.g-1); KS, shoot K^+ content (mmol.g-1); NKS, shoot Na^+/K^+ ratio

Table 4.2.4 Phenotypic correlation between different physiological and biochemical traits in rice germplasm under salt stress

	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS	PS
SL	0.619**	0.731**	0.508**	0.730**	0.750**	0.657**	-0.135	-0.015	-0.149	-0.087	0.010	-0.098	-0.388**
RL		0.534**	0.399**	0.536**	0.529**	0.434**	-0.083	0.023	-0.163	-0.119	0.111	-0.221*	-0.389**
SFW			0.682**	0.998**	0.957**	0.843**	-0.190	-0.067	-0.177	-0.146	-0.001	-0.100	-0.609**
RFW				0.729**	0.652**	0.628**	-0.114	-0.090	-0.125	-0.050	-0.063	-0.004	-0.330**
SEW					0.955**	0.846**	-0.189	-0.071	-0.177	-0.141	-0.007	-0.094	-0.600**
SDW						0.836**	-0.201	-0.038	-0.208	-0.166	0.020	-0.142	-0.560**
RDW							-0.133	-0.061	-0.134	-0.073	0.031	-0.046	-0.438**
NR								0.023	0.513**	0.821**	0.033	0.486**	0.189
KR									-0.516**	0.156	0.729**	-0.369**	-0.040
NKR										0.358**	-0.418**	0.704**	0.149
NS											0.110	0.556**	0.126
KS												-0.552**	-0.145
NKS													0.126

*, ** significant at p=0.01 and 0.05 respectively; SL, shoot length (cm); RL, root length (cm); SFW, shoot fresh weight (g); RFW, root fresh weight (g); SEW, seedling weight (g); SDW, shoot dry weight (g); RDW, root dry weight (g); NR, root Na^+ content ($mmol.g^{-1}$); KR, root K^+ content ($mmol.g^{-1}$); NKR, root Na^+/K^+ ratio; NS, shoot Na^+ content ($mmol.g^{-1}$); KS, shoot K^+ content ($mmol.g^{-1}$); NKS, shoot Na^+/K^+ ratio; PS, phenotypic salt tolerance score

Table 4.2.5 Details of marker allele counts and polymorphism information content

Marker	Chromosome	Allele count	PIC	Overall Frequency of multiple alleles
RM10800	1	2	0.284	9.76
RM590	10	2	0.371	
RM229	11	2	0.366	
RM7003	12	2	0.408	
RM10748	1	3	0.228	24.39
RM10843	1	3	0.343	
RM6711	1	3	0.418	
RM10825	1	3	0.507	
RM240	2	3	0.442	
RM475	2	3	0.459	
RM7200	4	3	0.456	
RM3367	4	3	0.479	
RM185	4	3	0.601	
RM31	5	3	0.587	
RM140	1	4	0.259	17.07
RM10852	1	4	0.373	
RM10827	1	4	0.544	
RM1234	2	4	0.393	
RM16	3	4	0.444	
RM168	3	4	0.497	
RM2935	12	4	0.527	
RM1287	1	5	0.564	17.07
RM84	1	5	0.624	
RM14	1	5	0.655	
RM154	2	5	0.640	
RM336	7	5	0.679	
RM1124	11	5	0.500	
RM1341	11	5	0.635	
RM10864	1	6	0.500	19.51
RM10890	1	6	0.526	
RM493	1	6	0.643	
RM3412	1	6	0.652	
RM562	1	6	0.705	
RM7075	1	6	0.709	
RM271	10	6	0.681	
RM7226	11	6	0.657	
RM10720	1	7	0.674	7.32
RM8094	5	7	0.770	
RM80	8	7	0.700	
RM10793	1	8	0.706	2.44
RM10871	1	10	0.882	2.44

Table 4.2.6 Population statistics of the estimated subpopulations

Population	Membership (%)	F_{ST}	Expected heterozgosity	Allelic frequency divergence
POP 1	22.7	0.5282	0.3369	
POP 2	77.3	0.081	0.5592	0.2504

Table 4.2.7. Significant principal components extracted using correlation matrix of the genotype data with percentage variation explained by each component

PC	Eigenvalue	Variance (%)	Cumulative variance (%)
1	5.16	12.59	12.59
2	3.85	9.40	21.99
3	2.54	6.20	28.19
4	2.48	6.07	34.25
5	2.26	5.52	39.77
6	2.08	5.10	44.87
7	1.88	4.58	49.45
8	1.77	4.32	53.77
9	1.67	4.06	57.83
10	1.44	3.51	61.34
11	1.40	3.41	64.75
12	1.30	3.18	67.93
13	1.20	2.94	70.86
14	1.08	2.63	73.49
15	1.02	2.48	75.97

PC, principal component

Table 4.2.8 Putative association of microsatellite marker loci with quantitative traits by LD mapping using (a) sub-population level ancestry coefficient (Q values) and (b) principal component scores as covariates. Common marker-trait associations identified by both the approaches are given in bold.

Trait	Marker Locus	Chromosome	Position (Mb)	p-adj	R^2 (%)*
(a) Using co-ancestry coefficients					
Phenotypic ST Score	**RM140**	**1**	**12.3**	**0.045**	**13.8**
Root length	**RM7003**	**12**	**6.7**	**0.000**	**23.5**
	RM1234	**2**	**11.3**	**0.000**	**33.4**
	RM229	11	18.4	0.002	16.8
	RM10800	1	12.7	0.000	18.2
	RM6711	1	16.4	0.015	15.9
	RM1287	1	10.8	0.023	19.9
	RM336	7	21.9	0.032	28.7
Root Na^+ content	**RM10927**	**1**	**15.7**	**0.000**	**29.1**
Shoot Na^+content	**RM10927**	**1**	**15.7**	**0.009**	**20.7**
Shoot Na^+/K^+ ratio	**RM1287**	**1**	**10.8**	**0.002**	**26.5**
(b) Using principal component scores					
Phenotypic ST Score	RM10871	1	14.4	0.002	38.6
	RM140	**1**	**12.3**	**0.009**	**16.6**
Root length	**RM7003**	**12**	**6.7**	**0.000**	**14.5**
	RM1234	**2**	**11.3**	**0.010**	**21.4**
Root dry weight	RM84	1	4.3	0.005	28.9
Shoot dry weight	RM84	1	4.3	0.000	32.2
Root Na^+ content	**RM10927**	**1**	**15.7**	**0.000**	**25.9**
Shoot Na^+ content	**RM10927**	**1**	**15.7**	**0.006**	**18.1**
	RM493	1	12.3	0.014	24.7
Root Na^+/K^+ ratio	RM493	1	12.3	0.000	39.0
	RM10825	1	13.3	0.002	27.8
Shoot Na^+/K^+ ratio	RM10890	1	14.7	0.003	31.1
	RM16	3	23.1	0.006	24.4
	RM1287	**1**	**10.8**	**0.017**	**22.5**
	RM493	1	12.3	0.045	26.5

R^2(%), percent variation explained (PVE)

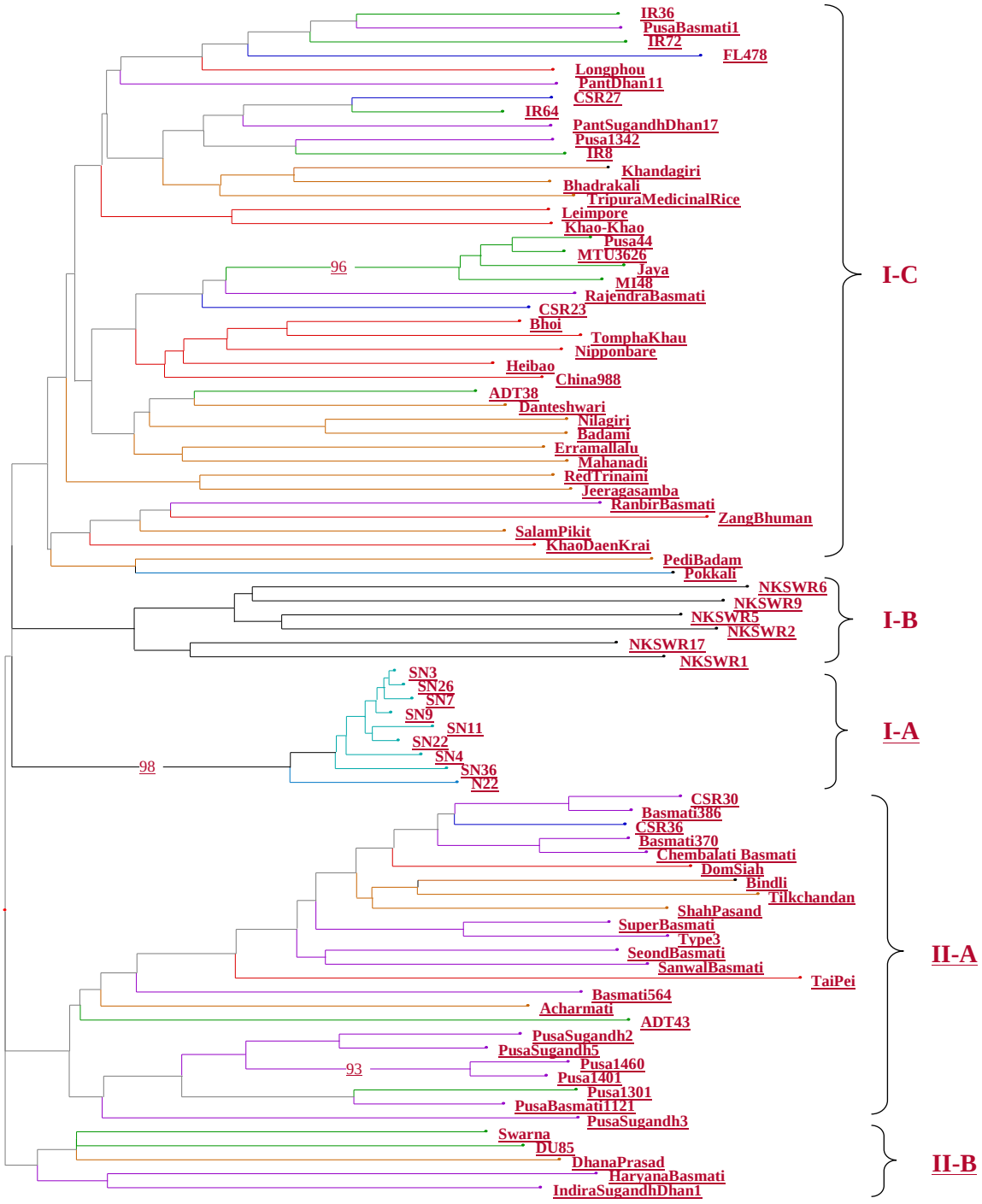
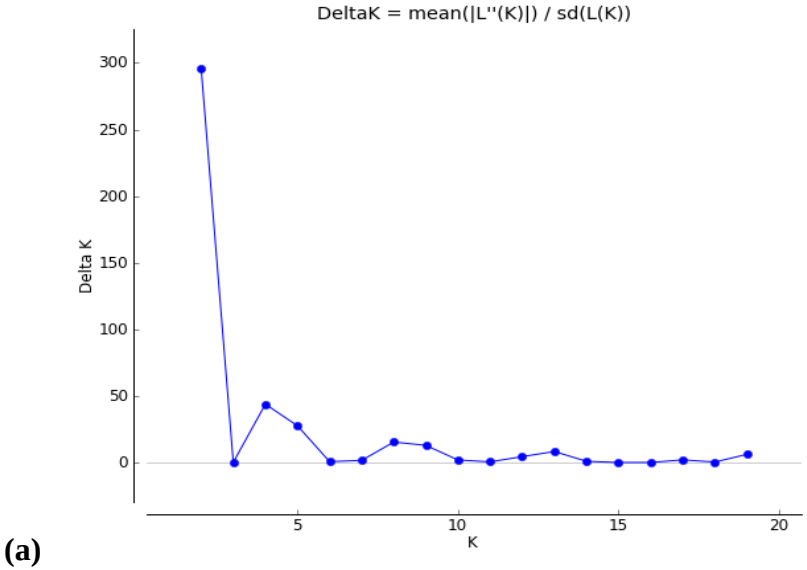
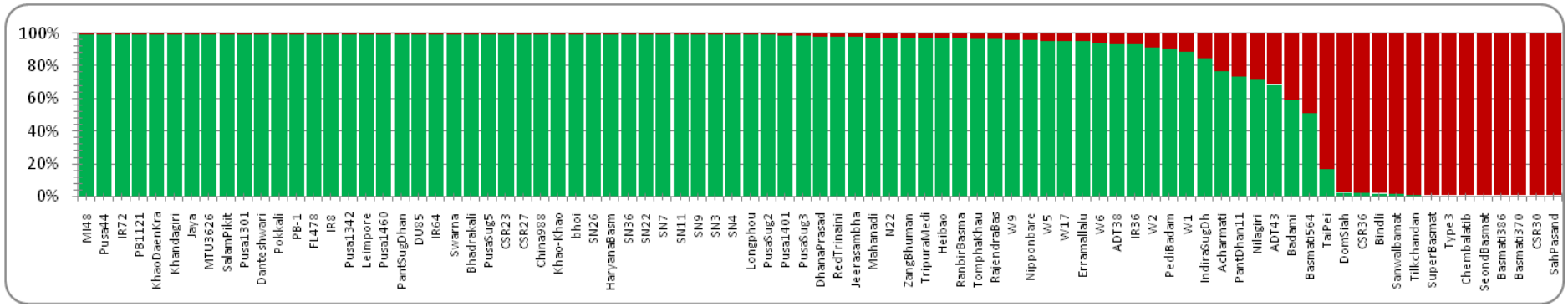


Fig 4.3.1. Genotype grouping of 85 germplams lines identified using random and *Saltol* linked microsatellite markers byneighbor joining analysis using simple matching coefficients



(a)



(b)

Fig 4.2.2. Analysis of population structure showing **(a)** values of Δ*K* for determining optimum number of subpopulations for total population **(b)** bar plot showing distributionof genotypes within subpopulations.

Appendix 2.

Details of the germplasm evaluated with morphological and biochemical data

No	Genotype	Feature	SL	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS	PS
1	Longphou	Japonica	30.25	12.00	0.79	0.10	0.89	0.05	0.02	1.63	0.55	2.96	2.69	0.74	3.62	9.00
2	TaiPei	Japonica	34.00	10.50	1.06	0.09	1.14	0.07	0.01	1.34	0.56	2.40	2.30	0.99	2.33	7.00
3	KhaoKhao	Japonica	43.25	14.75	1.15	0.11	1.25	0.07	0.02	0.99	0.50	1.98	1.21	0.89	1.36	7.00
4	China988	Japonica	47.35	11.40	3.57	0.47	4.04	0.18	0.03	0.39	0.36	1.10	1.64	0.50	3.30	5.00
5	Leimpore	Japonica	24.25	9.75	0.73	0.10	0.83	0.04	0.01	0.20	0.63	0.32	1.12	1.33	0.84	7.00
6	Heibao	Japonica	29.45	10.80	2.18	0.10	2.28	0.11	0.02	0.64	0.53	1.22	0.83	0.67	1.24	5.00
7	TomphaKhau	Japonica	33.20	15.05	2.55	0.30	2.85	0.14	0.02	0.57	0.43	1.31	0.86	0.54	1.58	3.00
8	Nipponbare	Japonica	27.70	9.90	2.20	0.08	2.28	0.14	0.02	0.59	0.51	1.17	1.13	0.60	1.90	3.00
9	Bhoi	Japonica	29.30	10.35	0.93	0.09	1.02	0.07	0.01	0.29	0.17	1.71	0.93	0.82	1.12	7.00
10	Zang Bhuman	Japonica	17.25	9.00	0.11	0.06	0.17	0.03	0.01	1.02	0.16	6.43	1.77	0.89	2.00	9.00
11	Khao Daen Krai	Japonica	35.70	10.25	0.53	0.08	0.60	0.04	0.01	0.11	1.15	0.09	0.84	1.25	0.67	7.00
12	Dom Siah	Japonica	30.50	10.50	0.57	0.08	0.65	0.04	0.01	0.76	0.57	1.33	0.91	1.56	0.58	7.00
13	Type 3	Basmati	27.50	8.25	0.56	0.07	0.63	0.04	0.01	1.08	0.55	1.95	1.26	1.05	1.20	7.00
14	Super Basmati	Basmati	19.80	12.20	0.32	0.09	0.41	0.02	0.01	0.69	0.17	4.13	0.86	0.89	0.97	7.00
15	Indira Sugandh Dhan 1	Basmati	15.25	6.00	0.30	0.05	0.35	0.02	0.01	2.77	0.82	3.37	3.81	0.96	3.98	9.00
16	Basmati 564	Basmati	32.75	11.00	0.93	0.08	1.01	0.06	0.01	0.17	0.63	0.27	1.42	0.87	1.62	7.00
17	Pusa Sugandh 5	Basmati	31.10	8.15	1.08	0.08	1.16	0.06	0.02	0.94	0.23	4.16	1.28	0.35	3.68	7.00
18	Haryana Basmati	Basmati	22.65	7.10	0.46	0.07	0.53	0.03	0.01	0.75	0.11	7.18	1.23	0.16	7.78	9.00
19	Pusa Sugandh 3	Basmati	35.50	11.75	1.92	0.22	2.14	0.11	0.03	0.69	0.32	2.17	1.85	0.48	3.84	5.00
20	Pant Dhan 11	Basmati	24.00	8.25	0.40	0.09	0.49	0.03	0.01	0.50	1.44	0.35	0.90	1.61	0.56	9.00
21	Pusa Sugandh 2	Basmati	14.50	8.00	0.08	0.07	0.14	0.01	0.01	1.05	0.52	2.02	1.74	0.67	2.58	5.00
22	Pusa 1401	Basmati	25.50	13.75	0.42	0.10	0.52	0.04	0.01	0.49	0.68	0.72	1.08	0.76	1.42	5.00

No	Genotype	Feature	SL	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS	PS
23	Pusa 1460	Basmati	31.35	10.90	1.04	0.09	1.13	0.06	0.01	0.81	0.78	1.04	1.15	1.58	0.73	5.00
24	Rajendra Basmati	Basmati	12.10	3.15	0.07	0.04	0.11	0.01	0.01	0.59	0.20	2.96	1.17	0.42	2.82	7.00
25	Ranbir Basmati	Basmati	25.10	7.75	0.57	0.07	0.64	0.04	0.01	0.89	0.26	3.39	1.06	0.61	1.73	7.00
26	Pant Sugandh Dhan17	Basmati	34.90	14.85	1.59	0.59	2.18	0.10	0.02	0.75	0.44	1.69	0.97	0.58	1.68	9.00
27	Chembalati Basmati	Basmati	31.85	10.25	0.54	0.08	0.62	0.04	0.01	2.19	0.52	4.25	2.84	0.79	3.60	9.00
28	Pusa Basmati 1	Basmati	31.00	13.50	1.12	0.08	1.20	0.08	0.02	0.93	0.49	1.88	1.15	0.89	1.29	9.00
29	Pusa Basmati 1121	Basmati	34.50	10.75	0.97	0.09	1.06	0.07	0.01	0.15	0.54	0.28	0.72	0.62	1.17	7.00
30	Sanwal Basmati	Basmati	26.25	7.50	0.88	0.07	0.95	0.06	0.01	0.13	0.44	0.29	0.23	0.64	0.36	9.00
31	Seond Basmati	Basmati	38.25	13.00	2.25	0.21	2.46	0.11	0.02	0.44	0.29	1.50	1.40	0.43	3.21	3.00
32	Basmati 386	Basmati	31.55	11.25	1.13	0.08	1.21	0.08	0.01	1.11	0.50	2.21	0.84	0.96	0.87	7.00
33	Basmati 370	Basmati	30.55	6.85	0.85	0.09	0.94	0.06	0.02	1.06	0.61	1.74	2.56	0.96	2.66	9.00
34	Pusa 1301	Basmati	35.25	8.00	1.12	0.07	1.19	0.13	0.01	0.39	0.59	0.66	0.62	0.64	0.98	7.00
35	CSR 30	Basmati	32.00	12.50	1.69	0.10	1.79	0.11	0.02	0.31	0.64	0.48	0.41	0.87	0.47	7.00
36	CSR 23	Salt tolerant Cultivar	31.80	13.05	1.60	0.10	1.69	0.09	0.02	0.64	0.21	3.04	0.77	0.81	0.95	3.00
37	CSR 27	Salt tolerant Cultivar	39.50	15.85	3.14	0.26	3.40	0.18	0.03	0.36	0.46	0.78	0.51	1.57	0.32	3.00
38	CSR 36	Salt tolerant Cultivar	45.00	9.75	2.08	0.20	2.28	0.14	0.03	0.40	0.32	1.24	0.78	0.55	1.41	5.00

No	Genotype	Feature	SL	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS	PS
39	FL 478	Salt tolerant Breeding line	51.00	13.00	5.78	0.47	6.24	0.34	0.05	0.24	0.27	0.89	0.54	0.99	0.55	3.00
40	IR 64	Cultivar	21.50	9.00	0.48	0.06	0.54	0.03	0.01	0.29	0.78	0.37	0.37	1.04	0.36	7.00
41	MI 48	Cultivar	27.25	9.00	2.28	0.06	2.34	0.10	0.02	0.42	0.19	2.23	0.53	0.75	0.71	7.00
42	Jaya	Cultivar	21.15	9.10	1.18	0.06	1.24	0.07	0.02	0.77	0.82	0.93	0.85	1.19	0.71	9.00
43	MTU 3626	Cultivar	35.75	15.75	2.26	0.08	2.34	0.15	0.02	0.51	0.59	0.86	0.94	1.46	0.64	7.00
44	Pusa 44	Cultivar	13.50	7.50	0.07	0.05	0.12	0.02	0.01	0.33	0.64	0.52	0.94	0.93	1.01	7.00
45	IR 8	Cultivar	23.20	9.50	0.79	0.08	0.86	0.05	0.01	0.40	1.19	0.34	0.65	1.34	0.48	7.00
46	Pusa 1342	Breeding line	33.25	10.50	0.88	0.08	0.96	0.06	0.01	0.08	0.43	0.20	0.15	0.81	0.18	7.00
47	ADT 43	Cultivar	24.85	11.00	0.89	0.07	0.96	0.07	0.02	0.40	1.52	0.26	0.91	1.07	0.85	9.00
48	IR 36	Cultivar	26.50	9.25	0.86	0.09	0.95	0.06	0.01	1.16	0.72	1.61	1.41	0.50	2.81	9.00
49	DU 85	Cultivar	41.75	12.00	0.24	0.09	0.33	0.10	0.02	0.76	0.57	1.34	0.96	0.90	1.08	9.00
50	Swarna	Cultivar	16.00	7.75	0.26	0.08	0.34	0.02	0.01	0.10	1.01	0.09	0.39	1.76	0.22	7.00
51	IR 72	Cultivar	25.00	11.25	0.56	0.08	0.64	0.04	0.01	0.51	0.43	1.19	0.69	0.64	1.08	9.00
52	ADT 38	Cultivar	27.40	13.00	1.42	0.08	1.50	0.09	0.01	0.47	0.43	1.09	0.73	0.81	0.90	9.00
53	Dhana Prasad	Landrace	22.75	12.25	0.55	0.08	0.63	0.04	0.01	0.76	0.19	4.08	0.81	0.32	2.54	9.00
54	Tilakchandan	Landrace	38.50	13.50	1.43	0.09	1.52	0.09	0.02	1.38	1.09	1.27	2.63	1.87	1.41	5.00
55	Jeeragasamba	Landrace	22.75	10.25	0.50	0.08	0.58	0.04	0.01	0.22	0.27	0.84	0.93	0.46	2.02	7.00
56	Shah Pasand	Landrace	50.25	10.00	2.77	0.15	2.91	0.17	0.02	0.28	1.32	0.21	0.80	1.44	0.56	3.00
57	Bhadrakali	Landrace	31.00	10.75	1.33	0.08	1.40	0.08	0.02	0.14	0.27	0.52	0.36	0.32	1.12	7.00
58	Danteshwari	Landrace	21.50	6.25	0.09	0.05	0.14	0.01	0.01	0.29	0.10	3.00	0.36	0.52	0.69	7.00
59	Red Trinaini	Landrace	16.50	8.00	0.78	0.05	0.83	0.05	0.02	0.29	0.90	0.33	1.73	1.17	1.48	5.00
60	Salam Pikit	Landrace	38.50	11.50	1.44	0.10	1.53	0.08	0.02	1.07	0.11	10.19	2.04	0.40	5.05	5.00

No	Genotype	Feature	SL	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS	PS
61	Khandagiri	Landrace	54.75	15.00	3.58	0.25	3.83	0.20	0.03	0.37	0.38	0.97	0.82	0.45	1.81	5.00
62	Mahanadi	Landrace	28.30	10.70	1.13	0.08	1.21	0.07	0.01	0.32	1.20	0.27	0.85	1.35	0.63	5.00
63	Badami	Landrace	39.75	14.25	2.39	0.17	2.55	0.14	0.02	0.37	0.40	0.92	0.65	0.66	0.98	3.00
64	Bindli	Landrace	48.25	14.50	1.93	0.09	2.02	0.09	0.02	0.52	0.81	0.64	1.20	1.11	1.08	7.00
65	Erramallalu	Landrace	34.00	10.00	1.43	0.08	1.51	0.09	0.02	0.47	0.13	3.57	0.76	0.15	5.03	7.00
66	Pokkali	Salt tolerant Landrace	28.00	12.25	1.53	0.08	1.61	0.09	0.02	0.93	0.87	1.07	1.49	1.88	0.79	3.00
67	Nilagiri	Landrace	30.00	13.50	1.18	0.09	1.27	0.07	0.02	0.56	0.37	1.52	0.92	0.47	1.96	5.00
68	Tripura Medicinal Rice	Landrace	34.15	11.10	0.88	0.07	0.95	0.06	0.01	1.18	0.14	8.41	1.29	0.37	3.53	7.00
69	Pedi Badam	Landrace	52.50	11.75	3.13	0.27	3.39	0.17	0.02	0.33	0.58	0.58	0.46	0.93	0.49	3.00
70	Acharmati	Landrace	19.25	9.75	0.25	0.08	0.33	0.01	0.01	0.48	0.28	1.72	0.57	0.69	0.82	7.00
71	Nagina 22	Landrace drought tolerant	47.35	14.35	2.04	0.26	2.30	0.12	0.03	0.57	0.70	0.81	1.12	1.35	0.82	7.00
72	SN36	Mutant of N22	42.25	11.00	1.54	0.08	1.62	0.10	0.03	0.59	0.34	1.73	0.49	0.68	0.72	7.00
73	SN26	Mutant of N22	35.55	7.55	0.69	0.07	0.75	0.05	0.01	0.50	0.51	0.98	1.83	0.81	2.26	9.00
74	SN22	Mutant of N22	44.65	11.10	1.37	0.08	1.45	0.10	0.02	0.31	0.21	1.47	0.59	0.93	0.64	7.00
75	SN7	Mutant of N22	28.75	7.50	0.74	0.06	0.80	0.06	0.01	0.38	0.19	2.00	0.85	0.24	3.57	7.00
76	SN11	Mutant of N22	43.00	11.50	1.02	0.08	1.10	0.07	0.02	0.39	0.64	0.61	0.64	1.01	0.63	9.00
77	SN9	Mutant of N22	19.25	8.50	0.15	0.08	0.23	0.07	0.01	0.15	0.72	0.20	0.65	1.21	0.53	7.00

No	Genotype	Feature	SL	RL	SFW	RFW	SEW	SDW	RDW	NR	KR	NKR	NS	KS	NKS	PS
78	SN3	Mutant of N22	29.45	8.50	0.62	0.07	0.69	0.06	0.01	0.11	0.11	0.96	0.30	0.23	1.28	9.00
79	SN4	Mutant of N22	29.55	8.70	0.45	0.08	0.52	0.03	0.01	0.25	0.35	0.73	0.36	0.96	0.37	7.00
80	NKSWR2	*O. rufipogon*	46.85	19.65	2.63	0.08	2.71	0.16	0.02	0.53	0.48	1.10	0.85	0.67	1.28	3.00
81	NKSWR5	*O. rufipogon*	33.50	11.00	0.77	0.14	0.91	0.05	0.01	0.56	0.56	1.00	0.95	0.90	1.06	7.00
82	NKSWR9	*O. rufipogon*	35.75	14.25	1.17	0.10	1.27	0.07	0.02	0.15	0.36	0.43	0.60	0.81	0.74	7.00
83	NKSWR6	*O. rufipogon*	40.25	16.75	1.63	0.12	1.74	0.11	0.01	0.54	0.65	0.84	0.90	0.75	1.20	7.00
84	NKSWR1	*O. rufipogon*	43.00	19.75	1.73	0.15	1.88	0.11	0.02	0.26	0.81	0.32	0.46	0.93	0.49	5.00
85	NKSWR17	*O. rufipogon*	38.75	13.00	1.12	0.12	1.23	0.08	0.01	1.16	1.08	1.07	2.96	1.47	2.02	3.00
	Mean		31.75	10.98	1.24	0.11	1.35	0.08	0.01	0.61	0.54	1.67	1.07	0.86	1.57	6.58
	SE		1.04	0.31	0.10	0.01	0.11	0.01	0.00	0.05	0.03	0.19	0.07	0.04	0.14	0.21
	CV(%)		30.10	26.38	77.49	83.10	75.92	67.33	44.31	73.74	58.97	106.96	62.63	45.79	83.30	29.34

SL, shoot length (cm); RL, root length (cm); SFW, shoot fresh weight (g); RFW, root fresh weight (g); SEW, seedling weight (g); SDW, shoot dry weight (g); RDW, root dry weight (g); NR, root Na^+ content ($mmol.g^{-1}$); KR, root K^+ content ($mmol.g^{-1}$); NKR, root Na^+/K^+ ratio; NS, shoot Na^+ content ($mmol.g^{-1}$); KS, shoot K^+ content ($mmol.g^{-1}$); NKS, shoot Na^+/K^+ ratio; PS, phenotypic salt tolerance score

4.3 Research Paper III

Marker assisted introgression of *Saltol*, a major QTL for seedling stage salinity tolerance into Pusa Basmati 1121, an elite Basmati rice variety

Abstract

Pusa Basmati 1121 (PB1121), an elite Basmati rice variety known for its unparalleled grain and cooking quality is susceptible to salinity. Marker assisted backcross breeding (MABB) was employed to introgress *'Saltol'*, a major QTL for seedling stage salinity tolerance into PB1121 using FL478 as donor. Foreground selection for *Saltol* was carried out using the tightly linked peak molecular marker RM 3412 and background selection was done using 90 polymorphic STMS markers providing genome wide coverage. Further, stringent phenotypic selection for agronomic, grain and cooking quality traits in each backcrossing and selfing generation was used to accelerate recovery of recurrent parent phenome (RPP). Fifty one BC_3F_2 superior *Saltol* homozygous lines were selected and advanced to BC_3F_4 generation to develop improved versions of PB1121 with salt tolerance. Background analysis revealed that the recurrent parent genome (RPG) recovery ranged from 93.33 to 99.44 per cent in selected families. The improved lines were on par or better than the original PB1121 in terms of yield, grain and cooking quality traits with salt tolerance at seedling stage. Further, the improved lines were characterised for shoot and root Na^+ and K^+ to understand the mechanism of salinity tolerance. Biochemical analysis of all selected salt tolerant lines for shoot and root Na^+ and K^+ concentration and their ratio showed significant differences in ionic concentrations. Interrelations between Na^+ and K^+ content of the *Saltol* carrier lines showed that root and shoot Na^+ concentration and root and shoot K^+ concentration were significantly associated, with later showing lesser magnitude of association than the former. Nine backcross derived lines showed yield and quality an par to recurrent parent while one of the improved lines, Pusa1734-8-3-26 showed significant superior in yield over the recurrent parent with seedling stage salinity tolerance level similar donor parent.

Key words: Saltol, Salinity tolerance, Marker assisted backcross breeding, Foreground selection, Grain and cooking quality

Introduction

Basmati, the unique quality rice, is a nature's gift to Indian sub-continent notable for its grain quality and delicate nuanced flavour. A superior Basmati variety Pusa Basmati 1121

(PB1121), developed by the Indian Agricultural Research Institute. New Delhi, occupies more than 60 per cent of the total Basmati rice area in India and almost 80 percent of the Basmati rice area in the state of Haryana, which has sizable proportion of rice lands affected by salinity. PB1121 commands a premium in the market over other Basmati rice varieties because of its excellent grain and cooking quality traits. However, this variety is sensitive to soil salinity, a major constraint to the production and sustainability of Basmati rice cultivation in states of Haryana and the neighbouring areas of state of Punjab.

Generally, rice plant is very sensitive to salt accumulation at seedling stage and less sensitive at reproductive stages (Lutts et al., 1995). However, Zeng et al., (2001) reported that the early reproductive stages *i.e*., panicle initiation or pollination (Khatun and Flowers 1995) are the most salt sensitive growth stages affecting the formation of grains and grain quality. In contrast, Khan et al., (1997) reported that rice is more tolerant to salt at germination than other stages. Salt tolerance is a complex trait to measure (Munns 2002) because phenological screens demand huge labour, space and laborious experiments coupled with ambiguity created by high environmental influence, which make them impractical for screening large number of genotypes (Flower and Yeo 1997; Gregorio et al., 2002; Yamaguchi and Blumwald 2005; Ismail et al., 2007; Thomson et al., 2010).

In the Basmati rice germplasm, only one variety namely, CSR30 (Yamini) is reported to have some degree of salinity tolerance (Singh et al., 2005), however the mechanism and genomic region involved in conferring salinity tolerance in CSR30 is not yet characterized. Conversely, in non-Basmati rices, remarkable variation exists for salt tolerance, which provides great opportunities to improve salt stress tolerance through genetic means (Akbar et al., 1987; Flowers and Yeo 1981). Although initial progress in breeding for salt tolerance in rice through conventional means was slow, accelerated development of molecular marker technologies in recent times and their rapid application in construction of genetic linkage map, molecular dissection of the important complex traits has aided in identifying several genomic regions associated with salt tolerance (Vinod et al., 2013). Recent advances in molecular marker assisted breeding in several crops have unequivocally proved the advantage in enhancing the efficiency and accuracy of breeding process through introgressing genes/QTLs governing target traits into high yielding varieties (Alpuerto et al., 2008).

A number of Genes/QTLs have been mapped for several salt tolerant parameters like Na^+ and K^+ uptake, Na^+ and K^+ concentration and Na^+/K^+ ratio in shoot on different chromosomes of rice (Koyama et al., 2001; Lin et al., 2004; Singh et al., 2007; Ammer et al.,

2009; Pandit et al., 2010; Haq et al., 2010). Lin et al., (2004) mapped two major QTLs namely shoot K^+ concentration on chromosome 1 (*qSKC-1*) and shoot Na^+ concentration on chromosome 7 (*qSNC-7*) in $F_{2:3}$ population derived from the tolerant *indica* landrace Nona Bokra with the susceptible *japonica* variety Koshihikari. The *SKC1* gene was subsequently cloned and found to encode a sodium transporter that helps control K^+ homeostasis under salt stress (Ren et al., 2005). A major QTL associated with the Na–K ratio and seedling-stage salt tolerance, named *Saltol* (Gregorio et al., 1997) was identified on chromosome 1 from using recombinant inbred line population developed by crossing a salt sensitive variety IR29 with a salt tolerant land race pokkali, explaining 43 to 70 per cent phenotypic variance for salt tolerance (Bonilla et al., 2002). Developed from this cross, IR 66946-3R-178-1-1 (also named FL478), a semi dwarf, early and photoperiod insensitive RIL with high level of seedling stage salinity tolerance has been extensively used as a donor for salinity tolerance in rice breeding. Further analysis of single feature polymorphisms in the *Saltol* region of FL478 revealed that it contained a <1 Mb DNA fragment from Pokkali at 10.6–11.5 Mb on chromosome 1, flanked by IR29 alleles (Kim et al., 2009). The region also contain many other Pokkali QTLs including that of *SKC1* (Thomson et al., 2010). The candidate markers within *Saltol* QTL region namely, AP 3206, RM 8094 and RM 3412 for use in marker assisted breeding for increased salt tolerance have been identified (Thomson et al., 2010; Aliyu et al., 2011). Although, work on QTL introgression of *Saltol* in major non-basmati rice varieties like BR11, BRRI Dhan 28, IR64, AS996 and Swarna has been initiated (Rahman et al., 2008; Ho et al., 2010; Linh et al., 2012, STRASA 2011), there is no report of similar attempt in Basmati rice varieties. Therefore, to reduce yield losses owing salinity stress in popular Basmati rice varieties there is a potential need to introgress salt tolerance.

In this study, we report introgresion of *Saltol* into the leading Basmati rice variety, PB1121, combining phenotypic and molecular marker aided foreground and background selection.

Material and Methods

Plant materials and development of improved lines

Plant materials used in the study include PB1121, a salt susceptible popular Basmati rice variety, as the recurrent parent and FL478 (IR 66946-3R-178-1-1), a *Saltol* QTL carrying non-Basmati RIL in the background of IR29 as donor parent. FL478 has seedling-stage salt tolerance upto 18 $dS.m^{-1}$ (Thomson et al., 2010). A single F_1 plant from cross PB1121 X

FL478 (designated as Pusa1734) was backcrossed with PB 1121 to generate the BC_1F_1s seeds. Marker-assisted foreground selection was employed using QTL linked markers to identify plants heterozygous for *Saltol* linked marker. Further, the selected plants were subjected to analysis of background recovery using polymorphic markers augmented with stringent phenotypic selection for agro-morphological, grain and cooking quality traits. A single BC_1F_1 plant with maximum recovery of the recurrent parent genome (RPG) as well as phenotypic similarity to recurrent parent was backcrossed to develop the BC_2F_1 generation. The BC_2F_1 plants were also subjected to foreground and background selection followed by phenotypic selection to identify one best plant heterozygous for *Saltol* and with maximum recovery of RPG, which was backcrossed to develop BC_3F_1s. The foreground, background and phenotypic selection cycle was repeated in BC_3F_1 as well to identify plants heterozygous for *Saltol* and with maximum recovery of RPG. These plants were then selfed to generate BC_3F_2 populations. In the BC_3F_2 generation, plants homozygous for *Saltol* were identified and then advanced to the BC_3F_4 generation with further selection for morphological traits, grain and cooking quality in the subsequent generation. RPG recovery was quantified at every step using STMS markers polymorphic between parents.

DNA extraction and polymerase chain reaction amplification

Total genomic DNA was extracted using CTAB method developed by Doyle and Doyle (1990). Polymerase chain reaction (PCR) was performed in a thermal cycler (G-Storm, Somerset, UK) using a 10 µl total reaction volume as described previously (Basavaraj et al., 2010). This contained 30 ng µl of template DNA, 5 pmol of each primer (synthesized from Sigma Inc., St. Louis, MO, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (MBI, Fermentas, Vilnius, Lithuania) and 0.5 U of Taq polymerase (Bangalore Genei, Bangalore, Karnataka, India). Polymerase chain reaction comprised one cycle of denaturation at 95 ^{0}C for 5 min, followed by 35 cycles at 95 ^{0}C for 30 s, 55 ^{0}C for 30 s and 72 ^{0}C for 1 min, with a final extension of 72 ^{0}C for 7 min. The amplified products were resolved on 3.5 % Metaphor™ gel (Lonza, Rockland, ME, USA) containing 0.1 mg/ml of ethidium bromide (Amresco, Solon, OH, USA) along with a DNA size standard 50bp ladder (MBI, Fermentas) and documented in a gel documentation system (BioRad, Hercules, CA, USA).

Molecular marker analysis

Foreground selection

Foreground selection was carried out using RM3412, which is the peak marker for *Saltol* QTL. Additionally, the markers RM35, RM1287, RM8094, RM10720, RM10748 and RM493 present on carrier chromosome (Chr 1) flanking the *Saltol* QTL were used for parental polymorphism between PB1121 and FL478 and the nearest flanking polymorphic markers were used for recombinant selection to minimize donor segment.

Background selection

A set of 600 SSR primer pairs distributed uniformly across the rice genome was used for parental polymorphism survey between the donor FL478 and the recurrent parent PB1121. The polymorphic SSR markers were then used to identify plants with maximum recovery of recurrent parent genome (RPG) in each backcross generation. The PCR products were amplified and resolved in 3.5 % Metaphor^{TM} gel. The genomic contribution of the parents in the elite selections in different backcross generation was estimated using the software Graphical GenoTypes (GGT) Version 2.0 (Van Berloo 1999).

Screening for seedling stage salinity tolerance

The homozygous BC_3F_4 lines and parents were screened for seedling stage salt tolerance under controlled environment in National Phytotron Facility at IARI, New Delhi and CSSRI, Kernal during *Kharif* and *Rabi* season of 2012. Pre-germinated (3 days after germination) seeds were sown in punch holes made on extruded polystyrene foam floats fitted with a nylon wire mesh on the bottom side and suspended on trays filled with Yoshida nutrient solution (Yoshida et al., 1976). Each tray carries 10 entries and parents as control and these trays formed fundamental unit of replication. Two replications were used for each experiment with nine plants per genotype per replication. In order to avoid border effect, the control parent, FL478 was sown along the border on all sides to normalize competition for light and space for next rows. Salt stress was imposed 14 days after germination by adding 60mM NaCl (ECe of 6.9 $dS.m^{-1}$) and salt concentration was increased to 120mM (ECe of 13.9 $dS.m^{-1}$) after 3 days in Yoshida nutrient solution and was maintained until final phenotypic scoring. The parents PB1121 (sensitive) and FL478 (highly tolerant) were used as checks. The pH of the nutrient solution was adjusted daily to 5.0, and the culture solution was replaced every 7 days. 16 days after salt stress, entries were scored based on visual symptoms using standard evaluation system (SES) for rice (IRRI 1996) as modified by Gregario et al., (1997), with scores ranging from 1 (highly tolerant) to 9 (highly sensitive). After, entries were scored for their vegetative vigor and observation of shoot length (cm) and root length (cm), shoot and root fresh weight and dry weight (g) was recorded on three randomly selected

plants per genotype from each replication. Dried samples of shoot and root were used for estimation of Na^+ and K^+ content in these tissues.

Estimation of salt ion concentrations in shoot and root

In the controlled hydroponic conditions, sampling of plant tissues for Na^+ and K^+ ion concentration in shoot and root was done immediately after phenotypic scoring. Whole plant was washed first for 1 min in tap water followed by 1 min in distilled water twice, blotted dry and recorded observations of length and fresh weight of shoot and root. Further root and shoot samples were kept in oven at 60°C for 3 days until completely dry and the dry weight was recorded. Dry samples (known weight) were mixed with 20 ml of 100 mM glacial acetic acid in 30ml test tube. The samples were kept in water bath for 2 h at 90°C which was followed by cooling, and the debris was removed by filtration. An aliquot of the digested material was taken and diluted ten times with 100mM glacial acetic acid to determine Na^+, and K^+ concentration by Flame photometer (ELICO CL360) by the equation:

$$\frac{Na \text{ or } K \text{ concentration}}{DW} = \frac{CdV}{100}$$

where, C is flame photometer reading, *d* is dilution factor, V is extraction volume (ml) and DW is oven dry weight (g). A standard curve of KCl and NaCl solutions of different concentrations, ranging from 10 to 100 $mg.l^{-1}$ was prepared and used for calibration. Na^+ and K^+ content of leaves and stem were expressed on $mg.kg^{-1}$ basis.

Correlation analysis

Among the *Saltol* carriers, to establish associated parameters of ion homeostasis to the salt tolerance, Pearson's correlation coefficients were worked out among root and shoot Na^+ and K^+ concentration, their proportions and salt tolerance score.

Evaluation of agronomic performance and grain quality

Advanced *Saltol* introgression lines were planted at a spacing of 30 × 20 cm in an augmented block design with six checks (Pusa Punjab Basmati 1509, Pusa Basmati 1, Pusa Sugandh 5, CSR30 and two parents, PB1121 and FL478) for evaluation of their agronomic performance during *Khairf* 2012 at the research farm of the Genetics Division, IARI, New Delhi. Data on five plants were recorded for various agronomic traits: days to 50 % flowering (DFF), plant height (PH), number of tillers (NT), panicle length (PL), filled grains per panicle (FGP), spikelet fertility (SF), 1000 grain weight (TW) and yield per plant (YP). The analysis of grain and cooking quality traits such as grain size (GS), kernel length before cooking (KLBC),

kernel length after cooking (KLAC), kernel breadth before cooking (KBBC), kernel breadth after cooking (KBAC), length/breadth ratio (LB), elongation ratio (ER) and aroma (ARM) was carried out as described in Basavaraj et al., (2010).

Results

Marker assisted selection for *Saltol*

Ten BC_1F_1 plants heterozygous for the *Saltol* linked marker RM3412 were selected from 20 BC_1F_1 plants generated by backcrossing a single F_1 plant to the recurrent parent PB1121. The *Saltol* heterozygotes had a recurrent parent genome (RPG) recovery ranging from 76.0 to 84.0 per cent when subjected to background selection using 90 polymorphic SSR markers spanning rice genome. One plant was finally selected among the *Saltol* heterozygous BC_1F_1 plants based on the agro-morphological similarity with PB1121 and RPG recovery and further backcrossed with PB1121 to generate fifteen BC_2F_1 plants. Foreground selection in BC_2F_1, resulted in 7 plants heterozygous for *Saltol* locus that were further subjected to background selection using 28 markers heterozygous in the selected BC_1F_1 plant(Fig 4.3.2). Two BC_2F_1 plants were recovered based on the morphological similarity to PB1121 and RPG recovery of 89.5 and 94.2 per cent coupled with reduced donor segment as determined by the recombinant selection using two markers flanking *Saltol* locus namely RM8094 (11.2Mb) and RM493 (12.3Mb). Twenty BC_3F_1 plants were produced from the selected two BC_2F_1 plants by another round of backcrossing. These BC_3F_1 plants were subjected to foreground, background, recombinant and morphological selection to identify three BC_3F_1 plants heterozygous for *Saltol* and having RPG more than 96 per cent, which were selfed and raise BC_3F_2 populations. Fifty one single plant homozygous for *Saltol* linked marker RM3412 were selected from BC_3F_2 populations that were initially shortlisted to 98 single plants based on stringent phenotypic similarity to PB1121, grain and cooking quality traits and were advanced to BC_3F_4 generation followed by selection of ten superior plants with grain and cooking quality characters of Basmati.

Recombinant selection was done using two polymorphic flanking markers (RM8094 and RM493) flanking *Saltol* locus(Fig 4.3.7). Most of the Saltol positive plants recovered the recurrent parent allele atthese markers loci in BC_3F_1. Further, additional polymorphic markers were used to analyse the background genome recovery on carrier chromosome of selected homozygous lines. Two *Saltol* homozygous genotypes Pusa1734-8-3-24 and

Pusa1734-8-3-26 has less than 0.3Mb donor segment in *Saltol* region (Fig 4.3.7) with 99 percent background genome recovery (Fig 4.3.1).

3.2 Screening for seedling stage salinity tolerance

Fifty one BC_3F_4 *Saltol* homozygous lines were screened in hydroponics at seedling stage in Yoshida nutrient solution containing NaCl having an EC of 13.9 dSm^{-1} along with parents and susceptible check IR 29. The check IR29 scored 9 showing high susceptibility to salt stress, while 18 *Saltol* homozygous lines were found to be tolerant with an average score of 3 *i.e*., as good as the donor parent FL478. While 17 lines possessed moderate tolerance of score 5 (?) and sixteen lines showed susceptible reaction similar to the recurrent parent PB1121 with a score of 7.

Field agronomic performance

The agronomic performance with respect to yield and yield components of fifty one BC_3F_4 *Saltol* homozygous lines evaluated during *Kharif* 2012(Fig 4.3.4) and ten selected lines data is presented in the Table 4.3.1. Most of the lines were found on par with PB 1121 with respect to yield and yield component except for filled grains per panicle and plant height. Days to 50 % flowering of the advanced lines ranged from 101 to 113 with mean of 106.8 days as compared to 109 days of PB1121. Significant variation was observed for the number of grains per panicle ranging between 48.6 (Pusa1734-8-3-31) and 113.2 (Pusa1734-8-3-26), as compared to 91.2 in PB1121. Similarly, plant height among the selected lines ranged between 83.2 cm (Pusa1734-8-3-31) and 157.0 (Pusa1734-8-3-3) as against 123.4cm of PB1121. The panicle length and spikelet fertility was better than recurrent parent in improved lines. Yield per plant ranged from 29.3g (Pusa1734-8-2-31) to 41.2g (Pusa1734-8-3-26), as compared to 29.1 g of PB 1121. One of the selections, Pusa1734-8-3-26 showed significant improvement for yield (41.7%), number of filled grains per panicle (50. 2%), spikelet fertility (11.5%) and days to 50% flowering (6 days earlier) over PB1121.

Grain and cooking quality

The data on grain and cooking quality traits of selected BC_3F_4 lines are presented in Table 4.3.2. All improved lines possessed extra-long (>12mm length), slender (<2mm width) grains similar to that of recurrent parent PB1121 (Fig 4.3.3). The KLBC of advanced lines ranged from 8.32mm in Pusa1734-8-3-37 to 9.26mm in Pusa1734-8-3-3 as compare to 9.32mm in PB1121. The LBR was in the range of 4.67 (Pusa1734-8-3-37) to 5.10 (Pusa1734-8-3-24) as compared with 4.78 of PB 1121. The two improved lines, Pusa1734-8-3-12 and

Pusa1734-8-3-66 showed superior kernel elongation ratio (≥2) as compared to the recurrent parent. High yielding lines, Pusa1734-8-3-26 and Pusa1734-8-3-52 had grain and cooking quality characters on par or superior to recurrent parent including aroma.

Analysis for salt ion concentration in the improved lines

Selected *Saltol* introgressed lines along with parents that were subjected to salt stress and analysed for root and shoot Na^+ and K^+ concentration (Table 4.3.3). The donor and recurrent parents significantly differed in their ionic concentration both in root and shoot under salt stress. Na^+ concentration in shoot (1.81mmol.g^{-1} of dry weight) and root (1.27mmol.g^{-1} of dry weight) of PB1121 under salt stress was three and five fold more than the donor parent FL478 (0.6 mmol.g^{-1} of shoot dry weight and 0.24mmol.g^{-1} of root dry weight) respectively. K^+ concentration in shoots of PB1121 (1.00 mmol g^{-1} of dry weight) was almost double the concentration of K^+ in FL478 (0.59 mmol g^{-1} of dry weight). In *Saltol* introgression lines, shoot Na^+ concentration ranged from 0.87 mmol.g^{-1} of dry weight (Pusa1734-8-3-24) to 2.83 mmol.g^{-1} of dry weight (Pusa1734-8-3-52) with a mean of 1.41 mmol.g^{-1} of dry weight, which was less than that of PB1121. The Na^+/K^+ ratio in shoot of FL478 was 1.01 as against 1.81 of PB112. The root Na^+/K^+ ratio in FL478 was 0.59 while PB1121 had almost 12 times higher Na^+/K^+ ratio(7.53). Among the introgressed lines, the shoot Na^+/K^+ ratio ranged from 1.13 (Pusa1734-8-3-26) to 11.72 (Pusa1734-8-3-34), while the root Na^+/K^+ ratio was as low as 1.00 in Pusa1734-8-3-24 to 11.00 in Pusa1734-8-3-14. The selected salinity tolerant advanced backcrossed lines like Pusa1734-8-3-24, Pusa1734-8-3-26 and Pusa1734-8-3-30 has Na^+/K^+ ratio similar to FL478. However, some of the introgression line recording higher Na^+/K^+ rates than PB1121, did express salinity tolerance (Table 4.3.4).

Simple correlation co-efficients between Na^+ and K^+ concentration of the *Saltol* carriers was also estimated (Table 4.3.5). Na^+ concentration in root showed significant negative association with K^+ concentration in root but it had significantly positive correlation with Na^+/K^+ ratio in root and shoot Na^+ concentration. K^+ concentration in root exhibited significantly negative correlation with root and shoot Na^+/K^+ ratio and Na^+ concentration in shoot while it showed negative correlation with K^+ in shoot (r=0.54). Root Na^+/K^+ ratio showed significantly positive association with shoot Na^+(r=0.66) and shoot Na^+/K^+(r=0.59). Shoot potassium concentration showed significantly negative association with shoot Na^+/K^+ (r = -0.89). Salt tolerance score was poorly associated with all ionic concentration and their ratios in shoot and root except with root potassium concentration.

Discussion

Marker assisted selection (MAS) offers remarkable advantage over conventional selection in improving genotypes for biotic and abiotic tolerance in crop plants. MAS is simpler and highly efficient and has been successfully employed for developing improved rice varieties with inbuilt resistance to bacterial blight (Huang et al., 1997; Joseph et al., 2004; Zhang et al., 2012; Gopalakrishnan et al., 2008; Sundaram et al., 2008; Basavaraj et al., 2009, 2010; Singh et al., 2012, 2013), blast (Hittalmani et al., 2000; Zhou et al., 2011; Singh et al., 2012; Singh et al., 2013), submergence tolerance (Neeraja et al., 2007; Jantaboon et al., 2011) and salinity tolerance (Rahman et al., 2008; Huyen et al., 2012). Due to faulty irrigation practices and injudicious use of chemical fertilizers, inland salinity is emerging as one of the major production constraints in Basmati growing regions of Indian subcontinent. The yield potential of PB1121, an elite Basmati variety grown on approximately 1.35 million hectare (60%) of Basmati area in india is not fully exploited in salt affected soils due to its susceptibility to seedling stage salinity stress. Therefore incorporation of seedling stage salinity tolerance in PB1121 is likely to improve its adaptability to salt affected soils and also sustain its productivity.

Developed from the cross of Pokkali, a salt tolerant landrace from Kerala and IR29, a salt sensitive rice variety, the line FL478 carries a major QTL *Saltol* on chromosome 1, which associated with Na^+ uptake, K^+ uptake, Na^+/K^+ ratio and seedling stage salinity tolerance in rice (Gregorio 1997; Thomson et al., 2010). FL478 has been extensively used for transfer of QTL *Saltol* through MAS into elite varieties (Thomson et al., 2010; Rahman et al., 2008; Ho et al., 2010; Linh et al., 2012; Hien et al., 2012). In the present study marker assisted backcross breeding (MABB) coupled with stringent phenotypic selection for agro-morphological, grain and cooking quality traits of PB1121 was used to hasten the recovery of recurrent parent genome and phenome.

Advantages and potentialities of modifications of MAS in backcross breeding strategies like foreground, background and recombinant selection for improvement of Basmati rice varieties were established by many previous works (Joseph et al., 2004; Gopalakrishnan et al., 2008; Basavaraj et al., 2010; Singh et al., 2012; Singh et al., 2013). MAS contributed to a paradigm shift in Basamti breeding (Singh et al., 2011), because foreground selection for target trait was driven by marker genetics, while parallel stringent phenotypic selection augmented marker based background selection helped to restore key Basmati quality traits to the selected lines thus hastening the entire breeding process. The

present study therefore, followed this well-established protocol for transferring *Saltol* to PB1121.

The best RPG recovery response obtained in the selected lines to the tune of 93.3 to 99.4 per cent categorically established the advantage of stringent phenotype augmented marker based background selection. This approach had not only helped in precise introgression of *Saltol* locus with reduced linkage drag but also help in keeping all Basmati grain and cooking quality traits intact. The results indicated that all this could be achieved through just three backcrosses, inspite of fact that the donor FL478 did not have good grain and cooking quality traits and possessed red pericarp colour. Some of the selected lines showed better yield performance owing to increased panicle length, filled grains per panicle and spikelet fertility. Improvement of recurrent parent with respect to non target trait has been demonstrated in MAS scheme with stringent phenotype augmented marker based background selection (Gopalakrishnan et al., 2008; Singh et al., 2011). In the present study, the donor FL478 had desirable traits like number of grains per panicle, spikelet fertility and sturdy stem and lodging resistant in addition to salt tolerance. This additional benefit recovery can be solely attributed to phenotypic selection. One of the selected lines, Pusa1734-8-3-26 has showed more than 40 per cent yield advantage over PB1121, with similar Basmati grain and cooking quality characters.

During phenotypic screening for seedling stage salinity, some of the *Saltol* homozygous families having nearly same level of RPG recovery, showed varied levels of salt tolerance. This deviation was not unexpected, since salinity tolerance by itself is a quantitative trait highly influenced by environment. Furthermore, since *Saltol* locus spans over a large genomic region(< 1Mb and possibly consists of many candidate genes including *SKC1*(Ren et al 2005; Walia et al 2005), recombination within *Saltol* region can also be expected as a possible reason for differential level salinity tolerance among RM3412 positive lines. Besides, minor genetic background differences among the selected lines cannot be ruled out due to undetected linkage drag since the background selection was based on a limited set of markers that were polymorphic between both parents. Interaction between the target gene/QTL with varying genetic backgrounds originating from common pedigree has been reported in previous studies (Koide et al., 2011; Singh et al., 2012; Singh et al., 2013). In the present study, the different advanced *Saltol* introgressed families showed differences in the level of tolerance at seedling stage due to differential interaction of introgressed region with

genetic background and recombination within *Saltol* region, which needs further in depth studies.

Salt tolerance of rice is the final manifestation of several components related to Na^+ and K^+ homeostasis. Physicochemical analysis of all selected salt tolerant lines for shoot and root Na^+ and K^+ concentration and their ratio showed significantly different ionic concentrations. Interrelations between Na^+ and K^+ content of the *Saltol* carriers (Fig 4.3.5), showed that root and shoot Na^+ concentration and root and shoot K^+ concentration were significantly associated, with later showing lesser magnitude of association than the former. This perhaps indicates that K^+ uptake and translocation was highly influenced by Na^+ ions having shared common ion porting channels. On the other hand, root Na^+ concentration had a negative impact on K^+ concentration implying that competition existed in ion uptake in roots, while in shoot Na^+ and K^+ concentration were poorly related. Further, the root Na^+/K^+ ratio was weakly associated to salt tolerance score, but there was hardly any association between these traits in shoots (Fig 4.3.6). Although, earlier reports show that *Saltol* offers salt tolerance mainly by maintaining low Na^+/K^+ concentration in shoot (Gregario 1997; Bonilla et al., 2002), the present study is in non-conformity to this. Interestingly, the shoot K^+ content was found to show a significant negative relation to salt tolerance score indicating that higher shoot K^+ offered better tolerance in the *Saltol* carriers in the present study. Identified initially as a QTL, *shoot K^+ content 1* (*SKC1*; Lin et al., 2004) is a transporter gene located at almost at the same location of chromosome 1 as *Saltol* does, codes for a protein that unloads Na^+ from the root xylem and preferentially expressed in the parenchyma cells surrounding xylem vessels. It was postulated that relative salt tolerance of rice landraces Pokkali and Nona Bokra is due to the presence of *SKC1* (Ren et al., 2005). Therefore, in this study we found that, maintaining shoot K^+ concentration offered relative salt tolerance in *Saltol* carriers including FL478, although Na^+ loading from roost to shoots were different among different lines. Salinity tolerance is not governed by single mechanism rather it is combination of different mechanisms such as low Na^+/K^+ ratio, Na^+ sequestration, tissue tolerance, osmotic adjustment and extrusion (Asch et al., 2000, 1999; Zeng et al., 2003; Vinod et al., 2013). The main site of Na^+ toxicity for most plants is in the shoot rather than in roots and therefore shoot Na^+ concentration and its relation with K^+ is more important in determining the salt tolerance. Under saline conditions, accumulation of Na^+ in shoots causes ionic imbalance particularly K^+, which is vital ion for plant growth and development. In the present observations, although root and shoot Na^+ loadings showed positive relations, corresponding loading to shoot K^+

concentration, was significantly lower indicating existence of alternate mechanisms of Na^+ sequestration and/or antiporting at root-shoot interphase. Therefore, a genotype which has an ability to maintain low Na^+/K^+ ratio in shoots by mechanisms that preferentially load relatively more K^+ than Na^+ would prove superior with salt tolerance. Notwithstanding this observation, some of the *Saltol* introgression lines in the present study showed increased Na^+/K^+ ratio implying either that the sequestered Na^+ ions in shoot cell vacuoles might have contributed to a relatively high estimate of shoot Na^+ content when all the cells are macerated for total Na^+ estimation, than the actual Na^+ ions that are functionally available or a different salt tolerant mechanism may be operational driven by the genomic complementation of the parents. Therefore in depth analysis is needed for deciphering the mechanism of salinity tolerance in the *Saltol* introgressed lines.

3. Conclusion

This is the first effort of marker assisted transfer of the QTL *Saltol* conferring seedling stage salt tolerance into Basmati rice. *Saltol* has been successfully introgressed into PB1121 through marker assisted backcross breeding (MABB) combined with stringent phenotype augmented marker based background selection for agronomic and quality traits. Most of the improved lines possessed seedling stage salt tolerance on par with the donor FL478, while a few had little lower level of salt tolerance. All of them recovered desirable Basmati grain and cooking quality traits and yield traits par with PB 1121and in some cases even yield higher than PB1121. Physiological basis for salt tolerance in the improved *Saltol* introgressed PB1121 lines indicates preferential K^+ loading into shoot tissues as the major mechanism for conferring tolerance, although other mechanisms to sequester or antiport Na^+ ions may be operational., These high yielding, salt tolerant improved PB1121 lines will be evaluated in multi-location trials for release to farmers for commercial cultivation as improved Basmati varieties. Additionally, these improved lines will also serve as valuable donors for salt tolerance in future Basmati breeding programmes.

Table 4.3.1: Agronomic traits, salinity tolerance score and % recurrent parent genome (RPG) recovery in improved lines in comparison to Pusa Basmati 1121

Sl. No.	Genotype	PH(cm)	NT	PL(cm)	DFF	FGP	SF	DM	TWg)	YPP(g)	SUP	ST	RPG
1	**Pusa 1734-8-3-3**	157.0	21.0	30.0	101.0	88.5	80.2	131.0	28.3	34.3	17.8	3.0	94.4
2	**Pusa 1734-8-3-14**	138.4	18.2	31.9	110.0	111.0	94.1	141.0	28.3	31.8	91.2	3.0	95.0
3	**Pusa 1734-8-3-24**	134.9	17.8	29.8	111.0	111.4	91.9	141.0	29.3	31.6	8.7	3.0	99.4
4	**Pusa 1734-8-3-26**	135.9	21.4	30.1	104.0	113.2	92.7	134.0	27.5	41.2	41.7	3.0	99.4
5	**Pusa 1734-8-3-30**	86.2	15.4	25.5	108.0	70.6	85.9	138.0	29.2	34.1	17.3	3.0	93.3
6	**Pusa 1734-8-3-31**	83.2	15.6	23.7	106.0	48.6	80.2	136.0	30.2	29.3	0.7	5.0	95.6
7	**Pusa 1734-8-3-34**	142.1	16.4	32.2	104.0	104.8	93.8	134.0	26.8	31.0	6.5	5.0	95.6
8	**Pusa 1734-8-3-38**	129.7	20.0	27.5	113.0	91.0	85.8	143.0	24.3	30.7	5.5	3.0	97.8
9	**Pusa 1734-8-3-52**	128.8	21.2	28.2	108.0	84.6	94.1	138.0	24.8	37.8	30.0	3.0	93.9
10	**Pusa 1734-8-3-66**	127.8	20.8	28.7	107.0	65.8	87.2	137.0	28.4	32.7	12.3	5.0	93.9
11	**Pusa Basmati 1121**	123.4	28.2	28.8	110.0	91.0	83.3	140.0	30.1	29.1		7.0	Recurrent
12	**FL478**	97.0	10.7	25.7	88.0	128.7	86.5	118.0	23.2	28.4		3.0	Donor
13	**SEd**	4.68	10.43	1.15	1.75	14.3	7.02	1.75	0.41	5.38			

DFF, Days to 50% flowering; **PH**, plant height in cm; **NT**, number of tillers; **PL**, panicle length in cm; **FGP**, number of filled grains per panicle; **DM**, days to maturity; **SF**, spikelet fertility in %, **TW**, weight of 1000 grains; **YPP**, grain yield per plant; **SUP**, yield superiority over PB1121 expressed in %; **ST**, salt tolerance score; **RPG**, recurrent parent genome recovered expressed in %. **Salt tolerance score 0-9 Scale:** Score **1** as Highly Tolerant, **3** as Resistance, **5** as Moderately Tolerant, **7** as Susceptible and **9** as Highly Susceptible

Table 4.3.2: Grain dimensions and quality traits of improved lines in comparison to Pusa Basmati 1121

Sl. No.	Genotype	KLBC	KBBC	LBR	KLAC	KBAC	KER	Aroma
1	**Pusa 1734-8-3-3**	9.26	1.89	4.90	16.99	2.77	1.83	3
2	**Pusa 1734-8-3-14**	8.33	1.67	5.00	17.00	2.67	2.04	3
3	**Pusa 1734-8-3-24**	9.28	1.82	5.10	18.42	2.86	1.98	3
4	**Pusa 1734-8-3-26**	8.69	1.81	4.80	16.12	2.45	1.86	3
5	**Pusa 1734-8-3-30**	8.65	1.76	4.91	16.48	2.54	1.86	3
6	**Pusa 1734-8-3-31**	8.66	1.83	4.73	15.98	2.66	1.85	3
7	**Pusa 1734-8-3-34**	8.66	1.83	4.73	15.98	2.66	1.85	3
8	**Pusa 1734-8-3-37**	8.32	1.78	4.67	16.52	2.68	1.99	2
9	**Pusa 1734-8-3-52**	9.11	1.94	5.06	15.90	2.56	1.71	3
10	**Pusa 1734-8-3-66**	9.00	1.88	4.79	18.00	2.44	2.00	3
11	**PB 1121**	9.32	1.95	4.78	17.10	2.54	1.83	3
12	**FL 478**	6.24	2.36	2.65	10.15	3.69	1.63	0

***KLBC**, Kernel length before cooking in mm; **KBBC**, kernel breadth before cooking in mm; **KLAC**, kernel length after cooking in mm; **KBAC**, kernel breadth after cooking in mm, **LBR**, length/ breadth ratio, **KER**, kernel elongation ratio.

Table 4.3.3: Analysis of Na^+ and K^+ concentration in the improved lines

Sl. No.	Genotypes	Root			Shoot			Salt tolerance Score
		Na (mmol/g)	K (mmol/g)	Na/K ratio	Na (mmol/g)	K (mmol/g)	Na/K ratio	
1	**Pusa 1734-8-3-3**	1.29	0.13	9.66	1.89	0.81	2.34	3.00
2	**Pusa 1734-8-3-14**	0.67	0.11	5.88	1.55	0.14	11.00	3.00
3	**Pusa 1734-8-3-24**	0.62	0.47	1.31	0.87	0.88	1.00	3.00
4	**Pusa 1734-8-3-26**	0.52	0.46	1.13	1.02	0.67	1.51	3.00
5	**Pusa 1734-8-3-30**	0.74	0.40	1.83	0.99	0.90	1.11	3.00
6	**Pusa 1734-8-3-31**	0.59	0.13	4.41	1.07	0.34	3.13	5.00
7	**Pusa 1734-8-3-34**	0.95	0.08	11.72	1.35	0.17	7.89	5.00
8	**Pusa 1734-8-3-37**	0.85	0.12	7.04	1.24	0.16	7.84	3.00
9	**Pusa 1734-8-3-52**	1.65	0.20	8.16	2.83	0.78	3.64	3.00
10	**Pusa 1734-8-3-66**	0.66	0.16	4.16	1.30	0.98	1.32	5.00
11	**PB 1121**	1.27	0.17	7.53	1.81	1.00	1.81	7.00
12	**FL 478**	0.24	0.41	0.59	0.60	0.59	1.01	3.00

Salt tolerance score 0-9 Scale: Score **1** as Highly Tolerant, **3** as Resistance, **5** as Moderately Tolerant, **7** as Susceptible and **9** as Highly Susceptible

Table 4.3.4: Physiological traits of improved lines

SI No	Genotype	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Root Fresh Weight (g)	Shoot Fresh
1	**Pusa 1734-8-3-3**	10.3	31.5	41.8	0.15	1.45
2	**Pusa 1734-8-3-14**	8.5	32.5	41	0.13	0.82
3	**Pusa 1734-8-3-24**	9.1	34.5	43.6	0.12	1.05
4	**Pusa 1734-8-3-26**	11.3	36.5	47.8	0.25	2.21
5	**Pusa 1734-8-3-30**	9	34	43	0.31	1.55
6	**Pusa 1734-8-3-31**	9.5	36.2	45.7	0.15	1.61
7	**Pusa 1734-8-3-34**	6.8	29.4	36.2	0.12	0.63
8	**Pusa 1734-8-3-37**	7.1	39.3	46.4	0.18	1.21
9	**Pusa 1734-8-3-52**	6.8	41.2	48	0.17	1.12
10	**Pusa 1734-8-3-66**	9.5	37.5	47	0.15	1.82
11	**PB 1121**	8.9	32.3	41.2	0.25	1.23
12	**FL 478**	10.1	39.5	49.6	0.35	3.82

Table 4.3.5: Correlation coefficients among ion parameters and salt tolerance

	Root Na^+	Root K^+	Root Na^+/K^+	Shoot Na^+	Shoot K^+	Shoot Na^+/K^+

Root K^+	-0.45*					
Root Na^+/K^+	0.73**	-0.83**				
Shoot Na^+	0.93**	-0.49*	0.66**			
Shoot K^+	0.12	0.54**	-0.41	0.07		
Shoot Na^+/K^+	0.18	-0.68**	0.59**	0.26	-0.86**	
ST Score#	-0.11	-0.49*	0.29	-0.10	-0.17	0.06

ST score – salt tolerance score;
*,** Critical values of two tailed test, $p<0.05 = 0.423$ and $p<0.01 = 0.537$ at df=20

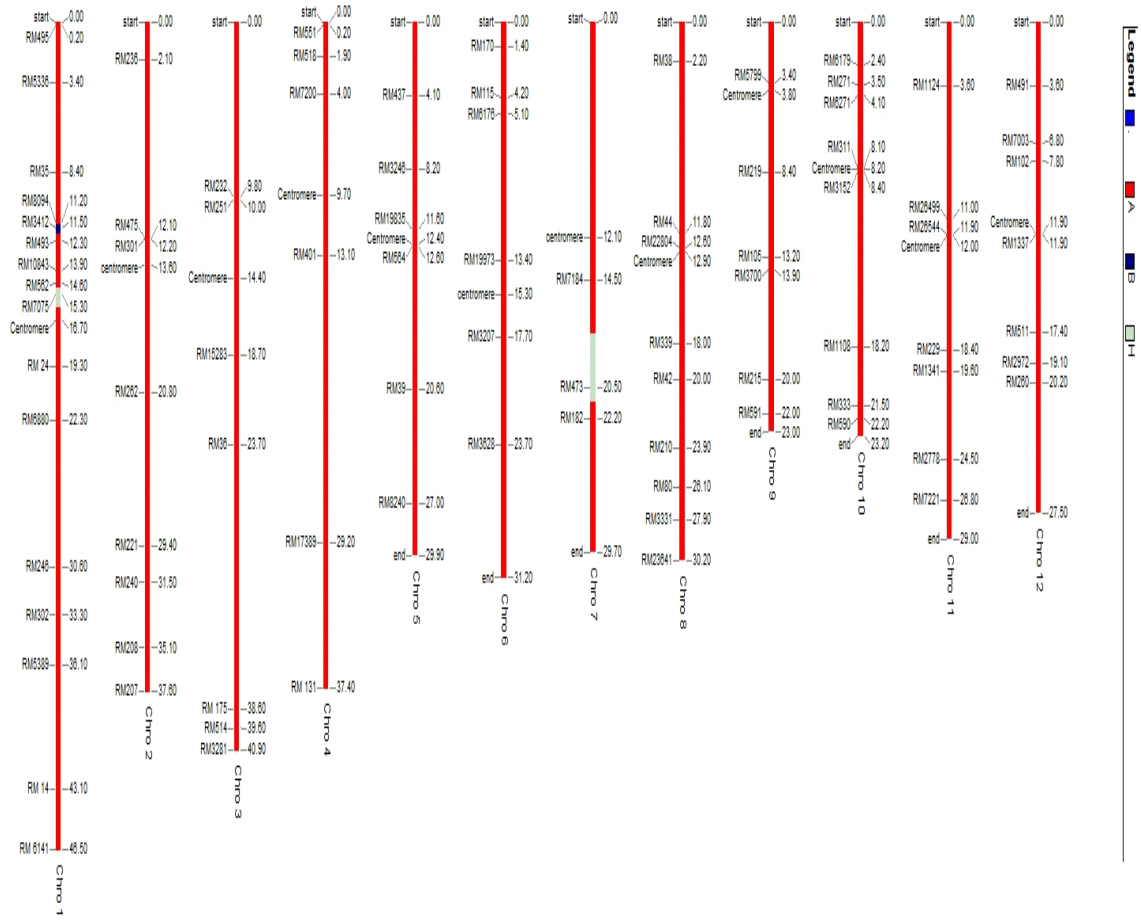


Figure 4.3. 1. Graphical representation of best Saltol introgressed High yielding line Pusa 1734-8-3-26

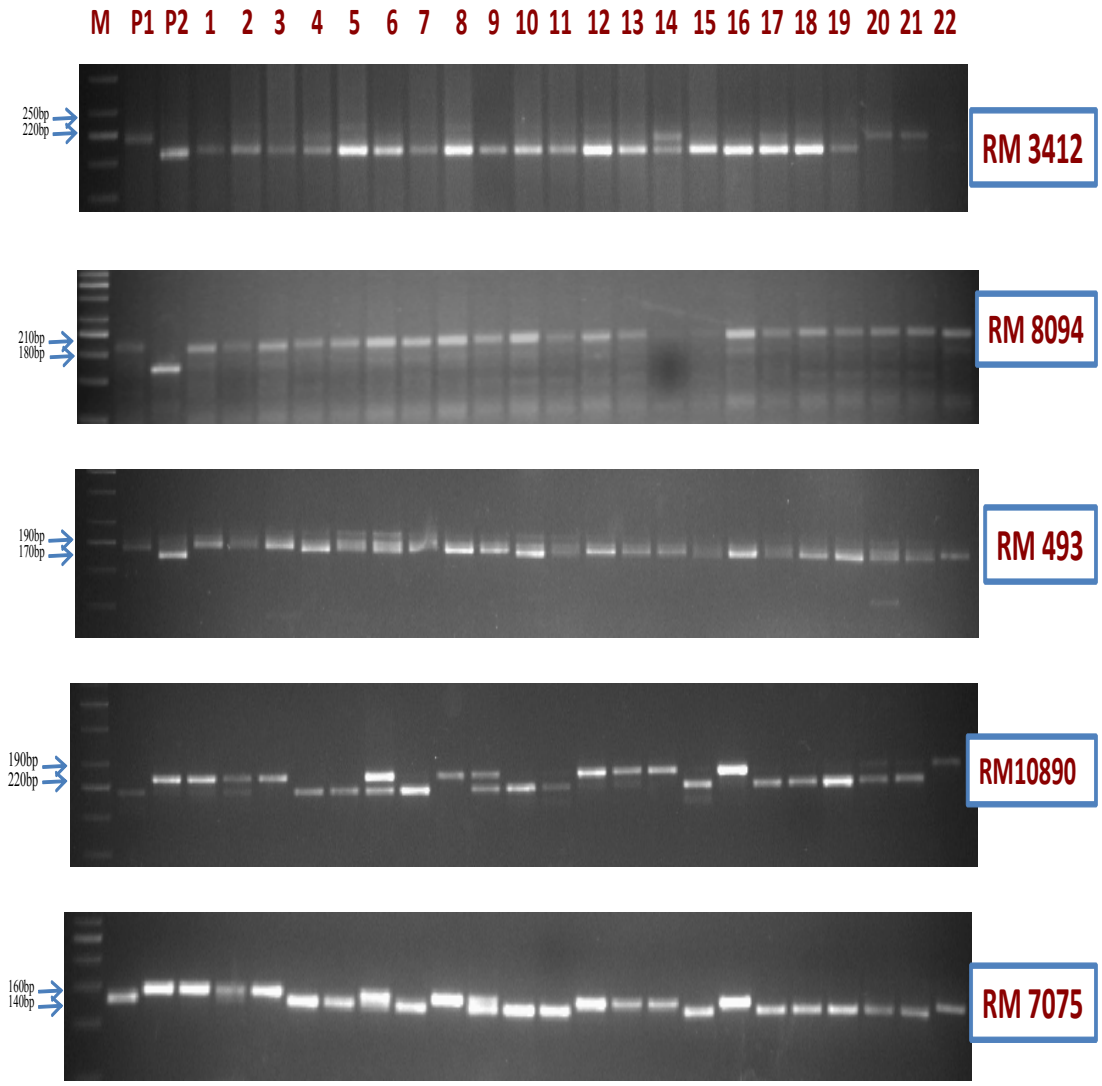


Fig 4.3.2. Foreground and Background selection of BC_3F_3 advanced lines in carrier chromosome

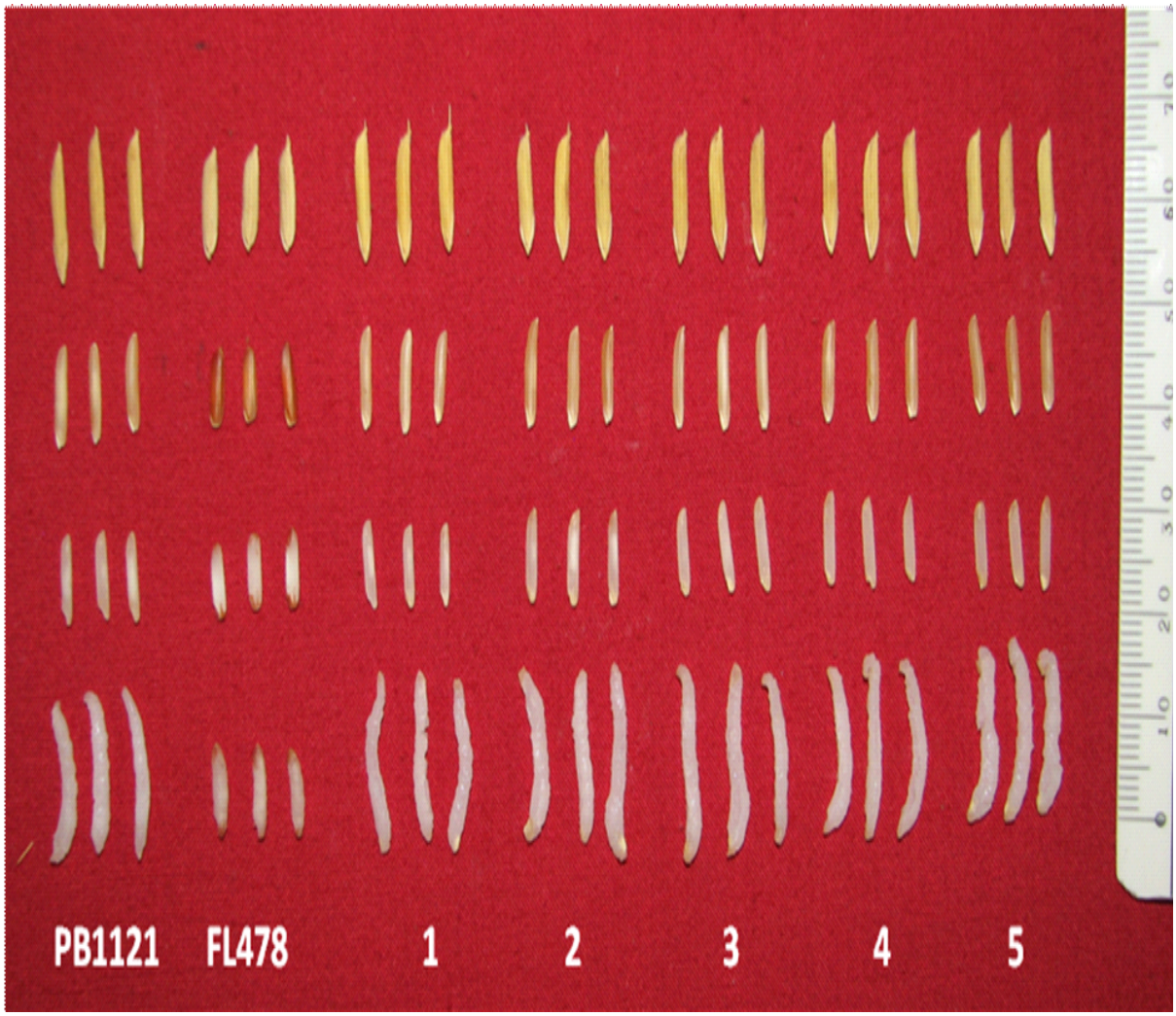


Fig 4.3.3. Cooking and grain quality of advanced derived lines viz **1:** Pusa1734-8-3-3, **2.** Pusa1734-8-3-24**, 3.** Pusa1734-8-3-26**, 4.** Pusa1734-8-3-34 and **5**. Pusa1734-8-3-52

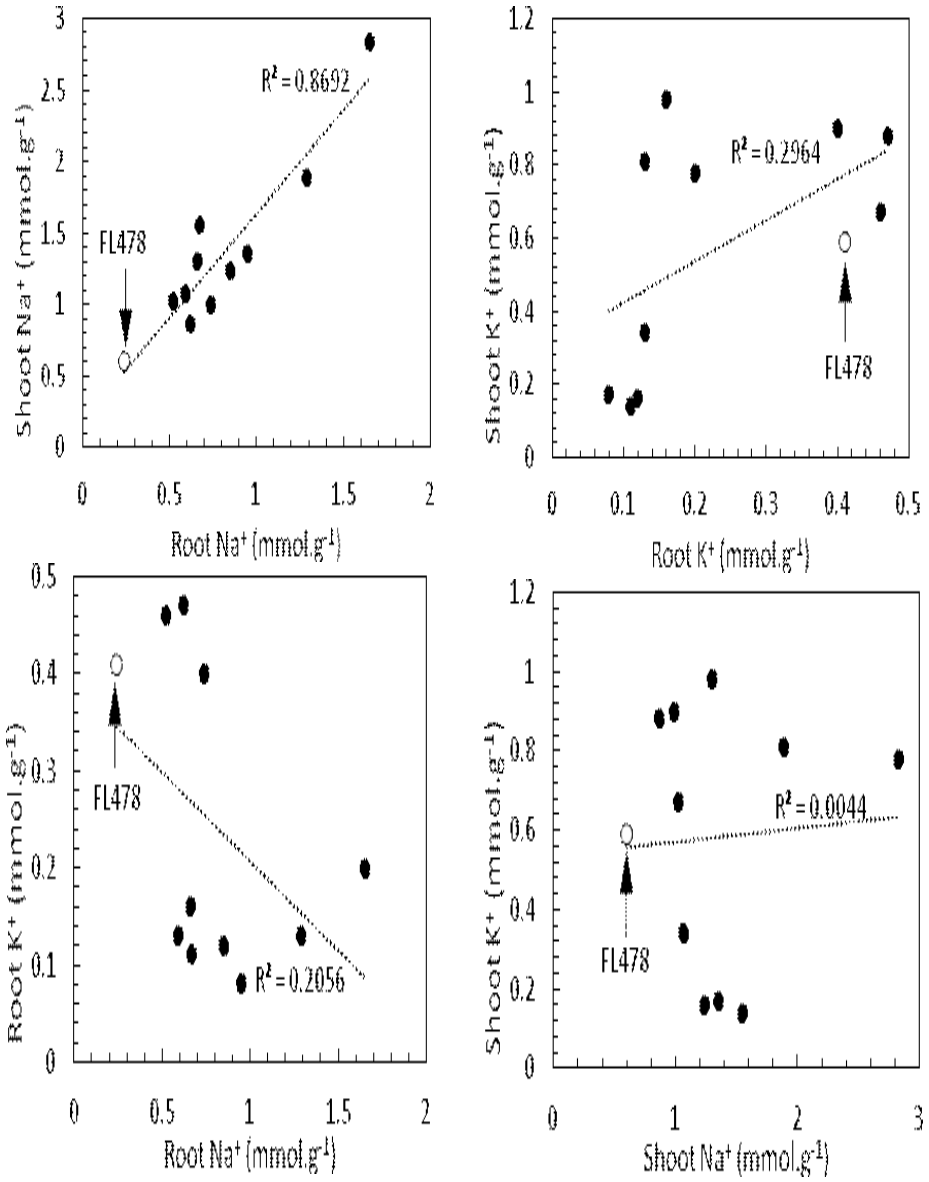


Fig 4.3.5: Interrelations of ionic concentration among *Saltol* introgressed lines and FL478

Abstract

Marker assisted improvement of Pusa Basmati 1121 for salinity tolerance

Rice is highly sensitive to salinity stress especially during early seedling and reproductive stages. The present study was undertaken to introgressa major QTL *Saltol*mapped on chromosome 1 for seedling stage salinity tolerance into the genetic background of sensitive Pusa Basmati 1121, a mega basmati rice variety known for its excellent grain, cooking quality traits and market dominance through MABB approach.

Twenty three diverse germplasm including land races, wild germplasm and improved varieties were used to characterize the haplotype diversity using 20 SSR markers in the *Saltol* region and it association with seedling stage salinity tolerance. Genotypes such as Kuthiru, Orkayama and Ezhome 1 from Kaipad region of Kerala showed salinity tolerance level and haplotypes of *Saltol* locus similar to Pokkali. Whereas salt tolerant wild rice genotypes NKSWR19 and NKSWR20,were found to possess a different haplotypes and therefore may be novel sources for salt tolerance.Altogether, 23 rice genotypes were found to possess 14 different haplotypes based on critical markers linked to *Saltol*QTL using Pokkali as the reference. The haplotypes possessing both of RM8094 and RM3412 markers could discriminate the salt tolerant genotypes from the susceptible genotypes and hence could be useful for marker-assisted selection of *Saltol*QTL.

Further a set of 85 diverse rice genotypes were used to ascertain their seedling stage salt tolerance.A set of 19 polymorphic SSR markers linked to *Saltol*QTL region and 22 random markers spread across the rice genome were used for genotyping the germplasm lines. Based on the phenotypic screening for salt stress tolerance under hydroponics, one basmati rice variety (Seond Basmati), two japonica linesTomphakhu and Nipponbare, three indica landraces viz., Badami, ShahPasandand Pedi Badamand two *Oryza rufipogon* accessions, NKSWR2 and NKSWR17 were identified to be highly salt tolerant genotypes, as that of Pokkali and FL478. The genotypic clustering and population structure divided the total germplasm into two sub-specific populations. The structured association mapping using co-ancestry coefficients and genotypic PC scoresrevealed six robust marker trait associations. The stable marker trait associations RM1287 (10.8Mb) associated with shoot Na^+/K^+ratio and RM140 (12.3Mb) associated with phenotypic salt tolerance score were detected. The locus around RM140 may be targeted for marker assisted improvement of Rice varieties for salt tolerance.

Marker assisted backcross breeding (MABB) was employed to introgress *'Saltol'* into PB1121 using FL478 as donor. Foreground selection for *Saltol* was carried out using the tightly linked peak molecular marker RM 3412 and background selection was done using 90 polymorphic STMS markers providing genome wide coverage. Further, stringent phenotypic selection for agronomic, grain and cooking quality traits in each backcrossing and selfing generation was used to accelerate recovery of recurrent parent phenome (RPP). Fifty one BC_3F_2 superior *Saltol* homozygous lines were selected and advanced to BC_3F_4 generation to develop improved versions of PB1121 with salt tolerance. Background analysis revealed that the recurrent parent genome (RPG) recovery ranged from 93.33 to 99.44 per cent in selected families. The improved lines were on par or better than the original PB1121 in terms of yield, grain and cooking quality traits with salt tolerance at seedling stage. Further, the improved lines were characterised for shoot and root Na^+ and K^+ to understand the mechanism of salinity tolerance. Biochemical analysis of all selected salt tolerant lines for shoot and root Na^+ and K^+ concentration and their ratio showed significant differences in ionic concentrations. Interrelations between Na^+ and K^+ content of the *Saltol* carrier lines showed that root and shoot Na^+ concentration and root and shoot K^+ concentration were significantly associated, with later showing lesser magnitude of association than the former. Nine backcross derived lines showed yield and quality at par to recurrent parent while one of the improved lines, Pusa1734-8-3-26 showed significant superior in yield over the recurrent parent with seedling stage salinity tolerance level similar donor parent.

The differential *SKC1* gene expression patterns and its association to *Saltol*was carried out using parental lines (FL478 and PB1121) and *Saltol* derived NILs of PB1121.Four genotypes were exposed to two levels of salt stress 21 days after of sowing. The results showed that level of *SKC1* expression at 24hr after salt exposure (50mM) is more in FL478 and lowest in PB1121. The NILs showed intermediate level of *SKC1* gene expression. The intermediate level gene expression in NILs may be mainly due to background genome interaction and position effect. The increased *SKC1* expression was observed in NILs and it clearly indicates the successful introgression of *Saltol* into PB1121 at molecular level.The results clearly provided the evidences for association *SKC1* expression to *Saltol* QTL under salt stress and proved that*SKC1*as one among many candidate gene associated to salinity tolerance. Pusa1734-8-3-3(NIL 1) showed almost similar level of *SKC1* expression like tolerant FL478 under salt stress.

लवण सहनशीलता हेतु बासमती 1121 का, चिह्नकों की सहायता से सुधार

सार

धान, लवण-प्रतिबल के प्रति, विशेष रूप से अगेती पौध एवं जनन-अवस्थाओं के दौरान, अत्यधिक संवेदनशील फसल है। लवण संवेदी पूसा बासमती 1121, जो बासमती धान की एक महत्वपूर्ण किस्म है और अपने उत्कृष्ट दाने, पकाने संबंधी गुणों एवं बाजार में प्रभाविता के लिए सुविख्यात है, की आनुवंशिक पृष्ठभूमि में, पौध अवस्था में लवण-सहनशीलता हेतु क्रोमोसोम 1 पर मैपिंग किए गए एक प्रमुख क्यूटी एल *सालटोल* का एम ए बी बी विधि के माध्यम से अंतर्वेशन हेतु प्रस्तुत अध्ययन किया गया।

सालटोल क्षेत्र में 20 एस एस आर चिह्नकों का उपयोग कर लवणग्रस्त विविधता के अभिलक्षण हेतु तथा पौध अवस्था लवणता सहनशीलता के साथ इसका साहचर्य जानने के लिए तैईस विविध प्रकार के जननद्रव्यों का उपयोग किया गया जिनमें लैंड रेसेज, वन्य-जननद्रव्यों एवं उन्नत किस्मों का समावेश था। जीनप्ररूपी प्रदा, बोरल के साइपड क्षेत्र की खुबीरू, ऑशीयामा एवं इक्रोम में पोक्कली के समान लवण सहनशीलता स्तर एवं *सालटोल* लोकस के लवणग्रस्त दर्शाए जबकि लवण सहनशील वन्य धान जीनप्ररूपों एन के एस डब्ल्यू आर 19 एवं एन के एस डब्ल्यू आर 20 में भिन्न प्रकार के लवणग्रस्त विद्यमान पाए गए और इसलिए लवण सहनशीलता हेतु ये नवीन स्रोत हो सकते हैं। पोक्कली का संदर्भ के रूप में प्रयोग कर *सालटोल* से जुड़े डिटिकल मार्कर्स के आधार पर धान के 23 जीनप्ररूपों में 14 विभिन्न प्रकार के लवणग्रस्त विद्यमान पाए गए। आर एम 8094 एवं आर एम 3412 दोनों चिह्नक रखने वाले लवणग्रस्त, लवण सहनशील जीनप्ररूपों का सुग्राही जीनप्ररूपों से विभेदन कर सके और इसलिए ये *सालटोल* क्यू टी एल के चिह्नक की सहायता से वरण में उपयोगी हो सकते हैं।

इसके अतिरिक्त, 85 विविध जीनप्ररूपों के एक सैट का उनकी पौध-अवस्था लवण-सहनशीलता सुनिश्चित करने के लिए उपयोग किया गया। *सालटोल* क्यू टी एल क्षेत्र से जुड़े 19 बहुरूपी एस एस आर चिह्नकों एवं जीनोम पर फैले जननद्रव्य क्रमों हेतु उपयोग किया गया। हायड्रोपोनिक्स के अन्तर्गत, लवण-प्रतिबल सहनशीलता हेतु लक्षणप्ररूपी विविक्तकर निरीक्षण के आधार पर एक बासमती धान किस्म (सेजौध बासमती), दो जापोनिका क्रम टोम्फावू एवं निप्पोनबेयर, तीन इंडिका लैंडरेसेज अर्थात बादामी, शाह पसंद एवं पेंडी धान तथा दो *ऑराइजा रुफीपोगोन* क्रम. एन के एम डब्ल्यू आर एवं एन के एस डब्ल्यू आर 17 की पोक्कली एवं एफ एल 478 के समान लवण सहनशील जीनप्ररूपों के रूप में पहचान की गई। जीनप्ररूपी समुच्चयन एवं आबादी संरचना ने कुल जननद्रव्य को दो उप-विशिष्ट आबादियों में विभक्त किया। को-एनसेस्ट्री गुणांकों एवं जीनप्ररूपी पी सी स्कोर्स का उपयोग कर स्ट्रक्चर्ड एसोसिएशन मैपिंग ने छह

दृढ़ विहनक गुण संगठन दर्शाए। प्रतीह Na^+/K^+ अनुपात के साथ संबंधित लवणी विहनक गुण पाकार्थी क्षार एस 1287 (10.8 एम बी) तथा लवणप्रकृती लवण सहनशीलता स्थापित के सम्बन्ध क्षार एस 140 (12.) एम बी) की पहचान की गई। लवण सहनशीलता हेतु धान की किस्मों के विहनक की सहायता से दृढ़ता के लिए आर एस 140 के आगे और लोकस को लक्ष्य किया जा सकता है।

एस एस 478 का दाता के रूप में उपयोग कर, पी बी 1121 में 'सालटोल' के आरोपण हेतु विहनकों की सहायता से बैकक्रास प्रजनन (एम ए बी बी) किया गया। दृढ़तापूर्वक जुड़े पीक आनुवंशिक विहनक क्षार एस 3412 का उपयोग कर सालटोल हेतु फोरग्राउंडसलेक्शन किया गया तथा बैकग्राउंडसलेक्शन, जीनोम विस्तृत कवरेज उपलब्ध कराने वाले 90 बहुरूपी एस टी एस एस विहनकों का उपयोग किया गया। इसके अतिरिक्त, आयनी जनक फेनोटेम (आर पी पी) की पुनर्प्राप्ति को तेज करने के लिए प्रत्येक बैकक्रासिंग एवं सेल्फिंग संतति में सरलविज्ञान संबंधी दाग एवं पकाने की गुणवत्ता संबंधी गुणों हेतु आवश्यक लक्षणप्रकृती चयन किया गया। इसमानव बीसी,एफ, बीज सालटोल सम्पदाता क्रमों का चयन किया गया और लवण सहनशीलता सहित पी बी 1121 के उन्नत संस्करणों के विकास के लिए उन्हें आगे बीसी,एफ, के लिए उपयोग किया गया। बैकग्राउंड विश्लेषण ने दर्शाया कि अग्र किए गए क्रमों में आयनी जनक जीनोम (आर पी जी) पुनर्प्राप्ति 93.33 से 99.44 प्रतिशत की सीमा में थी। उन्नत दाना एवं पकाने के गुणवत्ता संबंधी गुणों सहित पौध-अवस्था पर लवण सहनशीलता के सन्दर्भ में मूल्यांकन पी बी 1121 की तुलना में ये उन्नत क्रम या तो समकक्ष अथवा बेहतर थे। इसके अतिरिक्त, लवण-सहनशीलता की विधाविधि को समझने के लिए इन उन्नत क्रमों का प्रतीह एवं जड़ Na^+/K^+ हेतु अभिलाक्षण किया गया। प्रतीह जड़ Na^+/K^+ सम्पदता एवं उनके अनुपात हेतु चयन किए गए सभी लवण सहनशीलता क्रमों के जैव-रासायनिक विश्लेषण ने आयन-सम्पदताओं में महत्वपूर्ण अन्तर दर्शाए। सालटोल डेरिवेटिव क्रमों के Na^+/K^+ अंश के बीच अन्तरसंबंधों ने दर्शाया कि जड़ एवं प्रतीह Na^+ सम्पदता तथा जड़ एवं प्रतीह K^+ सम्पदता महत्वपूर्ण से संबंधित थी जहाँ पहले की तुलना में दूसरे ने संबंध का उल्ट परिणाम दर्शाया । नौ बैकक्रॉस व्युत्पन्न क्रमों ने आयनी जनक की समकक्ष उपज एवं गुणवत्ता दर्शायी जबकि उन्नत क्रमों में से एक, पूसा 1734-8-3-26 ने दाता जनक के समकक्ष पौध अवस्था लवण-सहनशीलता स्तर के साथ, आयनी जनक से महत्वपूर्ण रूप से अधिक उपज दर्शायी।

जनक क्रम (एस एस 478 एवं पी बी 1121) एवं पी बी 1121 के एन आई एल का उपयोग कर विभेदक एस के टी, जीन अभिव्यक्ति ढंग तथा सालटोल के साथ इसका संयोजन ज्ञात किया गया। चार जीनप्ररूपों को दो स्तरों के लवण-प्रतिबल में रखा गया। परिणामों ने दर्शाया कि लवण-प्रतिबल अवस्था (50 मिमी) में 24 घण्टे रहने के बाद एस के टी, अभिव्यक्ति का स्तर एस एस 478 में अधिक था पी बी 1121 में न्यूनतम था। एन आई एल ने एस के टी, जीन अभिव्यक्ति का स्तर मध्यवर्तिक था। एन आई एल में

सम्भव सांगिक जीन अभिव्यक्ति मुख्यतया बैकग्राउंड जीनोम पारस्परिक क्रिया एवं पोजीशन प्रभाव के कारण हो सकती है। परिणामों ने लक्षण-प्रतिबल के अन्तर्गत साल्टोल वयू टी एस के साथ एस के सी. अभिव्यक्ति के साहचर्य हेतु, स्पस्ट रूप से साक्ष्य उपलब्ध कराए तथा अनेक लवण-सहनशीलता से सम्बद्ध अनेक कैंडीडेट जीन्स में से एस के सी. एक है। लवण-प्रतिबल के अन्तर्गत पूसा 1734-8-3-3 (एन आई एल.) ने सहनशील एवं एल 478 के समान एस के सी. अभिव्यक्ति का लगभग समान स्तर दर्शाया।

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