GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF *'SALTOL'* INTROGRESSION LINES FOR RELEASE AS HIGH YIELDING SALT TOLERANT RICE

Ph.D. Thesis

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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
FACULTY OF BIOLOGICAL SCIENCES
UNIVERSITY OF DHAKA
DHAKA, BANGLADESH

APRIL 2016

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BY

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

FACULTY OF BIOLOGICAL SCIENCES
DEPARTMENT OF BIOCHEMISTRY
AND MOLECULAR BIOLOGY
UNIVERSITY OF DHAKA
DHAKA, BANGLADESH

REGISTRATION NUMBER: **110/2008-09**
RE-REGISTRATION NUMBER: **73/2014-15**

APRIL 2016

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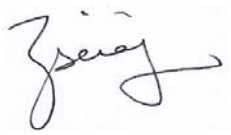
CERTIFICATE

This is to certify that Md. Sazzadur Rahman has conducted his thesis work entitled "Genotypic and phenotypic characterization of *'Saltol'* introgression lines for release as high yielding salt tolerant rice" under my supervision for fulfillment of the degree 'Doctor of Philosophy in Biochemistry and Molecular Biology' from the University of Dhaka. Furthermore, Dr. Abdelbagi M. Ismail, Principal Scientist, Crop and Environment Sciences Division, International Rice Research Institute (IRRI), Philippines, co-supervised Sazzad's work at IRRI. The work on any part thereof has not been submitted any where for any other degree.



Prof. Zeba Islam Seraj, Ph.D.
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DEDICATION

THIS THESIS IS
DEDICATED
TO MY PARENTS FOR THEIR ENDLESS LOVE,
SUPPORT AND ENCOURAGEMENT

ACKNOWLEDGEMENTS

All my praises and appreciations are for Almighty Allah for bestowing upon me the wisdom and potential for successful accomplishment of this manuscript. I also offer my humblest salutations upon the Holy Prophet (Peace be Upon Him), who is forever, a torch of guidance and knowledge for all mankind.

It is with a sense of immense pleasure that I express my heartiest gratitude to my esteemed supervisor, Prof. Zeba Islam Seraj, Ph.D., Department of Biochemistry and Molecular Biology, University of Dhaka. Without her guidance, proper direction and support, it would have never been possible for me to complete my Ph.D. Her cordial and friendly attitude has always inspired me thoroughly. Her devotion and philosophy of taking up the challenge to face the prevailing struggle for research works in the country is exemplary.

I am greatly indebted to my co-supervisor Dr. Abdelbagi M. Ismail, Principal Scientist, Crop and Environmental Sciences Division, International Rice Research Institute (IRRI), Philippines. I am very grateful to him for designing the country's first attempt at Marker Assisted Breeding within the facilities of Plant Biotechnology Laboratory of the Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh. I am also grateful his hosting me for a year as an 'On-The-Job" trainee for his lab at the International Rice Research Institute (IRRI), Philippines and sharing enormous knowledge, valuable advice and suggestions regarding the understanding of the use of Marker Assisted Breeding and Physiology of rice salt tolerance.

Grateful thanks are acknowledged for Dr. Md. Abdus Salam, Director Research, BRRI, Gazipur for his kind arrangements for the hybridization work of this Marker Assisted Breeding project at Plant Breeding Division of Bangladesh Rice Research Institute (BRRI). He also greatly encouraged this Marker Assisted Breeding research and shared enormous knowledge in rice breeding, which have been extremely helpful.

I wish to express my sincere thanks to Dr. Michael J. Thomson, Molecular Geneticist, Plant Breeding, Genetics and Biotechnology Division, IRRI for his scholastic guidance and active cooperation during the period of research at IRRI.

I would like to express my special thanks to Dr. Md. Rafiqul Islam, Principal Scientific Officer, Plant Breeding Division, Bangladesh Rice Research Institute (BRRI), Gazipur and his team for supporting all hybridization work at BRRI and also his valuable advice and suggestions for maintaining and advancing populations.

I deeply appreciate the kind cooperation of Prof. Dr. Haseena Khan, the Chairperson, Department of Biochemistry and Molecular Biology, University of Dhaka at the time of my work there. I would like to express my sincere gratitude to Prof. Dr. Mamun Rashid Chowdhury and Prof. Dr. Istiaq Mahmud for their encouragement and enthusiastic appreciation of my course and research work. I am very thankful to all the other teachers and officers of the Department for their kind help and cooperation.

I am very grateful to Suhaila Rahman, Aliya Ferdousi, Taslima Haque, Noorjahan Begum, Rumana Siddique, Md. Shamimuzzaman, Habibul Bari Shozib, Richard Malo, Rokeya Begum and Rejbana Alam for their help, cooperation and support to established Marker Assisted Breeding in Bangladesh.

My sincere thanks are due for Md. Shamim Hossain, Rabin Sarker and Tariqul Islam for their enormous help and cooperation in carrying all genotyping work at the Plant Biotechnology Laboratory, University of Dhaka. I am especially indebted to Md. Nazrul Islam and Md. Abdul Mazid Miah for their sincere cooperation in experimental trials, data collection and analysis at the Plant Physiology Division of BRRI. I am grateful to Mr. Shankar for his sincerity for all hybridization work as well as enormous helps in maintaining and advancing breeding populations at the Plant Breeding Division of BRRI.

I must not forget to thank the members of salinity group at IRRI, Marjorie de Ocampo, James Egdane, Rochelle Zantua, Mairish, Jonah, Meggy, Macay, Nolli, Ric, Jacinta, Rexie and Post Doctoral Fellow Dr. John Damien Platten for their kind helps during my work at IRRI.

This is a great opportunity to thank my friends, Dr. Ruhul Amin Sarker, Dr. Ebna Syod, Md. Harunur Rashid and Richard Malo for their kind help, moral support and companionship at IRRI, Philippines. I would like to thank all my respected senior and junior colleagues of BRRI for their encouragement and help for my Ph.D work.

I am grateful to the Generation Challenge Program (GCP) of CGIAR, BAS-USDA, GCP-Capacity Building for providing the funds for this work at University of Dhaka, BRRI and also at IRRI.

I express my sincere gratitude to the BRRI's present and past authorities especially Dr. Jiban K. Biswas, Dr. Md. Shahjahan Kabir, Dr. Md. Ansar Ali, Dr. M. A. Mannan, and Dr. M. A. Baqui for their encouragement and granting leave in deputation to pursue my Ph.D. at the Department of Biochemistry and Molecular Biology, University of Dhaka.

Finally, I have no words to thank my parents, who have always wished to see me successful and prosperous. My special gratitude to my brothers and sisters, Md. Bazlur Rashid, Md. Nazmul Rashid, Dr. Md. Asaduzzaman, Mina, Rina and Shahana for their financial and moral support throughout my career. I am also thankful to my wife Sabina Yeasmin (Popy) for her kind love, sacrifice, patience, inspiration and criticism.

--Md. Sazzadur Rahman

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List of Abbreviations

ABA	Abscisic acid
AEZ	Agro-ecological zones
AFLP	Amplified fragment length polymorphism
Aman	A rice growing season in Bangladesh (lowland ecosystem)
AMMI	Additive Main effects and Multiplicative Interactions
AOAC	Association of Official Analytical Chemists
APS	Ammonium per sulfate
Aus	A rice growing season in Bangladesh (upland ecosystem)
BARC	Bangladesh Agricultural Research Council
BAS	Bangladesh Academy of Science
BBS	Bangladesh Bureau of Statistics
BINA	Bangladesh Institute of Nuclear Agriculture
BLUE	Best Linear Unbiased Estimate
BLUP	Best Linear Unbiased Predictor
BOC	Bangladesh Oxygen Company
Boro	A rice growing season in Bangladesh (irrigated ecosystem)
bp	base pair
BRRI	Bangladesh Rice Research Institute
C	Celsius
C50	Concentration50 when yield is reduced by 50 percent
Ca^{2+}	Calcium ion
CB	Capacity Building Theme of GCP
cDNA	Complementary DNA/copy DNA
CGIAR	Consultative Group on International Agriculture Research
Cl^{-}	Chloride ion
cm	Centimeter
cM	Centi Morgan
Conc.	Concentration
CSR	Central Salinity Rice
CSSRI	Central Soil Salinity Research Institute

CTAB	Cetyl Trimethyl Ammonium Bromide
D	Day(s)
ddH_2O	distilled deionized water
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphate
dSm^{-1} or dS/m	Decisiemens per meter
e.g.	For example
EC	Electrical conductivity
ECe	Electrical conductivity of saturation paste extract
ECw	Electrical conductivity of water
EDTA	Ethylene diamine tetra acetic acid
ERF	Ethylene Response Factor
ESP	Exchangeable sodium percentage
ESTs	Expressed sequence tags
et al.	and others
EtBr	Ethidium bromide
etc.	Et cetera
FAO	Food and Agriculture Organization of United Nations
Fig.	Figure
G by E	Genotype by Environment
g or gm	Gram
GCP	Generation Challenge Program
GEI	Genotype by Environment Interaction
GGE	Genotype main effects and GEI model
GIS	Geographic Information System
GO	Government Organization/Public Organization
GWAS	Genome Wide Association Study
h or hr	Hour
H^+	Hydrogen Ion Concentration

ha	Hectare
HCO_3^-	bicarbonate ion
HKT	*High Affinity Potassium Transporter*
i.e.	That is
IAA	Isoamyl alcohol
InDel	Insertion/Deletion
IRGSP	International Rice Genome Sequencing Project
IRRI	International Rice Research Institute
JA	Jasmonic Acid
kb	kilobases
km	kilometer
L	Litre
LEA	*Late-embryogenesis abundant*
M	Molar
MABC	Marker assisted backcrossing
MAS	Marker assisted selection
mg	Milligram
µg	Microgram
mg/L	milligram/Liter
Mg^{2+}	Magnesium ion
Mha	Million hectare
min.	Minute
mL	Milliliter
µL	Microliter
mM	Millimolar
µM	Micromolar
µM	Micrometer
mmohos/cm	milli-mohos/cm
µS/cm	microsiemens/cm
MoA	Ministry of Agriculture
MoEF	Ministry of Environment and Forest
MV	Modern variety

MW/mol. wt.	Molecular weight
N	Normal
Na^+	Sodium ion
Na^+/K^+	Sodium ion/Potassium ion ratio
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NARES	National Agriculture Research and Extension Services
NCBI	National Center for Biotechnology Information
ng	Nanogram
NGO	Non-government Organization/Private Organization
NIL	Near Isogenic Line
nm	Nanometer
No. or no.	Number
NSCC	Non-selective Cation Channel
O.D.	Optical density
Os*NHX1*	*Oryza sativa* L. sodium-hydrogen antiporter
PAGE	Poly-acrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
pH	Negative logarithm of hydrogen ion concentration $^+$
PIL	Precision Isogenic Line
ppm	parts per million
QTL	Quantitative trait loci
RIL	Recombinant Inbred Line
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Rotation per minute
RT	Room temperature
SA	Salicylic Acid
Saltol	Salinity tolerant QTL
SDS	Sodium dodecyl sulfate
Sec.	Second

SKC	*Sodium Potassium Co-transporter*
SNPs	Single Nucleotide Polymorphisms
SO_4^{2-}	Sulfate ion
Sol^n	Solution
SOS	*Salt overly sensitive*
SRDI	Soil Resource Development Institute
SSR	Simple Sequence Repeat
Sub1/SUB1	Submergence tolerant QTL
T. Aman	*Transplanted Aman season*
t/ha	ton per hectare
TAE	Tris Acetate EDTA
TDS	Total dissolve solid
TE	Tris EDTA
TEMED	N,N,N,N,-tetrametlyl ethylene diamine
Temp.	Temperature
USDA	United States Department of Agriculture
USDA-ARS	USDA-Agriculture Research Service
UV	Ultraviolet
V/V	Volume by volume
V/W	Volume by weight
Viz.	Videlicet
Vol.	Volume
%	Percentage

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF *'SALTOL'* INTROGRESSION LINES FOR RELEASE AS HIGH YIELDING SALT TOLERANT RICE

-Md. Sazzadur Rahman

ABSTRACT

Rice is the staple food for more than 160 million people of Bangladesh. It is not just a cereal but the daily necessity for most of the people in the country. Rice is the synonym of food in Bangladesh since it provides about 72%, 62% and 17% of the calorie, protein and fat requirement of the population, respectively. Therefore, food security in Bangladesh may be regarded as rice security in the country. The country was at the brim of food security recently after attaining self-sufficiency in rice. But the sustainability of this sufficiency is encountering a lot of challenges like population growth, decreasing land, technological limitation, resource limitation, abuse of technologies, environmental pollution, climatic adversities, etc. However, among the different challenges, soil salinity is a serious environmental stress after submergence and drought which is threatening the rice productivity and food security of Bangladesh. Salinity intrusion has been increasing slowly but continuously. In the past four decades (1973 to 2012) it has been increased to more than 26% and the total affected area is now approaching >1.06 Mha. Rice is most sensitive to salinity but suited for growth the saline marshy land of the saline coastal belt in Bangladesh. The higher sensitivity of modern varieties to saline stress forces farmers to continue growing their traditional landraces, which however have low yields. Therefore, development of salt tolerant modern rice has been considered one of the feasible strategies to increase the total rice production of the country and maintain the sustainability of self-sufficiency.

Salinity tolerance of rice is a complex trait affected by various genetic and non-genetic factors, and its improvement via conventional breeding has been slow. Recent advancements of molecular breeding have led to the development of more efficient selection tools like molecular marker based selection to substitute phenotype based selection systems. The success of molecular breeding approach like marker-assisted backcrossing (MABC) has been demonstrated by the introgression of *Sub1*, a major QTL for submergence tolerance into several mega varieties. Therefore, a major QTL associated with the Na^+/K^+ratio and

seedling-stage salinity tolerance, named *Saltol*, was targeted to improve seedling stage salinity tolerance of two Bangladeshi mega rice variety BR11 (*for transplated aman* season) and BRRI dhan28 (*for boro* season) through MABC approach. Three-backcross generation MABC approach was used to introgress the *Saltol* QTL through SSR and InDel markers. Single Nucleotide Polymorphism (SNP) markers were also used to further check any potential background donor introgression of the selected lines from both MABC events. To study the phenotypic gain due to *Saltol* QTL in to the background of BR11 and BRRI dhan28, a number of studies were carried out with the developed lines at seedling and reproductive stages in controlled net house condition as well as in saline and non-saline field conditions respectively.

Three backcrosses and two selfing generations were carried out to transfer the positive alleles of *Saltol* QTL from FL378, a F_8 RIL developed from IR29/Pokkali. From first MABC event (BR11-*Saltol*), two Near Isogenic Line (NIL), NIL52 and NIL1 were selected at BC_3F_3 stage with 1.3 million bases/mega bases (Mb) introgression at the *Saltol* region (donor genome percentage is 3.33%) and about 96.67% of BR11 genome at the background. At later generations, 4 lines from NIL52 and 8 lines from NIL1 were selected phenotypically based on grain size for obtaining further homogeneity. To optimize the effect of *Saltol*, a different strategy was applied by increasing the size of the introgressed segment at the target *Saltol* region for the second MABC event (BRRI dhan28-*Saltol*). Four different size donor segments at the *Saltol* i.e. 1.3 Mb (2.73%), 2.4 Mb (3.64%), 3.3 Mb (4.55%) and 3.7 Mb (5.45%) were introgressed in to BRRI dhan28 background. Eleven NILs (NIL188, NIL204, NIL318, NIL341, NIL412, NIL434, NIL448, NIL607, NIL657, NIL683 and NIL807) at BC_3F_3 stage were selected with 91.82-95.45% recovery of the BRRI dhan28 genome. SNP assay identified single SNP (id1008684 at 12869918 bp) for 1.3 Mb and 2 SNP (id1008684 at 12869918 bp and id1009616 at 14592566 bp) for the larger segments i.e. 3.3-3.7 Mb at the *Saltol* region. For further checking background donor introgression through SNP markers, a number of background introgressions were detected for both BR11-*Saltol* NILs, having ~94% and ~80% recovery of BR11 genomes for NIL52 and NIL1 respectively. For BRRI dhan28-*Saltol* the recovery was better with respect to SSR and InDel markers for all NILs and the recovery ranged from 97% to 88%.

The phenotypic gain for the first MABC event with a maximum reduction of seedling shoot Na^+/K^+ ratio by about 20% due to 1.3 Mb donor segment at the target *Saltol* loci into BR11.

While the second MABC event the utmost reduction is about 32% by introgressing large donor segment (3.3 Mb) at the target *Saltol* loci in case of BRRI dhan28 for seedling stage salinity tolerance. Apparently, large introgression 3.7 Mb at the *Saltol* loci improves the overall salinity tolerance in terms of SES. A total of 13 lines/NILs (7 BR11-*Saltol* i.e. BR11-52-124, BR11-52-55, BR11-52-145, BR11-52-67, BR11-150, BR11-65, BR11-7 and 6 BRRI dhan28-*Saltol* i.e. NIL434, NIL412, NIL607, NIL618, NIL657, NIL683) were finally selected based on tolerance at seedling stage and for yield at field conditions. All agronomic and grain quality parameters for most of the developed lines/NILs from both MABC events showed successful recovery of the BR11 and BRRI dhan28 traits with an improvement of yield potential in both saline and non-saline field conditions. NIL52 of BR11-*Saltol* and NIL434, NIL657, NIL683 of BRRI dhan28-*Saltol* performed better in a moderate salinity stress (6-8 dSm^{-1}) condition during reproductive stage in controlled Net house condition. Genotype by environment interaction analyses of four-environment trials of BR11-*Saltol* lines showed a significant G by E interactions identified by AMMI-2 model. Stability analyses identified 3 BR11-*Saltol* lines such as BR11-52-124, BR11-52-67 and BR11-7 with superior performance in better environments. Therefore these could be suited for cultivation in better environments, whereas a line BR11-65 could be recommended for cultivation all over the country due to its stable and average performances across different environment. On the other hand, 3 NILs (NIL434, NIL607 and NIL683) of BRRI dhan28-*Saltol* showed better yields compared to the recipient while evaluated in saline and non-saline environments and could be recommended for further evaluation.

Salinity tolerance is a complex phenomenon, and several complementary biochemical and physiological adaptations are generally necessary to establish complete tolerance. The *Saltol* locus is just one component of a multifaceted strategy to improve rice yields on salt affected soils. The initial steps have been taken to use the MABC transfer of *Saltol* alleles into popular varieties to test the efficacy of this allele to provide seedling stage tolerance. Pyramiding of QTLs with complementary physiological and biochemical mechanisms could be the next step to forward further for getting higher and durable tolerances including the tolerance for the whole growth period.

Chapter 1: Introduction

Chapter 1
Introduction

1.1 General introduction

Rice is not just another grain. For three billion people, it is a daily necessity. Since its domestication some 8,000 years ago, rice has played a part in building civilizations, shaping societies and, most of all, feeding a growing world (Nature, 2014). So, rice is fundamental for food security for approximately three billion people, or about half of the world population. The major rice producers of the world grow more than enough rice to feed their own people. The excess ends up as exports, livestock feed, seed stock or waste (spoiled during transport or storage). Asia is the world's rice bowl in terms of production, consumption and export. Around 91% and 89% of world's rice was produced and consumed in Asia respectively (Fig. 1.1 and Table 1.1). However, 5 countries in Asia, such as China, India, Bangladesh, Vietnam and Thailand are the major producers, consumers and exporters. Contribution of the top 5 Asian countries to the world's rice production, consumption and exports were 67%, 67% and 76% respectively from the estimation of figures from 2007-2011 (Table 1.2).

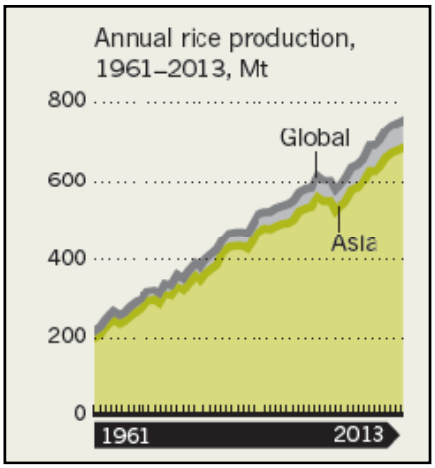

Fig. 1.1: Annual rice production in Asia and the world from 1961 to 2013 (Adapted from Elert, 2014)

Millions of people around the world rely on rice as the bulk of their daily diet and much of the poorest and most undernourished in Asia depend on rice as their staple food. Global

nutrition mostly relies on 3 cereals such as rice, wheat and maize. On average, every day, each person in the planet consumes 2,868 kcal, but most of the calorie or around 42% of our daily energy comes from 3 cereals (rice, wheat and maize) (Elert, 2014). However, in Bangladesh, the dependency of daily calorie derived from rice is much higher and remains nearly static since 1961 (80%) to 2011 (70%). This still tops the world consumption at 70% (Elert, 2014).

Table 1.1: World's average annual production and consumption from 2007 to 2011, measured in million tons (Mt). (Source: Elert, 2014)

Regions	**Production (% of total)**	**Consumption (% of total)**	**Surplus/Shortfall**	**Exports (% of total)**
Asia	417.00 (91)	318.20 (89)	+ 98.70	13.60 (84)
Ocenia	0.20 (0)	0.40 (0)	-0.20	-- (--)
Africa	15.80 (3)	20.60 (6)	-4.80	0.40 (2)
Europe	2.70 (1)	3.50 (1)	-0.80	0.30 (2)
Americas	23.20 (5)	15.80 (4)	+7.50	1.90 (12)
Total	**458.90 (100)**	**358.50 (100)**	**100.40**	**16.20 (100)**

Table 1.2: Top 5 Asian countries average annual production and consumption from 2007 to 2011, measured in million tons (Mt). (Source: Elert, 2014)

Country name	**Production**	**Consumption**	**Surplus/Shortfall**	**Exports**
China	130.40	106.80	+23.60	0.20
India	97.40	85.40	+12.00	2.30
Bangladesh	31.80	26.0	+5.90	--
Vietnam	26.10	12.80	+13.40	3.70
Thailand	22.10	7.60	+14.50	6.10
Total	307.80	238.60	+69.40	12.30
% of world's total	**67.00**	**67.00**	**+69.00**	**76.00**

In 2010, approximately 154 million ha of rice were harvested worldwide, of which 137 million ha (88 percent of the global rice harvested) were in Asia - of which 48 million ha (31 percent of the global rice harvested) were harvested in Southeast Asia alone (FAOSTAT, 2012). The Food and Agriculture Organization of the United Nations (FAO) estimates that food production will have to increase 70 per cent globally to feed an additional 2.3 billion

people by 2050. At the same time, food demand has been shifting towards more resource-intensive agricultural products, such as livestock and dairy products, thereby exerting additional pressure on land, water and biodiversity resources (UN World Economic and Social Survey 2013). An increase in food production will also require integrating sustainable practices, particularly regarding the use of natural resources. Many of the current agricultural practices have relied on cheap energy and abundant water and land, and are a leading source of greenhouse gas emissions (The Hague Conference, 2010). These practices are now proving unsustainable for the environment and health, due to contamination of air, land and water sources. At the same time, they have led to substantial productivity losses, thereby posing risks to food security (UN World Economic and Social Survey 2013).

1.2 Rice production: the Bangladesh perspective

Rice is life for 160 million Bangladeshis and it is the main component for food security of the country. Rice is the major source of food and nutrition. Therefore, it dominates in terms of area coverage and production throughout the year (Fig. 1.2 and 1.3). Rice alone can cover ~ 80% land area and thereby produce ~67% of food grains (BBS, 2013).

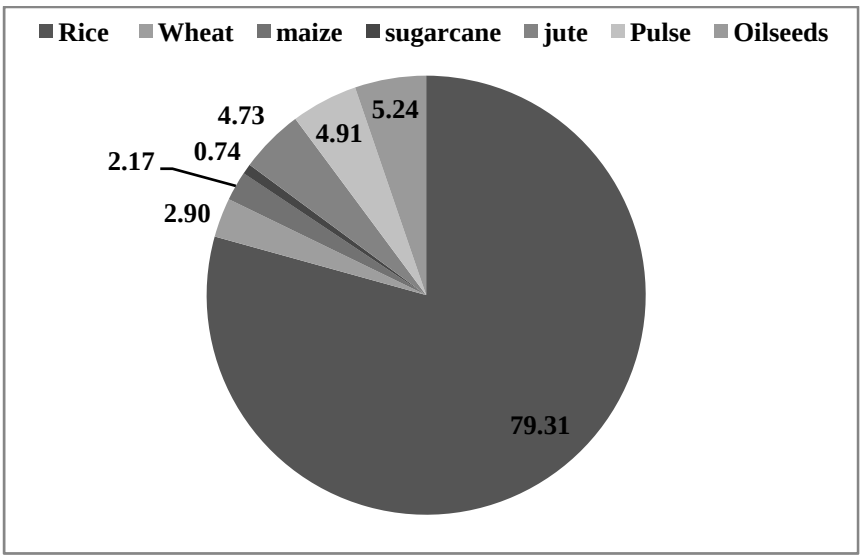

Fig. 1.2: Percent area coverage of rice compared to other crops.

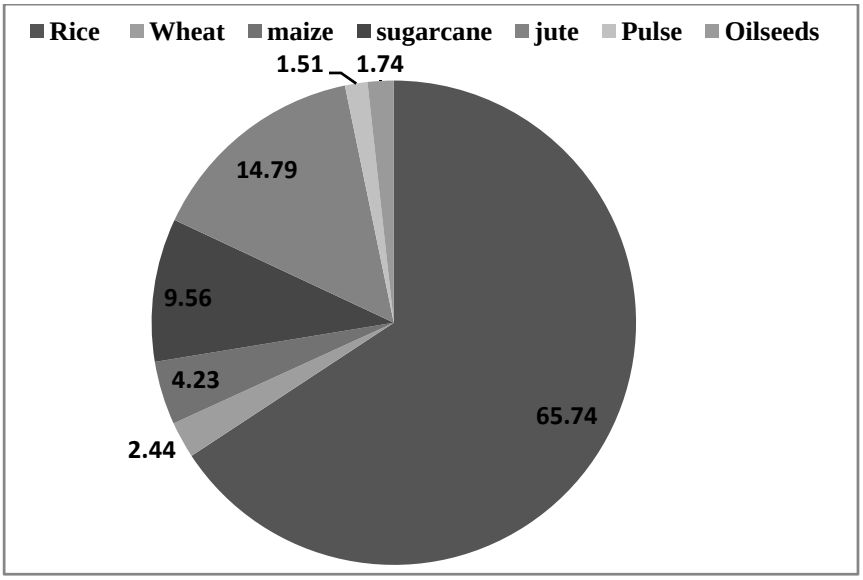

Fig. 1.3: Production percent of rice compared to that of the other crops.

Total cultivable land of Bangladesh is around 8.52 Mha, but the total cropped area is around 15.08 Mha. This is because of double and triple cropping systems in some areas. Rice is grown in Bangladesh in 3 seasons namely *Aus* (March/April – June), *Aman* (July – December) and *Boro* (November – May). The total rice area is about 11.42 million ha over 3 seasons and total production 33.83 million tons (clean rice) in 2013 (Table 1.3). Flexibility in sowing time and photo-insensitive nature of many modern rice varieties allows growth of three rice crops in the same parcel of land. However, only a tiny fraction of total farmers practice this. At present, *Aus*, *Aman* and *Boro* area occupies 9.22, 49.11 and 41.76%, respectively and contribute about 6.38, 38.12 and 55.50% to the total production. *Boro* rice is fully irrigated while other two are either rain-fed or partially irrigated. Underground water provides about 70% of irrigation water for rice.

Table 1.3: Rice statistics of Bangladesh.

Seasons	**Area**		**Production**		**Yield (t/ha)**	
	(million ha)	**%**	**(million ton)**	**%**	**Clean rice**	**Paddy**
Aus	1.05	9.22	2.16	6.38	2.05	3.10
Aman	5.61	49.11	12.89	38.12	2.29	3.48
Boro	4.76	41.67	18.78	55.50	3.95	5.98
Total	**11.42**	**100.00**	**33.83**	**100.00**	**Av. = 2.76**	**Av.= 4.19**

Source: BBS, 2013

1.3 Challenges and opportunities of rice production in Bangladesh

Food security in Bangladesh may be regarded as rice security in the country. The country attained the brim of food security recently with the self-sufficiency in rice. Against the total rice demand of 33.83 million tons, the country achieved a little excess in production. Given the different preferences of the people, BRRI scientists have already developed 72 modern varieties with diverse properties (BRRI, 2014). Aiming at export of rice, Bangladeshi scientists have also developed several premium quality varieties.

Stable growth in agriculture (around 3%) during the last decade and significant progress in food grain production especially in rice has been ensured. However, food security of the people of Bangladesh still remains a daunting challenge and food security is considered is the most major priority of the government. While the country has made impressive achievements over the last 40 years (notably, it has more than tripled its rice production), it still increasingly faces considerable challenges: shrinkage of arable lands, increasing population growth (Bangladesh is the most densely populated country of highest "man-land" ratios in the world and its population is still growing by over 2 million persons per year); impacts of climate change (intrusion of sea level into main land and increasing salinity, natural disasters like floods, cyclones, tornadoes every year); deteriorating access to increasingly scarce natural resources (water, land); vulnerability to price shocks, (as experienced in 2008); persistent poverty (leading to poor access to food); and one of the highest malnutrition rates in the world. The Government considers fighting against food and nutrition insecurity as a key strategy for Bangladesh to become a middle-income country. The National Food Policy and its Plan of Action has identified the objectives to be fulfilled to ensure food security and extended the concept of food security well beyond that of food availability. Since the stable supply of rice has great implication in food security, Bangladesh views food security as synonymous to achieve self-sufficiency in rice production. So the main theme of the development plan is to ensure and sustain rice self-sufficiency in future.

Bangladesh was badly hurt in the global food crisis during 2007-2008. This was when the rice price jumped to about 3 fold compared to the normal price. Moreover, the most important issue was the unavailability of rice, because most of the rice exporting countries banned their rice exports. They had even refused all sorts of treaties for exporting rice

elsewhere. Slayton (2009) explores the whole situation carefully and concluded "The fire was man-made, not the result of natural developments". However, a number of chronological events listed by Slayton (2009) in the working paper "Rice Crisis Forensics: How Asian Governments Carelessly Set the World Rice Market on Fire". Due to dramatic change in Asian rice exports specifically of India, Thailand, Vietnam, Pakistan, China and others by increasing price and banning of rice exports worsened the worlds' rice markets (Slayton, 2009). However, in November, 2007 cyclone '*Sidr* slammed into Bangladesh, destroying 2.1 million tons of paddy and necessitating an additional 1 million tons of imports. Bangladesh Government handled the crisis of unavailability of paddy in the world market by G-to-G treaty with the Indian Government (Slayton, 2009).

Considering unavailability and high price hike of rice related to the country's food security issues, the Government of Bangladesh has been giving special attention for securing rice production by supplying all sorts of inputs to the farmers in a proper and timely manner with substantial amounts of subsidies in the fertilizers and fuels for irrigation purposes. Considering the constant efforts from GO, NGO and private sectors, Bangladesh is now attaining self-sufficiency in cereal production especially in rice production. But this self-sufficiency is dependent on the non-occurrence each year of devastating natural calamities like- floods, cyclones, drought etc. Bangladesh is frequently cited as one of the country's most vulnerable to climate change, despite the country's insignificant contribution to climate change. Rice is the most susceptible to climate change and variability. Any changes in climate will, thus, increase uncertainty regarding rice production as climate is major cause of year-to-year variability in rice productivity.

1.4 Extent of soil salinity

Salinization is the accumulation of water soluble salts in the soil to a level that impacts on agricultural production. A soil is considered saline if the electrical conductivity of its saturation extract (ECe) is above 4 dSm^{-1} (USDA-ARS, 2008), which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa. This definition of salinity derives from the ECe that significantly reduces the yield of most crops (Munns and Tester, 2008). Loss of arable land via salinization is a major factor undermining the productivity of modern agricultural systems (Galvani, 2007). Salinization of agricultural soils occurs primarily due to agricultural practices, including poor water

management, high evaporation, heavy irrigation and previous exposure to sea water (Pitman and Lauchli, 2002). Currently, approximately 6% of the world's land area, which is equivalent to 800 million hectares, is affected either by salinity or sodicity (FAO, 2010). In addition, salinity affects 20% of the world's irrigated land, which accounts for one-third of the world food production (Chinnusamy, *et al.* 2005; FAO, 2008). It has been estimated that salinity is affecting 3 hectares of additional arable land each minute worldwide (FAO, 2008). This constant salinization of arable land is expected to have overwhelming global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang, *et al.* 2003). This progressive loss of arable land has potentially serious consequences for the expanding global population, which is steadily increasing towards seven billion, and set to increase by a further 50% by 2050 (FAO, 2009). Recent changes in global climate are likely to further exacerbate the problem of soil salinity. Variation in important climate variables including temperature and precipitation are expected to decrease water for irrigation and impose high evapo-transpiration losses (Yeo, 1999). The resulting drier conditions will further raise irrigation demands which are often met with poor quality of water containing dissolved salts. These conditions will be more critical for arid and semi-arid regions which are already at the limit with respect to water availability (Chartzoulakis and Psarras, 2005; Sivakumar, *et al.* 2005). The decrease in good quality of water in these areas will accelerate the use of saline water for irrigation which will raise salt accumulation in soils, and increase the extent of secondary salinization (Yeo, 1999).

In Bangladesh, more than 30% of the cultivable land is in the coastal areas. Soil salinity has mainly formed from sea water flooding or capillary rise from shallow ground water close to the coast. The intensity and spatial distribution of salinity widen to larger areas after a tidal surge or cyclone and this extra salinity remains for years. Soil salinity is more in areas of close proximity to the sea and tidal creeks. So far, 49 upazilas (Sub-districts) of 19 districts are affected by different degrees of salinity. Soil Resource Development Institute (SRDI) has temporal and spatial data on regular monitoring of soil and water salinity since 1989 besides reconnaissance survey data of 1973. It was estimated in 1973 and 2009 that the area coverage of soils with different degrees of salinity is about 0.833 and 1.056 million hectares respectively. Figure 1.4 represents the soil salinity map of Bangladesh with 5 different classes of salinity in different colors. Total spatial increase of saline area was about ~26% in 2009 over 1973. Besides this, there are areas of spectacular increase in different degrees of salinity in the 36 years from 1973 to 2009 (Table 1.4). Soils of moderately to strongly

salinity (S3 and S4: 8.1-16 dS/m) have increased by an area of about 272 ha (from 79 to 351 ha).Very strongly saline (S5: > 16 dS/m) soil has increased by 62 ha (from 39 to 101 ha). These are in areas of close proximity to the sea, mainly in western region and off- shore islands. Very slightly saline area (S1: 2-4 dS/m) increased by 41 ha (from 287 to 328 ha) from 1973 to 2009. Slightly saline (S2: 4.1-8 dS/m) area reduced from 426 to 274 ha over the 1973 to 2009 period (SRDI, 2012).

Table 1.4: Extent and distribution of saline soils (Adapted from Shoaib, 2013).

Districts	**Area ('000' ha)**		**Agro-ecological zone (Major)***	**Remarks**
	1973	**2009**		
Satkhira	146.35	153.11	HGRF,GTF	Increased
Khulna	120.04	147.96	HGRF,GTF	Increased
Bagerhat	107.98	131.12	HGRF,GTF,GKB	Increased
Pirojpur	20.30	35.83	HGRF,GTF,GKB	Increased
Barguna	103.55	95.62	GTF	Decreased
Patuakhali	115.10	155.18	GTF	Increased
Bhola	40.73	94.57	YMEF	Increased
Noakhali	49.60	52.52	YMEF	Increased
Laxmipur	20.30	18.43	YMEF	Decreased
Chandpur	1.50	0.00	LMRF	Decreased
Feni	9.00	5.75	CCF	Decreased
Chittagong	45.70	51.48	CCF	Increased
Cox's Bazar	54.70	55.35	CCF	Increased
Jessore	0.14	0.99	HGRF	New intrusion
Narail	0.00	18.71	HGRF,GKB	New intrusion
Gopalgonj	0.60	0.27	HGRF, GKB	New intrusion
Madaripur	0.00	0.72	HGRF,GKB	New intrusion
Barisal	0.13	0.96	GTF	New intrusion
Jhalakati	0.40	0.69	GTF	New intrusion
Grand Total	**833.45**	**1056.26**		**26% increased**

*HGRF: High Ganges River Floodplain; GTF: Ganges Tidal Floodplain; GKB: Gopalgonj-Khulna; Beel; YMEF: Young Meghna Estuarine Floodplain; CCF: Chittagong Coastal Plain, LMRF:Lower Meghna River Floodplain.

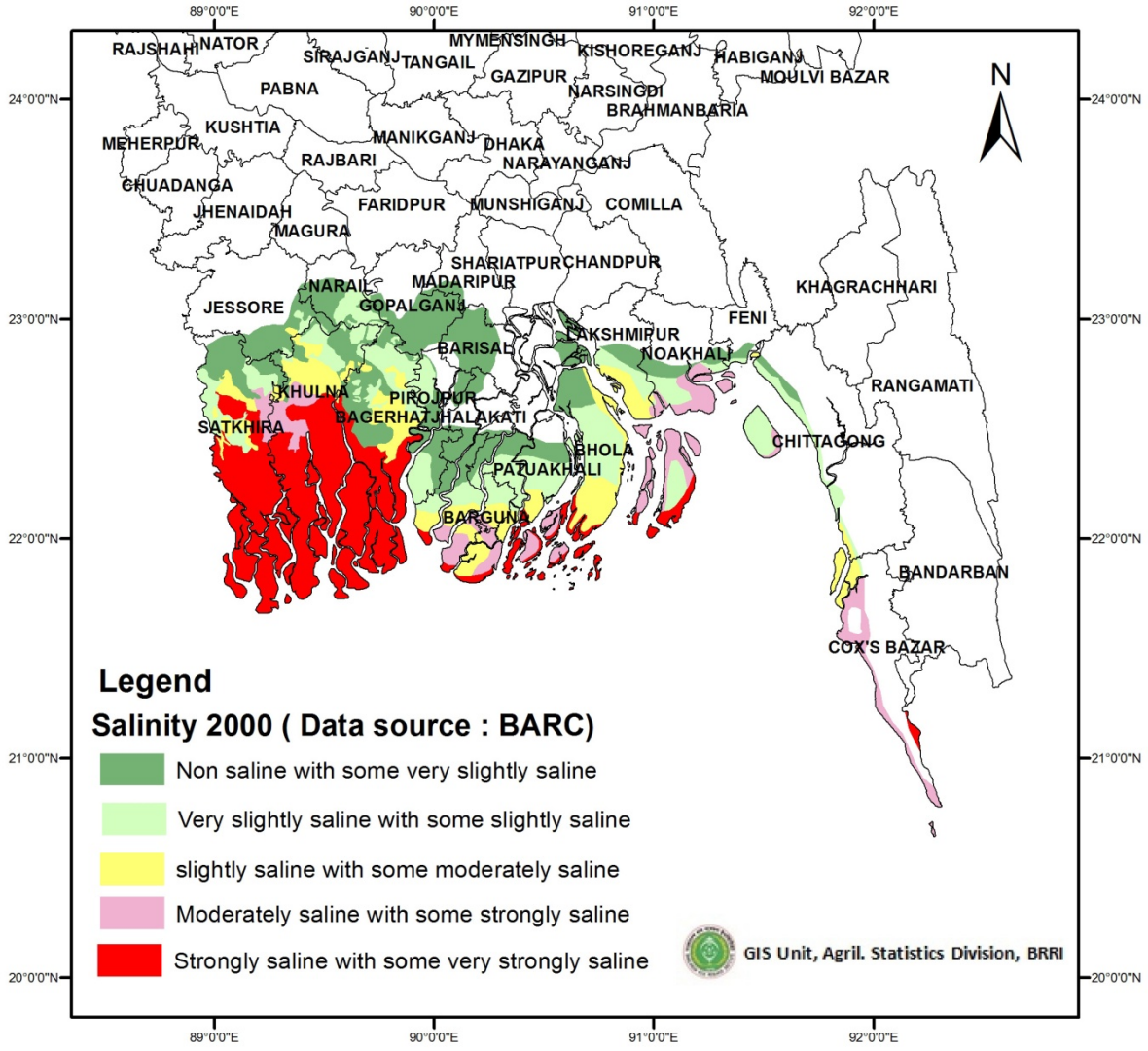

Fig. 1.4: Soil salinity map of Bangladesh, data source BARC 2000 (map redrawn by GIS Unit, Agricultural Statistics Division, BRRI).

1.5 Rice production under salinity

Soil salinity is the most widespread soil toxicity problem in rice growing countries. It is becoming an increasingly serious production constraint (Akbar and Ponnamperuma, 1980) and one of the major obstacles to increase crop production worldwide. One-third of rice growing area the world is affected by excess salt accumulation due to irrational management. Some effort has been given to overcome the effects of salinity in rice production such as leaching the field with fresh water, utilizing different effective land preparation techniques and applying organic materials to reclaim soil properties. But these

techniques were costly and time consuming. Breeding for salt tolerance was therefore adopted as a necessity.

Rice is generally reported as a salt sensitive crop, particularly when soil salinity in terms of electrical conductivity of saturation extract (ECe) is more than 3 dS/m (deci Siemens per meter) that significantly reduced yield with a rate of 12% per deci Siemens (Mass and Hoffman, 1977). Rice was categorized as sensitive by Maas and Hoffman (1977) and yet, rice has a large variability for tolerance of salinity (Flowers and Yeo, 1981; Lisa, *et al.* 2004; Mahmood, *et al.* 2004; Zeng, *et al.* 2004; Munns, *et al.* 2006). Defining salt tolerance of rice is very difficult because of the complex nature of salt stress and the wide range of plant responses. Rice responses to salinity also vary in different growth stages. In most cultivated rice, the young seedling is very sensitive to salinity (Pearson and Bernstein, 1959; Kaddah, 1963; Flowers and Yeo, 1981; Heenan, *et al.* 1988; Lutts, *et al.* 1995). Panicle length, spikelet number per panicle, and grain yield were significantly reduced after salt treatments when applied at panicle initiation stage (Sajjad, 1984; Heenan, *et al.* 1988; Mass and Grattan, 1994; Cui, *et al.* 1995; Khatun, *et al.* 1995). In addition salinity delayed the panicle emergence and flowering (Khatun, *et al.* 1995) and decreased seed set through reduced pollen viability also when applied from panicle initiation stage (Mass and Grattan, 1994; Khatun and Flowers, 1995; Khatun, *et al.* 1995). Akbar and Yabuno (1974) also reported that early seedling stage is very sensitive to salinity. Seedling and flowering stages of rice are more likely to be affected by salinity, with reduction in seedling growth and yield (Rood, 2000). Severe reduction in effective leaf area is the first symptom of salinity stress. In low stress condition, the dry weight of some cultivars often increases for some time and then decreases almost proportionally. Finally, rice seedling that survives has the older leaves losing viability with the youngest remaining green (Akita, 1986). The threshold level of salinity for rice beyond which yield begins to decline significantly is 3 dSm^{-1}, and the level called tolerant when the yield is reduced up to maximum by <50% (C50) (Maas and Hoffman, 1977; van Genuchten and Hoffman, 1984). A considerable degree of varietal difference exists in the salinity tolerance. The grain yield of many rice varieties being reduced by half at an electrical conductivity (EC) of 6 $mScm^{-1}$ (equivalent to 6 dSm^{-1}) (Maas and Hoffman, 1976; Yoshida, 1981): equivalent to an osmotic potential of about -0.23 MPa or 50 mol m^{-3} (50 mM) NaCl. Traditionally tolerant variety can produce reasonably good yields (35-40% reduction, relative to non-saline condition) even at 12.5 dSm^{-1}, while other varieties fail (Rana, 1985). Though rice is salt-sensitive, it is native species to swamps and

freshwater marshes therefore it is the only cereal that has been recommended as the first crop for reclamation because of its ability to grow well under flooded conditions, and because the standing water in rice fields can help leach the salts from the topsoil to a level low enough for subsequent crops (Bhumbla and Abrol, 1978).

1.6 Genetics and Breeding strategies for rice salt tolerance

Inheritance pattern and heritability i.e. the mode of gene action, association of different contributing traits, and gene expression that is interactions with the environment (G×E interaction) are the most important factors that determine success in plant breeding. Previous reports suggested the role of a few dominant genes for the inheritance of some of the contributing traits for salt tolerance in rice (Akbar, *et al.* 1972; Akbar and Yabuno, 1975, 1977), but later studies showed continuous variation in segregating generations, indicating polygenic inheritance for salt tolerance and its contributing traits (Moeljopawiro and Ikehashi, 1981; Mishra, *et al.* 1998; Singh, *et al.* 2001). Maternal effects could not be realized but there was little skewness in the distribution of the segregating progenies, suggesting the role of a few major genes along with numerous minor genes in the inheritance of salt tolerance (Mishra, *et al.* 1998; Singh, *et al.* 2001). Different studies suggested both additive and non-additive gene action for almost all the characters associated with salt tolerance (Narayanan and SreeRangasamy, 1991; Gregorio and Senadhira, 1993; Mishra, 1996; Gregorio, *et al.* 2002; Chauhan, 2007). Expression of the dominant components for grain yield is usually suppressed under stress. This suggests that to generate salinity-tolerant varieties, donors with more additive genes for grain yield should be used.

Breeding for salt tolerance has been reviewed by several workers. The possibility of breeding rice more tolerant than existing tolerant cultivars through cumulative crosses of tolerant cultivars has been suggested. Further improvement can be attained by crossing highly tolerant lines with donors of good agronomic traits, pest and disease resistance (Moeljopawiro and Ikehashi, 1981). Bennett and Khush (2003) reviewed the difficulties of enhancing crop salt tolerance through traditional and molecular approach. They reported that the low recovery efficiency of elite genome with salt tolerance, genetic complexity of the trait and also the strength of genotype × environment interactions (GEI) would make the trait untouchable for improvement through the traditional pedigree approach. Garcia, *et al.* (1995) suggested early selection for agronomic traits should be made after selection for

physiological traits such as leaf Na^+ concentration and, ideally, be delayed until the population has reached near homozygosity. Large G×E interactions for yield and yield components have been reported across a range of test environments (Mishra, 1996; Chauhan, 2007; Manneh, *et al.* 2007). So, selection under stress for grain yield has been reported as the best approach to breed stress-tolerant rice varieties (Mishra, 1996; Munns, *et al.* 2006; Venuprasad, *et al.* 2007; Kumar, *et al.* 2008). Selection for the component traits to breed a stress-tolerant rice variety have also been proposed (Yeo, *et al.* 1990) and long used (Singh, *et al.* 2004 & 2008; Serraj, *et al.* 2009). Morphological traits such as the proportion of filled/unfilled grains, grains per panicle, spikelet fertility, plant height, fertile tillers, and flowering in comparison to non-stress are good indicators associated with salt tolerance in rice. However grain yield is negatively correlated with physiological traits like Na^+ concentration and Na^+/K^+ ratio, whereas it had a positive correlation with K^+ concentration under both alkali and saline soil conditions (Chauhan, 2007; Rao, *et al.* 2008).

Breeding for salt stress tolerance in rice has been moderately successful (Mishra, *et al.* 2001; Senadhira, *et al.* 2002). The genetic base for salinity tolerance in internationally released cultivars has originated mainly from two common salt tolerance donors, Pokkali and Nona Bokra (Gregorio, *et al.* 2002). It is necessary to widen the genetic base for donors of salt tolerance traits for adaptability to different agro-ecological conditions common in most coastal areas. Conventional breeding practices are lengthy and require 10-15 years in order to release a new variety. Among the NARES institutes and IRRI, the major progress has been made by Central Soil Salinity Research Institute (CSSRI), Karnal, India, in breeding salt tolerant high-yielding rice varieties for various inland saline, coastal saline and alkaline soils of fragile ecosystems. To date about 32 salt tolerant high-yielding rice varieties with good grain quality have been developed and released for farmers (Mishra, *et al.* 2012). Almost all the conventional breeding methods have been followed for the development of the salt tolerant materials i.e. introduction, selection, hybridization, mutation and shuttle breeding approach. Most of the initial salt tolerant rice varieties like Damodar (CSR1), Dasal (CSR2), Getu (CSR3), Pokkali, Vytilla1, Vytilla2, Vytilla3, Vytilla4, Vytilla5 etc were developed through pureline selection from the local traditional cultivars prevailing in the Sundarban areas in West Bengal, India. Later on other salt tolerant varietal series like CSR10, CSR13, CSR23, CSR27, CSR30, CSR36**,** CR Dhan402, CR Dhan 403, TRY1, TRY2, TRY3, White ponni, CO43 etc. were developed through recombination breeding following methods like Pedigree method and Modified bulk pedigree method. In the latter

method, individual F_2 plant harvest is bulked up to the F_4 generation followed by panicle selection and handling the population as in pedigree method and Shuttle Breeding. The segregating materials are put in the long rows under salt stress with space planting in F_2. Selection pressure is gradually increased with generation advancement in moderate stress such as high stress of sodicity and salinity simultaneously (Reddy, *et al.* 2014). CORH2 was developed through the three line breeding method. Somaclonal variants of Pokkali with improved agronomic traits were identified. The variant (TCCP266-2-49-B-B-3) had desirable levels of all tested characteristics and retained salinity tolerance equal to Pokkali. The variant is semi-dwarf and has vigorous growth and high yield potential without lodging. TCCP266-2-49-B-B-3 had a white pericarp and also improved cooking quality, with medium gel consistency. High-yielding salt-tolerant AC-derived lines using this mutant (IR51500-AC11-1, IR51500-AC17, IR51485-AC6534-4, IR72132-AC6-1, IR69997-AC1, IR69997-AC2, IR69997-AC3 and IR69997-AC4) were generated in just 3 years (Senadhira, *et al.* 1994).

Bangladesh Rice Research Institute (BRRI)has so far released 7 salt tolerant varieties for *Transplanted Aman* (BRRI dhan40, BRRIdhan41, BRRI dhan53 and BRRI dhan54) and *Boro* and *Aus* season (BRRI dhan47, BRRI dhan61 and BRRI dhan67) respectively. However, Bangladesh Institute of Nuclear Agriculture (BINA) also released 2 salt tolerant varieties for *Boro* season (BINA dhan8 and BINA dhan10). All of these salt tolerant varieties originated from advanced salt tolerant IRRI lines (www.brri.gov.bd and www.bina.gov.bd). The *T. Aman* varieties are photoperiod sensitive and suitable for growth only in the monsoon season, while *Boro* varieties are suitable for growth in the dry winter season. However, the adoptions of these varieties are not encouraging due to some of their undesirable traits like shattering (BRRI dhan47), long awn (BRRI dhan40 and BRRI dhan41), poor grain qualities (bold grains of BRRI dhan47, BINA dhan8 and BINA dhan10), less resistance to diseases and insect-pests and most importantly low yield in high stress condition (less tolerance). BRRI dhan61 and dhan67 are still being adopted by farmers so their level of acceptability remains unknown.

The breeding successes observed in the examples above follows a long term goal to combine different traits conferring salt tolerance in to an elite background. However, the development is not straightforward because tolerance is controlled by multiple loci or QTLs. Grouping of the genotypes based on the inherent physiological mechanism responsible for salinity

tolerance, inter-mating of the genotypes with high degree of expression of the contrasting salinity tolerance mechanism and identifying/screening of the recombinants for pooling of the mechanisms is being followed to enhance the level of salt tolerance further. The genotypes are grouped into different categories based on the physiological mechanism for salt tolerance. Crosses are made between the parents/donors possessing contrasting physiological traits like tissue tolerance, Na^+ exclusion, K^+ uptake and Cl^- exclusion to pyramid the genes governing or contributing for salinity tolerance into one agronomically superior background. Moreover, the varieties developed had some performance faults and farmers did not accept all for their cultivation due to some unacceptable properties, like, grain quality coupled with poor performances in high salinity and other stresses. Moreover, the problem of salinity rarely occurs in isolation. Mineral deficiencies and toxicities frequently compound the problem of salinity. For inland salinity brought about by poor quality ground water irrigation, associated stresses are alkalinity, phosphorus and zinc deficiencies, and boron toxicity. In these soils, rice cannot be grown without good-quality irrigation water. For coastal salinity, on the other hand, tidal intrusion is the source of salinity, but there are also other associated problems.

1.7 Molecular markers and QTLs for rice salinity tolerance

Salinity affects the growth of plants in numerous ways: morphologically, physiologically, and biochemically. Tolerance to salt is a complex trait, both genetically and physiologically and is the sum affect of different contributory mechanisms, most of which are governed by polygenes (Moeljopawiro and Ikehashi, 1981; Mishra, 1996; Mishra, *et al.* 1998; Singh, *et al.* 2001). The discovery of quantitative trait loci (QTLs) associated with salt tolerance in various crops like tomato, wheat, barley, and rice, support this hypothesis (Mano and Takeda, 1997; Ma, *et al.* 2007; Cheng, *et al.* 2008; Läuchli, *et al.* 2008; Witcombe, *et al.* 2008; Zang, *et al.* 2008). By integrating physiological and genetic strategies, we can obtain a deeper understanding of the underlying molecular mechanisms, which opens the way towards a more targeted breeding approach for higher stress tolerance in crop plants. The breakthrough that has made this approach possible was the introduction of easy-to-use DNA markers that brought QTL mapping into the mainstream, making it possible to efficiently map the genetic loci controlling complex traits. This was made possible through genetic linkage analysis, allowing the construction of linkage maps, and the identification of QTLs

controlling particular traits based on statistical methods that help establish the association between molecular markers and phenotypic data.

A large number of studies have been carried out for mapping salinity tolerance and dissecting mechanisms underlying the tolerance traits since the discovery of molecular markers last century. A list of QTLs linked with salinity tolerance in rice can be found in Gramene (www.gramene.org). More detailed information on these QTLs has been compiled in the Rice module of the TropGene database (http://tropgenedb.cirad.fr) (Courtois, *et al.* 2009). Singh, *et al.* (2007) summarized the reports on QTL for salinity tolerance in rice starting from Claes, *et al.* (1990) on *Sal*T locus on chromosome 1 to Ammar, *et al.* (2009). Later several studies reviewed and listed the reported QTLs, genes and regulatory networks identified for salinity tolerance in rice such as Alam, *et al*, 2011; Negrao, *et al.* 2011; Gupta and Huang, 2014 and Deinlein, *et al.* 2014. The QTL studies also led to the conclusion that different *loci* were involved in the different plant responses under short *versus* long term salt stress (Haq, *et al.* 2010).The various components of salinity tolerance appear to be polygenically controlled. QTLs have been detected repeatedly on chromosomes 1, 4, 6, and 7. None have been found on chromosomes 8 and 11 and very few on chromosomes 2, 3, 5, 9, 10 and 12. A segment of the short arm of chromosome 1 concentrates a large number of QTLs (Negrao, *et al.* 2011). A major QTL designated '*Saltol*' was mapped on chromosome1 using a RIL population generated from a cross between the sensitive variety IR29 and a tolerant landrace, Pokkali. This QTL was responsible for Na^+ and K^+ absorption as well as Na^+/K^+ ratio and each accounted for more than 60% of the variation in this population (Gregorio, 1997; Bonilla, *et al.* 2002). Early studies reported the QTLs responsible for physiological parameters conferring seedling stage salinity tolerance in rice on different chromosomes (Flowers, *et al.* 2000; Koyama, *et al.* 2001; Lang, *et al.* 2001), and substantiated the independence of Na^+ and K^+ uptake as they were located on different linkage groups. Some of the studies have shown co-localization of QTLs for Na^+, K^+, and/or their ratios on chromosome 1 (Gregorio, 1997; Koyama, *et al*, 2001; Niones, 2004) and chromosome 12 (Lang, *et al.* 2001). Indeed, Na^+/K^+ ratio is a derived trait but a balance of both ions is very important within cytosol; thus it indicates that uptake of both the ions either could be due to the linkage or pleiotropic effects within the same gene complex that is quite desirable (where the QTLs for different traits are co-localized) or could be due to probable epistatic interactions.

QTL cloning is the best way to identify the genes underlying the QTLs and provide the markers needed to select for the favorable alleles of these genes. A number of rice QTLs for salinity tolerance have already been cloned, a gene underlying the major QTL on chromosome1 was isolated by map-based cloning (Ren, *et al.* 2005). The *OsHKT8* (*SKC1*) gene encoded a sodium transporter of HKT type (the favorable allele coming from Nona Bokra). Fine mapping work conducted in the same area of chromosome 1 seemed to indicate the presence of a cluster of QTLs and not just this gene alone (Haq, *et al.* 2010). Another QTL, *Saltol*, has been fine mapped on chromosome 1, initially a few Mb apart from *OsHKT*8 (the favorable allele coming from Pokkali). *Saltol* co-localized with the *Sal*T gene (Claes, *et al.* 1990) but there was no evidence that the gene and the QTL represented a unique genetic factor. Additional fine mapping work and the demonstration that *Saltol* acts to control shoot Na^+/K^+ homeostasis seemed to actually support the possibility that *OsHKT8* may be the causal gene underlying the QTL (Thomson, *et al.* 2010). Fine mapping though marker saturation of the *Saltol* QTL has incorporated more than 30 SSRs from the IRGSP and custom-designed insertion/deletion (InDel) markers at gene loci across the QTL peak region from 10.7 to 12.5 Mb (Thomson, *et al.* 2007). Four major genes, especially for transporters and membrane/stress proteins within *Saltol* region as annotated using genome browser of MSU Rice Genome Project (http://rice.plantbiology.msu.edu/), and an additional nearby gene (*Sal*T at 13.8 Mb) were targeted for the development of gene-based PCR markers. The availability of a large number of gene-based markers or fine-mapped QTLs underlying salinity-tolerance component traits could help in pooling of different tolerance mechanisms to enhance the level of salt tolerance in agronomically superior adapted varieties using MAS (Thomson, *et al.* 2007).

1.8 Molecular breeding for the development of high-yielding salinity tolerant variety

Classical breeding has supported the development of improved salt-tolerant genotypes up to a certain degree (Senadhira, 1994). To go further in improving salt tolerance of rice varieties, Yeo, *et al.* (1990) emphasized the importance of combining all favorable and complementary physiological traits in a variety, rather than considering salinity tolerance as a single trait. The recent advances in genomics have paved the way for clear and reliable methods for MAS in plants: from QTL identification, NIL development and fine-mapping to

transferring the QTL into popular varieties using a precise marker-assisted backcrossing (MABC) strategy (Thomson, *et al.* 2010; Mackill, 2006; Collard, *et al.* 2005; Collard and Mackill, 2008; Collard, *et al.* 2008). MABC involves the introgression of genomic regions involved in the expression of particular traits of interest through DNA markers, and combines the power of a conventional backcrossing program with the ability to differentiate parental chromosomal segments. So far, the greatest success in MABC for improving tolerance of biotic and abiotic stresses has been achieved with QTLs proven to provide high levels of tolerance in many different genetic backgrounds and environments (Thomson, *et al.* 2010; Collard and Mackill, 2008; Collard, *et al.* 2008). A good example in rice is the introgression of *SUB1*, the major QTL for submergence tolerance, into several popular rice varieties (Xu, *et al.* 2006; Neeraja, *et al.* 2007; Iftekharauddaula, *et al.* 2011).

The basis of MABC strategy is to transfer a specific allele at the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genomes (Neeraja, *et al.* 2007). The use of molecular markers, which permit the genetic dissection of the progeny at each generation, increases the speed of the selection process, thus increasing genetic gain per unit time (Hospital, 2003). The main advantages of MABC are (1) efficient foreground selection for the target locus, (2) efficient background selection for the recurrent parent genome, (3) minimization of linkage drag surrounding the locus being introgressed, and (4) rapid breeding of new genotypes with favorable traits. The effectiveness of MABC depends on the availability of closely linked markers and/or franking markers for the target locus, the size of the population, the number of backcrosses, and the position and number of markers for background selection (Frisch and Melchinger, 2005). The availability of the large-effect QTL *Saltol* for salinity tolerance in rice, a theoretical framework for MABC, and the existence of intolerant varieties that are widely accepted by farmers provided an opportunity to develop cultivars that would be suitable for larger areas of saline-prone rice (Mackill, 2006). Molecular breeding technologies have been widely applied in countries all over the world. It provides powerful tool for development of stress tolerant varieties that can deal with the adverse effects from climate change. However, application of molecular breeding such as MABC had only initiated sporadically in Bangladesh. Hence, the attempt of this study was to develop salinity-tolerant version of the widely grown BR11 and BRRI dhan28 by applying the MABC method. The improved cultivars may be useful for growing in the soil salinity of the coastal areas of Bangladesh.

1.9 Complexity of salinity tolerance mechanisms and signaling

Salinity tolerance of rice is a complex trait, both genetically and physiologically and is the sum of different contributory mechanisms, most of which are governed by polygenes (Moeljopawiro and Ikehashi, 1981; Mishra, 1996). Growth and yield of rice is reduced by salinity via several distinct processes, which are related either to the accumulation of salt in the shoot, or which are independent of shoot salt accumulation. Based on timing of responses, Munns and Tester (2008) reviewed the growth responses in two phases: a rapid response to the increase in external osmotic pressure (the osmotic phase), and a slower response due to the accumulation of Na^+ in leaves (the ionic phase). Plants have acquired different adaptive mechanisms to control the negative impacts of salinity and are classified into three categories by Munns and Tester (2008): osmotic tolerance, ion exclusion and tissue tolerance. In the simplest analysis of the responses of a plant to salinity stress, the reduction of growth and development occurs in a rapid and a slower phase (Munns and Tester, 2008), but the adaptation of plants to these responses depends on plant type, timing of stress, length of exposure, salt concentration, stage of growth, environmental conditions and balance between nutritional elements to toxic Na^+ & Cl^-. The two-phase effects of salinity on plants are not obvious if the salinity is high, or if the species is particularly sensitive to Na^+. The roots of some species, such as rice, are leaky and Na^+ may be taken up apoplastically (Gong, *et. al.* 2006). Then, the first phase, or osmotic effect, might last only a few hours or days at the most before the Na^+ levels build up to toxic levels within the leaves (Yeo, *et. al.* 1991). Pires, *et al.* (2015) termed the osmotic stress as shoot-ion independent stress based on the results obtained by Termaat, *et al.* (1985) and Choi, *et al.* (2014). They hypothesized that the growth reduction seems to be a specific signaling and sensing response to NaCl stress which is independent of osmotic pressure and also independent from other agents such as KCl or mannitol. However, for most plants in most conditions, the two phases are clearly separated in time (Munns, *et. al.* 1995), which facilitates the separation of the three tolerance mechanisms. Therefore, for salinity tolerance, the onset of the three mechanisms and their relative importance is complex and still not completely understood. In a recent study of Pires, *et al.* (2015) established that the three salinity tolerance mechanisms: shoot-ion independent tolerance (or osmotic tolerance), ion exclusion, and tissue tolerance appear among rice genotypes and that none of them is predominant. This suggests that different genotypes may be needed to study each of the different mechanisms of plant salinity tolerance. The study also demonstrates that in order to have a complete

understanding of salinity tolerance in plants researcher should focus on studying each tolerance mechanism independently by selecting an appropriate species or specific genotype that best exemplifies a specific mechanism. Roy, *et. al.* (2014) reviewed plant salt tolerance mechanisms and discussed with examples as a plant may have ion exclusion as its primary tolerance mechanism at moderate salinity, but then has tissue tolerance as its main tolerance mechanism when the exclusion processes are 'swamped' at high salinity. It is possible that some tolerance mechanisms are more effective in particular circumstances. For example, Na^+ exclusion may be more effective in conditions of higher salinity, whereas 'osmotic tolerance' may be more important in moderately saline conditions. Considering the above mentioned complexities of plant responses to salinity, it is probable that the efficient adaptations against different responses might depend on the temporal pattern of salinity signals and the role of these temporal patterns for the orchestration of cross-talks between signal transduction and signaling pathways. In general, a stress signal is first perceived at the membrane level by the receptors, which results in the generation of many secondary signal molecules such as Ca^{2+}, inositol phosphates, ROS and ABA. The stress signal then transduces and induce many stress responsive genes, the product of which ultimately lead to plant adaptation to stress tolerance. The stress responsive genes could be either early or delayed induced. Early genes are induced within minutes of stress perception, often express transiently, and their products can activate the expression of delayed genes. Overall, gene products are either involved directly in cellular protection against the stress or involved indirectly. Stress-induced gene products are also involved in the generation of regulatory molecules such as ABA, salicylic acid, ethylene, which can initiate second round of signaling (Tuteja, 2007). However, in recent development Ismail, *et. al.* (2014a) reviewed the salinity signaling with a model where they explained the two response modes (adaptation versus cell death) depend on the relative timing of two signal chains: one triggered by calcium and the other triggered by oxidative burst in the apoplast. They also hypothesized a delay in generation and dissipation of a salinity-triggered calcium-dependent signal relative to a signal conveyed by ROS will lead in the unconstrained activation of jasmonate (JA) signaling culminating in cell death. In contrast, the same molecular signal carrier (calcium) can, if properly timed, initiate adaptive processes such as sequestration and extrusion of sodium, and induce efficient constraint of JA signaling through the activation of abscisic acid (ABA) signaling. Recently, Ismail and his colleagues (2012, 2014b) also showed the role of early influx of Na^+ might act as signal to trigger salinity adaptation and also the role of Non-selective Cation Channels (NSCC) and their kinetic activities not only determine the

pattern of Na^+ influx but also modulate the cytoplasmic signatures of two crucial signaling elements, Ca^{2+} and H^+ and while working with grape varieties (*Vitis rupestris* and *V. riparia*). Therefore, a deeper understanding of the temporal patterns in signaling will help us to dissect adaptive from damage related events. Overall, the stress signal transduction requires exact coordination of all the signaling molecules, including protein modifiers, adaptors, and scaffolds (Xiong, *et. al.* 2002). The mechanism of salinity tolerance is a very complex phenomenon. Studies have shown that components of various pathways are involved in imparting the salinity tolerance to the plants (Tuteja, 2007). Salinity tolerance is too complex to be easily amenable to improvement through selection as a trait in itself, but traits that are hypothesized to contribute to salinity tolerance are more genetically tractable and genes underlying these can be discovered using molecular genetics tools and genomics. Alterations in crops can then be made using both marker-assisted selection and genetic modification, and the relevance of such traits on whole plant salinity tolerance can then be tested, as measured by yield maintenance in saline conditions.

1.10 Objectives of the study

The aim of the study is to introgress *'Saltol'* QTL into two Bangladeshi mega rice varieties BR11 and BRRI dhan28 through the MABC approach to improve the salt tolerance capacity of these two farmer's popular variety for cultivating in the coastal saline environments of Bangladesh.

Specific objectives are:

1) To develop salt tolerant version of BR11 and BRRI dhan28 containing only the specific *Saltol* QTL from salt tolerant donor with the aid of specific DNA markers.
2) To develop salt tolerant isogenic and recombinant lines with minimum background genotype from salt tolerant donor with the aid of specific DNA markers.
3) To quantify the effects of introgressed *Saltol* QTL in to the genetic background of BR11 and BRRI dhan28.
4) To evaluate the developed lines for recovery of mega variety traits including yield performances in saline and non-saline field conditions.

Chapter 2:

Review of Literature

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Review of Literature

2.1 Global food production and environmental constraints

To satisfy the needs of 9.2 billion people in 2050, overall food production will have to increase by about 70 percent and production in the developing countries will virtually need to double. Demand for cereals for both food and animal feed will reach around 3 billion tons by 2050, compared with 1.8 billion tons today, and with the advent of liquid biofuels, demand could increase even further (FAO, 2011). To achieve sufficient increases in food production, agriculture will be obliged to rely on a smaller rural workforce, adopting more efficient and sustainable production methods, while at the same time adapting to and mitigating climate change. Several environmental problems such as climate change, ozone depletion, drought, desertification, flooding, soil salinity, and soil erosion will further endanger our capacity to meet food demands (Godfray, *et al.* 2010). Specifically, changes in global climate due to increasing greenhouse gases are likely to aggravate current problems of global agricultural system and may erode all global efforts to achieve food sufficiency (Aggarwal and Singh, 2010). Climate change is likely to bring changes in global temperature and amount and the pattern of precipitation (IPCC, 2007). Agriculture, being intimately tied to nature, is likely to face severe losses due to these predicted changes in important climate variables as well as their associated impacts on water availability and increase in weeds and pests proliferation (Stern, 2007). Particularly, changes in the duration and pattern of rainfall will result in shortage of water in rain-fed regions, and will also indirectly reduce storage of water, thereby limiting water availability for irrigation (IWMI, 2007). Furthermore, changes in these key parameters of global climate are likely to deteriorate soil quality by influencing soil water content, runoff, erosion, soil temperature, salinization, and soil biodiversity leading to an adverse effect on crop production (Aggarwal and Singh, 2010). Also, climatic change is expected to accelerate the problem of ozone depletion allowing the penetration of UV-B radiation reaching the Earth surface leading to serious consequences for crop productivity (Caldwell, *et al.* 2007).

The prevalence of fewer natural resources, particularly land and water and their deterioration due to intensive agriculture is also a serious constraint to meeting global food needs (Khan and Hanjra, 2009). In order to feed the increasing population from existing natural resources,

significant advances are required in the field of agricultural production which is possible either by bringing more area under cultivation or increasing productivity from land already under cultivation. Recruiting new arable land under agricultural system is unlikely because of the limited amount of land suitable for agriculture (Kendall and Pimentel, 1994). This option is further offset by the urbanization, soil degradation, and depletion of water supplies (Khan and Hanjra, 2009). Fresh water supplies, essential for modern high-input agriculture, are dwindling because of the increased human and agricultural use, and are being polluted by agricultural run-off, and widespread use of agrochemicals (Khan and Hanjra, 2009). Increasing agricultural productivity from the existing arable land in an environmentally friendly manner is, however, a big challenge for the global agricultural system (Robertson and Swinton, 2005). A possible way forward is to increase efficiency and sustainability of current crop production practices along with incorporating modern agricultural biotechnology (McMichael, 2001; Giovannucci, *et. al.* 2012), and to take abrupt actions to preserve the natural resources in the form of soil and water (Khan and Hanjra, 2009).

2.2 Extent of soil salinity in global perspectives

Soil salinity is a major environmental issue threatening agricultural productivity worldwide (Wang, *et al.* 2003). It has been estimated that soil salinity, along with other abiotic stresses, is responsible for more than 50 percent crop production losses in major field crops (Mahajan and Tuteja, 2005). According to the USDA salinity laboratory, saline soil can be defined as soil having an electrical conductivity of solution extracted from the water-saturated soil paste ECe (Electrical Conductivity of the extract) of 4 dS m^{-1} (deci siemens per meter), where 4 dS m^{-1} ≈ 40 mM NaCl or more (Chinnusamy, *et al.* 2005; Kotuby-Amacher, *et al.* 2000), and soils with ECe's exceeding 15 dSm^{-1} are considered strongly saline (FAO, 1997). However, many crops are affected by soil with an ECe less than 4 dSm^{-1}. The moisture content of a drained soil at field capacity may be much lower than the water content of its saturated paste. Further, under dry land agriculture, the soil water content might drop to half of field capacity during the life of the crop. The actual salinity of a rain-fed field whose soil had an ECe of 4 dSm^{-1} could be 8-12 dSm^{-1} (www.plantstress.com/articles/salinity i/salinity_ i.htm). This would severely limit yield of most crops. The three common cations associated with salinity include Na^+, Ca^{2+}, Mg^{2+}; whereas the common anions include Cl^-, SO_4^{2-}, and HCO_3^-. However, the most damaging ions are Na^+ and Cl^- because excessive Na^+ causes deterioration of the soil structure, and both Na^+ and Cl^- can be toxic to plants (Dudley, 1994; Hasegawa, *et al.* 2000b).

There is another type of soil salinity generally termed as sodic soil. This soil has a low concentration of soluble salts, but a high percent of exchangeable Na^+; that is, Na^+ forms a high percent of all cations bound to the negative charges on the clay particles that make up the soil complex. Sodicity is defined in terms of the threshold ESP (exchangable sodium percentage) that causes degradation of soil structure. The USDA Salinity Laboratory defines a sodic soil as having an ESP greater than 15, but in Australia it is considered sodic when the ESP is greater than 6. This lower threshold is due to Australian soils having a low content of other soluble cations, particularly Ca^{2+}, which help to stabilize clay colloids during leaching (Munns, 2012, www.plantstress.com/articles/salinity_i/salinity_i.htm).If the concentration of soluble salts is sufficiently low, hydrolysis of the sodic clay will occur, creating a highly alkaline soil. *Alkaline soils* area type of sodic soil with a high pH due to carbonate salts, and are defined as having an ESP of 15 or more with a pH of 8.5-10.

Salinization of soils develop due to two sources; primary and secondary salinization. Primary salinization occurs due to natural processes including weathering of minerals and soils derived from saline parent rocks whereas secondary salinization results from improper agricultural management practices including poor water management, high evaporation, heavy irrigation and previous exposure to sea water (Galvani, 2007; Pitman and Lauchli, 2002). Of these two types of soil salinity, secondary salinization of arable land is a source of major concern because it has adversely affected approximately one third of the world's agriculturally productive land (FAO, 2008). Such increased salinization of productive land works against the needs of an expanding global population, which is projected to reach 9 billion by the year 2050 (FAO, 2009), and is expected to require an increase in food production of 20% in developed countries and 60% in developing countries over the next 30 years (Galvani, 2007).

2.3 Measuring soil and water salinity

Soil salinity is measured by electrical conductivity of a saturated soil paste extract (ECe) taken from the root zone of the plant and averaged overtime and depth. Soil paste extracts are soil samples that are brought up to their water saturation points (USDA, 1954).The SI unit of electrical conductivity (ECe) is dSm^{-1}. Previously it was measured as milli mhos per centimeter ($mmhocm^{-1}$).The relationships of different conductivity units as well as concentration of different salts are shown in Table 2.1 and 2.2 (adapted from Munns, 2012; www.plantstress.com/articles/salinity_i/salinity_i.htm). The advantage of using saturation

extracts as a method of measuring and referencing salinity is that this measurement is directly related to the field moisture range for most soils (USDA, 1954). The soluble salt concentration in a saturation extract is roughly one half as concentrated as the soil water at saturation for a wide range of soil textures from medium to fine. Thus, a measured ECe of 4 dSm^{-1} would be equivalent to an EC of about 8 dSm^{-1} in the soil water of a medium-textured soil at field capacity. For coarse, sandy soils, soil water EC would be higher (approaching 12 dSm^{-1}) (Shannon and Grieve, 1999). Soil to water extracts of 1:1 or 1:5 can be more easily made and measured than saturation extracts and back calculations can be developed to ECe for a given soil. New methods use electronic probes or electromagnetic pulses to calculate ECe with even less time and effort (Rhoades, 1976 & 1993).The electrical conductivity of irrigation or river water is measured with the same hand-held conductivity meter as above, but is expressed in units 1000 times magnified, as channel or river water would normally have a very low concentration of salts. River water quality is often expressed as dS/cm (1000 × dS/m). Irrigation water quality is often expressed as total soluble salts, an international convention being that 1 dS/m is equivalent to 640 mg/L of mixed salts (Table 2.1) (Munns, 2012).

Table 2.1: Units for measuring salinity, and conversion factors. (adapted from Munns, 2012; www.plantstress.com/articles/salinity_i/salinity_i.htm).

Measurement and units	**Application**	**1 dS/m is equal to**	**Equivalent units**
Conductivity (dS/m)	Soils	1	1 dS/m = 1 mS/cm = 1 mmho/cm
Conductivity (µS/cm)	Irrigation and river water	1000 µS/cm	1 µS/cm = 1 µmho/cm
Total dissolve salts (mg/L)	Irrigation and river water	640 mg/L (approx.)	1 mg/L = 1 mg/kg = 1ppm
Molarity of NaCl (mM)	Laboratory	10 mM	1 mM = 1 mmol/L

Note: *Conversion factors relating total dissolved salts or pure NaCl to an electrical conductivity (EC) of 1 dS/m (1 deciSiemens/metre) are given, along with equivalent units of various types, old and new. The conversion of EC of 1 dS/m to total dissovled salts (640 mg/L) assumes a composition of salts that is common in groundwater across the world. The exact factor varies from 530 (if the salt is predominantly NaCl) to 900 (if the salts are formed predominantly from divalent ions)*

Table 2.2: Electrical conductivity (EC) of pure solutions at 20 °C (dS/m). (adapted from Munns, 2012; www.plantstress.com/articles/salinity_i/salinity_i.htm).

Solution	EC (dS/m)
10 mM NaCl	1.00
100 mM NaCl	9.80
500 mM NaCl	42.20
10 mM KCl	1.20
10 mM $CaCl_2$	1.80
10 mM $MgCl_2$	1.60
50 mM $MgCl_2$	8.10

Note: *The solutions represent those of salts found in soils or in seawater. Data from the Handbook of Physics and Chemistry (CRC Press, 55th editition, 1975). (Note that 1 dS/m = 1 mmho/cm)*

2.4 Problems of soil salinity in Bangladesh

The coastal zone of Bangladesh lies in the southern part of the country approximately between 20 º34´ and 22º78´ N Latitude and 88º40´ and 92º30´ E Longitude. It spreads along the Bay of Bengal over approximately 2.83 million hectares in the southern districts in a strip of land few kilometres to 180 km along the sea coast and having a coast line of about 710 km (Shoaib, 2013). Depending on hydrology and geo-forming process it is broadly divided into three regions: the deltaic eastern region (Pacific type), the deltaic central and the stable deltaic western region (Atlantic type) (Minar, *et. al.* 2013). It occupies about 20% of total land and 30% of net cultivable area of Bangladesh. Due to salinity about 53% of coastal area limits crop production (Uddin, *et. al.* 2011). It is regularly experiencing sea water submergence by tidal water, tidal surges and cyclones. In addition, it is now under threat of sea level rise due to climate change. Saline water intrusion, water logging, late drainage due to slow water recession, desiccation of soil in dry season and capillary rises from shallow saline ground water have bearing on soil salinity in this area. It is patchy in nature, varies spatially and starts increasing from late November and attains its peak during May/June. Cyclone and tidal surge transport saline water into polders followed by breaching or collapse of polders in many cases. Water logging makes the situation more severe (Abedin, 2010). Nearly 40 million people are directly affected by soil salinity and another 20 million are at risk (Haque, 2006). There are strong evidences of increasing intensity and occurrence of soil-water salinity in coastal region due to limiting river flow in upper riparian areas and climate change that ingress saline water towards north, which is in inland. Several surveys

(Islam, 2013; Sabbir, 2013; SRDI, 2012) carried in different river points and regions far from the coastal periphery have been shown of increasing river water and soil salinity almost doubled in some cases.

Forty-nine upazilas (sub-districts) of 19 districts are affected by different degrees of salinity. These are in close proximity to the sea and tidal creeks. The intensity and spatial distribution widen to larger areas after tidal surge or cyclone and remain saline for years. Soil Resource Development Institute (SRDI) has temporal and spatial data after regular monitoring of soil and water salinity since 1989 besides reconnaissance survey data of 1973. It was estimated in 1973 and 2009 that the area coverage of soils with different degrees of salinity was about 0.833 and 1.056 million hectares respectively. Two different scenarios of area coverage, distribution and degree of salinity as mapped by SRDI are presented in Table 2.3 (SRDI, 2012). The coastal and off-shore areas are affected by different degrees of salinity. The severity of salinity problem has increased over time with the desiccation of the soil (MoEF, 2005).

Table 2.3: Extent and distribution of saline soils (Adapted from Shoaib, 2013).

Districts	Area ('000' ha)		Agro-ecological zone (Major)*	Remarks
	1973	2009		
Satkhira	146.35	153.11	HGRF,GTF	Increased
Khulna	120.04	147.96	HGRF,GTF	Increased
Bagerhat	107.98	131.12	HGRF,GTF,GKB	Increased
Pirojpur	20.30	35.83	HGRF,GTF,GKB	Increased
Barguna	103.55	95.62	GTF	Decreased
Patuakhali	115.10	155.18	GTF	Increased
Bhola	40.73	94.57	YMEF	Increased
Noakhali	49.60	52.52	YMEF	Increased
Laxmipur	20.30	18.43	YMEF	Decreased
Chandpur	1.50	0.00	LMRF	Decreased
Feni	9.00	5.75	CCF	Decreased
Chittagong	45.70	51.48	CCF	Increased
Cox's Bazar	54.70	55.35	CCF	Increased
Jessore	0.14	0.99	HGRF	New intrusion
Narail	0.00	18.71	HGRF,GKB	New intrusion
Gopalgonj	0.60	0.27	HGRF, GKB	New intrusion
Madaripur	0.00	0.72	HGRF,GKB	New intrusion
Barisal	0.13	0.96	GTF	New intrusion
Jhalakati	0.40	0.69	GTF	New intrusion
Grand Total	833.45	1056.26		26% increased

*HGRF: High Ganges River Floodplain; GTF: Ganges Tidal Floodplain; GKB: Gopalgonj-Khulna; Beel; YMEF: Young Meghna Estuarine Floodplain; CCF: Chittagong Coastal Plain, LMRF:Lower Meghna River Floodplain.

According to SRDI saline soils and waters are classified as:

Very slightly Saline (S1):	2-4 dS/m
Slightly Saline (S2):	4-8dS/m
Moderately saline (S3):	8-12dS/m
Strongly saline (S4):	12 -16 dS/m
Extremely saline (S5):	>16dS/m

On the other hand water salinity classified as:

Safe:	< 0.75 dS/m or 480 ppm
Unsafe or Harmful:	0.75-3.0 dS/m or 480-1920 ppm
Extremely harmful:	> 3.0 dS/m or 1920 ppm

Salt accumulation on top soils is highest in Ganges Tidal Floodplain (GTF) where range of soil salinity varies from 0.3 to 70.0 dS/m. Soil salinity decreases with depth. Identified cations are Na^+, Ca^{2+}, Mg^{2+} and K^+ and anions SO_4^{2-} and Cl^- indifferent areas (SRDI, 2012). In the dry season water salinity of major rivers is highest EC>5.0 dS/m in water near the coast and EC around 2.0 dS/m in northern parts of coastal zone. Influence of massive extraction of ground water and cut off of most of the river flow from the Ganges catchment has resulted in salinity ingression in a spectacular fashion. The ratio of this ingression is estimated to be 1:40 (Karim, *et al.* 1982). Soil salinity restricts normal crop production. Coastal lands of Bangladesh is used *inter alia* for agriculture, shrimp and fish farming, forestry (mainly Mangrove), salt production, ship-breaking yards, ports, industries, human settlements and wetlands and that has resulted in a different socio-economic environment of conflicts and competitions among different land users (Alam, *et. al.* 2002; Islam, 2006). Agricultural land has to contest with salt or shrimp farm and human settlements/urban area or sometimes to change status due to salinity invasion from shrimp or salt production areas. Most of the lands in the coastal regions are single cropped (Fallow-Fallow-*Transplanted Aman*), that is, cultivated in monsoon, when soils became non-saline. Double cropped lands with irrigation (*Boro/Transplanted Aus-Transplanted Aman*) also occur in this area. Dry land crops in *rabi* season have very narrow space due to salinity and also late drainage. Some *rabi* crops like soybean, cow pea, lentil etc are grown in the *rabi* season. The cropping intensity of the area is 159%. Saline soil reclamation in Bangladesh has mainly focused on polder intervention (protecting land from saline water flooding), land management (washing salt) or abiotic approaches and salt tolerant cultivar introductions by NARS institutions. Tidal River

Management (TRM), Integrated Coastal Zone Management and recent Master Plan for Agricultural Development in Southern Region of Bangladesh are the major drives to address the challenges of coastal region (Shoaib, 2013).

2.5 Definition of salt tolerance and extent of tolerance of different crop species

Lunin, *et al.* (1963) proposed a couple of ground rules for salinity studies: (1) the actual tolerance of a given crop to salinity will vary according to the growth stages at which salinization is initiated and the final level of salinity achieved; (2) Salt tolerance values should also take into consideration the portion of the plant to be marketed. Their study demonstrated that salinity caused greater reduction in beet roots than in the tops, whereas yield reductions for onion bulbs were less than those observed in the tops.

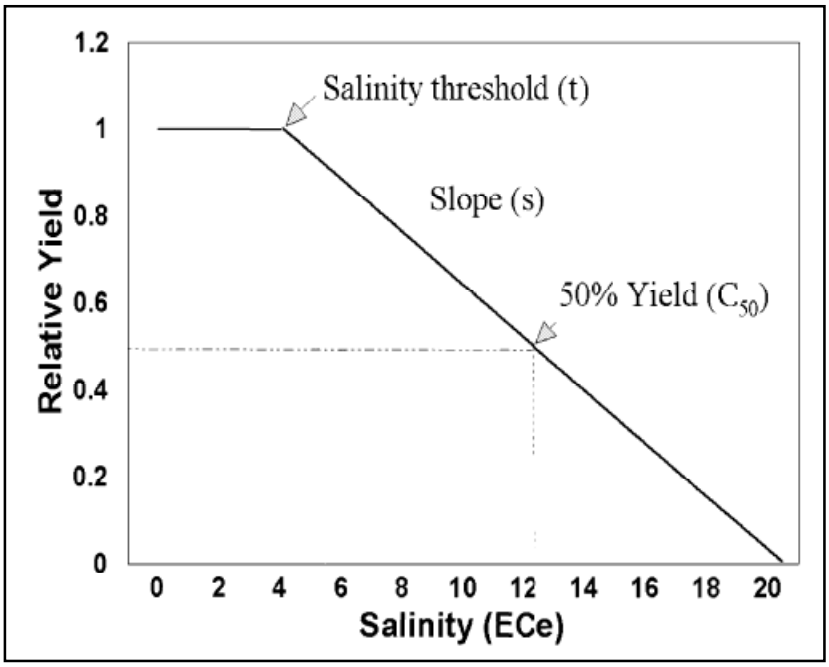


Fig. 2.1: Salt tolerance parameters relating relative yield to increasing salinity in the root zone (Adapted from Shannon and Grieve, 1999).

In addition, salt tolerance genes function in concert with other genes that influence both quantitative traits and environmental interactions. Hence, salt tolerance is a complex, quantitative, genetic character, controlled by many genes (Shannon and Noble, 1990; Shannon, 1996). In terms of its measurement, salt tolerance is described as a complex

function of yield decline across a range of salt concentrations (Maas and Hoffman, 1977; van Genuchten and Hoffman, 1984). Salt tolerance can be adequately measured on the basis of two parameters: the threshold (ECt), the electrical conductivity that is expected to cause the initial significant reduction in the maximum expected yield (Ymax) and the slope (s) (Fig. 2.1). Slope is simply the percentage of yield expected to be reduced for each unit of added salinity above the threshold value. Relative yield (Y) at any salinity exceeding ECt can be calculated:

$$Y = 100 - s(ECe - ECt) \quad \text{---------- (1)}$$

Where, ECe>ECt

The crop salt tolerance threshold, i.e. the salt concentration at which yield first declines with increasing concentration, is very sensitive to environmental interactions. The threshold value depends upon both the accuracy of salinity measurements and the method by which salinity measurements are integrated over area, depth and time. Because of this, there is a high degree of error in evaluating the slope at salt concentrations near the threshold; few salinity studies include enough replications to precisely determine the threshold value. In addition, there is a tendency for the slope to `tail-off' at the higher salt concentrations. For practical purposes, salt tolerance at high salinities has little economic importance and measurements made at high salt concentrations may disproportionately skew the salt tolerance curve (Shannon and Grieve, 1999). For these reasons the numerically most reliable value for crop salt tolerance response studies, and its applications, seems to be the value at which yield is reduced by 50 percent (C50). The C50-value may still be estimated when too few data points exist to provide reliable information on the threshold and slope (Fig. 2.1).The set of equations developed by van Genuchten and Hoffman (1984) takes advantage of the stability of C50. The C50 value, together with the p-value characterizing the steepness of the response function, may be obtained by fitting van Genuchten's function to observed salt tolerance response data.

Mass (1990) has been compiled data for 127 crop species which includes 68 herbaceous crops, 10 woody species and 49 ornamentals to provide information to growers concerning the potential hazards of a given saline water or soil. All above data has now been brought

together in to the USDA ARS website (www.ussl.ars.usda.gov).The data helps growers decide if they should substitute more tolerant species in their rotations when the potential hazards indicate that expected yield reductions may be economically disastrous. A brief examination of the threshold and slope parameters gives an indication of the potential range in variability that is found among the major domesticated plant species (Shannon and Grieve, 1999).Though the information that comprises this database is considered to be reliable, however the variability for salt tolerance based on yield criterion has not been adequately explored because of large environmental interactions of salinity tolerance. High temperatures and low humidities may decrease crop salt tolerance by decreasing the effective value of ECt in Eq. (1) and increasing the value of 's'. Thus, significant reductions in yields will be realized at lower salinities, and yields will decrease more rapidly with increasing salinity under hot, dry conditions. Root zone water-logging is another environmental hazard that can be exacerbated by salinity. Root zone salinity and water-logging greatly increase salt uptake compared with non-waterlogged conditions (West, 1978; West and Taylor, 1984). This effect may be due to anaerobic conditions that cause failures in active transport and exclusion processes in the root membrane. Salt tolerance in saline, drained conditions can be quite different from that in saline, water-logged conditions.

For accurately measuring salt tolerance, other indices have been suggested, these include tolerance during germination; conservation of shoot dry weight, root weight, or shoot number; resistance to leaf damage; maintenance of flowering, seed and fruit set, leaf size, canopy volume, or quality; and plant survival under salt stress. Some investigators have suggested using the tolerance of excised leaf or root tissues or the tolerance of tissue or callus cultures. Still other indices of tolerance have been proposed that are based on specific physiological characters; for instance, accumulation of specific ions in shoots or leaves, or the production of a metabolite. None of these artificial criteria have been unequivocally correlated with salt tolerance; however, maintenance of growth rate and leaf ion and metabolite changes that improve water balance while preserving nutrients and avoiding ion toxicities are probably the most common and universal characteristics of salt tolerant plants (Shannon and Grieve, 1999).

Yield components and growth parameters for different species of crops show differential responses to salinity stress. The variation occurs with ontogeny or growth stages. The degree of salt tolerance between and within species is likely to vary according to the criteria used

for evaluation i.e. the threshold salinity (ECt), the electrical conductivity that is expected to cause the initial significant reduction in the maximum expected yield (Ymax) and the slope (s) according to the equation provided by van Genuchten and Hoffman (1984). Fig. 2.2 represented salt tolerance of several vegetable species as rated by the salinity threshold and percent yield decline (Adapted from Shannon and Grieve, 1999).

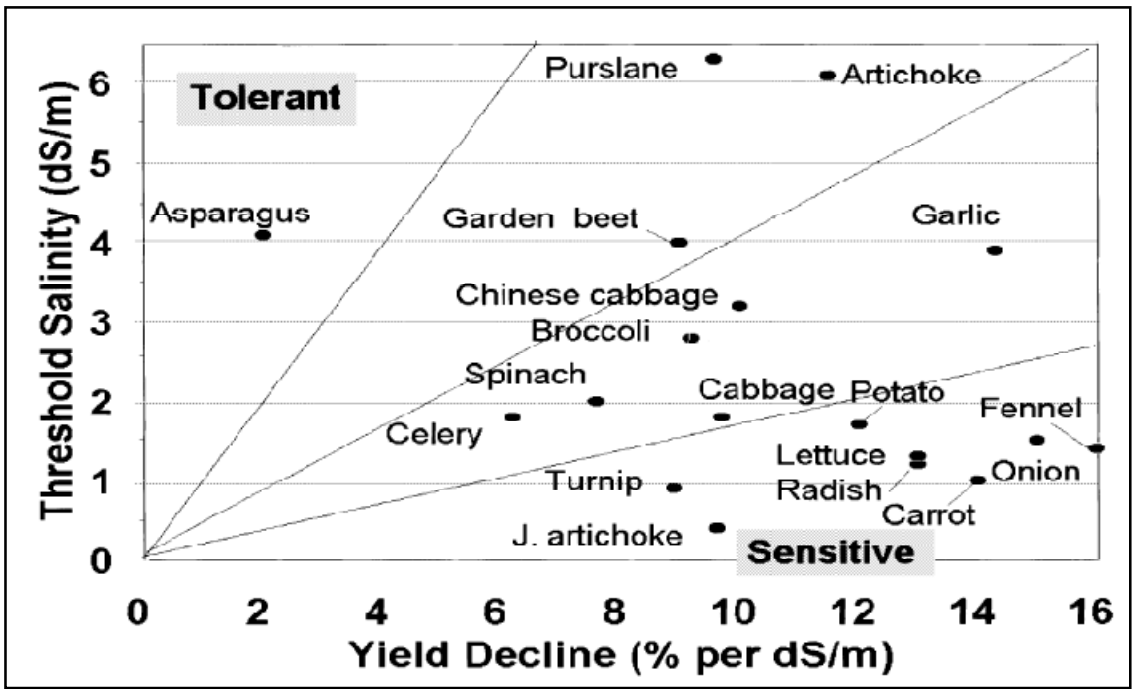


Fig. 2.2: Salt tolerance of several vegetable species as rated by the salinity threshold and percent yield decline (Adapted from Shannon and Grieve, 1999).

2.6 Diversity of salinity tolerance of cereals and rice

There is great variation for salt tolerance among plant species as reviewed by Munns and Tester (2008). Figure 2.3 shows the responses of salinity tolerances of different plant species, where among the cereals rice (*Oryza sativa*) is the most sensitive, bread wheat (*Triticum aestivum*) is moderately tolerant and barley (*Hordeum vulgare*) is the most tolerant. Tall wheatgrass (*Thinopyrum ponticum*, syn. *Agropyron elongatum*) is a halophytic relative of wheat and is one of the most tolerant of the monocotyledonous species. However, the variation in salinity tolerance in dicotyledonous species is even greater than in

monocotyledonous species. Some legumes are very sensitive, even more sensitive than rice; alfalfa or lucerne (*Medicago sativa*) is very tolerant, and halophytes such as saltbush (*Atriplex* spp.) continue to grow well at salinities greater than that of seawater (Munns and Tester, 2008).

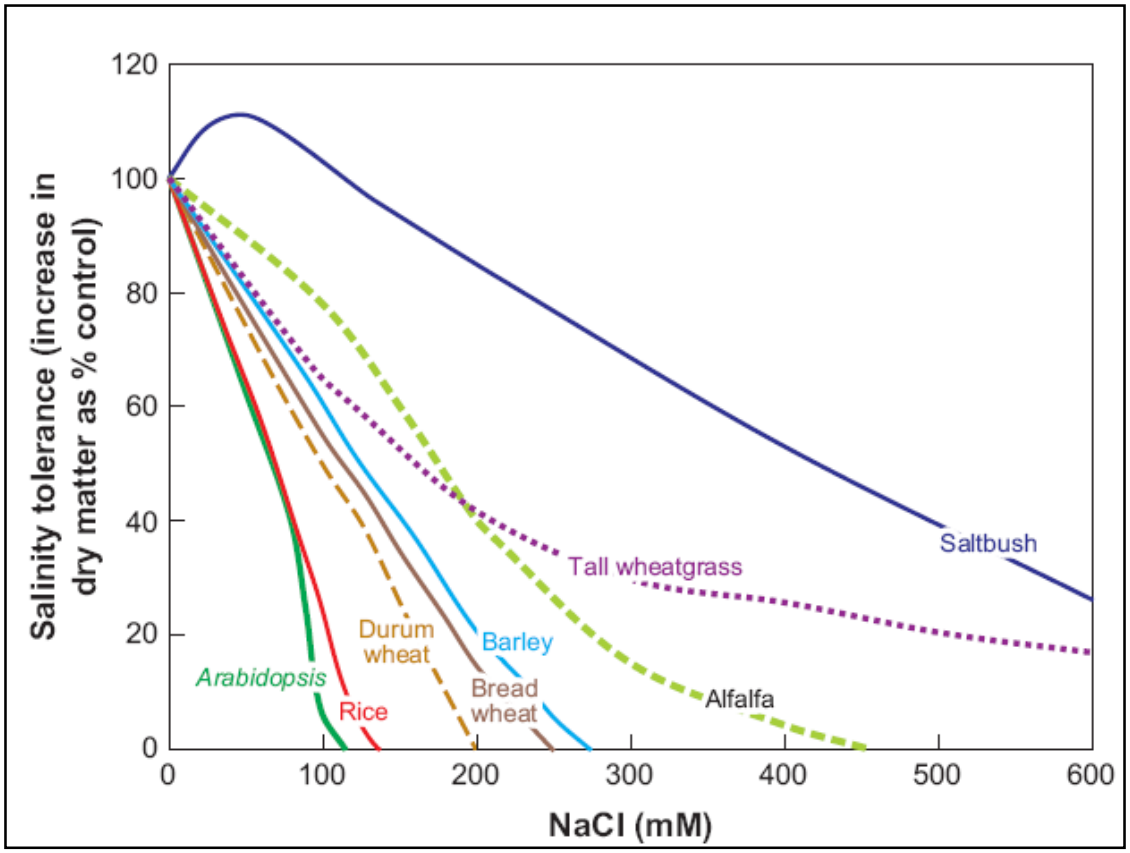


Fig. 2.3: Diversity in the salt tolerance of various species, shown as increases in shoot dry matter after growth in solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl (Adapted from Munns and Tester, 2008)

The three most important crops in the world are wheat, rice and maize. Wheat is one of the more salt-tolerant crop species, and many cultivars that have been selected for yield in water-limited conditions do not suffer a 50% reduction in biomass until salinities reach 15 dSm^{-1} (approximately 150 mM NaCl). Rice is more salt-sensitive, and many cultivars suffer a 50% reduction in growth at half this concentration of salts. Maize falls in between these two species in terms of salt sensitivity (Munns and Tester, 2008). Maas and Hoffman (1977) classified rice as sensitive, but it has a large variability for tolerance to salinity (Flowers and Yeo, 1981; Lisa, *et al.* 2004; Mahmood, *et al.* 2004; Zeng, *et al.* 2004; Munns, *et al.* 2006).

Several rice landraces from India and Bangladesh were identified as tolerant and subsequently used in mapping and breeding for salinity tolerance such as Pokkali, Nona Bokra, Cheriviruppu, Kalarata, Kuti Patnai, Horkuch, Jamai Nadu, Ashfal, Ashfal balam, Capsule etc. (Gregorio, *et al.* 1997; Lisa, *et al.* 2004; Singh and Flowers, 2010; Rahman, *et al.* 2016; Hossain, *et. al.* 2015). Hence, categorization of rice as sensitive may not be meaningful in terms of the possibilities of enhancing its resistance to salinity, especially as rice gene banks hold more than 100,000 accessions from around the world (Singh and Flowers, 2010).

The tolerance or sensitivity to salt not only varies between genotypes but between stages of plant development in case of rice. The germination stage is relatively tolerant, but growth becomes very sensitive during the early seedling stage (1–3 weeks), gains tolerance during active tillering, but becomes sensitive again during panicle initiation, anthesis, and fertilization, and finally is relatively more tolerant at maturity (Pearson, *et al.* 1966; Khatun and Flowers, 1995; Lutts, *et al.* 1995; Makihara, *et al.* 1999; Singh, *et al.* 2004; Shereen, *et al.* 2005). Singh, *et al.* (2008) has illustrated the variations of tolerance and sensitivity according to different developmental stages (Fig. 2.4). Abeysiriwardena (2004) showed the germination of tolerant rice genotypes can be separated from sensitive by soaking in very high salt concentration (45 dSm^{-1}) for 9 days. Germination of some varieties occurred at more than double sea water salinity (Sankar, *et al.* 2006): such concentrations are far beyond those tolerated during vegetative growth. In another study (Agnihotri, *et al.* 2006), landraces of rice could germinate in 200 mM NaCl (~20 dSm^{-1}), which again is a much higher concentration than that in which rice will grow. The threshold salinity at which growth of rice begins to be affected by the salt can be as low as 3 dSm^{-1} (~30 mM salt). In general, such low salt concentrations are unlikely to cause medium or long-term osmotic stress (Yeo, 2007). The sensitivity of the growth of rice to salinity is that some genotypes can be killed by just 50 mM NaCl over a period of just 2 weeks (Flowers and Yeo, 1981). Salinity affects plants by altering the water potential in the soil (the osmotic effects of the salt) and through the specific toxicity of the ions. That these ions bring about osmotic and specific toxic effects on plants is well documented (Munns and Tester, 2008) and it has been argued that these effects are sequential. Plants first respond to the change in water potential and later to the toxicity of the ions involved (Wilson, *et al.* 1970; Munns, *et al.* 1995; Munns and Tester, 2008); osmotic and ionic tolerances are not necessarily related (Munns and Termaat, 1986; Munns and Tester, 2008). Thus, seedling or early vegetative growth is very sensitive to

salinity and there is a large genetic variation in response, with many landraces in particular, found to be relatively tolerant. Consequently, traditional land races such as Pokkali and Nona Bokra have been used as donors of tolerance in breeding programs as well as other landraces and improved genotypes found to be tolerant to a salinity of 12 dSm^{-1} at the seedling stage (Singh and Flowers, 2010).

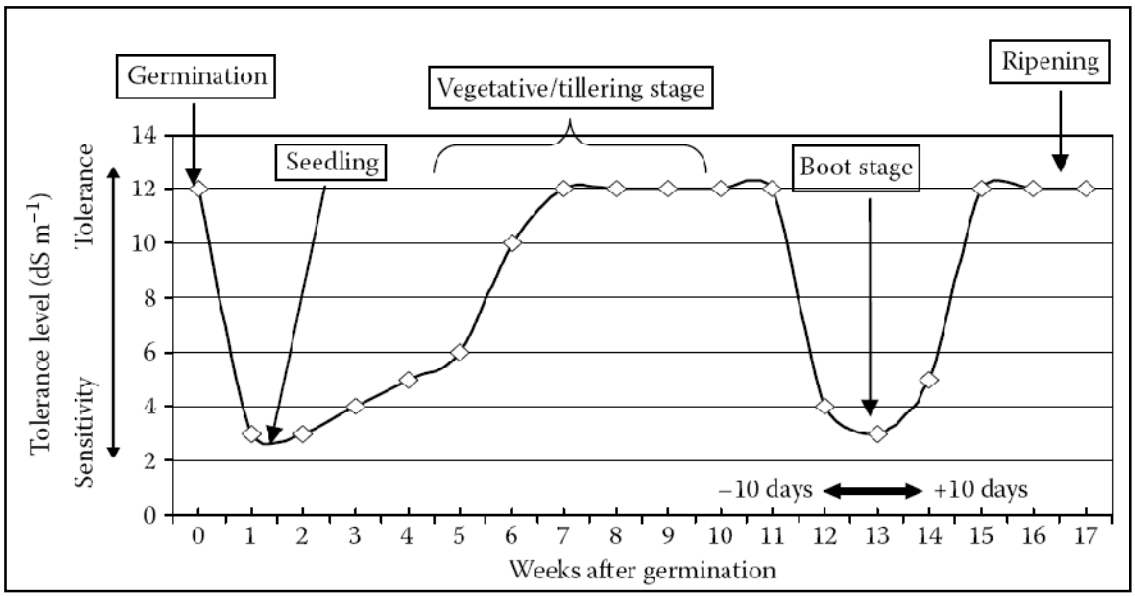


Fig. 2.4: Variation in the sensitivity of rice to salinity during its ontogeny. (Adapted from Singh, *et al.* 2008)

Reproductive stage is another developmental stage when rice is sensitive to salinity stress, more specifically this stage is the booting stage (7-10 days before and after booting stage) (Fig. 2.4). The reproductive stage is crucial as it ultimately determines grain yield, but the importance of the seedling stage cannot be underestimated as it determines crop establishment. There are few studies that address the effects of salinity on yield. Most research has been limited to the seedling or early vegetative stages or only reports parameters such as fresh or dry weight although the ultimate aim has been to increase grain yield with limited resources (Lee, *et al.* 2006 & 2007; Morsy, *et al.* 2007; Tajbakhsh, *et al.* 2006; Chen, *et al.* 2007; Moradi and Ismail, 2007; Singh, *et al.* 2007a; Cheng, *et al.* 2008; Jain, *et al.* 2008; Kanneganti and Gupta, 2008; Zang, *et al.* 2008). Khatun and Flowers (1995) reported a sharp decline of seed set with increasing salinity of a rice variety IR36. In another study where 5 rice genotypes were tested under varying salinity stress, Khatun, *et al.* (1995a) also found delayed flowering, reduced number of productive tillers, less number of

fertile florets per panicle, and reduced weight per grain and subsequently poor yield. Thus the effects on grain yield were much more severe than at vegetative growth and panicle length was also reduced as was the number of primary branches in the panicle. Zeng, *et al.* (2002) showed big varietal differences in salinity tolerance and found IR63731-1-1-4-3-2 to be much more tolerant at both the seedling and reproductive stage than M202. Grain yield per plant declined in linear fashion with increased salinity and as did the harvest index of M202 (Zeng and Shannon, 2000b). Mahmood, *et al.* (1999) reported average yield declined by 30% compared to normal soils under salinity (10 dSm^{-1}) using six rice varieties. Therefore, it is essential to name the genotype or variety when describing the degree of salinity tolerance or grain yield reduction. Rao, *et al.* (2008) categorized the effect of salinity as tolerant, moderately tolerant, and sensitive when grain yield was reduced by 27%, 46%, and 50% respectively at an ECe 8 dSm^{-1}.Hence, to know the response of the rice plant to salinity as a whole, it is imperative that the effects be observed in all the various stages of its development, that is at early seedling, vegetative and reproductive stages (Gregorio, *et al.* 1997).

2.7 Physiological responses of rice to salinity stress

The physiological responses of rice to salt stress vary according to their stages of development though the sensitivity to salt also varies between genotypes. These are indicative of the complex nature of the responses and the complexity of the tolerance trait. Moreover, to improve the salinity tolerance of rice it is necessary to understand the entire responses from single cell responses to whole plant level. In terms of duration, the responses can be divided in to two distinct phases: short term response and long term response (Negrao, *et al.* 2011).

A. Short-term responses: Munns and Tester (2008) defined short term response as two distinct phases, a rapid response to the increase in external osmotic pressure, and a slower response due to the accumulation of Na^+ in leaves.

a) Osmotic stress or Ion independent stress: The early response activated immediately after salt stress exposure is shoot-ion independent stress, also known as 'osmotic stress'. This fast response, which is independent from the accumulation of sodium in shoots, is related to Na^+ sensing and signaling in the root, and ends up in shoot growth reduction and

stomata closure under saline conditions (Munns and Tester, 2008; Roy, *et al.* 2014). Moradi and Ismail (2007) reported a rapid and temporary drop in stomatal conductance and growth rate when rice plant under salinity stress. The salt in the soil solution (the "osmotic stress") reduces leaf growth and to a lesser extent root growth, and decreases stomatal conductance and thereby photosynthesis (Munns, 1993). Osmotic stress has a direct and immediate effect on plant growth (Wilson, *et al.* 1970). However, this phase of response is relatively quick in rice; growth recovers (not necessarily to the original rate) over a period of 24 h, so any osmotic effects are transient (Yeo, *et al.* 1991; Roshandel and Flowers, 2009) and there is little time when osmotic stress can be equated with drought stress (Munns, *et al.* 1995). Ismail, *et al.* (2007) reported a substantial decrease in stomatal conductance before noticeable changes in leaf water potential, suggesting the involvement of some other type of root-shoot communication route. Moradi and Ismail (2007) reported genotypic differences in this fast response, with a relatively slower reduction in stomatal conductance rate in salt-sensitive genotypes as compared to salt-tolerant ones. This behavior suggests that stomatal closure may also be due to an increase in ABA concentration acting as a long-distance signal mediating the whole plant responses to salt (Zhang, *et al.* 2001 & 2006). Surprisingly, salt tolerant rice genotypes have a low level of constitutive and stress-induced ABA in leaves (Zhang, *et al.* 2006). Therefore, ABA signaling for stomatal closure could be the contrasting factor between drought and salinity induced osmotic stress. The signal for osmotic stress is perceived by stretch-activated channels, cytoskeleton-related mechanosensors, stretch-dependent ion (calcium) channels, redox-mediated systems and by transmembrane-proteinkinases, such as two component histidine-kinases (Kacperska, 2004; Marin, *et al.* 2003; Urao, *et al.* 1999) and wall-associated kinases (Kohorn, 2001). Yeo, *et al.* (1991) reported that these perception mechanisms seem to be activated not only by NaCl but also by KCl or mannitol and concluded that the initial growth reduction was due to a limitation in water supply caused by a variation in osmotic pressure. However, Termaat, *et al.* (1985) showed that the presence of NaCl on its own is enough to promote growth reduction. Plants under the same osmotic pressure caused by the presence of Na^+ and Cl^- ions, but not in the presence of either ion, did not exhibit growth reduction as plants that where in the presence of NaCl. More recently, Choi, *et al.* (2014) showed in *Arabidopsis thaliana* that NaCl around the roots elicits a calcium (Ca^{2+}) wave signal that propagates throughout the plant that might be responsible for the initial and fast plant responses to salinity. Choi, *et al.* (2014) also showed that exposing Arabidopsis root tips to sorbitol, an osmotic control for NaCl, did not elicit the same type of long-distance Ca^{2+} signaling as NaCl did. Therefore, in

light of the results obtained by Termaat, *et al.* (1985) and, especially by Choi, *et al.* (2014), Pires, *et al.* (2015) hypothesized that the growth reduction seems to be a specific signaling and sensing response to NaCl stress which is independent of osmotic pressure and also independent from other agents such as KCl or mannitol.

b) Ionic stress: Ionic stress develops over time and is due to a combination of ion accumulation in the shoot and an inability to tolerate the ions that have accumulated. In this phase of plant response to salinity starts when salt accumulates to toxic concentrations in the old leaves (which are no longer expanding and so no longer diluting the salt arriving in them as younger growing leaves do), and they die. If the rate at which they die is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate requirement of the young leaves, which further reduces their growth rate. Ionic stress impacts on growth much later, and with less effect than the osmotic stress, especially at low to moderate salinity levels. Only at high salinity levels, or insensitive species that lack the ability to control Na^+ transport, does the ionic effect dominate the osmotic effect (Munns and Tester, 2008). In rice, Na^+ ions make their way into roots through apoplastic and symplastic pathways. Symplastic transport requires movement through plasma membranes before off-loading to the xylem. Apoplastic transport, also called bypass flow occurs when ions move through cell walls and other extracellular spaces to the xylem. Such apoplastic pathways are commonly checked by physical barriers such as Casparian strips: discontinuities in such barriers are thought to allow direct access of solution to the stele. In rice roots, bypass flow has been shown to be a significant part of sodium entry under saline conditions (Yeo, *et al.* 1987; Yeo, 1992; Yadav, *et al.* 1996; Garcia, *et al.* 1997a; Yeo, *et al.* 1999; Ochiai and Matoh, 2002; Anil, *et al.* 2005; Gong, *et al.* 2006; Krishnamurthy, *et al.* 2009). The apoplastic leakage is the most important pathway in rice because, as an aquatic species, it has limited control of water loss, and its root anatomical development restricts cell to cell water movement (Ranathunge, *et al.* 2004). The anatomy and morphology of the rice root system differs not only from that of *A. thaliana*, (Coudert, *et al.* 2010; Rebouillat, *et al.* 2009) but also from hydrophobic monocots, as the cortical parenchyma of rice roots develops large lacunae to ensure shoot-to-root oxygen transfer. The magnitude of bypass flow in rice is thought to depend on the anatomical and morphological developments of the roots (Yeo, *et al.* 1987; Yeo, 1992; Yadav, *et al.* 1996; Garcia, *et al.* 1997a; Gong, *et al.* 2006; Krishnamurthy, *et al.* 2009). The magnitude of the apoplastic leakage was estimated to be about 10 times higher in rice than in wheat (Garcia, *et*

al. 1997a). It also accounts for the genotypic differences observed in Na^+ transport in rice roots (Garcia, *et al.* 1997a; Yadav, *et al.* 1996; Yeo, *et al.* 1987). Na^+ is taken up by plant cells still remains uncertain and to some extent controversial (Tester and Davenport, 2003; Wang, *et al.* 2007). For symplastic transport, where uptake of Na^+ involves a transporter, the situation is quite uncertain as there are a number of proteins capable of transporting Na^+ and K^+ (Tester and Davenport, 2003; Maathuis, 2007; Zhang, *et al.* 2010). The transporters commonly thought to play a role in low affinity uptake are nonselective cation channels (NSCCs), high-affinity K^+ transporters (*HKTs*), K^+ uptake permeases/high-affinity K^+/K^+ transporters (*KUP/HAK/KT*), cation-chloride co-transporters (*CCCs*) and possibly members of the Shaker family of K^+ transporter (*AKT*) (Flowers and Colmer, 2008; Zhang, *et al.* 2010). For instance, both *AtHKT1* and *OsHKT1*control the entry of Na^+ into the roots (Golldack, *et al.* 2003), and are responsible for maintaining a low Na^+/K^+ ratio. Ren, *et al.* (2005) used the map-based cloning to identify *SKC1*, a major QTL for shoot K^+ content, but later studies demonstrated that the gene underlying *SKC1* (*OsHKT*8) encodes a member of the HKT-type transporters that is preferentially expressed in the root parenchyma cells surrounding the xylem vessels. These physiological analyses revealed that *OsHKT8* is involved in maintaining low Na^+/K^+ ratios in rice plants submitted to salt stress (Ren, *et al.* 2005). In addition, they also proved that the regulation of K^+ selective intracellular uptake is also Ca^{2+}-dependent, since Ca^{2+} enhances K^+ selective accumulation (Maathuis, *et al.* 1996; Rains and Epstein, 1967).

Increasing evidence demonstrates the roles of a *Salt Overly Sensitive (SOS)* stress signaling pathway in ion homeostasis and salt tolerance (Hasegawa, *et al.* 2000a). Two major factors that maintain low cytoplasmic Na^+ concentrations in plant cells are the tonoplast-localized *NHX1* (Blumwald and Poole, 1985) and plasma membrane-localized *SALT OVERLY SENSITIVE 1* (*SOS1*, also known as *NHX7*) (Qiu, 2002; Yamaguchi, *et al.* 2013) Na^+/H^+ antiporters. Whereas most *NHXs* are essential for Na^+ detoxification via sequestration of Na^+ within the vacuole, the *SOS* signaling pathway was reported to export Na^+ out of the cell. *SOS* pathway identified in *Arabidopsis* is thought to be the Na^+ sensing process in any cellular system (Chinnusamy, *et al.* 2004; Zhu, 2002). The *SOS* signaling pathway consists of three major proteins, *SOS1, SOS2,* and *SOS3*. *SOS1* is expressed in many tissues, but particularly in the root epidermis and around the vascular tissue, and transcript levels are elevated after several hours or days of salt stress. After an initial increase in Na^+ in the plant root, a decrease in cytosolic Na^+ is likely mediated through the action of the *salt overly*

sensitive (SOS) pathway. *SOS1,* which encodes a plasma membrane Na^+/H^+ antiporter, is essential in regulating Na^+ efflux at cellular level. It also facilitates long distance transport of Na^+ from root to shoot. Overexpression of this protein confers salt tolerance in plants (Shi, *et al.* 2000 & 2002). *SOS2* gene, which encodes a serine/threonine kinase, is activated by salt stress elicited Ca^{2+} signals. This protein consists of a well-developed N-terminal catalytic domain and a C-terminal regulatory domain (Liu, *et al.* 2000). The third type of protein involved in the *SOS* stress signaling pathway is the *SOS3* protein which is a myristoylated Ca^{2+} binding protein and contains a myristoylation site at its N-terminus. This site plays an essential role in conferring salt tolerance (Ishitani, *et al.* 2000). Salt stress elicits a cytosolic calcium signal, which functions as a major secondary-messenger signaling molecule. A myristoylated-calcineurin B-like protein (*CBL4*), originally designated by *SOS3*, senses the salt-elicited calcium signal. Upon Ca^{2+} binding, *SOS3* undergoes dimerization and enhances the serine/threonine protein kinase activity of *CIPK24* (also known as *SOS2*). The *SOS3/SOS2 (CBL4/CIPK24)* complex is targeted to the plasma membrane via a myristoyl fatty acid chain covalently-bound to *SOS3 (CBL4)*, enabling the phosphorylation and thus the activation of the membrane-bound Na^+/H^+ antiporter *SOS1* (Martinez-Atienza, *et al.* 2007; Munns and Tester, 2008; Zhu, 2002b). Martinez-Atienza, *et al.* (2007) recently identified the rice orthologs of *SOS1, SOS2,* and *SOS3* and demonstrated that the *SOS* pathway of Na^+ control is structurally highly conserved in rice. The rice homolog of *Arabidopsis SOS2* and its Ca^{2+}-dependent activator *SOS3* acted co-ordinately to activate *OsSOS1* (*OsNHA1*) in yeast cells and they could be exchanged with their *Arabidopsis* counterpart to form heterologous proteinkinase modules that activated both *OsSOS1* and *AtSOS1* andsuppressed the salt sensitivity of *sos2* and *sos3* mutants of *Arabidopsis* (Martinez-Atienza, *et al.* 2007).

Besides conferring salt tolerance it also regulates pH homeostasis, membrane vesicle trafficking, and vacuole functions (Quintero, *et al.* 2011; Oh, *et al.* 2010). Thus with the increase in the concentration of Na^+ there is a sharp increase in the intracellular Ca^{2+} level which in turn facilitates its binding with *SOS3* protein. Ca^{2+} modulates intracellular Na^+ homeostasis along with *SOS* proteins. The *SOS3* protein then interacts and activates *SOS2* protein by releasing its self-inhibition. The *SOS3-SOS2* complex is then loaded onto plasma membrane where it phosphorylates *SOS1*. The phosphorylated *SOS1* results in the increased Na^+ efflux, reducing Na^+ toxicity (Martinez-Atienza, *et al.* 2007). Na^+ and Cl^- ions bring about osmotic and specific toxic effects on plants and these are well documented (Munns

and Tester, 2008) to be sequential. Plants first respond to the change in water potential and later to the toxicity of the ions involved (Wilson, *et al.* 1970; Munns, *et al.* 1995; Munns and Tester, 2008); osmotic and ionic tolerances are not necessarily related (Munns and Termaat, 1986; Munns and Tester, 2008).

B. Long-Term Responses: Long-term responses can be seen through increased osmotic tolerance and increased tissue tolerance and both will lead to an increased ability to maintain growth for a given accumulation of Na^+ in the leaf tissue. However, they can be distinguished because of their differential effects on younger versus older tissue. Increased osmotic tolerance will be mainly evident by an increased ability to continue production of new leaves, whereas tissue tolerance will be primarily evident by the increased survival of older leaves (Munns and Tester, 2008). Negrao, *et al.* (2011) explained the long-term responses by osmotic adjustment, Na^+ exclusion and K^+ homeostasis and regulation of antioxidants.

a) Osmotic adjustment: This is the first tolerance response happening during high salinity stress. When plant sense Na^+ in the soil solution or in the apoplastic solution then it activates mechanisms that prevent cell dehydration, protein denaturation (including proteins of photosystem II) and destabilization of cellular structures (Yancey, *et al.* 1982). Nontoxic metabolites, known as "compatible solutes" accumulate in the cytoplasm in order to adjust osmotic potential between the cytosol and apoplastic solution. Various sugars (fructose, glucose and sucrose), complex sugars (trehalose, raffinose and fructans), sugar alcohols (mannitol and glycerol) and amino acid and derivatives (proline, glycine-betaine, and proline-betaine) have been suggested to accomplish this function (Bohnert and Shen, 1999; Bohnert, *et al.* 1995; Flowers and Colmer, 2008). In addition to the accumulation of certain organic compounds known as 'compatible solutes' in the cytosol under salinity/osmotic stress conditions, ion accumulations in the cytosol (mainly K^+) and in the vacuole (Na^+, especially in salt tolerant cultivars/species) are also found to be important for the osmotic adjustment of plant cells (Gorham, *et al.* 1985). Compatible solutes were initially determined as compounds that are non-toxic even when they are highly accumulated in the cytosol and contribute to decrease the cytoplasmic water potential. In addition to the role in osmotic adjustments, compatible solutes seem to function as a chaperone protecting enzymes and membrane structures, and as a scavenger reducing radical oxygen species under stress conditions including salinity stress (Bohnert and Shen, 1999). Rice has two genes encoding

the betaine aldehyde dehydrogenase, which catalyzes betaine aldehyde to glycine betaine (GB), a compatible solute. However, rice cannot synthesize GB because of the lack of an upstream enzyme, the choline monooxidase (CMO), which convert a choline to a betaine aldehyde. Introductions of spinach CMO genes or the *Arthrobacter pascens* choline oxidase into rice plants promoted the synthesis of GB in the transgenic rice plants (Sakamoto, *et al.* 1998; Shirasawa, *et al.* 2006). However, only relatively small amount of GB accumulation and slight enhancement of salt tolerance of transgenic rice plants were observed in some conditions tested, probably because of low activities and/or miss-localization of the introduced enzymes (Shirasawa, *et al.* 2006). Rice transformed with BADH from barley and fed exogenous glycinebetaine shows increased salt tolerance (Kishitani, *et al.* 2000). Others have also shown exposure to exogenous glycinebetaine can enhance tolerance to salt (Harinasut, *et al.* 1996; Demiral and Turkan, 2004) and drought (Farooq, *et al.* 2008). In rice, the most remarkably enhanced compatible solute is proline. The proline accumulation rate increases under salt stress especially in tolerant rice genotypes, confirming its protective role against hyperosmotic stress (Demiral and Turkan, 2006). This accumulation of compatible solutes in the cytoplasm also helps to balance the ion osmotic pressure in the vacuole, where Na^+ and Cl^- are sequestered. Garg, *et al.* (2002) introduced *OtsA* and *OtsB* (*E. coli* trehalose-6-phosphate synthase) genes in rice and observed increased tolerance to salt, drought, and cold in the transgenic plants. The trehalose-6-phosphate synthase gene (*OsTPS1*) overexpressing rice lines showed improved tolerance to cold, high salinity and drought treatments without any morphological changes. These lines also exhibited higher expression of *RAB16C*, *HSP70*, and *ELIP* and *OsTPP1* and *OsTPP2* (Li, *et al.* 2011). The metabolic rearrangements and regulatory networks controlling osmolyte levels are therefore pivotal to understanding plant salinity tolerance. Molecular analyses have shown that salt stress stimulates proline synthesis whereas its catabolism is enhanced during recovery (Szekely, *et al.* 2008; Sharma and Verslues, 2010). During this recovery phase, proline may function as an essential signaling molecule and has been proposed to regulate cell proliferation, cell death and expression of stress-recovery genes (Szabados and Savoure, 2010). In *Arabidopsis*, knockout of the *P5CS1* gene, which encodes a Δ-1-pyrroline-5-carboxylate synthetase, impairs proline synthesis resulting in salt hypersensitivity (Szekely, *et al.* 2008). For many years, it was presumed that proline plays a crucial role in osmotic adjustment; however, alternative suggestions are that it acts as a reactive oxygen scavenger, redox buffer, or molecular chaperone, stabilizing proteins and membrane structures under stress conditions (Ashraf and Foolad, 2007; Verbruggen and Hermans, 2008). Like proline,

glycine betaine is an organic osmolyte synthesized by several plant families to balance the osmotic potential of intracellular ions under salinity. There is evidence that glycine betaine is a compatible solute involved in protecting major enzymes and membrane structures (Guinn, *et al.* 2011; Raza, *et al.* 2007). Although glycine betaine has been reported to play a vital role in maintaining the activities of ROS scavenging enzymes (Chen and Murata, 2011), there is no evidence showing whether or not glycine betaine has any direct ROS scavenging capability.

b) Na^+ exclusion and K^+ homeostasis: Over-accumulated Na^+ in the cytoplasm during salinity stress develops toxicity and disturbs essential cellular metabolisms such as protein synthesis, enzyme activity and, in the case of cells that compose the source organ, photosynthesis (Yeo and Flowers 1986; Glenn, *et al.* 1999; Tsugane, *et al.* 1999; Blaha, *et al.* 2000). At the whole plant level, salinity stress leads to Na^+ over-accumulation in shoots particularly in old leaves, and many reports have suggested that restricting Na^+ accumulation in shoots under salinity stress is associated with salt tolerance of wheat and barley (Jeschke, 1984; Gorham, *et al.* 1990; Munns and James, 2003; Garthwaite, *et al.* 2005). Moreover, it has been also reported that Na^+ accumulation in shoots is relatively well correlated with the survival of rice plants under salinity stress (Yeo, *et al.* 1990). Ionic stress eventually triggers premature senescence of older leaves with stress symptoms such as chlorosis and necrosis (Munns, 2002; Munns, *et al.* 2006), which in turn significantly reduces growth and productivity of cereals. Therefore, effective strategies for glycophytes to cope with salinity stress are to keep cytosolic Na^+ levels low at the cellular level and to keep shoot Na^+ concentrations low at the whole plant level. In addition to these factors, acquisition and maintenance of K^+ were found to have a considerable impact on plant salt tolerance (Wu, *et al.* 1996; Zhu, *et al.* 1998). Maintenance of high cytosolic K^+/Na^+ ratios especially in shoots have been strongly suggested to be crucial for salt tolerance of glycophyte plants (Gorham, *et al.* 1987; Gorham, *et al.* 1990; Blumwald, 2000; Ren, *et al.* 2005; Sunarpi, *et al.* 2005; Yamaguchi and Blumwald, 2005; Hauser and Horie, 2010). Under salinity stress, it is crucial for plant cells to maintain the low cytosolic Na^+ level while keeping the high level of K^+, resulting in a high cytosolic K^+/Na^+ ratio that is preferable for vital cellular metabolisms (Jeschke, 1984; Blumwald, 2000). It has been shown that the sensitivity of cytosolic enzymes from glycophytes and halophytes to increased salt levels is comparable; suggesting that keeping a high cytosolic K^+/Na^+ ratio is a basic requirement for plant cells under salinity stress irrespective of the innate difference in the salt sensitivity (Glenn, *et al.* 1999). Plants

use three main strategies to achieve a high cytosol K^+/Na^+: exclusion of Na^+ ions entry, extrusion of Na^+ ions out of the cells, and vacuolar compartmentation of Na^+ ions. At whole plant level, exclusion might occur in different ways: 1) selective uptake by root cells, 2) preferential loading of xylem with K^+ rather than Na^+, and 3) retention of Na^+ in the upper part of the root system and in the lower part of the shoot through exchange of K^+ for Na^+ by the cells lining the transpiration stream (Negrao, *et al.* 2011). A strong correlation exists between exclusion capacity and salt tolerance reported by Lee, *et al.* (2003) and Zhu, *et al.* (2004). The electrophysiology of the ion channels and transporters that work together to regulate the net movement of salt across cell membranes is well understood (Amtmann and Sanders, 1999; Hasegawa, *et al.* 2000a). Although rice is not a good excluder, it still excludes at least 94% of the soil Na^+ from the transpiration stream (Munns, 2005). According to the estimate of Yeo and Flowers (1986), even if 99% of arriving Na^+ is successfully sequestered into the expanded rice leaves during salinity stress, the apoplastic Na^+ concentration could reach 500 mM within 7 days, which would lead to severe cell dehydration and stomatal closure. Furthermore, shoot apoplastic Na^+ accumulations were found to be negatively correlated with the survival of rice varieties including a highly salt tolerant cultivar Pokkali (Krishnamurthy, *et al.* 2009; Krishnamurthy, *et al.* 2011). Therefore, reducing Na^+ transport to the shoots via apoplastic bypass flow is one of the primary subjects to solve in order to enhance salinity tolerance of rice plants. At tissue or organelle level, exclusion might occur through intracellular ion compartmentation. This adaptive mechanism is present in most species and is called tissue tolerance. In rice, similar salt concentrations in leaves were found to cause different toxicity levels according to genotypes (Flowers, *et al.* 1985; Yeo and Flowers, 1983; Yeo, *et al.* 1990). Tissue tolerance, involving the removal of Na^+ from the cytosol and compartmentalizing it in the vacuole before the ion has a detrimental effect on cellular processes, is also likely to require the synthesis of compatible solutes and higher level controls to coordinate transport and biochemical processes, thus having a role in both osmoprotection and osmotic adjustment (Munns and Tetser, 2008; Flowers and Colmer, 2008). Three main mechanisms contributing to shoot tissue tolerance have been targeted: accumulation of Na^+ in the vacuole, synthesis of compatible solutes and production of enzymes catalyzing detoxification of reactive oxygen species. Increasing the abundance of vacuolar Na^+/H^+ antiporters (*NHX*), vacuolar H^+ pyrophosphatases (e.g. *AVP1*), proteins involved in the synthesis of compatible solutes (such as proline and glycinebetaine) and enzymes responsible for the detoxification of reactive oxygen species have had differing degrees of success in improving crop salinity tolerance.

Although there is still uncertainty about the primary ions being transported by NHX proteins in planta (Bassil, *et al.* 2011a; Barragan, *et al.* 2012) (and the potential role of these proteins in K^+ transport needs to be kept in mind (Barragan, *et al.* 2012), and a new role has recently been proposed for *AVP1* (Ferjani, *et al.* 2011), salinity tolerant plants appear to have been developed by the overexpression of NHX and vacuolar pyrophosphatase genes. While approaches to improve the tissue tolerance of crops through increas-ing compatible solutes and enzymes involved in ROS metabolism also appear to have been successful, there are often reports of low performance by the transgenic lines in low stress environments (Romero, *et al.* 1997; Sheveleva, *et al.* 1997; Suarez, *et al.* 2009; Cortina, *et al.* 2005; Karakas, *et al.* 1997). Such negative effects might be avoided by use of tightly regulated stress-inducible promoters (Sheveleva, *et al.* 1997; Garg, *et al.* 2002; Vendruscolo, *et al.* 2007).

c) Regulation of antioxidants: Excessive production of cytotoxic reactive oxygen species (ROS) causes oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids (Haliwell, 1986; McCord, 2000). In salt conditions, the reduced rate of photosynthesis activates the Mehler Reaction to produce more harmful oxygen radicals (Hsu and Kao, 2003). Salinity can challenge plants to a degree that may even lead to cell death. Salt stress causes membrane disorganization, metabolic toxicity, formation of reactive oxygen species (ROS), inhibition of photosynthesis, and reduced nutrient acquisition (Hasegawa, *et al.* 2000a; Tuteja, 2007). Ismail, *et al.* (2014) reviewed and discusses the two response modes (adaptation versus cell death) depend on the relative timing of two signal chains: one triggered by calcium and the other triggered by oxidative burst in the apoplast. A delay in generation and dissipation of a salinity-triggered calcium-dependent signal relative to a signal conveyed by ROS will lead in the unconstrained activation of jasmonate (JA) signalling culminating in cell death. In contrast, the same molecular signal carrier (calcium) can, if properly timed, initiate adaptive processes such as sequestration and extrusion of sodium, and induce efficient constraint of JA signaling through the activation of abscisic acid (ABA) signaling. Plants must strictly maintain ROS homeostasis to mitigate the toxicity of ROS. Therefore, plants have employed different scavenging machineries that tightly control ROS levels, both enzymatic and non-enzymatic. Plant enzymatic antioxidant mechanisms include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX),

guaicol peroxidase (GOPX), and glutathione *S*-transferase (GST). The metalloenzyme SOD is the most effective intracellular enzymatic antioxidant and acts by dismutating superoxide to H_2O_2, which in turn can be detoxified by APX, GPX, and CAT. The non-enzymatic antioxidants comprise ascorbic acid (ASH), glutathione (GSH), phenolic compounds, alkaloids, non-proteinogenic amino acids, and α-tocopherols (Apel and Hirt, 2004; Gill and Tuteja, 2010). The quelling of ROS accumulation can also be achieved by other signals, such as NO. NO has the ability to neutralize Fenton-type oxidative damage by scavenging superoxide, therefore preventing the formation of oxidants (such as O^{2-}, H_2O_2, and alkyl peroxides), which makes it easier to recover a redox homeostasis (Lamattina, *et al.* 2003). A correlation between antioxidant capacity and salinity tolerance was also reported in several plant species including rice (Demiral and Turkan, 2004 & 2005; Dionisio-Sese and Tobita, 1998). A higher ascorbate redox state was found to increase the total open stomatal area of rice leaves, thus leading to increased stomatal conductance under salt stress (Chen and Gallie, 2004). In rice tolerant genotypes, the activity of enzymes, such as ascorbate peroxidase and peroxide dismutase, known to be involved in ROS scavenging was either constitutively high or up-regulated by salt stress (Moradi and Ismail, 2007). Halophytes appear to have a much greater ability to maintain net photosynthesis and protect and stabilize both photosystems under saline stress conditions, as compared with glycophytes. A comparison between rice (*Oryza sativa* L.) and its halophytic relative *Porteresia coarctata* has revealed that the latter was more efficient in the active protection of the photosynthetic machinery by increasing the abundance of (i) 33 kDa Mn-stabilizing proteins of the oxygen-evolving complex in PSII; (ii) a chlorophyll *a*/*b* protein (CP47) involved in stabilization of the reaction centre protein D1 of PSII; (iii) a PSI subunit IV protein essential for cross linking ferredoxin-$NADP^+$ oxidoreductase; (iv) RubisCo large subunit; and (v) RubisCo activase, when exposed to 400 mM NaCl stress (Sengupta and Majumder, 2009 and 2010). However, in a recent review, Bose, *et al.* (2014) argued that, truly salt-tolerant species possessing efficient mechanisms for Na^+ exclusion from the cytosol may not require a high level of antioxidant activity, as they simply do not allow excessive ROS production in the first instance. They also suggest that H_2O_2 'signatures' may operate in plant signaling networks, in addition to well-known cytosolic calcium 'signatures' only for halophytes. Finally, Bose, *et al.* (2014) emphasized the importance of non-enzymatic antioxidants as the only effective means to prevent detrimental effects of hydroxyl radicals on cellular structures. Therefore, most probably, increased antioxidant activity should be treated as a damage control mechanism rather than a trait directly conferring salinity stress tolerance.

C. Whole plant responses: Under salinity stress, over a period of acclimation through short-term and long-term responses, rice genotypes showed different types of adaptations based on inherent tolerance or sensitivity. Moradi and Ismail, (2007) reported that the tolerant genotypes had more responsive stomata that tended to close faster during the first few hours of stress, followed by partial recovery after a brief period of acclimation. However, in the sensitive lines, stomatal conductances (gs) continued to decrease for longer duration and with no recovery afterward. This finding is indicative of growth responses at the whole plant level. For better understanding of the responses at the whole plant level, this response is discussed below through vegetative and reproductive growth regulation:

a) Regulation of vegetative growth and development: Osmotic or water-deficit effect of salinity can cause overall growth reduction due to reduced uptake of water. Again, if excessive amounts of salt enter the plant in the transpiration stream there will be injury to cells in the transpiring leaves and this may cause further reductions in growth. This is called the salt-specific or ion-excess effect of salinity (Greenway and Munns, 1980). Within hours, cells regain their original volume and turgor owing to osmotic adjustment, but despite this, cell elongation rates are reduced (Cramer, 2002; Fricke and Peters, 2002; Passioura and Munns, 2000; Yeo, *et al.* 1991). Over days, reductions in cell elongation and also cell division lead to slower leaf appearance and smaller final size. Cell dimensions change, with more reduction in area than depth, so leaves are smaller and thicker (Munns and Tester 2008). If the intensity of the salt stress is high, and/or the expression of tolerance mechanisms is not sufficiently high to effectively exclude salt from the transpiration stream, leaves that have been transpiring for the longest time will accumulate salt to toxic levels, finally causing their death. As new leaf growth is supported through the export of carbon from mature leaves, the fate of the plant depends on the balance between the rate at which mature leaves die and young leaves are produced. Plant death may occur if the rate of leaf death overtakes the rate of new leaf initiation and surface expansion, since these supply thephotosynthates required for further growth and development (Munns and Tester, 2008). This is due to changes in cell osmotic and ionic status, increased concentration of organic osmolytes and growth regulators such as ABA, reduced membrane permeability, lower intercellular CO_2 partial pressure, lower guard cell turgor and stomatal conductance (Dionisio-Sese and Tobita, 2000), decreased efficiency of photosynthetic apparatus (Moradi and Ismail, 2007) and feedback inhibition due to reduced sink activity (Chaves, *et al.* 2009;

Munns and Tester, 2008). Apparently, under salt stress, shoot growth is usually more affected than root growth.

b) Regulation of reproductive growth and development: The ultimate aim of any crop plant isto complete the life cycle by reaching successful reproduction. In rice, if the stress is severe (NaCl >100 mM), plants die before maturity. Under less severe conditions (NaCl <50 mM) salt stress induces delayed panicle initiation and flowering (Grattan, *et al.* 2002) and also leads to poor seed set through reduced pollen viability. Yield parameters such as tillering, spikelet number, sterility and grain weight may be affected by the Na^+ concentration in the panicle (Khatun and Flowers, 1995). Pollen viability is much dependent on ionic toxicity. At plant level, an improved Na^+ partitioning between older tissues/leaves may protect the developing panicles from excessive Na^+ accumulation (Mohammadi-Nejad, *et al.* 2010). In fact, tolerant genotypes tend to exclude salt from flag leaves (crucial organs for carbohydrate synthesis at the reproductive stage) and panicles (Lisa, *et al.* 2011; Moradi, *et al.* 2003; Yeo and Flowers, 1986).

2.8 Mechanisms of salinity tolerance in rice

Considering above discussion about plant responses to salinity stress i.e. short-term, long-term and whole plant responses, in rice salinity tolerance may take place at three levels of the whole plant (Jeschke and Hartung, 2000, Munns, *et al.* 1983; Munns and Tester, 2008), cellular (Munns, *et al.* 1983; Munns and Tester, 2008), and molecular levels (Blumwald, 2000; Munns, *et al.* 2002), and includes physiological mechanisms:**(1) salt exclusion:** plants do not take up excess salt by selective absorption; **(2) salt reabsorption:** tolerant varieties absorb excess salt but it is reabsorbed from the xylem and Na^+ is not translocated to the shoot; **(3) root-shoot translocation:** salinity tolerance is associated with a high electrolyte content in the roots and a low content in the shoot; **(4) salt translocation:** tolerant plants have the ability to translocate a lesser proportion of Na^+ to the shoot; **(5) salt compartmentation:** excess salt is transported from younger to older leaves; **(6) tissue tolerance:** plants absorb salt but are properly compartmentalized in vacuoles within the leaves in order to lower the harmful effects on plant growth; and **(7) salt dilution:** plants take up salt but is diluted by fast growth rate and high water content in the shoot (Yeo and Flowers, 1984). Moreover, Yeo and Flowers, (1986) argued that, salinity resistance is not

conferred by a single factor, but is indeed the sum of many contributory physiological traits, which are not necessarily linked. They suggested that the growth of rice in saline conditions can be increased beyond the present phenotypic range by the use of physiological criteria to select independently for individual contributory genetic traits, which can subsequently be combined.

2.9 Physiological traits associated for salinity tolerance in rice: Current understanding

The above mentioned morpho-physiological mechanisms underlying salinity tolerance are important to understand how rice plants adapt to stressful conditions of salinity, but important questions remain regarding how to determine correct target traits for the improvement of salinity tolerance because there is no convincing evidence that any salinity tolerance mechanisms (traits) would be sufficient to confer rice plants' ability to adapt well to specific stress conditions. Studies of the effects of salinity on rice have indicated that salinity damage, and consequently adaptation to salinity, is complex. No single process can account for the variation in the plant's response to salinity; the subsequent distribution of salt within the plant is as important as the uptake of salt in the first place (Yeo and Flowers, 1984; 1986 and 1989). In a recent study of Pires, *et al.* (2015) analyzed phenotypic data of 56 rice genotypes and established that none of the three salinity tolerance mechanisms i.e. shoot-ion independent tolerance (or osmotic tolerance), ion exclusion, and tissue tolerance is predominant among rice genotypes. They also found that, the K^+ concentration was not significantly affected by salt stress in rice. However, in a previous study by Kanawapee, *et al.* (2012) observed a decrease in K^+ content combined with an increase in Na^+ content with growth in saline conditions. Consequently, the authors suggested that the K^+/Na^+ ratio is the most important mechanism controlling salinity tolerance in rice. It has been often observed that the accumulation of Na^+ in shoot tissues is accompanied by a reduction in shoot K^+ concentrations, resulting in decreased K^+/Na^+ ratios (Asch, *et al*. 2000). In fact, Garcia, *et al.* (1997a) concluded that in rice K^+/Na^+ is less relevant as a trait than the individual content of Na^+ and K^+, contrary to what might happen in wheat. Their conclusions were based on the fact that in rice Na^+ uptake is mechanistically different from K^+ uptake (Garcia, *et al.* 1997a). This observation is particularly important because several studies assume that a low K^+/Na^+ ratio is the most important goal in terms of ion concentrations in rice salinity tolerance and emphasize this value (Theerakulpisut, *et al.* 2011, Kanawapee, *et al.* 2012).

De Leon, *et al.* (2015) showed a significant genotypic variation and correlations among the traits such as salt injury score (SIS), ion leakage, chlorophyll reduction, shoot length reduction, shoot K^+ concentration, and shoot Na^+/K^+ ratio while studying with 49 rice genotypes. However, they also suggested that instead of considering only visual SISs, other parameters, such as ion leakage, chlorophyll concentration, shoot length, shoot K^+ concentration, and shoot Na^+/K^+ ratio could be unbiased parameters for assessing salinity tolerance. Interestingly, they did not find significant correlations among rice genotypes in the shoot sodium uptake. However, De Leon, *et al.* (2015) also pointed out and emphasized to use high shoot K^+ concentration and low Na^+/K^+ ratio traits from the donor such as FL378, Damodar, Hasawi, Ketumbar, PSBRC50, Cheriviruppu and IR2706-11-2 for improvement of salinity tolerance, which is contrary to the results presented by Pires, *et al.* (2015) (see above paragraph). Above disagreement could be due use of different groups of genotypes for example Pires, *et al.* (2015) used 59 genotypes having salinity tolerant genes mostly Os*NHX*1 and Os*HKT*1;5 are Na^+ and K^+ transporters, Os*CPK*17 and Os*RMC* seem to be involved in salt stress signal perception and transduction pathways, and Os*Sal*T is possibly associated with the production of compatible solutes (Negrão, *et al.* 2013). This suggesting the salinity tolerance among the tolerant varieties used is not a function of restricting sodium uptake, but more likely in the compartmentalization of sodium to alleviate its toxic effect (Blumwald, 2000). However, this could be due to lesser exposed under salinity experimental condition, because Ul Haq, *et al.* (2014) showed that earlier measurements of Na^+ or of injury had lower correlations, the most sensitive growth components were tiller number $plant^{-1}$ and shoot water content (g water g^{-1} dry weight), and these were correlated significantly with Na^+ and, to a lesser extent, with K^+/Na^+. These studies also showed that exposure for at least 42 days may be needed to clearly demonstrate the beneficial effect of the trait for Na^+ exclusion on growth under salinity. Pires, *et al.* (2015) showed the level of growth reduction is not directly associated with salinity tolerance as measured by the SES score while combined analysis of traits and salinity tolerance indices of 56 rice genotypes. They found growth reduction can be the same in plants with differing biomass, but the plant with more biomass will, in general, be able to tolerate salt much better. They also found that, higher the Na^+ levels in the 3rd leaf, the higher the SES score (Platten, *et al.* 2013), genotypes with similar levels of Na^+ accumulated in leaves can exhibit differences in their physiological responses to salt stress. Considering above differences while analyzing different groups of genotypes, Pires, *et al.* (2015) recommended some common and important aspects such as proper controls have to be used and analyzed;

when there is *a priori* information on the function of a certain gene under study, experiments should be designed to look into the specific salinity tolerance mechanism; quantitative data on the trait of interest should be presented; tolerance scores measure a combination of several tolerance mechanisms each with an unknown percentage of contribution to the final phenotype of the plant/genotype under study and genome-wide analysis (GWA) where the trait being associated is a tolerance score are hard to interpret. Because a tolerance score is the end result of a combination of salinity stress tolerance mechanisms, genes implicated in each of those mechanisms can associate with phenotype, which makes it hard to identify the candidate gene(s) for each peak and its (their) function, therefore it is suggested to used specific traits rather than combined trait.

Previous attempts to characterize salt-tolerant rice varieties were done using morphological traits (Caldo, *et al.* 1996; Zeng, *et al.* 2003; Sanni, *et al.* 2012). In most breeding strategies, the simple visual salt injury scoring (Gregorio, *et al.* 1997) is widely used for characterization because it reflects the overall plant's response to salt stress. However, the inherent subjectivity and the quantitative nature of salinity tolerance complicate the evaluation for salinity tolerance. Thus, other studies suggest the use of Na-Ca selectivity (Zeng, *et al.* 2003), tiller number and Na-K selectivity (Zeng, 2005), and proline concentration (Kanawapee, *et al.* 2012) as criteria for classification of rice varieties for salt tolerance. However, varietal differences showed that it is natural for varieties to be superior in one trait and inferior in others (Yeo, *et al.* 1990). Though there is no single definite morphological marker available for salt tolerance or sensitivity in rice, but a combination of criteria give a good indication toward the salt response of crop plants. Therefore, several parameters are used in combination for the effective and reproducible results for salt tolerance and sensitivity.

2.10 Breeding strategies to improve salinity tolerance

Understanding of the genetic control of the trait and the physiology of tolerance is the basis to improve salinity tolerance. Breeders need a simple and efficient mass screening technique, access to adequate genetic variability.

(a) Screening techniques: Breeding for salinity tolerance in rice requires reliable screening techniques. These techniques must be rapid to keep pace with the large amount of breeding

materials generated. Screening under field conditions is difficult due to stress heterogeneity, presence of other soil-related stresses, and the significant influence of environmental factors such as temperature, relative humidity, and solar radiation. These complexities, together with the degree of control of salinity and reproducibility, cause difficulties in developing and using reliable methods of screening voluminous materials. A screening method for salinity tolerance could be readily acceptable when based on a simple criterion for selection; it provides rapid screening of large number of materials and reproducible results (Gregorio, *et al.* 1997; Yeo, *et al.* 1990). Hydroponic screening is fast, easy to control, and well adapted to the high volume of material coming from breeding programs. But in such conditions, transpiration is too low to be representative of field conditions, and the imposed stress is either often not gradual enough or is too severe (Flowers, *et al.* 2000).

As mentioned above, the plant development stage at which the stress is imposed is also important, with the most sensitive stages corresponding to early seedling (2–3 leaf stage), pollination, and fertilization (Gregorio, *et al.* 1997). Detailed screening procedures have been proposed (Gregorio, *et al.* 1997) and discussed (Singh, *et al.* 2007; Yeo, *et al.* 1990). These procedures generally target salinity tolerance as a whole or assess the effect of salt on yield components or various organs. Few specific large scale physiological screening techniques targeting individual mechanisms have yet to be proposed to enable dissecting the different plant reaction types. An example of such a specific technique is the method based on electrical potential differences between the external solution and the root vacuoles to measure sodium exclusion allowing the evaluation of 50 to100 plants a day (Chowdhury, *et al.* 1995). Another interesting and recent technique was used by (Rajendran, *et al.* 2009), who combined image capture and analysis equipment, with flame-photometry for quantitative assays of osmotic tolerance, Na^+ exclusion and Na^+ tissue tolerance. Although applied in *Triticum*, this may be an interesting technology to explore in rice. Depending on the objectives and resources available for the experiments, the traits most commonly assessed include salinity-induced injuries, Na^+ and K^+ uptake and their concentrations in various tissues and organs. Injuries can be measured by visual scales or survival rates, relative development, growth rates, or biomass production of different organs (e.g., tillers, leaves, or roots) in saline *versus* control conditions over a period of time. To take into account correlations between these parameters, clustering based on multiple traits has also been used (Zeng, *et al.* 2002). The correlation of survival and visual assessment of salt damage with physiological parameters, however, seems to have limited links, and Yeo, *et al.*

(1990) suggest using overall performance to identify tolerant accessions for breeding programs.

(b) Collection and characterization of genetic variability for salinity tolerance in rice: Rice is generally considered as a salt sensitive species but natural variability for salt tolerance is encountered. The International Rice Research Institute alone screened approximately 100,000 varieties for salt tolerance during the1969–1984 periods using visual damage scoring. The materials included traditional varieties from salt prone regions and progenies from general and specific breeding programs. Of these, about 20% were rated tolerant (score 1 to 3), indicating that salinity tolerance was not a widespread trait (Senadhira, 1994). *Oryza sativa* accessions are known to be genetically organized into two major groups (*indica* and *japonica*) and four several minor groups (Garris, *et al.* 2005; Glaszmann, 1987). Understanding the relationships between varietal groups and phenotypic variation for salt tolerance is particularly important to know where to mine for interesting alleles. Previous studies enabled the identification of a few landraces or improved varieties tolerant by one mechanism or another (Gregorio, *et al.* 2002; Ismail, *et al.* 2007; Lisa, *et al.* 2004; Mohammadi-Nejad, *et al.* 2010; Yeo, *et al.* 1990). The traditional tolerant varieties from coastal areas of South Asia such as Nona Bokra (India), Pokkali (India and Sri Lanka), Getu (India), Kalarata 1-24 (India), Cheriviruppu (India), SR26B (India), Damodar (India), Pat(India) and Solla (India) or from coastal areas of Bangladesh like Jamainaru, Lakshmikajal, Patnai Balam, Horkuch, Morichshail, Ashfal, Raniselute and Kajalshail and other countries such as Ketumbar (Indonesia), Khao Seetha (Thailand), or Soc Nau (Vietnam) most of all belongs to the *indica* group. IRRI breeding lines such as IR4630-22-2-5-1-3 (a donor of leaf compartmentation), IR45427-2B-2-2B-1-1, IR51500-AC11-1 (later named as PSBRc50), IR51500-AC17 named as CSR21, IR51485-AC6534-4 named as CSR28, IR52724-2B-6-2B-1-1, IR60167-129-3-4 (a donor for tissue tolerance), IR63731-1-1-4-3-2 or IR65192-4B-10-3 (mentioned as checks for tolerance from IRRI), IR66946-3R-178-1-1 and IR66946-3R-78-1-1 also known as FL478 and FL378 respectively, or breeding lines from other breeding programs such as CSR10, CSR13, CSR27 or CSR30 from CSSRI-Karnal in India, having often one of the above-mentioned traditional varieties as director remote parent, are also *indica* type. More recently, another *indica* type variety from Saudi Arabia, Hasawi, showed a surprisingly high salt tolerance, as compared to the other well-known salt-tolerant varieties Pokkali and Nona Bokra (Glenn Gregorio, IRRI, personal communication in 2010).

Recent studies with diverse set of germplasms were characterized to identify novel sources of salt tolerance through physiological and SNP assays. Based on SES score, vigor, chlorophyll content and Na^+/K^+ ratio several germplasms i.e. Akundi, Ashfal, Capsule, Cheriviruppu, Chikirampatni, Kalarata, and Kutipatnai from Bangladesh and India were proposed for good donors and alternative novel sources of tolerance to salinity at seedling stage (Rahman, *et al.* 2016). Another study was conducted with 180 *Japonica* accessions from the European Rice Core collection (ERCC) and revealed that under moderate salinity stress some accessions achieved the same level of control of Na^+ concentration and Na^+/K^+ equilibrium as the *indica* reference variety for salinity tolerance Nona Bokra, although without sharing the same alleles at several loci associated with Na^+ concentration. These indicating differences between *indica* and *japonica* in the effect of salinity tolerance and further potential for the improvement of tolerance to the salinity above the level of Nona Bokra (Ahmadi, *et al.* 2011).

Another study was carried out recently at IRRI with 103 accessions from *O. sativa* and 12 accessions from *O. glaberrima* to infer diversity of mechanisms used by these species to cope with salt stress, and to identify varieties displaying additional physiological and/or genetic mechanisms to confer higher tolerance. Results revealed that the association of leaf Na^+ concentrations with cultivar-groups was very weak, but association with the *OsHKT1;5* allele was generally strong. Seven major and three minor alleles of *OsHKT1;5* were identified, and their comparisons with the leaf Na^+ concentration showed that the *Aromatic* allele conferred the highest exclusion and the *Japonica* allele the least. The majority of accessions display a strong quantitative relationship between tolerance and leaf lade Na^+ concentration, and thus the major tolerance mechanisms found in these species are those contributing to limiting sodium uptake and accumulation in active leaves. However, there appears to be genetic variation for several mechanisms that affect leaf Na^+ concentration, and rare cases of accessions displaying different mechanisms also occur. These mechanisms show great promise for improving salt tolerance in rice over that available from current donors (Platten, *et al.* 2013).

Other than cultivated accessions from the primary gene pool, some work has also been conducted on wild relatives. From those, only *Porteresia coarctata* Tateoka, found in brackish water in Asian coastal mangrove areas, has been extensively studied for its potential as donor of salt tolerance to rice (Bal and Dutt, 1986; Flowers, *et al.* 1990; Latha, *et*

al. 1998; Latha, *et al.* 2004; Sengupta and Majumder, 2010; Garg, *et al.* 2013). However, so far, the use of *P. coarctata* in rice breeding programs is limited due to the difficulty in recovering viable embryos (Gregorio, *et al.* 2002). But a recent report claims successful hybridization of *Porteresia coarctata*to modern rice IR36 and IR28 through a special embryo rescue technique (K. K. Jena) (http://archive.gramene.org/newsletters/rice genetics/rgn11/v11p78.htm). The crossability was found very minimum ranged from 0.009% to 0.13%. Chromosome counts of the hybrids were made at diakinesis and metaphase-I stages of meiosis and were found to have 36 chromosomes therefore the hybrids are male sterile and being multiplied vegetatively for backcrossing with *O. sativa* as recurrent parents.

(c) Molecular markers and Quantitative trait loci (QTL) identification: Markers provide signs or flags through which a species can be recognized or identified in a particular trait of interest. In our daily life a particular sign that are permanent would be or not heritable readily used for identification of a particular person in a number of cases. Likewise in crop plants there are three major types of genetic markers: (1) morphological (classical or visible), (2) biochemical (allelic variants of enzymes called isozymes) and (3) DNA (Deoxyribo Nucleic Acid) (molecular) markers, which reveal sites of variation in DNA (Winter and Kahl, 1995; Jones, *et al.*1997). Genetic markers represent genetic differences between individual organisms or species. Genetic markers that are located in close proximity to genes may be referred to as gene 'tags'. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait (Collard, *et al.* 2005).The major disadvantages of morphological and biochemical markers are that it may be limited in number and are influenced by the environmental factors or the developmental stage of the plant. DNA markers are the most widely used type of marker predominantly due to their abundance and are not affected by the environment as well as the development stages of the plant (Winter and Kahl, 1995). Paterson, (1996) reported that it arise from different classes of DNA mutations, such as substitution mutations, rearrangements or errors in replication of tandemly repeated DNA. These markers are selectively neutral because they are usually located in non-coding regions. DNA markers may be broadly divided into three classes based on the method of their detection: (1) hybridization-based; (2) Polymerase Chain Reaction (PCR)-based and (3) DNA sequence-based (Winter and Kahl, 1995; Jones, *et al.* 1997; Gupta, *et al.* 1999; Joshi, *et al.* 1999). DNA markers readily used in breeding RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), RAPD (Randomly

Amplified Polymorphic DNA) and SSR (Microsatellites/Simple Sequence Repeats). Most recently, the discovery and use of SNPs (Single Nucleotide Polymorphisms) would become very popular to the breeders. Recent advances in genotyping single nucleotide polymorphism (SNP) markers have introduced more efficient and cost-effective marker systems with varying levels of multiplexing to suit a range of genetics and breeding applications. SNPs are the most abundant type of polymorphism in plant genomes, with recent studies identifying approximately five SNPs/kb across 20 Arabidopsis accessions and nine SNPs/kb across 517 resequenced rice varieties (Clark, *et al.* 2007; Huang, *et al.* 2010). Thus SNP markers are ideal for high resolution genotyping for association studies, rapid genome-wide scans for genomic selection, as well as routine genetic diversity analysis, linkage mapping, and marker-assisted selection (MAS) (Tung, *et al.* 2010; McCouch, *et al.* 2010). One key benefit is that modern SNP genotyping techniques combined with improved informatics algorithms provide robust automated allele calling, producing high quality data that can be easily merged across groups and stored in databases no matter which genotyping platform is used. Further facilitating computational analysis is the fact that SNPs are usually bi-allelic (having two alleles per locus) due to the low frequency of single nucleotide substitutions at SNP loci and a bias towards transitions over transversions (Vignal, *et al.* 2002). Moreover, many of the recent SNP genotyping systems enable efficient, high-throughput processing of samples with increased speed and lower cost per data point compared to previous marker platforms. For many genetics and breeding applications involving large populations, the cost per sample is more important than the cost per data point, highlighting the need to select an optimal number of loci for each application. The choice of using DNA markers may depend on the availability of characterized markers or the appropriateness of particular markers for a particular species (Collard, *et al.* 2005).

Linkage map can only be used to detect the gene(s) or QTL(s) controlling the trait of interest thus, Paterson, (1996) called it the ‘road map’ of chromosomes derived from two different parents. It indicates the position and relative genetic distances between markers along chromosomes, which is analogous to signs or landmarks along a highway (Collard, *et al.* 2005). The detection of QTLs (Quantitative Trait Loci) has enabled large progress in understanding the genetic control of the trait.QTL mapping is based on the principle that genes and markers segregate via chromosome recombination during meiosis, thus allowing their analysis in the progeny (Paterson, 1996). Linked markers are grouped together into ‘linkage groups’, which represent chromosomal segments or entire chromosomes. Referring

to the road map analogy, linkage groups represent roads and markers represent signs or landmarks (Collard, *et al.* 2005). The distance on a linkage map is not directly related to the physical distance of DNA between genetic markers, but depends on the genome size of the plant species (Paterson, 1996).

A gene(s) or chromosomal region that influences in quantitative attributes is called quantitative trait locus/loci (QTL). Most agronomically important traits are controlled by more than one gene called quantitative traits. Conventional method cannot detect genetic loci associated with quantitative traits, their location and linkage relationship with other genes, but can be easily done with molecular markers. The process of constructing linkage maps and conducting QTL analysis to identify genomic regions associated with trait is known as QTL mapping, which is also known as 'genetic', 'gene' or 'genome' mapping (McCouch and Doerge, 1995; Paterson, 1996; Mohan, *et al.* 1997). QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured (Tanksley, 1993; Young, 1996).

Single-marker analysis, simple interval mapping and composite interval mapping are the three widely used methods for QTL detection (Tanksley, 1993; Liu, 1998). Among the following method the composite interval mapping combines both interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping (Jansen, 1993; Zeng, 1993 & 1994; Jansen and Stam, 1994). An individual QTL may be described as major or minor based on the proportion of the phenotypic variation explained by a QTL (R^2 value), major and minor accounts for >10% &<10%, respectively. QTL that are stable across the environment sometimes referred as major while sensitive to the environment called as minor, especially for QTL that are associated with disease resistance (Li, *et al.* 2001; Lindhout, 2002; Pilet-Nayel, *et al.* 2002).

Surprisingly few studies, of heterogeneous quality, have been conducted on salinity tolerance in rice (Ammar, *et al.* 2007; Bonilla, *et al.* 2002; Flowers, *et al.* 2000; Haq, *et al.* 2010; Kim, *et al.* 2009; Koyama, *et al.* 2001; Lee, *et al.* 2007a; Lin, *et al.* 2004; Prasad, *et al.*

1999; Sabouri, *et al.* 2009; Takehisa, *et al.* 2004; Alam, *et al.* 2011). A list of QTLs linked with salinity tolerance in rice can be found in Gramene (http://www.gramene.org). More detailed information on these QTLs has been compiled in the Rice module of the TropGene database (http://tropgenedb.cirad.fr) following a procedure identical to that used to compile QTLs of drought tolerance (Courtois, *et al.* 2009). Singh, *et al.* (2007b) summarized the reports on QTL for salinity tolerance in rice starting from Claes, *et al.* (1990) report on *Sal*T locus on chromosome 1 that was responsible for "Jacalin-like lectin domain protein" of 146 amino acids. The review extended to the QTL work on seedling stage salinity tolerance of Lee. *et al.* (2006) and this list was updated with several more recent studies (Singh and Flowers, 2010). Most mapping populations used for QTL analysis were *indica* × *japonica* populations originally developed for other purposes, such as IR64 x Azucena or Co39 × Moroberekan. A few of those were developed specifically for salinity tolerance but involved a very limited number of resistant parents (either Nona Bokra or Pokkali or varieties derived from these parents such as CSR27). Most studies used RIL, DH or $F_{2:3}$ mapping population, but Kim, *et al.* (2009) used an advanced backcross population more interesting for the rapid development of pyramiding approaches. The most interesting studies are those computing indices that link parameters of stress and control conditions or measure ion concentrations in shoots or roots. Many of these studies, however, still consider salinity as a single character. The various components of salinity tolerance appear to be polygenically controlled. QTLs have been detected repeatedly on chromosomes 1, 4, 6, and 7. None have been found on chromosomes 8 and 11 and very few on chromosomes 2, 3, 5, 9, 10 and 12. However, it should be highlighted that most of these studies have been conducted under hydroponic conditions and only one used field conditions (Takehisa, *et al.* 2004). None of the QTLs designated as the most significant under field conditions co-localized with QTLs detected under hydroponics. Similar evidence was obtained in barley where salinity stress was shown to have a different impact in plants grown under hydroponics *versus* soil systems (Tavakkoli, *et al.* 2010). The QTL studies also led to the conclusion that different *loci* were involved in the different plant responses under short *versus* long term salt stress (Haq, *et al.* 2010).

(d) Major salinity tolerance QTL (Saltol): A major QTL designated "*Saltol*" was mapped on chromosome1 using a RIL population generated from a cross between the sensitive variety IR29 and a tolerant landrace, Pokkali. This QTL was responsible for Na^+ and K^+ absorption as well as Na^+/K^+ ratio and each accounted for more than 60% of the variation in this population (Gregorio, 1997; Bonilla, *et al.* 2002). Early studies reported the QTLs

responsible for physiological parameters conferring seedling stage salinity tolerance in rice on different chromosomes (Flowers, *et al.* 2000; Koyama, *et al.* 2001; Lang, *et al.* 2001), and substantiated the independence of Na^+ and K^+ uptake as they were located on different linkage groups. Some of the studies have shown co-localization of QTLs for Na^+, K^+, and/or their ratios on chromosome 1 (Gregorio, 1997; Koyama, *et al.* 2001; Niones, 2004) and chromosome 12 (Lang, *et al.* 2001). Indeed, Na^+/K^+ ratio is a derived trait but a balance of both ions is very important within cytosol; thus it indicates that uptake of both the ions either could be due to the linkage or pleiotropic effects within the same gene complex that is quite desirable (where the QTLs for different traits are co-localized) or could be due to probable epistatic interactions (where QTLs for the different traits are on separate linkage group). So far, the most systematic study for QTL identification and map-based cloning of genes responsible for salt tolerance was the identification of *qSKC1* controlling K^+/Na^+ homeostasis under salt stress and encodes an *OsHKT*-type transporter (Lin, *et al.* 2004; Ren, *et al.* 2005). Nona Bokra was used as the salt-tolerant donor and the population was derived from an *indica*/*japonica* (Koshihikari) cross. *SKC1* (*Os01g20160*) is located within the *Saltol* locus. This is a large-effect QTL with LOD score of 11.7 and explained more than 40% of the phenotypic variation (Lin, *et al.* 2004). Ren, *et al.* (2005) reported the further progress of map-based cloning of *qSKC1*. They identified a NIL with a very small Nona Bokra introgression involving *SKC1* regionin the Koshikari background that had substitution of 6 nucleotides. The substitution is responsible for the altered protein by 4 amino acids, which probably is responsible for the functional difference of *SKC1* from Nona Bokra and Koshihikari. Two very closely flanking CAPS (cleaved amplified polymorphic sequence) markers, K159 and K061, were developed to identify *SKC1*.

The *Saltol* QTL was frequently mapped and cited by a number of researchers by using original RIL & NIL populations (Elahi, *et al.* 2004; Alam, *et al.* 2011) and different population (BRRI dhan40/NSICRc106) by Islam, *et al.* (2011). But the effect and position was differed due to use of different number of populations, markers and methods of QTL detection. The S*altol* QTL of Pokkali was further confirmed in three breeding populations by 20 SSR and two EST markers and finds a good parallel among the linkage maps of the three populations to the previous QTL map that identified *Saltol* (Islam, 2006). A number of research have been carried out to characterize *Saltol* QTL through haplotyping, marker segregation analysis, and delineation the size of *Saltol* in to the short arm of chromosome 1 for direct use in breeding programs (Elahi, *et. al.* 2004; Islam, 2006; Mohammadi-Nejad, *et.*

al. 2008; Aliyu, *et. al.* 2011, Islam, *et al.* 2012). However, the number and position of markers were found different from different researches and none of the researcher's able to provide concrete decision for the most tightly linked markers and size of the QTL segment.

Fine mapping of the *Saltol* QTL at IRRI using several sets of NILs derived from the cross IR29 × Pokkali. Marker saturation of the region has incorporated more than 30 SSRs from the IRGSP and custom-designed insertion/deletion (indel) markers at gene loci across the QTL peak region from 10.7 to 12.5 Mb (Thomson, *et al.* 2007). Four major genes, especially for transporters and membrane/stress proteins within *Saltol* region as annotated using genome browser of erstwhile online TIGR rice genome project (which is now moved to Michigan State University (MSU) and can be browsed using MSU Rice Genome Project (http://rice.plantbiology.msu.edu/), and an additional nearby gene (*Sal*T at 13.8 Mb) are targeted for the development of gene-based PCR markers (encircled in Figure 2.5). The availability of a large number of gene-based markers or fine-mapped QTLs underlying salinity-tolerance component traits could help in pooling of different tolerance mechanisms to enhance the level of salt tolerance in agronomically superior adapted varieties using MAS.

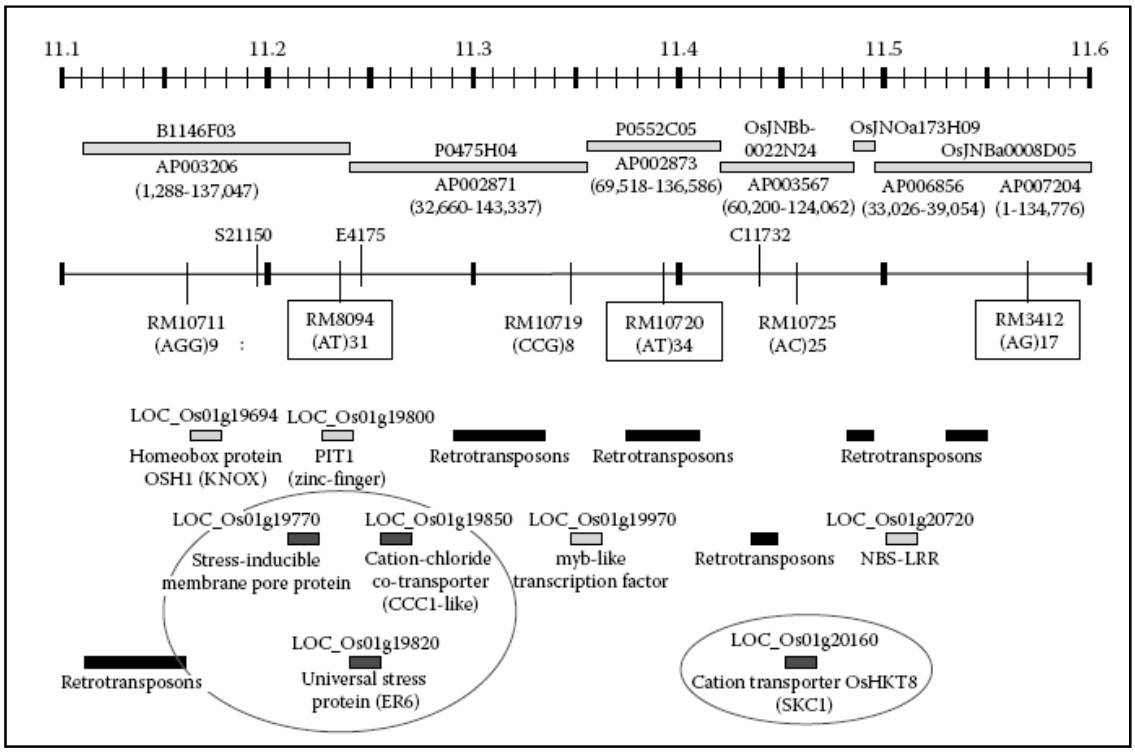


Fig. 2.5: Physical map on the short arm of chromosome 1 between 11.1 and 11.6 Mb showing polymorphic SSR markers and four genes targeted for indel marker development: a stress-inducible membrane pore protein, universal stress protein ER6, cation-chloride co-transporter, and *SKC1*. (Adapted from Thomson, *et al.* 2007).

To further characterized Pokkali-derived *Saltol* QTL for seedling stage salinity tolerance for use in marker-assisted breeding. A complementary effort was taken by Thomson, *et al.* (2010a) with IR29/Pokkali recombinant inbred lines (RILs) confirmed the location of the *Saltol* QTL on chromosome 1 and identified additional QTLs associated with tolerance. Results of analysis of a series of backcross lines and Near-Isogenic Lines (NILs) developed to better characterize the effect of the *Saltol* locus revealed that *Saltol* mainly acted to control shoot Na^+/K^+ homeostasis and multiple QTLs were required to acquire a high level of tolerance. Again, unexpectedly, multiple Pokkali alleles at *Saltol* were detected within the RIL population and between backcross lines, and representative lines (Fig. 2.6) were compared with seven Pokkali accessions to better characterize this allelic variation. Thus, while the *Saltol* locus presents a complex scenario and it provides an opportunity for marker assisted backcrossing to improve salt tolerance of popular varieties followed by targeting multiple loci through QTL pyramiding for areas with higher salt stress.

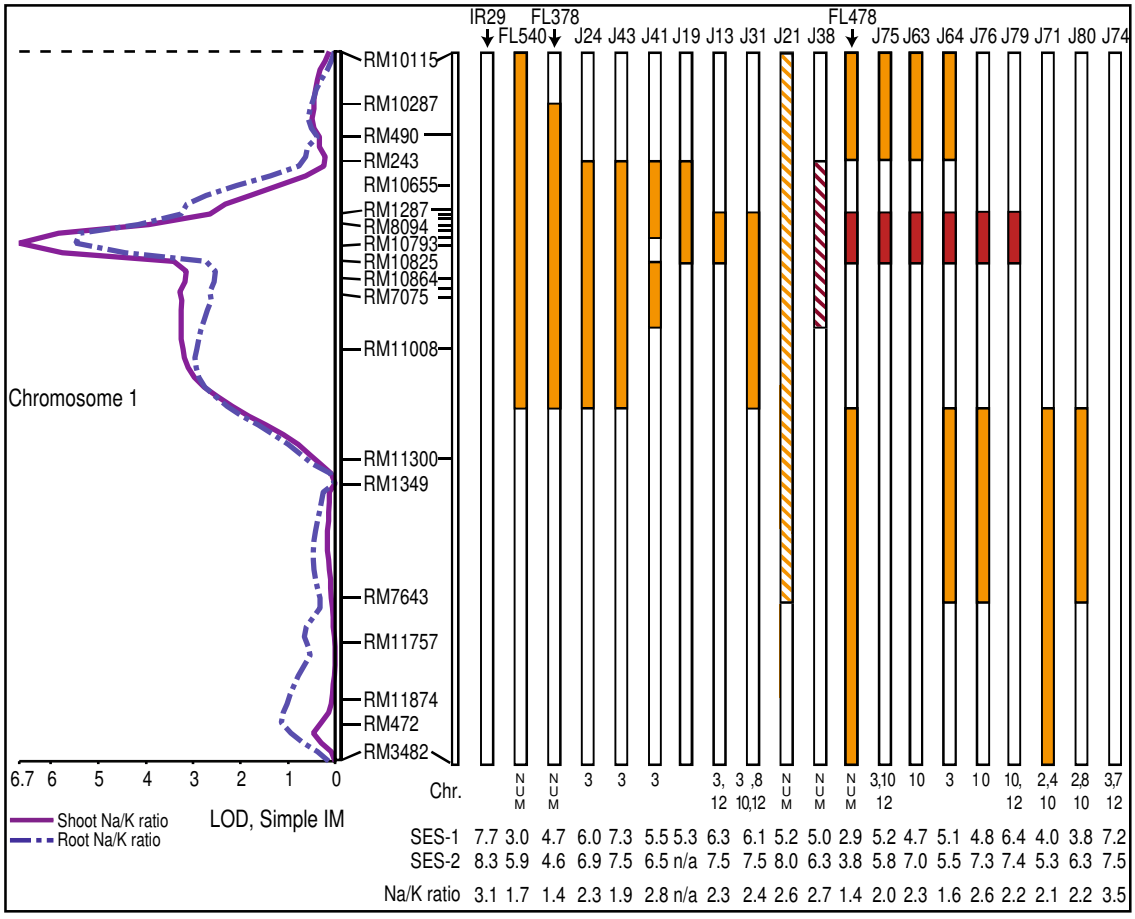


Fig. 2.6: A set of 16 IR29/Pokkali and FL478/IR29 backcross lines and three RILs screened with 38 markers to define their Pokkali introgressions. The original QTL interval plots for the shoot Na–K ratio (solid line) and

root Na–K ratio (dotted line) are shown on the left to indicate the location of the *Saltol* QTL peak. Additional markers were genotyped at the *Saltol* locus to more precisely define the introgression boundaries, as indicated by hash marks between RM1287 and RM7075, representing RM10694 (11 Mb), AP3206 (11.2 Mb), SKC1b (11.4 Mb), RM3412(11.5 Mb), RM493 (12.2 Mb), RM10793 (12.5 Mb), RM10864, (14.2 Mb), and RM562 (14.6 Mb). The most common Pokkali introgressions are shown as lightly shaded boxes, the FL478 *Saltol* allele is shown as darkly shaded boxes, and two additional alleles derived from NIL-17 (J21) and NIL-30 (J38) are shown by striped boxes. The presence of background Pokkali introgressions is indicated by the chromosome numbers below each line (with several lines having numerous background introgressions, indicated as NUM), and the SES tolerance scores for each line are shown below each line for experiments under standard phytotron conditions (SES-1) and in growth chambers under high temperature conditions (SES-2), along with the Na–K ratio from the growth chamber experiment. (Adapted from Thomson, *et al.* 2010a)

Based on the present above complete analysis of multiple Pokkali alleles at *Saltol* within the RIL population, backcross lines, and representative lines and the presence of complete segment of Pokkali introgression at the *Saltol* QTL with moderately salinity tolerance (SES 4.6-4.7 and Na^+/K^+ ratio 1.4) at seedling stage, FL378 (a F_8 RIL) lines was taken as donor for the present molecular breeding work (see Fig. 2.6).

(e) Genome wide association study **(GWAS)*:*** In vertebrate systems, association mapping (also known as linkage disequilibrium mapping) is increasingly being adopted as the mapping method of choice (Myles, *et al.* 2009). Association mapping involves searching for genotype-phenotype correlations in unrelated individuals and often is more rapid and cost-effective than traditional linkage mapping. However, linkage and association mapping are complementary approaches and are more similar than is often assumed. Unlike in vertebrates, where controlled crosses can be expensive or impossible (e.g., in humans), the plant scientific community can exploit the advantages of both controlled crosses and association mapping to increase statistical power and mapping resolution. While the time and money required for the collection of genotype data were critical considerations in the past, the increasing availability of inexpensive DNA sequencing and genotyping methods should prompt researchers to shift their attention to experimental design (Myles, *et. al.* 2009; Ingvarsson and Street, 2011).

The first examples of marker-aided selection based on QTLs in rice have shown that the QTL confidence interval was often too large to reach good breeding products (Ahmadi, *et al.*, 2002). Association mapping in natural populations overcomes this disadvantage by enabling the position QTLs with better precision. This precision increase is due to the lower linkage disequilibrium generally observed in natural populations in comparison with that of

mapping populations. This method is more and more widely used in plants (Zhu, *et al.* 2008), notably because sequencing and resequencing efforts now provide SNPs and InDels at the right density for such studies (Delseny, *et al.* 2010). Examples of targeted or genome-wide applications are so far limited in rice. The first studies concerned yield components (Agrama, *et al.* 2007), size and shape of milled grains (Iwata, *et al.* 2007), and diverse qualitative and quantitative agronomic traits (Huang, *et al.* 2010; Yang, *et al.* 2014; Begum, *et al.* 2015). However, because of the resolution of association mapping that makes it a useful tool towards positional cloning between QTL analysis and fine mapping, and the availability of an increasing number of polymorphisms (McNally, *et al.* 2009), no doubt that the number of association studies will soon escalate exponentially in rice (Spindel, *et al.* 2015).

Concerning salt tolerance in rice, one study targeted chromosomal segments carrying candidate genes or QTLs for salinity tolerance (Ahmadi, *et al.* 2011) focusing on accessions with temperate japonica background, and identifying significant associations for 19 independent *loci* distributed on chromosomes1, 2, 4, 5, 6 and 9. Association mapping has started to yield insights into the genetic architecture of complex traits in plants, and future studies with greater genome coverage will help to elucidate how plants have managed to adapt to a wide variety of environmental conditions (Ingvarsson and Street, 2011). A recent genome-wide association study (GWAS) was carried out using the genotyping by sequencing (GBS) data for 130 S_4 MAGIC *indica* populations at IRRI to identify the novel QTL for heat, anaerobic germination and salinity tolerance traits (Rashid, 2014). The study identifies 41 QTL for three stresses where 28 were previously identified and 13 were new putative QTL. For high temperature tolerance, major QTLs were found for grain number, plant height and panicle length, which were correctly mapped by many strong association signals. For AG tolerance, four novel QTLs were identified through Mixed Linear Model (MLM) analyses with high mapping resolutions for chromosomes 1, 4, 6 and 7 where three QTL were correctly overlapped with recently published QTLs. For seedling stage salinity tolerance, QTL were identified at EC 12 dSm^{-1} (score based salt injury scale), root dry weight, shoot dry weight, shoot fresh weight, shoot sodium and sodium potassium ratio. The study concludes with comments about the combined use of MAGIC population and association mapping were very effective in identifying QTLs with better accuracy. This proves that an approach integrating the multiple parent-derived populations and GWAS can be used as a powerful strategy for understanding complex traits in rice (Rashid, 2014).

Another genome wide association study for salinity tolerance have been conducted at IRRI with 44K SNP panel of 325 lines and identified higher tolerance of *indica*-tropical *japonica* sub-population with a number of QTLs in all chromosomes except Chr. 8 and 11 for SES scores, root biomass, shoot biomass, root length, shoot height, Chlorophyll a%, Chlorophyll b%, Chlorophyll (a+b)%, Na^+% in the shoots, K^+% in the shoots, Na^+% in the roots, K^+% in the roots, Na^+/K^+ ratio in the shoots and Na^+/K^+ ratio in the roots (de Ocampo, *et al,* 2014). A very recent genome-wide association study (GWAS) was implemented to identify loci controlling salinity tolerance in rice. A custom-designed array based on 6,000 single nucleotide polymorphisms (SNPs) in as many stress-responsive genes, distributed at an average physical interval of <100 kb on 12 rice chromosomes, was used to genotype 220 rice accessions. Genetic association was found for 12 different traits recorded on these accessions under field conditions at reproductive stage and identified 20 SNPs significantly associated with Na^+/K^+ ratio, and 44 SNPs with other traits observed under stress condition. The region harboring *Saltol*, a major quantitative trait loci (QTLs) on chromosome 1 in rice, which is known to control salinity tolerance at seedling stage, was detected as a major association with Na^+/K^+ ratio measured at reproductive stage. In addition to *Saltol*, GWAS peaks representing new QTLs on chromosomes 4, 6 and 7. The current association mapping panel contained mostly *indica* accessions that can serve as source of novel salt tolerance genes and alleles (Kumar, *et al.* 2015).

(f) Molecular breeding approaches: Marker Assisted Selection (MAS)/Marker Assisted Backcrossing (MABC): DNA (or molecular) markers has irreversibly changed the disciplines of plant genetics and plant breeding. While there are several applications of DNA markers in breeding, the most promising for cultivar development is called marker assisted selection (MAS). MAS refer to the use of DNA markers that are tightly-linked to target loci as a substitute for or to assist phenotypic screening. By determining the allele of a DNA marker, plants that possess particular genes or quantitative trait loci (QTLs) may be identified based on their genotype rather than their phenotype. Marker-assisted selection may greatly increase the efficiency and effectiveness for breeding compared to conventional breeding. The fundamental advantages of MAS compared to conventional phenotypic selection are: Simpler compared to phenotypic screening, Selection may be carried out at seedling stage, and Single plants may be selected with high reliability.

These advantages may translate into (1) greater efficiency or (2) accelerated line development in breeding programs. For example, time and labour savings may arise from the substitution of difficult or time-consuming field trials (that need to be conducted at particular times of year or at specific locations, or are technically complicated) with DNA marker tests. Furthermore, selection based on DNA markers may be more reliable due to the influence of environmental factors on field trials. In some cases, using DNA markers may be more cost effective than the screening for the target trait. Another benefit from using MAS is that the total number of lines that need to be tested may be reduced. Since many lines can be discarded after MAS at an early generation, this permits a more effective breeding design. The greater efficiency of target trait selection which may enable certain traits to be 'fast-tracked', since specific genotypes can be easily identified and selected. Moreover, 'background' markers may also be used to accelerate the recovery of recurrent parents during marker-assisted backcrossing (Collard and Mackill, 2006).

There are three levels of selection in which markers may be applied in backcross breeding. In the first level, markers may be used to screen for the target trait, which may be useful for traits that have laborious phenotypic screening procedures or recessive alleles. The second level of selection involves selecting backcross progeny with the target gene and tightly-linked flanking markers in order to minimize linkage drag. That refers as 'recombinant selection'. The third level of MAB involves selecting backcross progeny (that have already been selected for the target trait) with 'background' markers. In other words, markers can be used to select against the donor genome, which may accelerate the recovery of the recurrent parent genome (Collard and Mackill, 2006).

The use of molecular markers, which permit the genetic dissection of the progeny at each generation, increases the speed of the selection process, thus increasing genetic gain per unit time (Tanksley, *et al.* 1989; Hospital, 2003). The basis of marker assisted backcrossing strategy is to transfer a specific allele at the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome. The main advantages of MAB are: efficient foreground selection for the target locus, efficient background selection for the recurrent parent genome, minimization of linkage drag and rapid breeding of new genotypes with favorable traits. The effectiveness of MAB depends on the availability of closely linked markers and /or flanking markers, the size of the population, the number of backcrosses and the position and number of markers for

background selection (Frisch, *et al.* 1999; Frisch and Melchinger, 2005). MAB has previously been used in rice breeding to incorporate a number of traits viz. bacterial blight resistance gene *Xa21* (Chen, *et al.* 2001), waxy gene (Zhou, *et al.* 2003), root trait QTLs (Steele, *et al.* 2006) and a very recent submergence tolerance *Sub1* QTL was incorporate into several mega rice variety Swarna, IR64, BR11, Samba Mahsuri, Ciherang (Neeraja, *et al.* 2007; Iftekharauddaula, *et al.* 2011). Various other successful experiments reported the manipulation of known genes with indirect (linked) markers, including pyramiding of several major resistance genes in rice (Huang, *et al* 1997; Hittalmani, *et al.* 2000; Sanchez, *et al.* 2000; Singh, *et al.* 2001; Jiang, *et al.* 2004; Sharma, *et al.* 2004), wheat (AnLi, *et al.* 2005; XiangYan, *et al.* 2005) and tomato (Barone, *et al.* 2005; Yang and Francis, 2005). Several authors also reported introgression of one or more QTLs in different crops. The decision of which QTL regions to transfer with MAB and/or to consider in a selection index should be based on QTL effects verified in an independent validation sample.

The advantages of using MAS in plant breeding have been well documented (Xu and Crouch, 2008). The essential basis for successful breeding with MAS is an in-depth knowledge of genetic traits and variability within the desired plant species (Ashraf and Foolad, 2013). One example is *Saltol*, a favorable QTL identified in rice that is responsible for the bulk of genetic variation in ion uptake under saline conditions (Ashraf and Foolad, 2013; Gregorio, *et al.* 2002). Remarkable achievements through the discovery and cloning of the *SUB1A* gene facilitated the introgression of the gene to several high yielding varieties that have now been released and demonstrated for submergence tolerance in South and South-East Asian countries. Considerable progress was made in mapping the major QTL such as *Saltol* thus providing opportunities to fast track the introgression of *Saltol* QTL to popular varieties in Bangladesh, India, Vietnam and Philippines (Thomson, *et al.* 2010b; Gregorio, *et al.,* 2013). After getting success of *SUB1A*introgression and its benefits to the farmers of Asian and African regions, a comprehensive effort have been taken at IRRI for introgression of major salinity tolerance QTL *Saltol* in to several mega varieties of Bangladesh and Vietnam (Ismail, A.M. personal communication). Three mega varieties for Bangladesh i.e. BR11 and BRRI dhan28 (Rahman, 2010) and BRRI dhan29 (Sarker, 2012) and 3 varieties from Vietnam i.e. BT7 (Linh, *et al.* 2012), Bachthom7 (Vu, *et al.* 2012) and AS996 (Huyen, *et al.* 2012) were considered for introgression and successfully completed conversion process and the improved lines are now under field trial. All above 6 introgression works used FL478 (a F_8 RIL lines from IR29/Pokkali) as donor for *Saltol* QTL.

Most of the report showed a full recovery of elite background at 3rd backcross generation in terms of background marker recovery percentage and important agronomic traits (Rahman, 2010; Sarker, 2012; Linh, *et al.* 2012; Vu, *et al.* 2012; Huyen, *et al.* 2012). However, only 3 reports (Rahman, 2010; Sarker, 2012; Huyen, *et al.* 2012) showed a significant improvement of salinity tolerance either in terms of SES or Na^+/K^+ ratio at seedling stage but none of them released for farmers cultivation (Personal communication with authors). The above works only was getting success for seedling stage tolerance. However, to go further in improving salt tolerance of rice varieties, Yeo, *et al.* (1990) emphasized the importance of combining all favorable and complementary physiological traits in to single elite background, rather than considering salinity tolerance as a single trait. According to Flowers, *et al.* (2010) the traits needing to be pooled in barley or wheat through MAS including Na^+ exclusion, K^+/Na^+ discrimination, ion retention in sheaths, tissue tolerance, ion partitioning in different leaves, osmotic adjustment, enhanced vigor, water use efficiency and early flowering. The same traits are likely to be relevant in rice. Since markers have been found as linked to some traits of interest and already cloned genes, MAS offers the possibility to pyramid efficiently the QTLs or genes contributing to the different plant tolerance strategies. A major bottle-neck remains the selection of appropriate markers (i.e., key players in the context of salinity tolerance). Many studies are attempting to improve plant salt tolerance by genetic manipulation of certain genes; however, some of these genes might not have sufficient impact to improve crop viability significantly in highly saline environments. It has been proposed that enhancing plant stress tolerance is practicable by manipulating only one or a few main components of the regulatory gene network instead of engineering several molecular mechanisms (Golldack, *et al.* 2011). Besides conventional breeding methods, stacking of traits (also known as pyramiding) is a promising approach based on the introduction of several beneficial genes to improve plant performance. However, this approach is limited by independent segregation of traits, which complicates breeding strategies. An emerging method to circumvent this issue involves the use of trait landing pads, whereby engineered sequence-specific nucleases, such as zinc finger nucleases, are used to target multiple transgenes to the same locus (Ainley, *et al.* 2013). Although so far only zinc finger nucleases have been used in practice for generating trait landing pads, it is expected that the rapidly emerging CRISPR/Cas method will further facilitate targeted insertion of promoters and genes of interest (Belhaj, *et al.* 2013).

2.11 Candidate genes for salt stress response

To engineer more salt-tolerant plants it is crucial to unravel the key components of the plant salt-tolerance network. Recent studies have shown that stress sensing and signaling components can play important roles in regulating the plant salinity stress response (Deinlein, *et al.* 2014). In addition to that, key Na^+ transport and detoxification pathways and the impact of epigenetic chromatin modifications on salinity tolerance is important. Recently, a number of transcriptomic studies have been published reporting the use of cultivars with a range of salt sensitivity (Kumari, *et al.* 2009; Senadheera, *et al.* 2009; Walia, *et al.* 2005 and 2007). These studies showed that genes from many functional classes were found to be differently regulated in different organs, emphasizing the existence of diverse mechanisms in shoots and roots for a salt tolerance response.

(a) Sensory mechanisms and signaling in salt stress: An effective response to cope with salt stress, plants has developed the ability to sense both the hyperosmotic component and the ionic Na^+ component of the stress. These two sensory modalities are evident in that some responses to NaCl remain distinct from responses to purely osmotic stress. A high salt concentration in the soil solution produces hyperosmotic stress on roots. The first reactions of a plant to salinity occur within seconds to hours upon exposure to salt stress. In saline conditions, a rapid influx of Na^+ from the soluble phase of the soil into the cortical cytoplasm of plant roots occurs through NSCCs, and, later, through the high affinity K^+ transporter (*HKT1*) (Demidchik and Maathuis, 2007; Essah, *et al.* 2003; Tester and Davenport, 2003). This initial and rapid Na^+ uptake through NSCCs could serve as an efficient signal to activate adaptation to the osmotic part of salinity (Munns and Tester, 2008). Therefore, the elevated intra- and extracellular Na^+ are partially able to inhibit the K^+ outward rectifiers and thereby prevent the loss of cellular K+, maintaining cellular K^+/Na^+ homeostasis (Shabala, *et al.* 2006). Thus, in the tolerant line, rapid influx of Na^+ by the more effective NSCCs has advantageous effects, at least during the first phase of the salt stress response. The slower influx in sensitive cells will be less efficient in adjusting the water potential such that cells will lose water. These differences in sodium content are very short lived and therefore their connection with differential responses at the later stages must be conveyed by signals of a different molecular nature. Therefore, the rapid influx of sodium along with calcium ions and protons through NSCCs represents the first signal that allows the discrimination of salinity stress from mere osmotic stress. The appropriate target for the

sodium signal is actually the concomitant influx of calcium, which will carry on signalling even after the sodium signal has been dissipated by the deactivation of the NCCS through cAMP/cGMP-dependent signals, as well as deactivation of the slower HKAT channel (Ismail, *et al*. 2014a).

Calcium ions (Ca^{2+}) are considered the most prominent ubiquitous second messenger in cells ranging from bacteria and plants up to specialized neurons (Clapham, 1995). Ca^{2+} signals are shaped by influx or efflux of ions from the extracellular space through a couple of different channels in the plasma membrane, some of which seem to be mechanosensitive, whereas others are voltage gated and might be identical to the NCCS (Swarbreck, *et al.* 2013). Different channels are localized at the surface of intracellular compartments. Slow vacuolar channels, such as TPC1, are targets of different signaling molecules including Ca^{2+}, calmodulin (CaM), and nucleotides, and play a crucial role in raising cytosolic Ca^{2+} under a wide range of environmental and developmental cues (Pottosin, *et al.* 2009; Hedrich and Martena, 2011; Peiter, 2011). The spatial pattern of Ca^{2+} signals (e.g. cytosol, nucleus, organelles, or other specific regions of the cell), the temporal propagation of Ca^{2+} levels, the amplitude of the signal, and the frequency of Ca^{2+} oscillations are all informative aspects of Ca^{2+} signals, which are perceived by adaptor proteins or Ca^{2+}-modulated proteins that regulate downstream signaling events (Bouché, *et al.* 2005; Kudla, *et al.* 2010). Interestingly, Ca^{2+} signals participate in virtually all developmental, hormonal, and stress cues (Reddy, *et al.* 2011). The apparent ambiguity of this signal is even amplified by the fact that nitric oxide (NO), a small, uncharged, short-lived, water- and lipid-soluble, highly diffusible, ubiquitous, volatile, highly reactive free radical, can act as a Ca^{2+}-mobilizing messenger (Neill, *et al.* 2003; Besson-Bard, *et al.* 2008; Siddiqui, *et al.* 2011). The appropriate target for the calcium signal is on the one hand the SOS system driving the elimination of sodium ions from the cytoplasm, and the CAX transporters that will contribute to the shut-off of the calcium signal. Calcium-triggered activation of the NADPH oxidase will relay the signal to the next player apoplastic ROS (Ismail, *et al*. 2014a). Delayed sodium/calcium influx, activation of the SOS system as well as the block of sustained sodium influx will not be efficient. As a result, the sequestration of calcium into the vacuole will be slowed down, and the calcium signal is conveyed to other calcium adaptor proteins, such as CBL9. This will impede the ABA 'status' as a dynamic product of synthesis and signaling.This situation might even become accentuated by sustained calcium released from the vacuole through the slowly activated TPC1 channels (Ismail, *et al*. 2014a).

Protons (H^+) play crucial roles for cell signaling either directly or in cross-talk with phytohormones or Ca^{2+} (Gao, *et al.* 2004a). In addition, protons directly regulate enzymatic conformations and thus metabolic activities (Roberts, *et al.* 1980). However, intracellular pH can also act as a second messenger for several signaling pathways. Proton influx can occur concomitantly with calcium, and the resulting apoplastic alkalinization has been used extensively as a robust reporter for the rapid activation of calcium influx channels by elicitors (Felix, *et al.* 1993 and 1999) or abiotic stresses including salinity stress (Ismail, *et al*. 2012 and 2014; Geilfub and Mühling, 2013). In addition, the elevated steady-state level of apoplastic superoxide as a further relevant signal will be enhanced if the level of protons is low. Furthermore, stress-induced pH changes in the xylem sap might act as a root signal through ABA anions that redistribute and accumulate due to the low membrane-permeability of the charged anion, promoting stomatal closure (Taiz and Zeiger, 2010).

ROS plays a bi-functional role in the response to salinity stress. The accumulation of ROS causes oxidative damage to DNA, proteins, carbohydrates, and lipids. However, they also could function as signaling molecules regulating responses of development and various aspects of stress. Therefore, they must be closely regulated by orchestrated mechanisms (Miller, *et al.* 2010). For different stimuli, the elevated levels of ROS are sensed at the plasma membrane, for instance by two-component signaling systems (membrane-localized histidine kinases) that, in turn, activate the mitogen-activated protein kinase (MAPK) signaling cascades. Under salinity challenge, different MAPK elements are activated such as MAPK4, MAPK6, and MAPKK1 (Taj, *et al.* 2010). Although MAPK4 regulates the cross-talk between SA and JA, supporting the JA/ethylene signaling pathway (Brodersen, *et al.* 2006). In addition to the MAPK pathways, ROS can modulate gene expression by modifying transcription factors (Apel and Hirt, 2004). A third mechanism is the reversible oxidation of critical thiols in key signaling enzymes (Forman and Torres, 2002). However, ROS production needs to be tightly controlled to act as a signal, otherwise an excessive oxidative burst would result in cell death. The quelling of ROS accumulation can also be achieved by other signals, such as NO. NO has the ability to neutralize Fenton-type oxidative damage by scavenging superoxide, therefore preventing the formation of oxidants (such as O_2^-, H_2O_2, and alkyl peroxides), which makes it easier to recover a redox homeostasis (Lamattina, *et al.* 2003). In addition, NO is considered a redox regulator of the NPR1/TGA1 system, a key redox controlled regulators in plant systemic acquired resistance in plants (Lindermayr, *et al.* 2010). As an additional regulator, hydrogen sulfide (H_2S) has emerged as a signaling

molecule in plants that increases GSH levels, alters enzyme activities, and interacts with NO and ROS metabolism (Paul and Snyder, 2012; Lisjak, *et al.* 2013). As NO is acting as a secondary messenger of ABA signaling (Hancock, *et al.* 2011), this molecule provides cross-talk between oxidative and phytohormonal signaling. This crosstalk is even bi-layered, because also ROS deriving from the activity of the NADPH oxidase in the plasma membrane are essential for ABA induced signaling (Kwak, *et al.* 2003).

ABA regulates numerous plant biological processes including adaptive stress responses, such as drought, salt, ozone, and pathogen infection, and therefore is seen as a stress-related hormone (Xiong and Zhu, 2003). ABA activates genes that encode enzymes for the biosynthesis of compatible osmolytes (as shown for water-stress-induced betaine in pear leaves; Gao, *et al.* 2004b), proline (Strizhov, *et al.*1997), and cellular chaperones (dehydrins and LEA-like proteins) that protect proteins and membranes under stress (Liu, *et al.* 2013; Hasegawa, *et al.* 2000a, Shinozaki and Yamaguchi-Shinozaki, 2007). In addition, ABA causes induction of Ca^{2+} via ROS or IP3 recruitment (Murata, *et al.* 2001; Taiz and Zeiger, 2010). Moreover, ABA and JA play pivotal roles in controlling stomatal closure, which is considered a fast response in stressed plants. Both synthesis (Xiong and Zhu, 2003) and signaling (Kwak, *et al.* 2003) of the ABA pathway are promoted by ROS, whereas calcium, through calcineurin B, constrains both synthesis and signaling of ABA (Pandey, *et al.* 2004).

Jasmonic acid function as a master switch in plant responses to several abiotic and biotic stresses such as wounding (mechanical stress), drought and salt stress, ozone and pathogen infection, and insect attack (Wasternack, 2007; Wasternack and Hause, 2013). Ismail, *et al.* (2014a) reviewed and suggests the role of JA signaling in conferring tolerance to drought and salinity, or oxidative stress. The importance of JA tuning is corroborated by analysis of the rice mutant *rice salt sensitive 3* (*rss3*), where root growth is more severely inhibited under salinity compared with the wild type (Toda, *et al.* 2013). This growth phenotype is accompanied by elevated expression of JA-dependent genes. RSS3 binds to JAZ and non-MYC-type bHLH transcription factors, and has been proposed to repress an exaggerated JA response in the root tip (Toda, *et al.* 2013). Collectively, these data suggest that fine-tuning JA signalling is important for the growth and viability of plants under salinity stress. The existence of a multimeric transcriptional co-repression complex machinery to inactivate JA signalling (Chini, *et al.* 2007; Thines, *et al.* 2007; Pauwels, *et al.* 2010), in addition to JA-dependent repression of MYC2 via the MEK2/MAPK6 pathway (Petersen, *et al.* 2000), is

evidence that suppression of hazardous side effects of JA signaling is crucial for survival. It should be kept in mind that ABA and JA signaling are antagonistic on several levels partially by mutual competition for shared signaling factors such as MYC2 (Anderson, *et al.* 2004).

There are major groups of Ca^{2+}-binding proteins in plants, including calmodulins (CaM) and CaM-like proteins (CMLs); Ca^{2+}-dependent protein kinases (CDPKs); and calcineurin B like proteins (CBLs) (DeFalco, *et al.* 2010). Calcium sensors have been divided into two groups: the sensor relays, including calmodulin (CaMs) and calcineurin B-like (CBLs) proteins, and the sensor protein kinases, such as calcium-dependent protein kinases (CDPKs) as well as calcium and calmodulin dependent protein kinases (CCaMKs). CaMs and CBLs do not possess any intrinsic activity and have to transmit the calcium-induced modification to target proteins, whereas CDPKs and CCaMKs are directly activated upon calcium binding. Calmodulin is the most important Ca^{2+} transducer in eukaryotes and regulates numerous proteins with diverse cellular functions, including protein kinases. Plants also possess specific multigene families of protein kinases that play crucial roles in mediating calcium signaling. The multiplicity and diversity of plant calcium sensors, as well as the interconnections between various signal transduction pathways, constitute a tightly regulated signaling network that induces specific stress responses to improve plant survival (Boudsocq and Sheen, 2010). CaMs are found in all eukaryotes, while CMLs, CDPKs, and CBLs are restricted to plants and some proteins (DeFalco, *et al.* 2010). CaMs/CMLs bind Ca^{2+} and undergo conformational changes that regulate the function of target proteins. In rice, five putative CaMs and 32 CMLs proteins were identified in a genome-wide gene-sequence homology study (Boonburapong and Buaboocha, 2007). Such a big array of identified putative CaM/CMLs illustrates their importance in plants and suggests a mechanism where different stimuli can be perceived by different CaM/CMLs, leading to specific cellular responses. Slow vacuolar channels, such as TPC1, are targets of different signalling molecules including Ca^{2+}, calmodulin (CaM), and nucleotides, and play a crucial role in raising cytosolic Ca^{2+} under a wide range of environmental and developmental cues (Pottosin, *et al.* 2009; reviewed by Hedrich and Martena, 2011; Peiter, 2011). The spatial pattern of Ca^{2+} signals (e.g. cytosol, nucleus, organelles, or other specific regions of the cell), the temporal propagation of Ca^{2+} levels, the amplitude of the signal, and the frequency of Ca^{2+} oscillations are all informative aspects of Ca^{2+} signals, which are perceived by adaptor proteins or Ca^{2+}-modulated proteins that regulate downstream signaling events (Bouché, *et al.* 2005; Kudla, *et al.* 2010).

CDPK proteins are usually constituted by five domains, namely, very variable N- and C-terminus domains, a highly conservedserine/threonine kinase domain next to the N-terminus, an auto-inhibitory junction-domain and a CLD (CaM-like domain), usually with four functional EF-hands (Asano, *et al.* 2005; Klimecka and Muszynska, 2007).Within the CDPK family, *OsCPK*13 (*OsCDPK*7) is the best candidate gene for rice salt stress tolerance improvement. *OsCPK*13 (*OsCDPK*7) mRNA levels are increased in both cold and salt stress conditions (Saijo, *et al.* 2000; Wan, *et al.* 2007). More importantly, the over-expression of this protein increases the plant tolerance to salt, cold and drought (Saijo, *et al.* 2000). In over-expressing transgenic rice lines, the induction of mRNA levels of important stress responsive genes is exacerbated in roots (*SalT*, *OsLEA*29) and in shoots (*OsLEA*29), in response to salt stress, but not too cold (Saijo, *et al.* 2000). This observation suggests the existence of different response pathways for salt and cold stress with a common upstream CDPK. There is a growing list of CDPK substrates, underlying their importance in diverse cellular functions, but until now the possible targets for *OsCPK13 (OsCDPK7)* have not been identified. CDPKs activity may be modulated by Ca^{2+}, by reversible phosphorylation, by phospholipid interaction, and by association with adapter proteins such as 14-3-3 (see Klimecka and Muszynska, 2007). In rice, five different isoforms of 14-3-3 protein were identified and several abiotic stresses (including salt) found to induce their mRNA expression levels (Chen, *et al.* 2006). NaCl-induced cytosolic Ca^{2+}, in turn, activates the plasma-membrane ATPases mediated by Ca^{2+}/CaM-dependent protein kinases, restoring membrane voltage after Na^{+}-induced depolarization, maintaining membrane integrity and ionic homeostasis, promoting H^{+} influx, and inhibiting both K^{+} and H^{+} efflux (Klobus and Janicka-Russak, 2004; Shabala, *et al.* 2006; Wolf, *et al.* 2012).

As it happens with CaM, CBL proteins bind Ca^{2+} through four EF-hands. These CBL proteins are mainly regulatory subunits for plant-specific serine/threonine protein kinases, known as CBL-interacting protein kinases (CIPKs) (Luan, 2009; Weinl and Kudla, 2009) for recent reviews). The classical example of CBL/CIPK interaction in salt stress response leading to Na^{+} detoxification is integrated in the *SOS* pathway. *AtCBL4* (*SOS3*) is known to bind *AtCIPK24* (*SOS2*) in a Ca^{2+} dependent manner (Sanchez-Barrena, *et al.* 2007), although further *in vivo* evidence for the exact role of Ca^{2+} in the interaction is still lacking. Recently, the rice homologues for *SOS*2 and *SOS*3, *OsCIPK*24 and *OsCBL*4 were identified (Martinez-Atienza, *et al.* 2007). In *Arabidopsis*, another CBL protein *AtCBL10* was shown to bind *AtCIPK24 (SOS2)* revealing a role in salt stress response (Kim, *et al.* 2007; Quan, *et al.*

2007). *AtCBL4* or *AtCBL10* binding to *AtCIPK24* recruit the kinase for the plasma membrane or the tonoplast, respectively, illustrating CBL regulation of CIPK through different cellular localization (Kim, *et al.* 2007). So far, no functional homologue for *AtCBL*10 in rice has been identified. Besides*OsCBL*4, so far, only *OsCBL*8 was shown to be directly involved in salt stress response, since its over-expression induces rice salt stress tolerance (Gu, *et al.* 2008). Six other *OsCBL* transcripts have been shown to be up-regulated in response to salt stress (Gu, *et al.* 2008). Several *OsCIPK* genes were also shown to be up-regulated in response to salt stress (Xiang, *et al.* 2007). *OsCIPK31 (OsCK1)*, a salt stress inducible CIPK (Kim, *et al.* 2003), was recently described as modulating the expression of salt stress–inducible genes (*OsLEA29*, *OsDip1*, and *SalT*) under stress conditions (Piao, *et al.* 2010). Furthermore, loss-of-function mutation of *OsCIPK*31 (*OsCK1*) in rice plants rendered the plants hypersensitive to salt (Piao, *et al.* 2010). Finally, *OsCIPK*15 overexpression in rice resulted in enhanced salt tolerance. Furthermore, the transcript level of this gene was studied in a rice salt tolerant *vs.* a sensitive variety (FL478 and IR29, respectively) in response to salt stress, revealing that the gene was down regulated in FL478, while it suffered no change in IR29 (Senadheera and Maathuis, 2009). Although further functional studies are necessary to understand the true potential of this gene as a target for salt stress improvement, these two studies put together, strongly support *OsCIPK*15 role in salt stress response.

Mitogen-activated protein kinase (MAPK) cascades are typically composed by three types of kinases that can transducer environmental signals through processes of reversible phosphorylation into adaptive and programmed cellular responses.MAP kinase kinase kinase (MAPKKK) are activated by signal plasma membrane receptors, and activate downstream MAPkinase kinases (MAPKK), that in turn activate MAPK. The downstream targets for MAPK can be TFs, phospholipases, or cytoskeletal proteins, among others (for extended review on current literature on MAPK signaling in plants, see Rodriguez, *et al.* 2010). In *Arabidopsis*, a salt stress responsive MAPK cascade has been identified (Teige, *et al.* 2004). The authors verified that overexpression of *AtMKK2* induces tolerance to salt and cold, while null-mutants have reduced tolerance to salinity. Furthermore, they identified two downstream MAPK proteins (*AtMPK4* and *AtMPK6*) and a potential upstream MAPKKK (*AtMEKK1*). The over-expression of *AtMKK*2resulted in the constitutive activation of *AtMPK4* and *AtMPK6*with resulting enhanced salt and freezing tolerance, while*mkk2* mutants had the opposite effect (Teige, *et al.* 2004). In rice, there is no completely described

MAPK cascade with involvement in salt stress response, although a good candidate component is *OsMAPK5a*. The over-expression of *OsMAPK5a*leads to salt, drought, and cold stress increased tolerance (Xiong and Yang, 2003). Recently, a Mn^{2+}-dependent receptor-like kinase (RLK), *Os-SIK*1, induced by salt, drought and H_2O_2 treatments, was propose to mediate a stress signaling pathway that may involve scavenging and detoxification of reactive oxygen species (ROS) (Ouyang, *et al.* 2010). Over-expression of this gene induces salinity and drought tolerance by increasing rice antioxidant activity (Ouyang, *et al.* 2010). The downstream components of the proposed stress signaling pathway have not yet been identified, but, nevertheless, *OsSIK1* appears as a potential candidate for future crop improvement, since rice over-expression lines also display higher vigor than wild type plants (Ouyang, *et al.* 2010).

(b) Responses of root ion uptake and transport: Transcription factors are integral in linking salt sensory pathways to many tolerance responses. Core sets of transcription factor family genes are differentially expressed in response to elevated external salinity (Golldack, *et al.* 2011), including basic leucine zipper (bZIP) (Yang, *et al.* 2009), WRKY (Jiangand Deyholos, 2009), APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) (Kasuga, *et al.* 1999), MYB (Cui, *et al.* 2013), basic helix–loop–helix (bHLH) (Jiang, *et al.* 2009), and NAC (Tran, *et al.* 2004) families. These transcription factors, in turn, regulate the expression levels of various genes that may ultimately influence the level of salt tolerance of plants Fig. 2.7. To counteract the water potential decrease resulting from the osmotic component of enhanced salinity, genes relevant for inorganic ion uptake and osmolyte synthesis are up-regulated (Geng, *et al.* 2013). To some extent, transcriptional regulation of these stress response genes in plants is mediated by dynamic changes in hormone biosynthesis (Geng, *et al.* 2013; Dinneny, *et al.* 2008) (Fig. 2.7). After stress induction an initial quiescence period is followed by a growth recovery phase, both of which correlate with changes in the levels of the plant hormones abscisic acid (ABA), jasmonate (JA), gibberellic acid (GA), and brassinosteroid (BR). In response to high salinity, most stress-induced transcriptional changes occur approximately 3 h after application of salt stress (Geng, *et al.* 2013). The expression of 5590 genes was reported to be salt-regulated in roots of *A. thaliana* seedlings (Chen, *et al.* 2002), and fluorescence-activated cell sorting (FACS) has revealed that root cortex cells were the most transcriptionally active (Geng, *et al.* 2013). Furthermore, molecular analyses have revealed that the root endodermis is the pivotal cell layer in the context of lateral root development under salt stress conditions. ABA prevents lateral root

elongation into surrounding media with high salt concentrations (Duan, *et al.* 2013). In addition to the above mentioned hormones, ethylene was recently shown to confer plant salt tolerance in soil grown Arabidopsis plants by improving the Na^+/K^+ ratio in shoots (Jiang, *et al.* 2013). Knockout of *ETHYLENE OVERPRODUCER1 (ETO1)* resulted in elevated ethylene levels, which stimulated root stele ROS production by the respiratory burst oxidase homolog F (RBOHF). The increase in stele ROS accumulation led to reduced net Na^+ influx in roots, decreased Na^+ xylem loading and to root K^+ retention and subsequent enhanced salinity tolerance (Jiang, *et al.* 2013).

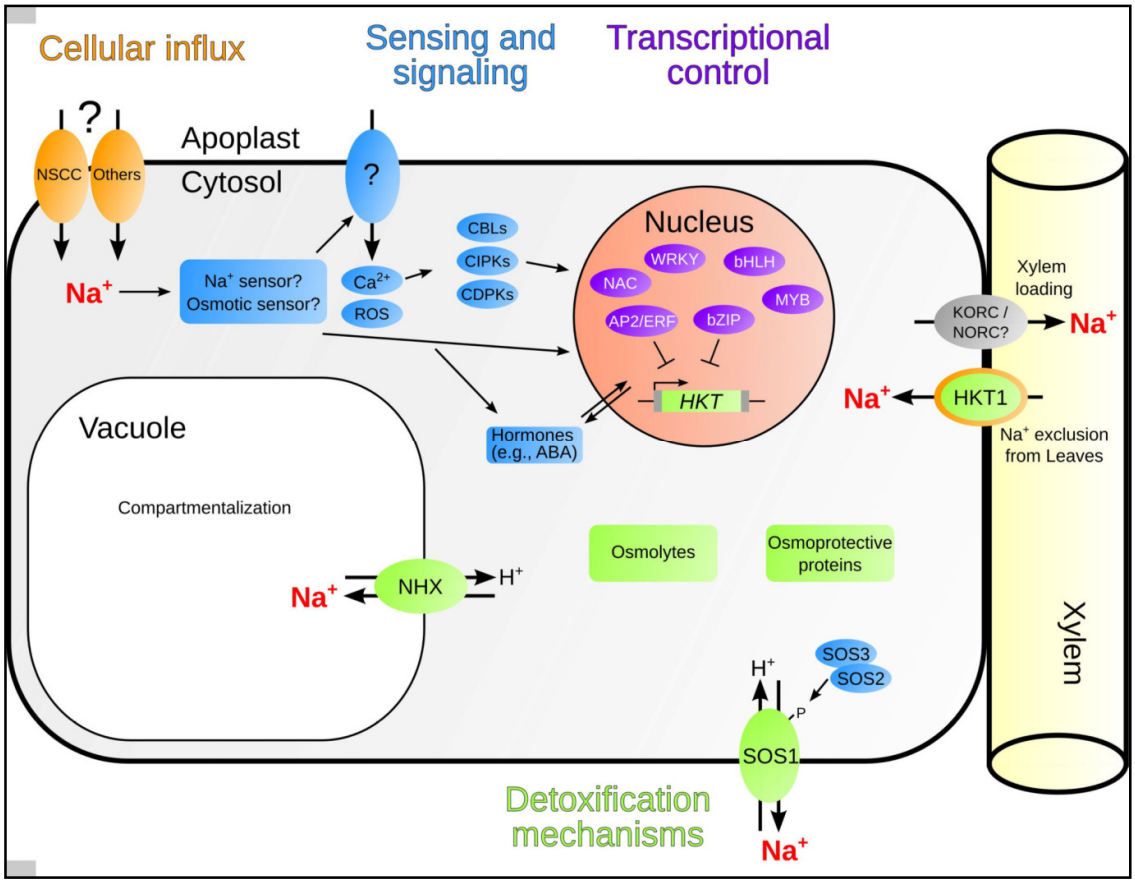


Fig. 2.7: Overview of cellular Na^+ transport mechanisms and important components of the salt stress response network in plant root cells. Na^+ (depicted in red) enters the cell via NSCCs and other, as yet largely unknown membrane transporters (cellular Na^+-influx mechanisms highlighted in orange). Inside the cell, Na^+ is sensed by an as yet unidentified sensory mechanism. At the next step, Ca^{2+}, ROS, and hormone signaling cascades are activated. CBLs, CIPKs, and CDPKs are part of the Ca^{2+}-signaling pathway (sensing and signaling components highlighted in blue), which can alter the global transcriptional profile of the plant (transcription factor families in the nucleus depicted in purple; an AP2/ERF and a bZIP transcription factor that negatively regulate HKT gene expression are shown as an example). Ultimately, these early signaling pathways result in expression and activation of cellular detoxification mechanisms, including HKT, NHX, and the SOS Na^+ transport mechanisms as well as osmotic protection strategies (cellular detoxification mechanisms highlighted in light green). Furthermore, the Na^+ distribution in the plant is regulated in a tissue-specific manner by unloading of

Na^+ from the xylem. Abbreviations: NSCCs, nonselective cation channels; ROS, reactive oxygen species; CDPKs, calcium-dependent protein kinases; CBLs, calcineurin B-like proteins; CIPKs, CBL-interacting protein kinases; AP2/ERF, APETALA2/ETHYLENE RESPONSE FACTOR; bZIP, basic leucine zipper; NHX, Na^+/H^+ exchanger; SOS, SALT OVERLY SENSITIVE. (Adapted from Deinlein, *et al.* 2014).

Several plant membrane transporters play key roles in resistance mechanisms to biotic and abiotic stress, particularly Na^+ and K^+ transporters for resistance to salt stress (Schroeder, *et al.* 2013). Multiple Na^+-influx pathways into roots exist. Na^+ may cross the plasma membrane via nutrient channels and transporters. Some channel and transporter mutants reduce Na^+ accumulation in plant cells, but only a few transporter mutants have been directly shown to impair Na^+ influx into roots. Calcium-permeable nonselective cation channels (NSCCs) (Tester and Davenport,2003; Tyerman and Skerrett,1999) including the CYCLIC NUCLEOTIDE-GATED CHANNEL (CNGC) (Hua, *et al.* 2003; Gobert, *et al.* 2006; Guo, *et al.* 2008) and the GLUTAMATE-LIKE RECEPTOR (GLR) (Tapken and Hollmann, 2008) families, are permeable to Na^+ and, thus, represent a likely entry point of Na^+ into the cell (Fig. 2.7). Furthermore, the rice (*Oryza sativa*) Na^+ transporter *OsHKT2;1* has been shown to mediate Na^+ influx into roots under K^+ starvation (Horie, *et al.* 2007). In addition, *AtCHX21*, a cation/H^+ antiporter expressed in the root endodermis, is involved in Na^+ transport from endodermal cells to the stele (Hall, *et al.* 2006). Na^+ enters the xylem by efflux out of stellar cells and is subsequently transported to aerial plant tissues. Potential candidates for the control of xylem loading of Na^+ are the outward-rectifying K^+ channels KORC and NORC (de Boer and Wegner, 1997; Wegner and de Boer, 1997). *AtSKOR*, an ortholog of KORC in Arabidopsis, is involved in xylem loading of K^+ (Gaymard, *et al.* 1998). Furthermore, class I HKT transporters have an important function in removing Na^+ from the xylem (Ren, *et al.* 2005; Sunarpi, *et al.* 2005).

(c) Ion homeostasis: Membrane transport proteins are crucial for cell function. They regulate the fluxes of ions, nutrients, and other molecules across the membranes of all cells, and their activities underlie numerous physiological processes (Ashcroft, *et al.* 2009). In a stress situation, these transporters have a crucial role on the control of the cell homeostasis. An important mechanism of plant salt tolerance seems to be a selective uptake of K^+ over Na^+ by root systems (Maathuis and Amtmann, 1999). Therefore, transporters controlling the distribution of K^+ and Na^+ in plants are considered as one of the key determinants of plant salt tolerance due to their capacity to maintain a high cytosolic K^+/Na^+ ratio (Maathuis and Amtmann, 1999). One of the most important transport systems is the *HKT* (High-Affinity K^+

Transporters) family. The *HKT* family isactive at the plasma membrane and permeable to either $K^+$and Na^+ or to Na^+ only (Rodriguez-Navarro and Rubio, 2006) and plays an important role in plant salt tolerance or growth in conditions of K^+ shortage (Horie, *et al.* 2007). The *HKT* family comprises a much larger number of members in rice, with seven to nine genes depending on the cultivar (Garciadeblas, *et al.* 2003). The function of *OsHKT*8 gene seems to be analogous to *AtHKT*1;1 by regulating shoot K^+ homeostasis and protecting leaves from Na^+ toxicity (Rus, *et al.* 2004; 2005). In addition, it was found by mutations in the rice and *Arabidopsis* HKT Na^+ transporters, that the reduced K^+ accumulation in the xylem sap and shoots of *hkt* mutants was inverted to enhanced Na^+ accumulation in the same tissues. Specifically, enhanced Na^+ levels in the xylem sap of *athkt1;1* mutants was accompanied by reduced xylem sap K^+ levels (reviewed by Horie, *et al.* 2009). These findings suggests that *AtHKT*1;1 and *OsHKT*8 indirectly propel K^+ release from xylem parenchyma cells into the xylem vessels (Horie, *et al.* 2009; Ren, *et al.* 2005). Taken together, these results support the idea that *OsHKT*8 is involved in regulating K^+/Na^+ homeostasis in the shoots.

QTL analyses for Na^+ resistance have suggested that similar xylem Na^+-unloading mechanisms are essential for salt tolerance in rice and wheat (*Triticum turgidum*) (Ren, *et al.* 2005; James, *et al,* 2006). In both cases, major salt tolerance QTL map to regions that include *HKT1;5* orthologs, encoding a more Na^+-selective class I HKT transporter (Ren, *et al.* 2005; Byrt, *et al.* 2007). Na^+-tolerance QTL analyses of wheat led to the identification of another strong salt tolerance QTL named *Nax1* (Munns, *et al.* 2003). The *Nax1* locus, which maps to the region of the *TaHKT1;4* gene that also encodes a class I HKT transporter, was found to contribute to Na^+ removal from xylem in the leaf sheath to protect leaf blades from Na^+ over-accumulation (Huang, *et al.* 2006). Recently, comparative analyses using salt tolerant *indica* cultivars and a sensitive *japonica* cultivar have led to the hypothesis that *OsHKT1;4* restricts leaf sheath-to-blade Na^+ transfer in rice plants under salinity stress (Cotsaftis, *et al.* 2012).The recent HKT marker-assisted introduction of a wheat *HKT1;5* from an ancestral wheat relative *Triticum monococcum* into commercial durum wheat (*T. turgidum* ssp. *durum* var. Tamaroi) has led to significant increases in grain yields in field trials on natural saline soil in Australia (Munns, *et al.* 2012). Similar to wheat *HKT1;5*, a recent study with diverse rice accessions identified seven major and three minor alleles of *OsHKT1;5* with strong association of leaf Na^+ concentrations and these probably indicating the existence of additional highly effective exclusion mechanisms in rice (Platten, *et al.*

2013). Together, these findings demonstrate that xylem parenchyma-localized class I HKT transporters are an essential mechanism for plants to protect photosynthetic organs from Na^+ over-accumulation during salinity stress.

Allelic differences at *OsHKT*8 gene between a salt-tolerant Nona Bokra and a sensitive variety Koshihikari showed six nucleotide substitutions in the coding region leading to four amino acid changes which seems to enhance the overall Na^+ transport activity in heterologous systems (Ren, *et al.* 2005). On the other hand, the *OsHKT*1 gene seems to act in a different way under salinity stress. Under high Na^+ concentrations there is a rapid inactivation of *OsHKT*1, however, the regulation mechanisms underlying this system are still not understood. Previous works (Horie, *et al.* 2007) showed that *OsHKT*1 is rapidly down regulated by salinity stress and also by protein kinase inhibitors, suggesting that Na^+ transport activity of this gene is post-translationally regulated by still unknown cellular signaling pathways. Furthermore, analysis of *oshkt1* mutant alleles confirmed the reduction of the Na^+ influx into plant roots (Horie, *et al.* 2007).

High levels of salts can be tolerated by plants through internal distribution of ions for osmotic adjustment and by keeping Na^+ away from the cytosol (Zhang and Blumwald, 2001). The antiporters Na^+/H^+ are widespread membrane proteins that catalyze the exchange of Na^+ for H^+ and can be found in animals, yeasts, bacteria, and plants. The increased expression of antiporter genes by salt stress has been reported for both glycophytes (*A. thaliana*, *Hordeum vulgare*, *Beta vulgaris* and rice) and halophytes (*Mesembryanthemum crystallium* and *Atriplexgmelini*) (Blumwald and Poole, 1985; Chauhan, *et al.* 2000; Fukuda, *et al.* 1999; Garbarino and Dupont, 1988; Gaxiola, *et al.* 1999; Hamada, *et al.* 2001). The presence of large, acidic-inside, membrane-bound vacuoles in plant cells allows the efficient compartmentation of Na^+ in the vacuole through the operation of vacuolar Na^+/H^+ antiporters (Apse, *et al.* 1999). These antiporters use the proton-motive force to catalyze the exchange of Na^+ for H^+ across vacuolar membranes (Zhang and Blumwald, 2001). Vacuolar sequestration of Na^+ not only lowers Na^+ concentration in the cytoplasm but also contributes to osmotic adjustment in the cytoplasm to maintain water uptake from saline solutions (Zhu, 2003). Among the Na^+/H^+ antiporters involved in salt stress tolerance, the *NHX*1 encodes a vacuolar Na^+/H^+antiporter. In rice, it was shown that *OsNHX*1 encodes vacuolar(Na^+, K^+)/H^+ antiporters localized in the tonoplast, and treatments with high a concentration of NaCl (200 mM) increased the transcripts levels of *OsNHX*1 in rice roots and shoots, up to four-fold

(Fukuda, *et al.* 2004). They had already clearly demonstrated that overexpression of *OsNHX*1 improved the salt tolerance of transgenic rice plants. In addition, it was found that the Na^+ content in transgenic seedlings was similar to that found in wild-type plants. There was, however a clear difference in Na^+ content between wild-type and transgenic rice plants in the mature leaves, but not in young ones. Based on these findings, they proposed that Na^+ compartmentation in mature tissues could prevent young tissues from being more severely injured. Interestingly, other genes such as *Sal*T also respond in an analogous way suggesting the importance of the growth state in salinity tolerance (Garcia, *et al.* 1998). Apparently, an expression shift associated with leaf aging can be related to salinity tolerance.

In the work performed by Senadheera, *et al.* (2009), the authors performed a transcriptomic study in roots of two well characterized cultivars with different salinity tolerance, IR29 (sensitive) and FL478 (tolerant). Physiological studies showed that FL478 has a lower Na^+ influx together with a reduced translocation to the shoot thus maintaining a low Na^+/K^+ ratio. In these two cultivars, some transcripts encoding genes for cation transport (namely *OsCHX*11 and *OsCAX*4) have shown differential expression, particularly after salt treatment.

(d) LEAs and other stress-induced proteins: Salt stress results in a water deficit condition that takes the form of physiological drought. Therefore, some of the genes/proteins responsive to salt stress are also involved in drought. In plants, a group of very hydrophilic proteins, known as Late Embryogenesis Abundant (LEA), accumulates to high levels both during the last stage of seed maturation(when the embryo acquires desiccation tolerance) and during water deficit in vegetative organs, suggesting a protective role during water limitation (Battaglia, *et al.* 2008). LEA proteins are known to play important roles in water stress tolerance by protecting specific cellular structures or ameliorating the effect of drought stress through ion sequestration and maintenance of minimum cellular water requirements (Dure, *et al.* 1989).

In rice, 25 new LEA genes have been identified (Wang, *et al.* 2007). These genes can be grouped in seven distinct families. The induction by salinity of four *OsLEA* genes (each from a different family) was clearly demonstrated by semi quantitative RT-PCR analysis (Hu, 2008; Wang, *et al.* 2007). In general, LEA proteins are structurally well characterized (reviewed by Battaglia, *et al.* 2008), however, their abundance in different tissues/organs, and their diverse responses to ABA among other aspects, has complicated the definition of

possible function(s). As compared to other LEA proteins, *OsLEA5* and *OsLEA12* belong to LEA 2 and LEA 3 groups, respectively, they contain a significantly higher proportion of hydrophobic residues, are not soluble after boiling and experimental data confirmed globular conformation (Singh, *et al.* 2005). *OsLEA19a* belongs to LEA group 4, a group that appears to be regulated by ABA at specific developmental stages and/or upon stress conditions (reviewed by Battaglia, *et al.* 2008). *In vitro* assays have shown that LEA 4 group is effective in protecting several enzymes such as lactate dehydrogenase, malate dehydrogenase, citrate synthase, fumarase etc. (reviewed by Battaglia, *et al.* 2008). Together, these results suggest a role for this LEAgroup as protector molecules under water limitation and also in providing a water-like rich environment to their target enzymes. The last gene, *OsLEA*29 belongs to the designated Pfam class: dehydrins (Wang, *et al.* 2007). For some proteins of this group, namely acidic dehydrins, ion-binding activity is most likely one of the major biochemical functions, acting as calcium buffers or as calcium-dependent chaperone like molecules (Alsheikh, *et al.* 2005). However, since *OsLEA*29is a basic dehydrin it probably has a different role. Alternatively, a metal binding activity could be related to a scavenger function of radicals under oxidative stress (Hara, *et al.*, 2004).When referring to salt stress in rice, the *Sal*T gene is one of the most reported, co-localizing with the QTL *Saltol* (Salt Tolerance) on chromosome 1. The *Sal*T gene was first isolated and characterized from the roots of salt-treated rice plants (Claes, *et al.* 1990) and it seems to be regulated by ABA-dependent and ABA-independent pathways (Garcia, *et al.* 1998). In addition, the recombinant SALT lectin is a potent agglutinin (Branco, *et al.* 2004), suggesting a potential role in pathogen agglutination (Zhang, *et al.* 2000). Evidence suggests the correlation of *Sal*T expression with osmoprotectants, such as trehalose and proline. Rice treatment with trehalose improved saline tolerance but suppressed *Sal*T up-regulation, while proline treatment increased growth inhibition of salt-treated rice and up-regulated *Sal*T (Garcia, *et al.* 1997). In another study, it was suggested that lower *Sal*T expression in Nona Bokra (salt-tolerant variety) could be mediated by trehalose and a sign of salt tolerance (Chao, *et al.* 2005). *Sal*T transcripts were found to accumulate with wounding and heat (although with lower accumulation than with salt), with no response to cold and dark treatments (de Souza, *et al.* 2003). In addition, *Sal*T expression is also induced by fungal elicitors, jasmonic acid and abscisic acid (deSouza, *et al.* 2003; Kim, *et al.* 2004; Moons, *et al.* 1997). Taken together, these results suggest that the role of *Sal*T protein is not restricted to environmental stress responses and that it may be included in a broader response/sensor mechanism to the imposed stress (de Souza, *et al.* 2003). The *Sal*T gene is highly expressed

in the youngest not fully expanded leaves, but as plants get older, the higher expression levels are found in the oldest leaves of mature plants (Garcia, *et al.* 1998).

(e) Transcription regulators: Proteins with sequence-specific DNA binding and capable of activating and/or repressing gene expression is usually defined as transcription factors (TFs). Regulation of gene expression controls many biological processes, such as cell cycle, metabolic and physiological balance, and responses to the environment (Riechmann, *et al.* 2000). During the last decade, many TFs, belonging to different TF families and subfamilies, have been shown to be involved in plant responses to adverse environmental conditions, such as high salinity, drought, heat, and low temperatures (Saibo, *et al.* 2009; Yamaguchi-Shinozaki and Shinozaki, 2006). Among the TF families present in plants, AP2/EREBP (Apetala2/Ethylene Responsive Element BindingProtein), NAC (NAM, ATAF and CUC), ZF-HD (zinc-finger homeodomain), AREB/ABF (ABA-responsive element binding protein/ABA binding factor), and MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene) have been the most involved with the abiotic stress responses. Interestingly, AP2/ERF and NAC genes are widely present in land plant genomes. However, no homologue has been so far identified in other eukaryotes (Riechmann, *et al.* 2000). More than 40 TFs were so far shown to be somehow involved in rice responses to high salinity and we believe that many more will be soon unveiled. Although these TFs belong to different families, such as AP2/EREBP, bZIP, HD, Zinc Finger, MYB, CCAAT-HAP2 and NAC, members of the latter have been by far the most reported as related to the rice responses to high salinity.

Several DREB proteins, belonging to the AP2/EREBP family, were also reported as involved in rice salt stress responses. Both *OsDREB*1A and *OsDREB*1F are induced by high salinity and also by cold (Dobouzet, *et al.* 2003; Liu, *et al.* 2007). On the other hand, *OsDREB*2A is induced by salt, drought and high temperature, but not by low temperature (Dubouz, *et al.* 2003; Matsukura, *et al.* 2010). This behavior agrees with our knowledge that most DREB1/CBFs are ABA-independent and have been more associated with low temperature responses while DREB2 is usually ABA-dependent and more related to osmotic stress. The genes *OsBIERF*2 (*AP*37), *OsBIERF*3 (*AP*59) and *Os-BHD*1 encode TFs shown to be highly involved in plant disease resistance (Cao, *et al.* 2006; Luo, *et al.* 2005a and 2005b). Nevertheless, the transcript level of *OsBIERF*2 (*AP*37) and *OsBIERF*3 (*AP*59) were shown to be slightly induced by salt and their over expression in plants also led to increased

tolerance. Regarding *OsBHD*1, its effect is not yet known, although its overexpression in plants reduces salt tolerance (Cao, *et al.* 2006a; Luo, *et al.* 2005b; Oh, *et al.* 2009). This suggests that these TFs, although not playing a key role on salt stress signaling, are involved in rice responses to high salinity and may mediate a cross-talk between abiotic and biotic stress responses.

Three genes encoding zinc fingers proteins have been reported as responsive to high salinity (Huang, *et al.* 2007 & 2008; Sun, *et al.* 2010). The *ZFP*179 and *ZFP*182 transcripts are induced, while *SRZ*1 is repressed by salt stress. The irresponsive to ABA is similar to that observed for salt treatment. In addition, when *SRZ*1 was over expressed in tobacco plants it repressed the expression of salt-responsive genes and increased plant sensitivity to salt and cold stress, while the over expression of *ZFP*179 and *ZFP*182 improved stress tolerance. This indicates a putative function as positive or negative regulators of salt stress response. Among the four genes encoding bZIPs (*OsABF*1, *OsABI*5,*OsbZIP*23, and *OSBZ*8;) associated with salt stress responses, all are induced by salt treatment and also positively responsive to ABA, and only *OsABF*1 is induced by cold (Hossain, *et al.* 2010; Nakagawa, *et al.* 1996; Xiang, *et al.* 2008; Zou, *et al.* 2008). Curiously, although both *OsABI*5 and *OsbZIP*23are induced by high salinity, only the over expression of *OsbZIP*23 improves salt tolerance, while *OsABI*5 over expression leads to high salt sensitivity. Given that, in rice, repression of*OsABI*5 improves stress tolerance and reduces fertility, it was proposed that this gene is a negative regulator of the salt stress response in rice (Zou, *et al.* 2008). TFs belonging to different families, such as MYB, HSF, Trihelix or CCAAT-HAP2 have also been associated with salt stress responses. *OsMYB*3*R*-2, *OsGTγ* -1 and *OsHsfA*2*e*genes are induced by high salinity and, when over expressed in *Arabidopsis*, all improve salt tolerance (Dai, *et al.* 2007; Fang, *et al.* 2010; Liu, *et al.* 2010). *Arabidopsis* plants over expressing *OsMYB*3*R*-2 showed also increased tolerance to low temperature and drought, suggesting that MYB acts as a master switch in stress tolerance (Dai, *et al.* 2007). Unlike most plant R2R3MYB transcription factors, OsMYB3R-2 has three imperfect repeats in the DNA-binding. Whether this is associated with abiotic stress responses, is not yet known. The plant-specific NAC (NAM, ATAF1/2, CUC2) family, which plays regulatory roles in diverse developmental processes and stress responses (Riechmann, *et al.* 2000), is one of the largest rice TF families with approximately 150 members (http://drtf.cbi.pku.edu.cn/). Curiously, most of the TFs shown to be associated with rice responses to salt stress (25/42) belong to this family. In 2008, Fang *et al.* had already reported that more than 40 *OsNAC*

genes were responsive to salt and/or drought stress (Fang *et al.*, 2008). Phylogenetic analysis showed that NAC family can be divided into five groups (Fang, *et al.* 2008). Although many salt responsive *OsNAC* genes encode TFs that belong to group III (stress-related NAC genes, *SNAC*), salt-responsive OsNAC are spread into several groups (I–IV). The fact that all *OsNAC* genes published until now as being associated to salinity are induced and not repressed by salt stress is also striking. This expression pattern is also observed when these genes are modulated by other stresses, such as cold, drought, heat, mannitol, and ABA, suggesting that *OsNAC* genes are normally expressed at low levels and may be induced when plants are subjected to adverse environmental conditions. The over-expression of several *OsNAC* salt-responsive genes in rice such as *SNAC*1 (Hu, *et al.* 2006), *SNAC*2 (also known as *NAC*6 or *NAC*48) (Nakashima, *et al.* 2007) *OsNAC*5 (Takasaki, *et al.* 2010) and *OsNAC*10 (Jeong, *et al.* 2010) was shown to improve salt and drought tolerance. This fact seems to indicate that these genes are positive regulators of the salt stress response and must activate mechanisms involved in salt and drought tolerance. For instance, transgenic plants over expressing *SNAC*1 are more sensitive to ABA and lose water more slowly by closing more stomatal pores (Hu, *et al.* 2006). The *SNAC*2 gene (also known as *NAC*6 or *NAC*48) is also induced by wounding and blast and rice transgenic plants over expressing this gene exhibited increased blast disease tolerance (Nakashima, *et al.* 2007), thus indicating that some *OsNAC* members may also be involved in a cross-talk between salt stress and biotic stress responses. Notably, a subset of the NAC members, collectively designated NTLs, is membrane-associated, signifying that membrane-mediated regulation is an important molecular scheme functioning in rapid transcriptional responses to external stimuli (Kim, *et al.* 2010). The transcript level of *OsNTL*2, *OsNTL*3, *OsNTL*4, *OsNTL*5*,* and *OsNTL*6 is induced by high salinity and mannitol. The *OsNTL*2 and *OsNTL*3 genes are both induced by cold and heat. Although the *OsNTL* genes may play a role in a broad spectrum of plant stress responses, they are particularly responsive, at transcriptional level, to high salinity and drought (Kim, *et al.* 2010). Other stresses such as cold may have a membrane-mediated control of their activity. However the possibility that high salinity and/or drought also control *OsNTL* activity through membrane association cannot be ruled out. Although many TFs have been reported as involved in salt stress responses, still not much is known regarding their epigenetic and post-transcriptional/translational regulation, or their target genes, under salinity. Small RNAs have been shown to play an important role controlling the transcript level of many TFs involved in abiotic stress responses (Covarrubias and Reyes, 2010; Liu, *et al.* 2010). During the last years, many miRNAs, siRNAs and their targets have

been identified in rice (Li, *et al.* 2010; Sunkar, *et al.* 2005; Sunkar and Jagadeeswaran, 2008) and many of them are certainly involved in salt stress responses. Post-translational regulation mediates many responses to high salinity and the activity of many TFs is known to be regulated at this level. In addition, many genes involved in salt stress response mechanisms have been reported to be differentially regulated under salinity, but little is known concerning the TF transcription regulation.

(f) Protein modifications involved in salt stress response: Post-translational modifications (PTMs) fast regulate the cell proteome allowing it to rapidly respond to new cellular needs. Thus, PTMs are particularly important in the response to environmental changes in plants. As described above, a key example of the importance of PTMs in plant response to salt stress is the regulation of the *Salt Overly Sensitive (SOS)* pathway. In *Arabidopsis*, the activity of the Na^+/H^+ antiporter *SOS1* is regulated through protein phosphorylation by the *SOS2/SOS3* kinase complex (Qiu, *et al.* 2002; Quintero, *et al.* 2002). More recently, rice *SOS1* was isolated and shown to also be phosphorylated and activated by the *SOS2/SOS3* protein kinase complex, indicating conservation in the *SOS* pathway of both species (Martinez-Atienza, *et al.* 2007). The plant *SOS* pathway is not only dependent on phosphorylation; it also requires the N-myristoylation of *SOS3* to function. N-myristoylation is a co-translational protein modification that occurs in the N-terminal glycine residue of some proteins. Additionally, this modification can also occur post-translationally, when internal glycine residues become exposed by protease activity. N-myristoylation results from their reversible addition of the lipid myristate (C:14) and is catalyzed by N-myristoyl transferase (NMT) and predicted to affect 2% of all plant proteins (Meinnel and Giglione, 2008). N-myristoylation function is to target proteins to membranes which is very important in the control of membrane signaling pathways (Traverso, *et al.* 2008). In *Arabidopsis*, this process was shown to be essential for plant viability, since *AtNMT*1 knock-out mutants show severe defects during embryonic development at the shoot apical meristem, leading to early embryonic growth arrest (Pierre, *et al.* 2007). The authors linked the effect to the protein complex *SnRK1*, a *SNF1*-related proteinkinase,that appears to be involved in the control of the molecular mechanisms leading to plant sensing and adaptation to severe environment changes (Traverso, *et al.* 2008). The Ca^{2+}sensor *AtSOS3*, belonging to the family of CBL proteins, is also N-myristoylated (Gong, *et al.* 2004; Ishitani, *et al.* 2000). Nevertheless, besides *OsCBL4 (SOS3)*, two other rice CBLs (*OsCBL7* and *OsCBL8*) with high homologies to *AtSOS3* are predicted to be N-myristoylated (Ishitani, *et al.* 2000), again pointing to the

importance of this modification in the rice *SOS* pathway regulation. Besides playing an important role in ion homeostasis, due to the regulation of *SOS1* activity, the phosphorylation central role in plant response to salinity is also well established due to its involvement in the signaling cascades that connect stress perception to cellular response. The specific MAPK signaling pathway is a clear example of the importance of phosphorylation. Furthermore, specific proteins involved in different cellular mechanisms also become phosphorylated upon stress. Analysis of the rice phosphoproteome resulted in the identification of over 30 phosphoproteins differentially regulated in response to salt stress (Chitteti and Peng, 2007; Khan, *et al.* 2005). Among them are cytoplasmic malate dehydrogenase and calreticulin, with phosphorylation induced by salt stress (Khan, *et al.* 2005). Also, the typical stress response proteins *SalT* and mannose-binding rice lectin show upregulated phosphorylation (>two-fold) in rice roots, after 10h salt stress (Chitteti and Peng, 2007).

Other important PTMs in plant abiotic stress response are ubiquitination and binding of Ubiquitin-like proteins, such as SUMOylation. Protein modification by Ubiquitin and Ubiquitin-like proteins is important in the regulation of several cellular processes due to their involvement in protein stability and cellular localization, and in protein-protein interactions. Ubiquitination involves the covalent attachment of Ubiquitin to substrate proteins, usually targeting them for degradation. It involves the concerted activity of ubiquitin-activating enzyme (E1), Ubiquitin-conjugating enzyme (E2), and Ubiquitin ligases (E3). Several studies suggest that ubiquitination plays an important role in plant abiotic stress response, as it is the case of the involvement of *Arabidopsis* E3 ligases CHIP in temperature stress response (Luo, *et al.* 2006; Yan, *et al.* 2003) and *HOS1* in cold stress response (Ishitani, *et al.* 1998; Lee, *et al.* 2001). Over-expression of *SDIR1*, also an E3 ligase, was shown to lead to increased drought stress tolerance (Zhang, *et al.* 2007). Heterologous over-expression of *AtSDIR*1 in rice also increased drought stress tolerance (Zhang, *et al.* 2008). Recently, the first report of ubiquitination involvement in salt stress response showed that the soybean ubiquitin-conjugating enzyme (E2), *GmUBC*2, is induced by both salt and drought stress and that its heterologous over-expression in *Arabidopsis* resulted in tolerance improvement to both stresses (Zhou, *et al.* 2010). The authors also showed that *GmUBC*2 over-expression in *Arabidopsis* significantly increased the transcripts of *AtNHX*1 and *AtCLC*a known to be involved in ion homeostasis. SUMOylation is the conjugation of the Small Ubiquitin related

MOdifier (SUMO) to proteins through an enzymatic pathway similar to ubiquitination, but with unique E1, E2, and E3 equivalents. In plants, SUMOylation has been implicated in several important cellular processes, (Miura and Hasegawa, 2010), including in abiotic stress response. In rice seedlings, 24-h treatments with salt, cold, or ABA were shown to dramatically increase protein SUMOylation, most probably through conjugation with rice SUMO1/2 (Chaikam and Karlson, 2010). This phenomenon was already observed in *Arabidopsis* in response to temperature, drought, salt, and oxidative stresses (Catala, *et al.* 2007; Conti, *et al.* 2008; Kurepa, *et al.* 2003; Miura, *et al.* 2007). The induction of SUMOylation in response to abiotic stress uses mainly the cellSUMO1/2 pool. So far, only three proteins directly involved in abiotic stress response in *Arabidopsis* have been identified as SUMOylated, ICE1, ABI5, and RD29B (Budhiraja, *et al.* 2009; Miura, *et al.* 2007; Miura, *et al.* 2009). ICE1 and ABI5 are good examples of crosstalk between SUMOylation and ubiquitination, in plants. ICE1, for instance, is stabilized when SUMOylatedby SIZ1 (a SUMO-E3 ligase). SUMOylation blocks ICE1polyubiquitination induced by the Ub-E3 HOS1, resulting inCBF3 (DREB1A) activation and increased cold stress tolerance (Miura, *et al.* 2007). The regulation of the rice homologousICE1 protein by PTMs also seems to involve rice SIZ1 and HOS1 homologues, but its involvement in cold/abiotic stress tolerance was not yet established. Protein deSUMOylation is crucial in the maintenance of the correct levels of SUMOylation during stress response. Recently, studies with two functionally redundant SUMO-proteases OTS (Overly Tolerant to Salt)1(ULP1d) and OTS2 (ULP1c) show the importance of maintaining SUMO-conjugated proteins during *Arabidopsis* salt-stress response (Conti, *et al.* 2008). Double *ots*1 *ots*2 mutants are phenotypically similar to wild type plants under normal conditions, but extremely salt sensitive. On the other hand, over-expression of OTS1 increased salt stress tolerance with decreasedSUMO1/2 protein-conjugation (Conti, *et al.* 2008). These observations show the complexity of the regulation of salt stress response by SUMOylation. In rice, some evidences exist relating SUMOylation to abiotic stress response (Chaikam and Karlson, 2010; Nigam, *et al.* 2008; Park, *et al.* 2010). Recently, Srivastava, *et al.* (2016) showed direct evidences of SUMOylation in rice plants, overexpressing *OsOTS1* have increased salt tolerance and a concomitant reduction in the levels of SUMOylated proteins. Here, *OsOTS1* confers salt tolerance in rice by increasing root biomass. High salinity triggers *OsOTS1* degradation indicating that increased SUMO conjugation in rice plants during salt stress is in part achieved by down-regulation of OTS1/2 activity. *OsOTS1* is nuclear localized indicating a direct requirement of *OsOTS1* dependent deSUMOylation activity in rice nuclei for salt tolerance. This massive

SUMOylation seems to follow the transcript profile of *OsSIZ*2 (an E3 SUMO ligase) detected during salt stress imposition, pointing to an important role of this protein in rice salt stress response (Chaikam and Karlson, 2010). Other PTMs, such as histone acetylation are also expected to play important roles in the control of gene expression, during salt stress conditions, as well as during other types of abiotic and biotic stresses. For instance, in *Arabidopsis*, histone modifications mediated by *HDA6* (a histone deacetylase) modulate the expression of the salt stress–induced *DREB2A*, *RD29A*, and *RD29B*, among other abiotic stress responsive genes. The *HDA6* mutant *axe1-5* showed hypersensitivity to both ABA and salt stress (Chen, *et al.* 2010). In rice, an expression analysis of members of the histone deacetylase (HDAC) family, failed to reveal central players in salt stress response (Fu, *et al.* 2007). Overall, little is still known regarding how PTM modulates salt stress response in rice. However, the available data, mostly from the model plant *Arabidopsis*, leave no doubt about the significance of these mechanisms, unlocking interesting and important research lines that may ultimately lead to crop stress tolerance improvement.

(g) Genes overlapping with QTLs: Several rice genes have been shown to be differentially expressed when plants are subjected to high salinity. However, many of them do not co-segregate with known QTLs associated with salt stress responses. Negrao, *et al.* (2011) prepared a list to gain new insights into the putative relation of rice candidate genes (CG) and salinity-related QTLs, summarized in three main groups of QTL traits (Ion uptake/concentration, Plant development and Salt tolerance/damage). Many QTLs found to overlap with CGs falling in the categories of Signaling, Ion homeostasis and LEAs and other proteins, are characterized as related to processes of ion uptake/concentration (Na^+ or K^+ uptake, quantity/concentration or Na^+/K^+ ratios). Interestingly, the QTLs that co-segregate with CGs encoding transcription factors concentrate in a small region in the long arm of chromosome 1, in which two QTLs have been identified (NQR 2092 and DSD 2085). Particularly remarkable is the number of QTLs that overlap with some CGs, namely: *OsHKT*8, which co-segregates with 13QTLs and is involved in Na^+ removal from the xylem sap (guaranteeing salt exclusion from leaves); SALT, a mannose-binding lectin that co-segregates with 10 QTLs; and *Os01g25280*, coding for a protein with still unknown function, and also co-segregating with 9 QTLs. From the traits listed in the group Salt tolerance/damage, clearly the most interesting QTL is *Saltol*, for its relation to increased plant survival under salinity. As previously explained, this particular QTL is derived from Pokkali and largely used for breeding purposes. Recently, Thomson, *et al.* (2010) suggested

OsHKT8 (Na^+ selective transporter) as the major gene of this QTL responsible for shoot Na^+/K^+ homeostasis. However, besides *OsHKT8*, also *Sal*T and the unknown gene *Os01g25280* co-segregate with *Saltol* and with other QTLs involved in ion uptake/concentration. Therefore it is believe that these two latter genes deserve close attention and a more detailed study. The faster and cheaper new sequencing techniques now available (see the recent review by Delseny, *et al*. 2010) are allowing resequencing several species, covering a number of different varieties. This new effort is also being extended to rice (http://www.OryzaSNP.org) with the analyses of 100Mbp of non-repeated sequences in 20 cultivated rice accessions (McNally, *et al.* 2009). One of the outcomes of these projects will be the easier identification and tracking of new allelic variations present in QTL regions. This study will have to be combined with the screening of the diverse rice germplasm available, and detailed phenotype characterization, to identify alleles putatively interesting for breeding programs.

(h) Engineering salt tolerance (Transgenic approach): In spite of the complexity of salt tolerance, claims are common in the literature that the transfer of a single or a few genes can increase the high level of tolerance of plants to saline conditions. Transgenic plants are those that contain gene(s) that are artificially inserted from another unrelated organism and commonly known as genetically modified (GM) plants. For salt stress, transgenics have been produced since1993 (see Flowers, 2004) with the preponderance involving *Arabidopsis*; about 45 papers, however, report transgenic rice with claims for altered salt tolerance. A study conducted by Flowers, (2004) showed that, of the 68 papers produced between 1993 and early 2003, only 19 reported quantitative estimates of plant growth. About half of all the papers showed data on experiments conducted under conditions where there was little or no transpiration: such experiments may provide insights into components of tolerance, but they cannot qualify for the claims of enhanced tolerance at the whole plant level. A comprehensive list of nearly 40 papers in which rice has been transformed and where a claim is made in the title or the abstract of the paper that the transformant showed enhanced salt tolerance have been incorporated in to a review paper Singh and Flowers, (2010). The papers have been categorized according to the criteria developed by Flowers, (2004) as it is important to distinguish between assessments of tolerance made *in vitro* (category 3) and those where experiments provide quantitative data on transformants and a control under saline and non-saline conditions (category 1). Where there is little or no transpiration,

experiments have less relevance to field situations than assessments made in greenhouses (or even fields) although such in vitro experiments do provide some information on the action of the gene product. It is apparent from the experiments classed as category1 that increasing the synthesis of compatible solutes such as glycine betaine (Su, *et al.* 2006) or proline does enhance growth (Anoop and Gupta, 2003) and yield (Wu, *et al.* 2003) under saline conditions. Improvements in tolerance have also been seen in transgenic plants that express a Na^+/H^+ antiporter from yeast (*SOD2* Zhao, *et al.* 2006) and various transcription factors (*SNAC1* Hu, *et al.* 2006; ZFP252 Xu, *et al.* 2008; *ONAC045* Zheng, *et al.* 2009) as does the production ofsedoheptulose-1,7-bisphosphatase in chloroplasts (Feng, *et al.* 2007). Beside the above mentioned transgenic list and examples given by Singh and Flowers, (2010), there are a number of genes have been recently cloned and transformed to rice and subsequently claimed for enhanced salt tolerance listed below in table 2.4. However, the expression of some genes can enhance sensitivity to salt. While some researchers continue to assess tolerance using germination or plantlets *in vitro*, what is really needed is field assessment, since after more than 10 years of research using transgenic rice to alter salt tolerance, the value of this approach has yet to be established in the field (Singh and Flowers, 2010).

Table 2.4: List of recent updates of transgenic developed for salinity tolerance in rice

Sl No.	Gene	Encoding Protein /function	Gene source	Target Stresses	Reference
1	*AtBAG4*, *Hsp70* and *p35*	*Programmed cell death-associated antiapoptotic genes*	*Arabidopsis(AtBAG4)*, *Citrus tristeza virus (Hsp70)* and *Baculovirus* (*p35*)	Salt	Hoang, *et al.* 2015
2	*SNAC1*	*Transcription factor*	*Oryza sativa* L.	Salt and drought	Parvin, *et al.* 2015
3	*OsBAT1*	*DExD/H-box protein involved in messenger RNA (mRNA) splicing*	*Oryza sativa* L.	Salt	Tuteja, *et al.* 2014
4	*OsSUV3*	*Improving the antioxidant machinery*	*Oryza sativa* L.	Salt	Tuteja, *et al.* 2013
5	*Psp68*	*Pprototypic member of DEAD-box protein family*	*Pisum sativum*	Salt	Banu, *et al.* 2014
6	*SfIAP*	*Programmed cell death-associated antiapoptotic gene*	--	Salt	Hoang, *et al.* 2014
7	*OsRab7*	*Vesicle Trafficking Gene*	*Oryza sativa* L.	Salt	Peng, *et al.* 2014
8	*OsMSR2*	*Rice calmodulin-like gene*	*Oryza sativa* L.	Salt	Xu, *et al.* 2013

9	*AtEm6*	*Late embryogenesis abundant gene (LEA)*	*Arabidopsis thaliana*	Salt	Tang and Page, 2013
10	*AeHMDER*	*Monodehydroascorbate reductase*	*Acanthus ebracteatus*	Salt	Sultana, *et al.* 2012
11	*OsDREB2A*	Transcription factor	*Oryza sativa* L.	Salt	Mallikarjuna, *et al.* 2011
12	*PDH45*	*DEAD-box helicase*	*Pisum sativum*	Salt	Amin, *et al.* 2012
13	*OsNHX1*	*Vacuolar Na^+/H^+ antiporter*	*Oryza sativa* L.	Salt	Islam, *et al.* 2009a
14	*PgNHX1*	*Vacuolar Na^+/H^+ antiporter*	*Pennisetum glaucum*	Salt	Islam, *et al.* 2009b

There are numerous candidate genes that might be usefully used to transform rice for salinity tolerance reviewed by Singh and Flowers, (2010) and also some recent events from above list illustrating a sporadic effort to develop tolerances by inserting either a single gene/multiple/fusion gene/s by through a single physiological mechanisms either direct or indirect effects. However, the discussion above for the genetic and physiological complexity throughout the life cycle of rice for salt tolerance is simply explains a number of traits should be incorporated for getting complete tolerance including ion exclusion, osmotic tolerance and tissue tolerance proposed by Munns and Tester, (2008). Roy, *et al.* (2014) reviewed the three main traits that are proposed by Munns and Tester, (2008) to be the primary mechanisms for salinity tolerance - shoot ion exclusion, shoot tissue tolerance and 'osmotic' tolerance - and proposed genes that might contribute to each of these traits (Table 2.5 gives the comprehensive lists of genes for the three main traits proposed, adapted from Roy, *et al.* 2014). A particular gene (or gene family) may well contribute to more than one trait, just as one trait can be conferred by more than one gene. They discussed a particular gene family in this review where it is likely to be making a significant contribution - this does not preclude members of this gene family also contributing to other traits. They have taken this approach to try to encourage a focus more on the traits that contribute to salinity tolerance, rather than on the traditional functional categorizations of gene families. This is an attempt to come more 'from the plant's perspective' of functional effects of the genes at the whole plant level, rather than categorizing genes based on the immediate activity of their encoded proteins.

Table 2.5: Genes that have been overexpressed to improve specific salinity tolerance traits in crops (adapted from Roy, *et al.* 2014).

Transgenes	Gene isolated from	Promoters used	Transgenic crop	Reported transgenic plant performance during salt stress
Ion exclusion (transporters)				
Na^+/H^+ antiporter (*SOS1*)	*Arabidopsis*	Constitutive	Tobacco	Altered shoot and root accumulation of Na^+ and K^+
Na^+/H^+ antiporter (*SOD2*)	*Salicornia brachiata*	Stress inducible	Rice	Improved biomass production
Na^+ transporter (*HKT*subfamily 1)	Yeast	ABA responsive	Barley	Improved germination
Na^+/K^+ transporter (*HKT*subfamily 2)	Barley			
Na^+ ATPase (*ENA*)	*Physcomitrella patens*			
Tissue tolerance (transporters/proton pumps)				
Na^+/H^+ antiporter (*NHX*)	*Arabidopsis*	Constitutive	Buckwheat	Improved shoot and root biomass production
Na^+/H^+ antiporter (*nhaA*) Vacuolar H^+pyrophosphatase (*vacuolar H+-PPase*)	*Atriplex gmelini*		Cotton	Altered $Na^+$and K^+accumulation
	Rice		Tomato	Increased proline content
	Cotton		Poplar	
	Hordeum brevisubulatum		Kiwifruit	
	Pennisetum glaucum		Fescue	
	Aeluropus littoralis		Rice	
	Salicornia brachiata		Wheat	
	Salsola soda		Brassica	
	Malus domestica		Bentgrass	
	E. coli		Sugar beet	
			Alfalfa	
			Tobacco	
			Apple	
Tissue tolerance (Compatible solutes)				
Trehalose-6-phosphate synthase (*TPS*)	Yeast	Constitutive	Alfalfa	Increased compatible solute accumulation
Trehalose-6-phosphate	Rice	Stress	Tomato	Improved plant

phosphatase (*TPP*)		inducible		survival
Mannitol-1-phosphate dehydrogenase (*mt1D*)	*E. coli*	Shoot expression	Rice	Increased growth
L-myo-Inositol-1-phosphase synthase (*MIP*)	*Porteresia coarctata*	Protein often targeted to chloroplast	Tobacco	Reduced wilting
Myo-inositol O-methyltransferase	*Mesembryanthemum crystallinum*		Wheat	Maintenance of photosynthetic efficiency
Betaine aldehyde dehydrogenase (*BADH*)	Spinach		Sweet potato	
Choline oxidase/dehydrogenase (*codA*/*betA*)	Moth bean		Wheat	
Δ1-pyrroline-5-carboxylate synthetase (*P5CS*)			Potato	
Tissue tolerance (Degradation of reactive oxygen species)				
Ascorbate peroxidase (*APX*)	*Arabidopsis*	Constitutive	Tobacco	Maintenance of photosynthetic efficiency
Glutathione S-transferase (*GST*)	Tomato	Protein often targeted to chloroplast or cytosol	Rice	Maintenance of growth
Superoxide dismutase monodehydroascorbate reductase (*MDR*)	Tobacco			Improved maintenance of photosynthesis
Catalase	Mangrove			Improved germination
	Pea			Improved growth of seedlings
	E. coli			Increased antioxidant enzyme activity
Signaling/regulatory pathways				
Calcineurin-B like interacting protein kinases (*CIPK*)	*Arabidopsis*	Constitutive	Barley	Altered Na^+, K^+ and Cl^-accumulation
Mitogen-activated protein kinase (*MAPK*)	Chickpea		Tobacco	Improved biomass production
Sucrose nonfermenting1-1 type protein kinase	Rice		Rice	Reduced leaf senescence
	Apple		Tomato	
	Cotton			
	Tomato			
Transcription factors				
DREB	*Pennisetum glaucum*	Constitutive and inducible	Tobacco	Improved germination
AP2/ERF	Cotton		Wheat	Improved biomass
MYB	Soybean		Tomato	Improved chlorophyll retention

NAC	Tomato		Rice	Altered Na^+accumulation
	Rice			
	Chrysanthemum			
	Wheat			

(i) Concluding remarks: After drought, salinity is the second major obstacle to rice production worldwide. The enormous importance of rice as staple food crop, and its high sensitivity to saline soils makes the study of salt stress tolerance particularly important in this crop. The high sensitivity during growth stages such as seedling and flowering/seed set strongly compromises plant survival and good yields. Salt tolerant varieties have been described in several countries. Tolerant *indica* varieties were mainly found in South Asia, while tolerant *japonica* varieties are more widespread (Europe, Asia, Africa). The tolerance mechanisms and strategies adopted by those varieties seem to be quite diverse and polygenically controlled. Many studies on QTL analysis are based on controlled hydroponic assays, with limited relation to field conditions. In spite of the obvious difficulties for controlling a field experiment considering all the factors influencing plant growth, clearly more field work should be conducted. The main technical problem of field assays is the existence of multiple stresses that simultaneously impact plant development. Numerous crosstalks have been identified between the response to salt stress and other abiotic (e.g., drought or cold), or biotic factors (beneficial or detrimental). To illustrate this, recent findings demonstrated that, by closing stomata, ABA plays important roles in the plant defense system against pathogens. The improvement of rice culture under salinity certainly requires a deeper comprehension of all the putative field interactions.

This review presented the current understanding of the multiplicity of perception, signaling and whole plant response mechanisms that rice uses to deal with salinity. In the salt stress response, the temporal and cross-scale (from the individual cell to the whole-plant) coordination of these responses is regulated by hormonal signals and by a large array of gene families, although with variations among genotypes. Some of these gene families and interaction networks have been uncovered. However, many processes are still obscure. This review presented a comprehensive map integrating salt tolerance QTLs and candidate genes. This reinforces the relevance for breeding for salt-tolerance of certain chromosome regions where both co-localize. An interesting aspect of this revision was the observation that

although many salt responsive genes overlap with the confidence interval of known QTLs, many others are distributed along the rest of the genome. Still *Saltol* QTL is the most promising and major effect QTL for seedling stage tolerance from Pokkali. This is, for instance, frequently identified in original and different populations and even unrelated populations for salinity tolerance. The case of *SKC1* in Nona Bokra co-localize within the same genomic region as *Saltol*. But the region is still mysterious to uncover the real controlling factors. Still several QTL regions were found to overlap with less characterized candidate genes and these certainly deserve a deeper study. An updated list of salt-tolerant varieties useful as donors for breeding programs is also presented. At this point only limited information is available regarding the salinity tolerance mechanisms present in the different varieties, which requires further attention to ultimately combine different mechanism in a single variety and achieve a higher tolerance level.

Chapter 3:

Materials and Methods

Chapter 3
Materials and Methods

The present work comprises two major research components: 3.1 and 3.3 Introgression of salinity tolerance QTLs (*Saltol*) in to two Bangladeshi mega rice varieties through Marker-assisted backcrossing and 3.2 and 3.3 phenotypic characterization for salinity tolerance and yield of introgression lines at controlled Green house/Net house and field conditions.

3.1 Introgression of salinity tolerance QTL Saltol into Bangladeshi mega rice variety BR11 through Marker-assisted backcrossing.

3.1.1 Plant Materials: Recipient and Donor parents

Mukta is the popular name of BR11 and it was developed and released by Bangladesh Rice Research Institute (BRRI) in 1980. The ancestry of BR11 is BR52-87-1-HR88 with the parentage IR20/IR5-47-2. BR11, is a semi-dwarf (~115 cm), medium duration (~ 145 days in the transplanted *Aman* season), medium bold grain with yield potential about 5.5 t/ha (BRRI, 2014). It is the most popular *T. Aman* season variety widely cultivated in the rain-fed ecosystem. Due to its enormous popularity and wider adaptability, this variety was chosen first for conversion in to salt tolerant-BR11 by introgressing *Saltol* QTL through a molecular breeding approach called Marker-assisted backcrossing.

FL378, a F_8 Recombinant Inbred Line (RIL) was used as *Saltol* QTL donor for this introgression work. The pedigree of FL378 is (IR66946-3R-78-1-1) which was developed at the International Rice Research Institute (IRRI) from IR29/Pokkali. This line was chosen due to its higher level of tolerance to salinity at the seedling stage. The tolerance of FL378 was derived from the well known Indian salt tolerant cultivar Pokkali (formerly it was called Pokkali B but it is now known as IRRI Gene Bank accession no. IRGC108921). The tolerance of Pokkali was mapped at IRRI and the identified major effect seedling stage tolerance QTL was called *Saltol* that can explain 45.00% phenotypic variation for seedling stage salinity tolerance (Thomson, *et al.* 2010a; Bonilla, *et. al.* 2002).

3.1.2 Marker Assisted Backcrossing (MABC) Scheme

The Marker Assisted Backcrossing (MABC) approach used in this study was followed first by Neeraja, *et. al.* (2007) for the development of a submergence tolerant variety Swarna-

Sub1 through the introgression of *Sub1* QTL in to an Indian mega variety *Swarna*, and then the MABC method was published as a manual for MABC in rice by Collard, *et. al.* 2008a. Hybridization of recipient to donor and then 3-step backcrossing with recipient parent followed by marker-assisted backcross approach was used in this study. Each backcross generation was subjected to a 3-step marker-aided selection i.e. Foreground selection (FGS), Recombinant selection (RBS) and Background selection (BGS). In the marker-aided selection process for each backcross generation, first selection is the foreground selection where tightly linked markers were used to select progenies which had the QTL present in heterozygous form. This was also referred as positive selection. After the first selection, the selected progenies were subjected to RBS, where the nearest-unlinked markers bordering both sides of the QTL were used for precise introgression (none or minimum additional segment adjoining the target loci) of the QTL. The selected progenies were subjected to BGS after RBS, where markers were used for quick recovery of rest of the recipient genome. This selection is also called negative selection. The best progeny/progenies, which have the QTL in heterozygous form, recombination on either or both side of the QTL and has maximum number of recipient loci at the background were crossed again with the recurrent parent to obtain BC_2F_1. The same procedures were followed to obtain BC_3F_1. The calculation of minimum population was carried by POPMIN software. At BC_3F_1 stage, the progenies with the maximum recovery of background genome were selfed to BC_3F_2. All these progenies were genotyped by foreground markers for fixation of the QTL loci (selection of progenies which have homozygous QTL loci). This step also increased the proportion of the recurrent parent loci, some of which were still heterozygous. The selected progenies, which had the QTL in homozygous forms and had clean background, were referred to as Precision Isogenic Lines (PILs). These are also commonly referred to as Near Isogenic Line (NILs). BC_3F_3 seeds of the selected plants were used to screen for salinity tolerance at the seedling stage to confirm the tolerance gained in the newly developed NILs or in other words, BR11-*Saltol* in comparison with their parents.

3.1.3 Molecular Marker Analysis:

a) Validation and Selection of Molecular Markers for FG and RB selection

The success of any MABC works exclusively depends on the effects of the corresponding QTL and its efficient transfer from donor to recipient. Therefore use of most tightly linked

markers for the locus of interest is needed. Similarly closely adjacent unlinked and robust markers are required to avoid introgression of loci outside of the region of interest (termed as minimization of linkage drag). These two criteria are the key to efficient, easy and clean transfer of the target QTL loci into the recipient genome. So, the validation and selection of markers prior to use in MABC system is a pre-requisite and most important necessary step. Before starting this research, a marker validation work was carried out in the Plant Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka. The research was carried out in F_6 breeding population developed from the original *Saltol* donor Pokkali. From this validation work two markers namely RM493 and RM1287 was found tightly linked to *Saltol* QTL and one marker RM3627 was found unlinked to the *Saltol* and were selected for use in the present MABC work (Seraj, *et al.* unpublished data). Again to compare the selected markers with previous work carried out at IRRI (Islam, 2006a; Thomson, *et al.* 2007) the following markers were found to have tight linkage with *Saltol, e.g.* RM1287, RM8094, RM3412, RM493 and CP3970 and no/weak linkage with RM3627, RM10825, RM10864 and RM562. However, based on the above comparison, the following 3 SSR markers RM1287, RM3412 and RM493 were selected for foreground selection due to their tight linkage with *Saltol* and for recombinant selection due to no/weak linkage RM3627 at the telomeric end and RM10825, RM10864 and RM562 at the centromeric end were selected.

b) Identification of Polymorphic Markers for BG selection

Molecular markers are particularly useful if they reveal differences between individuals of the same or different species. These are called polymorphic markers, whereas markers that do not discriminate between genotypes are called monomorphic markers (Collard, *et al.* 2005). SSRs are co-dominant markers and therefore can easily be used in genotyping work like QTL mapping as well as in MAS/MABC. Initially a set of 153 SSR and InDel markers distributed throughout rice genome were screened for polymorphism of the parental combination of BR11/FL378. The positions of the SSR markers were inferred from the Physical map of IRGSP 2005 through BLAST searchers or found in the Gramene SSR database (www.gramene.org). The order of the SSR markers were considered based on the Physical map of IRGSP (IRGSP 2005) and the corresponding genetic distances (cM distances) were computed through software called cM Converter v.1.0.0.4 (http://mapdisto.free.fr/cMconverter/). From the first polymorphism survey 52 were found polymorphic between BR11/FL378 and 48 of them used to genotype the first backcross

generation. Later, in second and third backcross generation 29 and 10 additional polymorphic SSR and InDels were used to genotype progenies. Finally, a total of 87 SSR and InDel were included in to the panel of BGS (see the BGS polymorphic markers in a diagram in the result section Fig. 4.1.2 and lists in the Appendix II, Table 8.2.4).

3.1.4 Marker Genotyping

a) Collection and storage of leaf sample

Newly developed leaves were collected after establishment of seedlings at 6-7 leaves stage (Fig. 3.1). About 1.0 g fresh leaf was collected and wrapped with foil paper as well as masking tape and marked with a marker pen and stored immediately in Liquid Nitrogen (Fig. 3.1). Then in the laboratory half of the collected leaf samples were used for DNA isolation and rest of them were packed and sealed in a plastic bag and stored into -80 ^{0}C for future use.

Fig. 3.1: The BC_1F_1progenies at 6-7 leaf stage in the field of Plant Breeding Division of BRRI (left) and the leaf collection by researcher and technicians (right).

b) Isolation of genomic DNA from leaves

Genomic DNA was isolated separately along with the parents. DNA was extracted using two protocols i.e. CTAB method described by Doyle and Doyle (1990) and the DNeasy® plant mini kit from QIAGEN (www.qiagen.com). The procedures for isolation of genomic DNA

from rice leaves are given below. Preparation and maintenance of reagents are placed in appendix III.

c) Procedure of plant DNA isolation

The CTAB method is less expensive procedure and is characterized by high yields of purified genomic DNA from a small amount of tissue (Doyle and Doyle, 1990). The procedure of CTAB method is outlined below:

1. Approximately 1.0 g of leaf tissue was chilled with liquid nitrogen and ground to a very fine powder by mortar and pestle.
2. The powdered tissue was transferred in a 15 ml screw cap tube containing 5 ml of preheated (65 ^{0}C) CTAB buffer.
3. Incubation at 65 ^{0}C in water bath continued for 30 min with occasional vigorous shaking.
4. After incubation, same amount of Phenol: Chloroform: Isoamyl alcohol (24:24:1) was added to the extract.
5. The mixture was centrifuged for 10 min at 6000 rpm (>5000 × *g*)
6. The aqueous phase was transferred to another fresh screw cap tube and Isopropanol (2/3 of the aqueous phase) was added and mixed gently to precipitate DNA.
7. The mixture was centrifuged for 10 min at 14000 rpm (20000 × *g*)
8. The supernatant was discarded and the pellet was washed with 3-5 ml of ice-cold 70% Ethanol.
9. The pellet was dried completely and dissolved in minimum TE buffer.
10. DNase free RNase (Invitrogen, USA) was added (0.10 µl RNase for 100µl DNA) and incubated at 37 ^{0}C for 3 min.
11. Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added in equal volume of the DNA stock.
12. The stock was centrifuged for 10 minute at 14000 rpm (20000 × *g*)
13. The aqueous phase was taken and again equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added.
14. The mix was centrifuged for 10 min at 14000 rpm (20000 × *g*) and the aqueous phase containing the DNA separated.
15. Na-acetate (3.0 M, pH 8.0) (1/10 of the aqueous phase) and ice cold 99% Ethanol (2 times of the aqueous phase) were added in order to precipitate DNA.

16. This mixture was kept on ice for 30 minutes and then centrifuged for 10 minutes at 14000 rpm (20000 × *g*)
17. The supernatant was discarded.
18. The DNA pellet washed with 3-5 ml ice cold 70% Ethanol. The washing and precipitation steps repeated thrice.
19. Finally the pellet was completely dried and dissolved in minimum TE solution and the DNA stock was stored at –20 ^{0}C.
20. Quality and quantity of DNA for all extracted samples then checked by gel electrophoresis and NanoDrop spectrophotometer.

d) Isolation of total DNA from plant tissue using the DNeasy® plant mini kit (Qiagen)

DNA isolation through CTAB method can produce good quality and quantity of DNA while it is quite laborious and can't handle large population. Therefore, a very short and easy kit method of DNA extraction (Qiagen, USA) from plant tissues was used. The kit method was registered and patented according to the company as DNeasy® plant mini kit method *(www.qiagen.com)* is quite easy compared to CTAB method because all the reagents and filters required for this method are readymade but are expensive. The procedures were as follows:

1. Approximately 0.10 g of leaf tissue was chilled with liquid nitrogen and ground to a very fine powder by mortar and pestle.
2. The powdered tissue was transferred in an eppendorf tube.
3. 400µl of buffer AP1 and 4 µl of RNase a stock solution (100 mg/ml) were added to the powdered tissue.
4. The mixture was incubated at 65 ^{0}C for 10 minute and mixed 2-3 times during incubation by inverting tube.
5. 130 µL of buffer AP2 was added to the lysate, mixed and incubated on ice for 5 minutes. This step precipitates detergent, proteins, and polysaccharides.
6. Centrifuge the lysate for 5 min at 20,000 × *g* (14,000 rpm).
7. The aqueous phase was then transferred into QIA-shredder Mini Spin Column and the column centrifuged at 14000 rpm (20000 × *g*) for 3 minutes, where the flow-through is collected in the tube placed at the bottom

8. From the previous step the flow-through fraction was transferred into a 1.5 ml eppendorf tube (without disturbing the pellet debris).
9. Then 1.5× volume of buffer AP3/E (for example, if the lysate is 450µl, buffer is 675µl) was added to the cleared lysate and mixed. The ratio of buffer AP3/E: Ethanol was 1:2.
10. From the previous step 650 µl of mixture was collected in the DNeasy Mini Spin Column sitting in a collection tube (supplied) and centrifuged at 8000 rpm (>6000 × *g*) for 1 minute and the flow-through was discarded.
11. Step 10 was repeated with remaining sample.
12. The DNeasy Mini Spin Column was placed in a 2 ml collection tube.
13. 500 µl of buffer AW was added to the DNeasy Mini Spin Column and centrifuged at 8000 rpm (>6000 × *g*) for 1 minute. The flow-through was discarded and the collection tube was reused in the next step.
14. Again 500 µl of buffer AW was added to the DNeasy Mini Spin Column and centrifuged at 20,000 × *g* (14,000 rpm) for 3 minutes.
15. The membrane was dried at room temperature for 5-10 minutes.
16. The DNeasy Mini Spin Column was transferred to a 1.5 ml eppendrof tube and 100 µl of buffer AE/TE was added to the DNeasy membrane and incubated for 15 minutes at room temperature.
17. Then centrifugation was performed at 8000 rpm (>6000 × *g*) for 1 minute.
18. Step 17 was repeated

e) Quality checking and quantification of DNA

Once the isolation of DNA is completed, it is very important to check the quality of DNA for obtaining good results and for long-term storage. It is also important to know that how much of the DNA is available for genotyping and exact concentration of DNA in known volume of Tris-Buffer.

f) Procedures for DNA quantification

i) Using spectrophotometer

1. The spectrophotometer was set at the wavelength of 260 nm and 280 nm.
2. The cell was washed with distilled water and TE buffer and then dried.

3. Calibration was carried out using TE solution (10 mM Tris-HCl + 0.1 mM EDTA).
4. Optical density (OD) of the TE buffer was taken as blank and then it was discarded.
5. In a Cuvette 5.0 µl of each DNA sample was added in 995.0 µl of TE. It was mixed well and the Cuvette was placed in the compartment and the leakage of light was avoided. The OD values were read directly from the display.
6. DNA concentration was calculated using the following formula:

Concentration of DNA= $OD_{260} \times 50 \times$ dilution factor µg/ml

N.B. Dilution factor = $\frac{1000}{5} = 200$

For OD_{260} 1, DNA concentration is 50 µg/ml

OD_{260}= Absorbance at 260 nm.

Comparison of sample DNA with λ-DNA standard

i. Stock DNA preparations were diluted to 10× and 20×.
ii. 1-2 µl of diluted samples (10× and 20×) were loaded in the wells of 0.8% Agarose gel followed by 25, 50, 100, 200 ng of λ-DNA standard.
iii. Electrophoresis and staining with Ethidium Bromide was carried out. DNA concentration was estimated by visually comparing the fluorescence in any of the standard with the fluorescence of diluted DNA sample.

ii) Using NanoDrop spectrophotometer

After availability of NanoDrop spectrophotometer (NanoDrop™1000) the quality and quantity of each of isolated DNA samples were checked and diluted for the required concentration. The instrument was from Thermo Scientific, USA and measures 1 µl of sample containing DNA or RNA with UV light absorbance with high accuracy and reproducibility. It was controlled by PC based software. The procedure of DNA quantification through NanoDrop was as follow:

1) Open the sample arm and double click on to NanoDrop PC software
2) Pipette small amount (1-2 µl) of PCR grade water onto the upper and lower measurement pedestal and wipe the water by soft tissue.

3) Again pipette small amount (1-2 µl) of PCR grade water onto the lower measurement pedestal and close the sampling arm and initiate a spectral measurement using the operating software on the PC.
4) Pipette small amount (1-2 µl) of PCR grade water/TE buffer (those used to diluting DNA) onto the lower measurement pedestal and close the sampling arm and click to PC for setting blank measurement.
5) After setting the blank as zero, wipe the water/TE buffer by soft tissue from lower and upper pedestal.
6) Again pipette small amount (1-2 µl) of DNA sample onto the lower measurement pedestal and close the sampling arm and initiate a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement made.
7) After completion of each measurement the lower and upper pedestal were washed through PCR grade water and wipe out by soft tissues.
8) All the measurements were saved in to file to the PC.
9) From saved file, the calculation was made for desired dilution of each sample for PCR reaction.

g) DNA amplification using SSR and designed primers through Polymerase Chain Reaction (PCR)

For PCR reaction at first genomic DNA, 20% DMSO and ultra-pure water were dispensed in the labeled PCR tubes prior to adding the master mixture as follows in Table 3.1. The DNA, DMSO and water mixture was then denatured at 95 ^{0}C for 5 min and immediately transferred into ice. After thorough mixing and momentary spin 5.0 µl of above master mixture was dispensed to thin walled PCR tubes containing genomic DNA, 20% DMSO and ultra- pure water. The volume was made 15 µl by adding varying amounts of sterilized ultra-pure water. Taq DNA polymerase was added just before the start of the reaction. Finally, the tubes were subjected to momentary spin and transferred to Thermo-cycler for the amplification reaction (Appendix IV for composition and preparation of all the PCR components).

Table 3.1: Preparation of sample and control tube with DNA, DMSO and ddH_2O

Tube usage	DNA (50ng/µl)	DMSO (20%)	Ultra-pure H_20 (adjustable)	Total volume
Sample tube	1.0 µl	2.0 µl	7.0 µl	10 µl
* Control tube	0.0 µl	2.0 µl	8.0 µl	10 µl

[N.B. Control tube is required to detect any kind of contamination, which causes false positive amplification. Ice was used for thawing and placing the components for maintaining efficient working condition.]

h) Thermal cycling profile used in PCR

The thermal cycling profiles programmed in PCR machine (Fig. 3.2) to amplify the marker/gene by polymerase chain reaction (PCR) for 35 to 40 cycles are as follows in Table 3.2.

Table 3.2: Thermal cycling program for PCR amplification

Steps	Temperature (^{0}C)	Time (minutes)	No. of cycles
Initial denaturation	95	5	1 (First)
Denaturation	95	1	35 to 40
Annealing	55 (adjustable)	0.30-0.45 (±)	
Elongation	72	0.45 (±)	
Final extension	72	7	1 (Last)

[N.B. For different primers different anneal temperatures were employed.]

Fig. 3.2: Two PCR machine (left BIORAD and right EPPENDORF) at Plant Biotechnology Laboratory (PBT Lab) of the Department of Biochemistry and Molecular Biology in the University of Dhaka.

i) Visualization of the amplified PCR product

The amplified PCR products (using microsatellites/SSRs and gene specific primers) can be resolved and visualized with the help of Agarose gel electrophoresis or Polyacrylamide gel electrophoresis. Type of gel selection generally depends on the length, nature and quality of the amplified product.

j) Agarose gel electrophoresis

By using the technique of Agarose gel electrophoresis (Fig. 3.3) amplified DNA fragments can be size wise fractionated by comparing with standard DNA markers. For confirmation of PCR amplification 0.8% Agarose gel was used. But for separating different size depending on the size of the DNA band, different concentrations of Agarose were used. For SSR markers 2% Agarose was ideal. The standard method was used to separate and identify DNA fragments through Agarose gel electrophoresis (Sambrook and Russell, 2001). (See Appendix V for composition, preparation and maintenance of Agarose gel).

k) Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel has much higher resolution than Agarose gel. To obtain unambiguous polymorphic bands between Pokkali and IR29 Polyacrylamide gel electrophoresis (Fig. 3.4) was performed. Depending on the polymorphic nature different concentrations (8%) of PAGE were used for easy analyzing or scoring. (Please see Appendix VI for composition, preparation and maintenance of PAGE).

Fig3 .3: Agarose gel electrophoresis with Power Pac

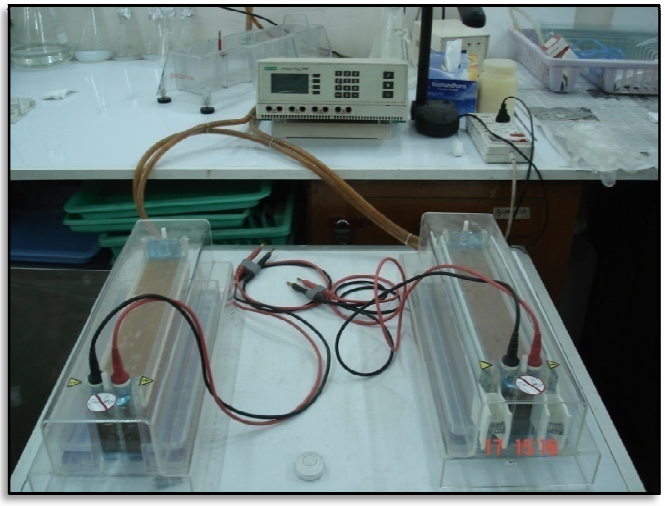


Fig. 3.4: Polyacrylamide gel electrophoresis with Power Pac

l) Gel documentation for scoring DNA band patterns

i) Gel preparation

After completion of electrophoresis, staining in Ethidium Bromide solution and de-staining in ddH_2O for a short moment, both the Agarose gel and Polyacrylamide gel become ready for visualization.

ii) Capturing of image

DNA band patterns visualized in the gel was photographed using highly sophisticated Alpha Ease FC imaging system (Fig. 3.5) (www.alphaimager.com). Special software was included in this system. Before taking photograph, Agarose gel and Polyacrylamide gel was placed under UV-illumination.

Fig. 3.5: Alpha Innotech gel documentation system

iii) Scoring for polymorphic bands

SSR markers that amplified polymorphic bands in 8% Polyacrylamide gel and 2% Agarose gel in BR11 and FL378 were used to compare the populations. Parents were loaded along with the populations for several times. When a population showed the same level of band pattern of either parents or both then the progeny with particular marker loci was referred to as recipient or donor or heterozygous allele, respectively. Progenies were scored by notation A (recipient), B (donor) and H (heterozygous).

Genetic similarities of the developed NILs were determined using Simple Matching Coefficient (SMC) (Sneath and Sokal 1973 and vanBerloo, 1999). The SMC is the sum of

the proportion of shared positive bands plus the proportion of shared negative bands. The true value for the Simple Matching Coefficient is given by-

$SMC = sp + (1-p)s$

Where, s = total similarity, p = proportion of similarity due to shared positive bands

Recurrent parent genome recovery in the selected segregants of the backcross population was determined using the Graphical GenoTypes Software (GGT; vanBerloo, 2009).

3.1.5 SNP genotyping

For SNP genotyping, genomic DNA was extracted from leaves of young seedlings using a DNeasy Plant Mini Kit (Qiagen, www.qiagen.com). For each genotype, leaf tissue was ground through mortar and pestle by using liqid-N and about 0.10 g of powdered tissue was processed. Purified genomic DNA was quantified at 260 nm using a NanoDrop 1000 spectrophotometer. Detailed methodology for DNA extraction through Qiagen Plant Mini Kit was described earlier in this section.

SNP assay on the Illumina system

The Illumina GoldenGate assay was performed as per the manufacturer's protocol and as described in Fan and co-workers (2003). Illumina BeadXpress 384-plex SNP plates GS0011861 (customized for *Indica–Indica*). The custom oligo pool assay (OPA), which contained 384 well-distributed SNPsper assay, was designed by Cornell University (Thomson, *et al.* 2012; Zhao, *et al.* 2010) from a high-quality subset of the SNPs discovered in 20 diverse *O. sativa* landraces (McNally et al. 2009). SNP Assay was carried out at the Molecular Marker Application Laboratory (MMAL) of IRRI, Philippines (Dr. Michael Thomson, MMAL, PBGB, IRRI, Philippines). Illumina technology is based on a two-step hybridization design. Basically, denatured genomic DNA is first hybridized with three primers linked to magnetic beads. Two primers (called Allele Specific Oligo, ASO) are specific to the upstream region of the SNP with the exact nucleotide corresponding to the SNP present at the 3'-end; the third one corresponds to the exact downstream region of the SNP (called Locus Specific Oligo, LSO). Besides being specific to the SNP flanking regions, each of the primers harbors a sequence corresponding to universal primers and the

LSOs, a unique sequence. After the hybridization of the DNA to the primers, a DNA polymerase synthesizes the few nucleotides separating the ASOs from the LSOs. Matrix DNA is eliminated and only the new DNA strand is kept to serve as template DNA for a PCR step using universal primers. The second hybridization step is performed on 384 beads arrays carrying the unique sequences for the LSOs. Thus by at the same time 384 SNP can be typed on a single DNA. Universal primers are labeled with *Cy3* and *Cy5*. After amplification, the products are hybridized to a SentrixBeadChip/InfiniumBeadChip for detection. An Illumina BeadStation 500G (Illumina, San Diego, CA) is then used to analyze hybridized SentrixBeadChip. The automatic allele calling for each locus is accomplished with GenCall software (Illumina, San Diego, CA). A genotype that is homozygous for one or the other SNP alleles will display a signal in either the *Cy3* or *Cy5* channel, whereas a genotype that is heterozygous will display a signal in both channels. All GenCall data were manually checked and re-scored if any errors in calling the homozygous or heterozygous clusters were evident (Fig. 3.6). Graphical genotyping and genomic similarity of BR11-*Saltol* introgression lines, recurrent parent BR11 and donor FL378 were done by using Flapjack software (http://www.bioinf.hutton.ac.uk/flapjack) developed by the Hutton Institute.

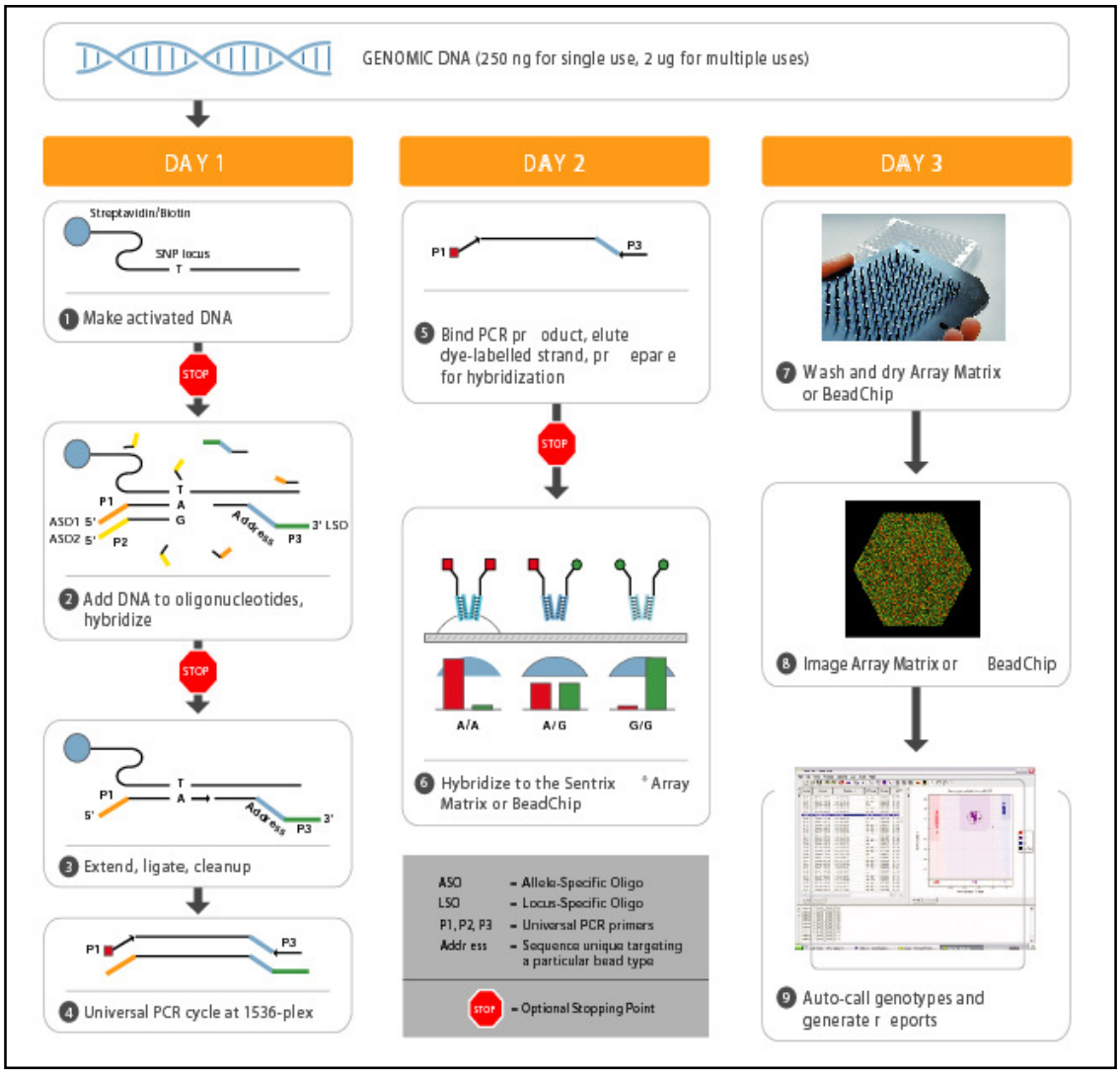


Fig. 3.6: Schematic representation of Illumina GoldenGate SNP Assay (www.illumina.com).

3.2 Phenotypic characterization of BR11-Saltol introgression lines

3.2.1 Experiment location and climatic conditions

BR11-*Saltol* lines were tested at seedling stage in two growing conditions i.e. at BRRI, Gazipur ($24^0$09' N; $90^0$41' E, 12.83 meter), Bangladesh, and at IRRI, Los Banos ($14^0$11' N, 121^0 15' N; 21 meter) Philippines, on several occasions during the years 2009-2013.The weather conditions during the experimental periods are presented in Appendix-VII.

3.2.2 Characterization for salinity tolerance at seedling stage in net house condition

The phenotypic screening for the salinity tolerance at seedling stage was done by the method described by Gregorio, *et al.* (1997) in the Net house of Plant Physiology Division of BRRI (Fig. 3.7 and 3.8). Sprouted seeds of the test BR11-*Saltol* lines along with parents (BR11 and FL378) and standard tolerant and susceptible checks Pokkali and IR29 were sown in the Styrofoam sheets floating in trays containing Yoshida's culture solution (Yoshida, *et al.* 1976) under net house condition (see Appendix I for preparation and nutrient composition of Yoshida solution). At 12 days of seedling age, NaCl was applied in the screening trays to attain the electrical conductivity (EC) of 6 dSm^{-1} of the culture solution. Then the EC of the culture solution increased @ 2 dSm^{-1} in every 2 days until it finally reached 12 dSm^{-1}. The culture solution was changed once a week throughout the experimental period. The pH and EC of the culture solution was checked daily and maintained at 5±0.5 and 12±0.5, respectively (Fig. 3.9). The sensitivity of each seedling was scored when the sensitive checks were almost dead. The scoring was done by the scoring system described Gregorio, *et al.* 1997 (Table 3.3). Immediately after scoring, the data of seedling height and root lengths were recorded and then the seedlings were sampled for dry mass and mineral content (Na^+ and K^+ content of shoot and root).

Fig. 3.7: Screening for salinity tolerance at seedling stage of rice in the net house condition of Plant Physiology Division, BRRI, Gazipur

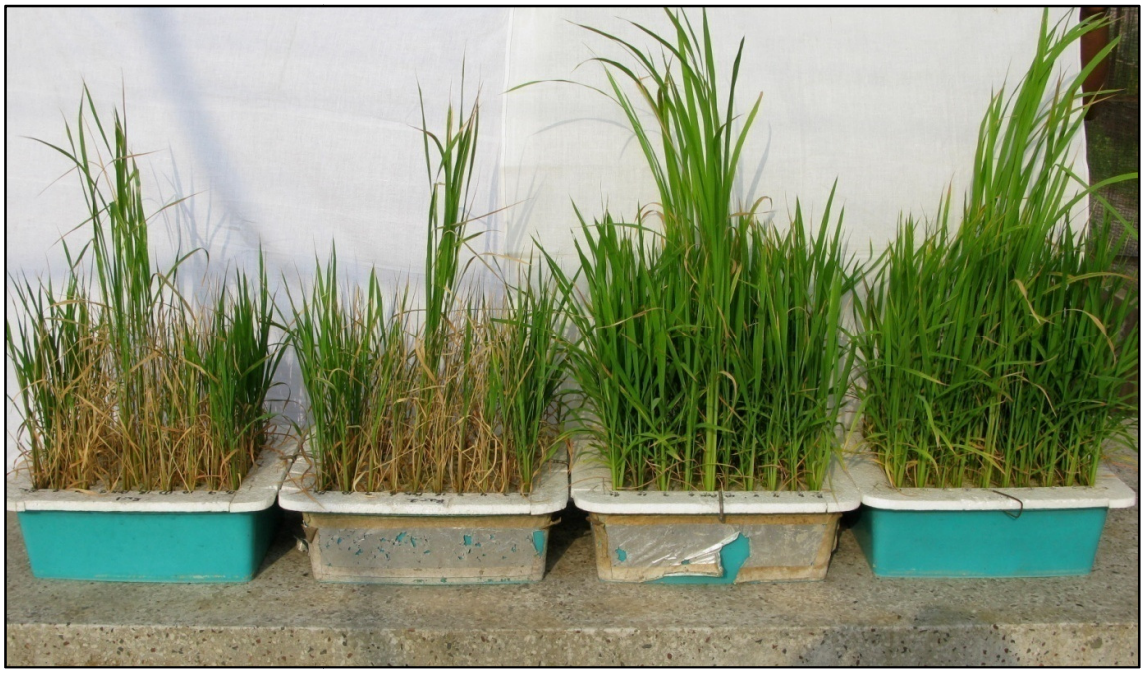


Fig. 3.8: Experimental set-up for seedling stage screening of BR11-*Saltol* NILs in the Net house condition of BRRI, Bangladesh. (May-June, 2010) [left two trays are for stress and right two trays are control (without stress)]

Fig. 3.9: pH (5±0.5) and EC (12±0.5) dSm^{-1} for seedling stage screening in hydroponics' culture were checked daily by pH meter and EC meter.

Table 3.3: Scoring system of rice seedling against salinity, proposed by Gregorio, *et al.* 1997.

Observations	Leaf damage (%)*	Score	Remarks
Normal growth, no leaf symptoms.	<10	1	Highly tolerant
Nearly normal growth, but leaf tips or few leaves whitish and rolled	10-30	3	Tolerant
Growth severely retarded; most leaves rolled; only a few are elongating	30-50	5	Moderately tolerant
Complete cessation of growth; most leaves dry; some plants dying	50-70	7	Moderately susceptible
Almost all plants dead or dying	>70	9	Susceptible

*Adapted from Ponnamperuma, 1978

3.2.3 Sampling and sample preparation for physiological characterization

Sampled seedlings were dried in a hot air circulating oven for 3 days @70 ^{0}C. Dried samples were then weighted for dry mass. For mineral content (Na^+ and K^+) dried samples of shoot and roots were ground by a rotor grinding mill to make powder. Each powdered sample was ere then weighed and added to a 50 ml conical flask for digestion. Na^+ and K^+ extraction and estimation were done according to the method described by Yoshida, *et. al.* 1976. One gram of powdered samples (shoot/root) were digested by 25 ml 1N HCl for 24 hrs at room temperature and then filtered by Whatman1 filter paper. The extract was then diluted by 1:20 with 1N HCl and then aspirated in a Flame Photometer (Sherwood 410) with setting proper filter (Na^+/K^+). A standard series for both Na^+ and K^+ were prepared and reading taken before the samples. Samples' reading were then plotted in the standard curve to estimate the content of Na^+ and K^+ in the samples.

3.2.4 Characterization for salinity tolerance at reproductive stage in net house condition

This experiment was carried out at Plant Physiology Division Net house of BRRI during the *T. Aman* season 2012 by the soil based method described by Gregorio, *et. al.* 1997. Two BR11-*Saltol* NILs (NIL52 and NIL1) along with parents BR11, FL378 and standard salt

tolerant, sensitive HYV check BRRI dhan47 and IR29 were used. Sprouted seeds were sown to the Styrofoam sheets in Yoshida solution (Yoshida, *et. al.* 1976) to raise the seedlings. At 5-leaf stage single seedling was transplanted in the perforated plastic pots filled with fertilized puddle soil and the pots were kept in to big plastic bowl with a capacity to accommodate 6 pots. Tap water was added to the bowls for the establishment of the seedlings. After establishment of the seedlings and at tiller initiation stage salinity stress (NaCl) at EC @ 6 dSm^{-1} was applied to the bowls by replacing tap water with saline water and maintained by keeping the water volume same on a daily basis until maturity. The dish and pots were arranged in RCBD with 3 replicates (Fig. 3.10 and 3.11). Heading date was recorded for each of the lines/variety when panicles of mother tiller start emerging. At physiological maturity all plants were harvested and different phenotypic parameters including yield and its components were measured and recorded. During harvesting of the plants, electrical conductivity of the crop standing water was recorded by an EC meter and soils of each pot was sampled for saturation paste extract to measure level of salinity developed in the pot soil. Grain, straw and soil samples were sun dried for 3 days and then dried by a Hot Air Circulating Oven (ALP, Japan) for 3 days at 70 ^{0}C. After drying grain weight was taken by a Mettler Balance and at the same time grain moisture was measured by a moisture meter (Digital Grain Moisture Meter, Model: YM-8E, Yamamoto, Japan) and the moisture content was adjusted to14%.

Fig. 3.10 and 3.11: Experimental set-up of reproductive stage characterization of BR28-*Saltol* lines at BRRI Net house.

3.2.5 Field trials and phenotypic selection

The performance of the two Near Isogenic Lines (NIL1 and NIL52) of BR11-*Saltol* was tested in the non-saline field condition at BRRI farm, Gazipur. All seeds of NIL1 and NIL52 along with the recurrent parent BR11 were grown with normal cultural practices recommended by BRRI for modern rice cultivation (BRRI, 2004). At this stage, due to unexpected segregation of grain size a head-row selection for both of the NILs were made and finally 8 lines from NIL1 and 4 lines from NIL52 were selected for further evaluation. All 12 BR11-*Saltol* NIL1 and NIL52 derivative lines were evaluated in the non-saline field condition in *T. Aman* season 2012-2013. BR11-*Saltol* 12 derivative lines were also evaluated in the *T. Aman* season 2013 along with BR11 (parent) and two salt tolerant *T. Aman* variety BRRI dhan53 and BRRI dhan54. Seedlings were raised in the seedbed of all the tested lines, parents and checks in the respective year of *T. Aman* season. Seedlings of 5-leaf stage were transplanted in the plots 3m × 3m of the main field. Single seedling per hill and 20cm × 20cm spacing were maintained. The field was divided in to 3 homogenous blocks along the fertility gradient and all lines and/or varieties were completely randomized with each block. All crop management e.g. fertilizer, weed, pest, irrigation managements were followed as per recommendation of BRRI (BRRI, 2004). Days to 50% flowering was recorded when 50% tillers of each plot flowers. Plot yield was measured from 5 square meters of inner areas of each plot. Other morphological parameters like plant height, tiller number, productive tillers, panicle length and yield components were measured and recorded from randomly selected 10 hills (Gomez, 1972). Three more field trials were also conducted by using same lines and varieties with recommended cultural managements (BRRI, 2004) in the non-saline Gazipur *Boro* 2011-12 and in the saline areas of Satkhira and Koyra, Khulna at respective *T. Aman* season of the year 2012 and 2013, respectively.

3.2.6 Grain quality assessment

Grain samples of BR11-*Saltol* lines along with recurrent parent BR11 and donor parent FL378 were analyzed for physical and chemical attributes at the Grain Quality and Nutrition Division (GQN) of BRRI.

a) Grain quality parameters (Physico-Chemical properties of rice grains):

i) Milling of rice: Rough rice was cleaned to remove leaves, rice stems, and other foreign materials and was sun dried before milling. Duplicate 200 g rough rice was de-hulled by

Satake THU-35A (Japan) sheller to brown rice. The resulting brown rice was milled in Satake TM-05. mill with #5330 abrasive disc at 1730 rpm to obtain approximately 10.0% by weight bran-polish removal for all samples (the part of brown rice which includes pericarp, embryo, aleurone and sub–aleurone layer). The broken grains were separated manually. Milled rice yield and head rice recovery was expressed as percentage of rough rice and milled rice basis. Rice powder was prepared by grinding milled rice in Udy Cyclone Mill (Udy Corporation, USA) to pass through 60-mesh net.

Calculation: The percent of hull, brown rice, degree of milling, total milled rice and head rice was calculated as follows:

$$\text{Hull (\%)} = \frac{\text{weight of hull}}{\text{weight of rough rice}} \times 100$$

$$\text{Brown rice (\%)} = \frac{\text{weight of brown rice}}{\text{weight of rough rice}} \times 100$$

$$\text{Degree of milling} = \frac{\text{weight of total milled rice}}{\text{weight of brown rice}} \times 100$$

$$\text{Total milled rice (\%)} = \frac{\text{weight of total milled rice}}{\text{weight of rough rice}} \times 100$$

$$\text{Head rice (\%)} = \frac{\text{weight of head rice}}{\text{weight of rough rice}} \times 100$$

ii) Appearance: Grain appearance was done by visual observation. Size and shape, presence or absence of endosperm opacity and level of chalkiness were collectively considered for the classification of excellent, very good, good and fair grain.

iii) Chalkiness: Amount of chalkiness, either on the dorsal side of the grain (white belly) or in the center (white center) influences the grain appearance of milled rice. An international standard scale was used for classifying chalkiness of milled rice (Khush, *et al.* 1979).

Scale	Percent of area with chalkiness
0	None
1	<10%
5	10-20%
9	>20%

iv) Grain size and shape: Grain length and breadth was measured using digital slide calipers.

Based on length, size of milled rice was classified into 3 classes.

Size	Length in mm
Long	>6.0
Medium	5.0-6.0
Short	<5.0

Based on length to breadth ratio, shape of milled rice was again classified into 3 classes.

Shape	Length/Breadth ratio
Slender	>3.0
Bold	2.0-3.0
Round	<2.0

v) Amylose content (AC): Amylose in rice is released by treatment with dilute alkali. By the addition of Tri-iodide ion, amylase produces blue color. The absorbance of blue color produced in aqueous solution is measured by UV-spectrophotometer at 620 nm as described by Williams, *et al.* (1958).

vi) Protein content: Micro Kjeldahl procedure of AOAC (1995) was used for the determination of nitrogen and crude protein was calculated by multiplying the nitrogen content by a factor 5.95 (Juliano, 1985). Nitrogen present in the sample is converted to ammonium sulphate by digestion at 380 ^{0}C with sulphuric acid in presence of a catalyst

mixture. Ammonia liberated by distilling the digest with NaOH solution is absorbed by boric acid and is titrated for quantitative estimation (AOAC, 1995).

vii) Elongation ratio (ER): The principle is to presoak the un-cooked rice in water, then cook the soaked rice for a set time by putting it directly into hot water. The ratio of the average length of the cooked grain to that of the uncooked grain is the elongation ratio. Elongation ratio is the ratio of length of cooked rice over the length of raw milled rice. Some milled rice show extreme elongation when cooked, especially after presoaking. Formation of transverse cracks during presoaking improves grain elongation during cooking. Fissures that form during presoaking increase grain elongation during cooking.

viii) Cooking time: It is amount of time when starch granules are fully disappearing in the boiling water inversely with increasing time (Ranghino method).

3.2.7 Data analysis

a) Genotypic data analysis: Genotypic data obtained from SSR, InDel and Gene-based markers were computed and analyzed for percent recovery of recurrent genome through Global Statistics and graphical genotypes by using the software Graphical Genotype (GGT 2.0) (vanBerloo, 2008). SNP marker data for similarity index and graphical genotypes were computed and analyzed by Flapjack software (http://www.bioinf.hutton.ac.uk/flapjack). Numerical optimization for population minimization of a marker assisted backcrossing program was computed by POPMIN software developed by Hospital and Decoux (2002).

b) Phenotypic data analysis for individual trial: Data obtained from single experiment/trial were analyzed by the following linear model for fixed effects of the genotypes:

$$y_{ij} = \mu + g_i + b_j + \varepsilon_{ij} \quad (1)$$

Where y_{ij} is the observation of the ith genotype in the jth block; μ is the grand mean; g_i is the effect of the ith genotype; b_j is the effect of the jth block; and ε_{ij} is the residual. The assumptions of the residual ε_{ij} isdistributed with zero mean and constant variance. The variables were further checked through Fishers' LSD test to declare significant differences

among treatments when the variable was found significant at least 5% level of probability. All analyses were carried out by CropStat v7.2, statistical software developed by IRRI (www.irri.org).

is the observation of the ith genotype in the jth block; is the grand mean; is the effect of the ith genotype; is the effect of the jth block; and is the residual, block effects and residuals are random and

c) Data analysis for multi-environment trials for G by E and stability:

The data from single trial was analyzed through linear mixed model by Residual Maximum Likelihood (REML) method:

$$\underline{y_{ij}} = \mu + \underline{g_i} + \underline{b_j} + \underline{\varepsilon_{ij}} \quad ---------- (2)$$

Where $\underline{y_{ij}}$ is the observation of the ith genotype in the jth block; μ is the grand mean; $\underline{g_i}$ is the effect of the ith genotype; $\underline{b_j}$ is the effect of the jth block; and $\underline{\varepsilon_{ij}}$ is the residual.All effects i.e. genotype effect $\underline{g_i}$, block effects $\underline{b_j}$and residuals $\underline{\varepsilon_{ij}}$ are random and distributed with zero mean and constant variance. Underlined parameters are considered as random. Here, REML was used to estimate genetic variance components and heritability. REML analyses were carried out through Breeding View statistical software from Breeding Management System v2.0, integrated software package developed by Generation Challenge Program of CGIAR (https://www.integratedbreeding.net).

Adjusted means i.e. Best Linear Unbiased Estimates (BLUEs) were calculated from above REML analysis and used for G by E interaction and stability analyses. G by E interaction and stability analyses were carried out through the methods adopted in to Breeding View software i.e. Finaly-Wilkinson modified join regression analysis (Finlay and Wilkinson, 1963; Yates and Cochran, 1938), the Additive Main effects and Multiplicative Interactions model (AMMI) (Gauch, 1992) and Genotype main effects & GEI model (GGE) (Yan and Kang, 2003). Stability analyses were carried out for Static stability (Type 1), Wricke's ecovalence (Wricke 1962 & 1964) or Dynamic stability (Type 2), Finlay-Wilkinson sensitivity and Mean Square Deviation (Deviations with respect to the FW regression line) or Eberhart-Russel stability (Eberthart and Russell, 1966) and Lin & Binns (1988) cultivar

superiority measures which use stability parameters in combination with parameters of performance per se, and finally select best variance-covariance model for constant variances and co-variances across environments (Malostti, *et.al.* 2013). All above analyses were done through Breeding View statistical software from Breeding Management System v2.0, integrated software package developed by Generation Challenge Program of CGIAR in the GeneStat software environment (https://www.integratedbreeding.net).

3.3 Introgression of salinity tolerance QTL Saltol in to Bangladeshi mega rice variety BRRI dhan28 through Marker-assisted backcrossing.

3.3.1 Plant Materials: Recipient and Donor parents

BRRI dhan28 (BR28 hereafter) was developed and released by Bangladesh Rice Research Institute (BRRI) in 1994. The ancestry of BR28 is BR610-3-3-4-2-5 with the parentage BR6 (IR28)/Purbaci. BR28, is a semi-dwarf (~90 cm), medium duration (~140 days in the *Boro* season), slender grain with yield potential about 5.5 t/ha (BRRI, 2004). It is one of the most popular *Boro* season variety widely cultivated in the irrigated ecosystem. Due to its enormous popularity and wider adaptability, this variety was chosen for conversion in to salt tolerant-BR28 by introgressing *Saltol* QTL through a molecular breeding approach Marker-assisted backcrossing.

FL378, a F_8 Recombinant Inbred Line (RIL) was used as *Saltol* QTL donor for this introgression work. The pedigree of FL378 is (IR66946-3R-78-1-1) which was developed in International Rice Research Institute (IRRI) from IR29/Pokkali. This line was chosen due to its high tolerance to salinity at seedling stage. The tolerance of FL378 was derived from the well known Indian salt tolerant cultivar Pokkali. The tolerance of Pokkali was mapped in IRRI and named the major effect QTL *Saltol* that can explain 45% phenotypic variation for seedling stage salinity tolerance (Bonilla, *et. al.* 2002).

3.3.2 Marker Assisted Backcrossing (MABC) Scheme

The Marker Assisted Backcrossing (MABC) approach used in this study was followed first as described by Neeraja, *et. al.* 2007 and then published electronic manual for MABC in rice

by Collard, *et al.* 2008a. Hybridization of recipient to donor and then 3-step backcrossing with recipient parent followed by marker-aided selection approach was used in this study. Each backcross generation underwent a 3-step marker-aided selection i.e. Foreground selection (FGS), Recombinant selection (RBS) and Background selection (BGS). In the marker-assisted backcrossing process for each of backcross generation, first selection is the foreground selection where tightly linked markers were used to select progenies which had the QTL in heterozygous form, also referred as positive selection. After the first selection, the selected progenies were subjected to RBS, where the nearest-unlinked markers bordering both sides of the QTL were used to precisely introgress (no or minimum additional segment adjoining the target loci) of the QTL. The selected progenies after RBS was subjected to BGS, where markers were used for quick recovery of the rest of the recipient genome, this selection also called negative selection. The best progeny/progenies, which have the QTL in heterozygous form, as well as recombination on either or both side of the QTL and have maximum number of loci at the background, were crossed with recurrent parent to obtain the BC_2F_1 progenies. The same procedures were followed to obtain BC_3F_1. For the calculation of population minimization was carried by POPMIN software. At the BC_3F_1 stage, the maximum recovered progenies were selfed to produce BC_3F_2. All progenies were genotyped by foreground markers for fixation of the QTL loci (selection of progenies which have the homozygous QTL loci) and to increase the proportion of the recurrent parent loci, which were still heterozygous. The selected progenies, which have the qtl in homozygous form and have clean background, were referred to Precision Isogenic Lines (PILs). These are also commonly referred to as Near Isogenic Line (NILs). BC_3F_3 seeds of selected plants were used to screen for salinity tolerance at seedling stage to confirm the tolerance gained in the newly developed NILs or BR28-*Saltol* as compared their parents.

3.3.3 Molecular Marker Analysis:

a) Selection of Molecular Markers for FG and RB selection

Validation of molecular markers used in Foreground and Recombinant selection was described above in the section 3.1.3 (a)

b) Identification of Polymorphic Markers for BG selection

Molecular markers are particularly useful if they reveal differences between individuals of the same or different species. These markers are called polymorphic markers, whereas markers that do not discriminate between genotypes are called monomorphic markers (Collard, *et al.* 2005). SSRs are co-dominant markers thus it can easily be used in genotyping work like QTL mapping as well as in MAS/MABC. Initially a set of 153 SSR markers from Plant Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka and later 373 SSR, InDels (Insertion/Deletion) marker and some Gene based markers distributed throughout the rice genome were screened for polymorphism of the parental combination of BR28/FL378. The positions of the SSR markers were inferred from the Physical map of IRGSP 2005 through BLAST searchers or found in the Gramene SSR database (www.gramene.org). The order of the markers were considered based on the Physical map of IRGSP (IRGSP 2009) and the corresponding genetic distances (cM distances) were computed through a software called cM Converter v.1.0.0.4 (http://mapdisto.free.fr/cMconverter/). From the first polymorphism survey 52 were found polymorphic between BR28/FL378 and 49 of them used to genotype first backcross generation. Later, in second and third backcross generation 20 and 38 additional polymorphic SSR and InDel markers were used to genotype progenies. Finally, a total of 107 SSR and InDel markers were included in to the panel for BGS (see the BGS polymorphic markers in a diagram in the result section Fig. 4.2.2 and lists in the Appendix-II, Table 8.2.9).

3.3.4 Marker Genotyping

All processes for marker genotyping is same for MABC of BR11-*Saltol* as described above in Study 1a except the DNA isolation protocol. To reduce the genotyping efforts and cost, a new Mini Preparation DNA isolation method was used for genotyping of MABC of BRRI dhan28-*Saltol* (this Mini Prep method is the modified method described by Zheng, *et.al.* 1995; M.J. Thomson, Molecular Marker Application Laboratory, IRRI, personal communication). Different steps of this method are shown in the photographs 3.12 in the next page.

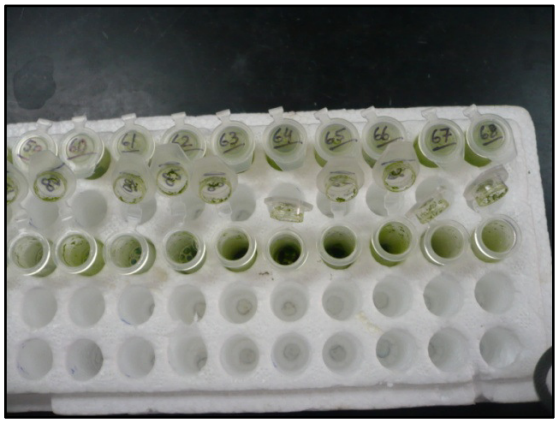
Rice DNA Extraction Miniprep Protocol

1. Microfuge tubes of 2.00 mL was placed in to tube rack and labeled the sample names for harvesting tissue.
2. Tube racks were placed in to a plastic ice box and filled with liquid nitrogen right below the level of the tube rack (this was done immediately before harvesting).
3. Fresh young leaf tissues of approximately 1 cm wide by 3 cm long, or 0.5 by 6 cm was collected in to the labeled microfuge tubes.
4. After collection, all samples were chopped by inserting small scissors in to the microfuge tubes and then put two small stainless steel balls each of the tube.
5. Microfuge tubes were then placed in to two iron rack with the capacity of 48 per rack; both racks were then placed in to a plastic bowls containing liquid-N.
6. Both racks were placed in to a Geno/Grinder® 2000 (SPEX Sample Prep, NJ, USA) after proper locking the lid of the Grinder, the frozen tissues were pulverized with 6000 rpm strokes for 10 minutes.
7. Tubes were then centrifuge for 3 minutes at 11,000 rpm in a micro-centrifuge.
8. Sodium bi-sulfite was added @ 0.38 g/ml to the extraction buffer and pre-heat in the microwave for 30 sec.
9. After grinding and centrifuging, the tubes were then kept in to the lab condition for warming-up at room temperature.
10. Approximately 800 µL pre-heated extraction buffer was added to each tube and mixed well by vortexing and inverting.
11. The tubes were placed in a 65 ^{0}C heated water bath in a tube holder for 20 minutes (after 10 minutes mixed by inverting and returned again to the water bath).
12. The tubes were removed, mixed by inverting, and brought to a chemical fume hood.
13. Chloroform: Isoamyl alcohol (24:1 mixture) was added to each tube @ 800 µL.
14. The tubes were then placed in tube rack and closes tightly, covered with paper towels and hold a second tube rack against the top of the tubes and inverted repeatedly for 3 minutes.

15. Centrifuged the tubes for 8 minutes at 11,000 rpm in a micro-centrifuge.
16. Pipet-out of approx. 500 µL of the upper aqueous layer to a new 1.5 mL tube.
17. Ice-cold ethanol @ 1000 µL was added and mixed by inverting.
18. Centrifuged for 12 minutes at maximum speed (13,200 rpm).
19. The solution was then removed by pouring the solution into a beaker and then touched the tip of the tube to a tissue to remove the excess solution.
20. Again 1000 µL ice-cold 70% ethanol was added to all tubes and centrifuged for 3 minutes at 13,200 rpm.
21. Ethanol was removed by pouring off into a beaker from all tubes.
22. The DNA pellets were then drying by inverting the tubes on a bench top on top of tissue for 30-45 min.
23. After drying, the pellets were then re-suspended in 100 µL TE buffer, and dissolved pellet by warming in a 65° C water bath for up to 1 hr (with frequent mixing). After the pellet was dissolved, store the concentrated DNA at -20°C.
24. Checked the concentrated DNA in to a NanoDrop 2000 Spectrophotometer and dilute DNA as per optimal PCR concentration 20-35 ng/µl.

N.B.: Stock solution preparation for Rice DNA Extraction Miniprep Protocol was placed in the Appendix-III


CORNING




MIKE /
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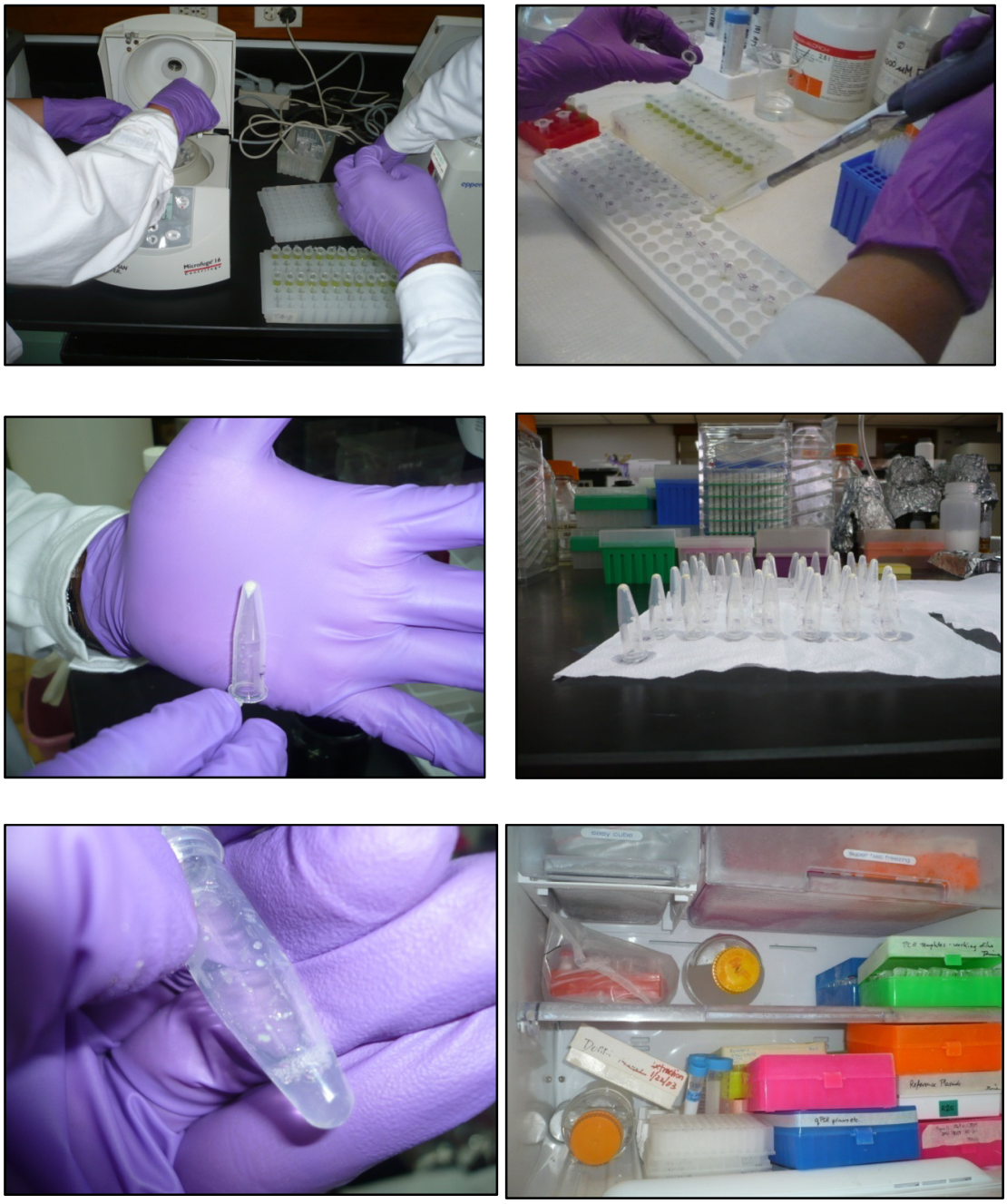


Fig. 3.12: Above 12 photographs showing the different steps for DNA isolation procedures of IRRI's Rice DNA Mini Prep protocol.

3.3.5 SNP genotyping

All processes for SNP genotyping is same for SNP assay on the Illumina system as described above in the section 3.1.5

3.4 Phenotypic characterization of BRRI dhan28-Saltol lines

3.4.1 Experiment location and climatic conditions

BR28-*Saltol* lines were tested at seedling stage in two growing conditions i.e. at BRRI, Gazipur ($24^0$09' N; $90^0$41' E, 12.83 meter) Bangladesh and at IRRI, Los Banos ($14^0$11' N, 121^0 15' N; 21 meter) Philippines for several occasions during the year 2011-2013. The weather conditions during the experimental periods were recorded in the Appendix-VII.

3.4.2 Characterization for salinity tolerance at seedling stage in controlled net house condition

As described in above experiment in the section 3.2.2

3.4.3 Characterization for salinity tolerance at reproductive stage in net house condition

This experiment was carried out at BRRI Plant Physiology Division Net house during the *Boro* season 2012-13 by the soil based method described by Gregorio, *et. al.* 1997. For phenotyping at reproductive stage, seven BR28-*Saltol* lines along with parents BR28 and standard salt tolerant HYV check BRRI dhan47were used. All other procedures for seedling raising, transplanting, different management practices, data recording and harvesting were similar to described in 1b except the applied salinity level. Here we applied $8dSm^{-1}$. Additional data like different photosynthetic parameters i.e. photosynthesis rate, stomatal conductance, internal CO_2concentration and transpiration rate were recorded on the flag leaf of the mother tiller during the flowering by a Portable Photosynthesis System Li-6400 (LICOR Biosciences, Lincoln, Nebraska, USA).

3.4.4 Field trials and phenotypic selection

The performance of Near Isogenic Lines of BR28-*Saltol* was tested in the non-saline field condition at BRRI farm Gazipur. Seven NILs along with the recurrent parent BR28, donor FL378 and standard salt tolerant check BRRI dhan47 were grown with normal cultural practices recommended by BRRI for modern rice cultivation (BRRI, 2014). All 7 BR28-*Saltol* lines were evaluated in the non-saline field condition for respective growing season

i.e. *Boro* 2012-2013. Seedlings were raised in the seedbed of all the tested lines and check varieties in the respective season. Seedlings of 5-leaf stage were transplanted in a plot size 3m × 3m at main field. Single seedling per hill and 20cm × 20cm spacing were maintained. The field was divided in to 3 homogenous blocks along the fertility gradient and all lines and/or varieties were completely randomized with each block. All crop management practices, e.g., fertilizer, weed and pest management, irrigation were followed as per recommendation of BRRI (BRRI, 2014). Days to 50% flowering was recorded when 50% tillers of each plot flowered. Photosynthetic parameters i.e. photosynthesis rate, stomatal conductance, internal CO_2 concentration and transpiration rate were recorded for the flag leaf of the mother tiller during the flowering by a Portable Photosynthesis System Li-6400 (LICOR Biosciences, Lincoln, Nebraska, USA). Plot yield was measured from 5 square meter of inner areas of each plot. Other morphological parameters like plant height, tiller number, productive tillers, panicle length and yield components were measured and recorded from randomly selected 10 hills (Gomez, 1972). Before weighing and counting of straw and grains, the samples were dried in sun for 2-3 days first and then dried in a hot air circulating oven (ALP, Japan) for 72 hrs at 70 ^{0}C.All NILs (except NIL448) along recipient BRRI dhan28, standard *Boro* check BRRI dhan29 and HYV tolerant check variety BRRI dhan47 were evaluated in the actual saline field condition in the Satkhira during respective *Boro* season, 2012-13 with BRRI recommended cultural managements (BRRI, 2011).

3.4.5 Grain quality assessment

Same as described in previous study in the section 3.2.6

3.4.6 Data Analysis

All genotypic and phenotypic data were analyzed for BRRI dhan28-*Saltol* lines are very similar to described in previous study in the section 3.2.7 except G by E interaction and stability analyses, these analyses were not carried out, because no such multi-environment trials were conducted for BRRI dhan28-*Saltol* lines.

Chapter 4: Results

Chapter 4
Results

This chapter contains details of all experimental findings obtained from the entire research. In order to present the results in a sequential manner and easily understandable way, the chapter has been divided in to 3 different parts: where, part-1 or 4.1 contains development of BR11-*Saltol* and characterization of developed lines both at Greenhouse/Nethouse condition and also their field evaluation; part-2 or 4.2 contains development of BRRI dhan28-*Saltol* and characterization of developed lines both at Greenhouse/Nethouse condition and also their field evaluation and part-3 or 4.3 contains the results and analysis of multi-environment trials for G by E interactions of BR11-*Saltol* lines evaluated in 4 environments.

4.1: Introgression of the salinity tolerance QTL 'Saltol' into Bangladeshi mega rice variety BR11 through Marker-assisted backcrossing.

4.1.1 Polymorphism between parents

Both parents (BR11 as recipient and FL378 as donor) are from the *indica* group and share some common parents. More specifically all HYV (high yield variety) traits originate from IR8. Therefore, a high degree of genetic similarity between the two is expected. Initially, 151 microsatellites, sequence tagged site (STS) and gene-based markers were surveyed for polymorphism between the two parents. Out of 153, only 51 were found polymorphic between BR11 and FL378 i.e. percent polymorphism was 33.33%. So, in the 1st backcross generation, the identified 51 polymorphic markers were used to genotype all backcross progenies. Later during genotyping of the 2nd backcross generation, an additional 39 polymorphic SSRs and InDels were added by surveying another 150 markers. So, finally a total of 90 SSRs, STS and InDels were used for the MABC of *Saltol* introgression into BR11.

The target *Saltol* region spans a physical distance of 10.31-15.10 mega bases (Mb) in the short arm of chromosome 1. Fourteen markers were found polymorphic within and around this region (Fig. 4.1.1). Three highly polymorphic, robust and frequently- linked markers RM1287 (10.90 Mb), RM3412 (11.50 Mb) and RM493 (12.20 Mb) were used for foreground selection (to confirm the presence of *Saltol* QTL in the backcross progenies). Five highly polymorphic, robust and frequently-loosely linked or un-linked markers

RM3627 (10.31 Mb) at the telomeric end and RM10825 (13.30 Mb), RM10864 (14.20 Mb), RM562 (14.60 Mb), RM7075 (15.10 Mb) at the centromeric end were used for the recombinant selection. A total of 87 including the recombinant markers were used for background selection. The distribution of 87 background markers was found not to be equal in the genome. The highest and the lowest number of markers was found to be 13 and 4 in chromosome 1 and chromosome 5 respectively (Table 4.1.1) (See the name, position and distribution of background polymorphic marker in Fig. 4.1.2 and lists are given in Appendix-II, Table 8.2.4). Total coverage of the background markers is 1445.25 cM with an average genetic distance between markers throughout the genome being 16.61 cM (Table 4.1.1).

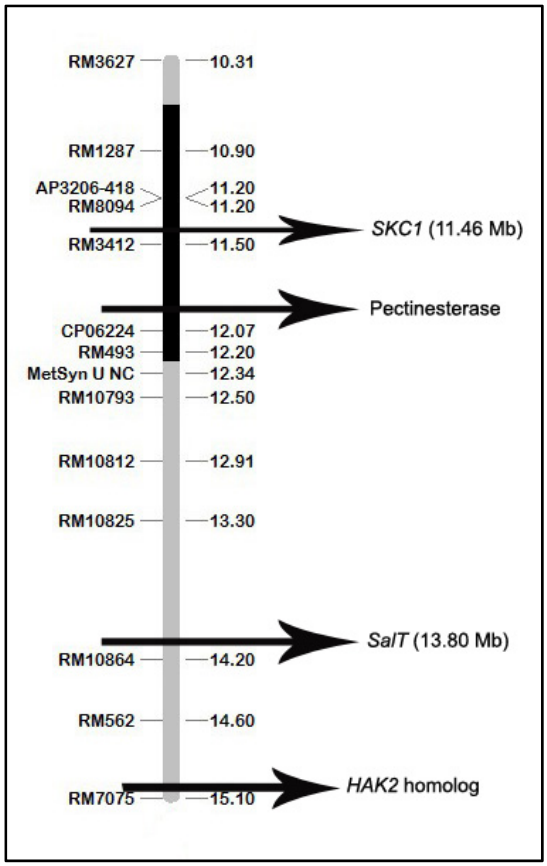


Fig. 4.1.1: *Saltol* QTL region in the short arm of chromosome 1, markers within and around the region with physical position in million bases (Mb) and 4 possible candidate genes within *Saltol*.

Table 4.1.1: Number, distance coverage's and average genetic distances of markers used in background selection for the recovery of recurrent genome in the BR11-*Saltol* work.

Sl. No.	Chromosome	No. of markers	Genetic distance coverage in cM	Average genetic distances between markers in cM
1	Chr1	13	189.87	14.61
2	Chr2	8	128.43	16.05
3	Chr3	8	195.56	24.45
4	Chr4	5	131.68	26.34
5	Chr5	4	65.62	16.41
6	Chr6	8	83.71	10.46
7	Chr7	7	111.89	15.98
8	Chr8	9	144.61	16.07
9	Chr9	5	114.14	22.83
10	Chr10	5	90.72	18.14
11	Chr11	7	71.39	10.19
12	Chr12	8	117.62	14.70
Whole genome		**87**	**1445.25**	**16.61**

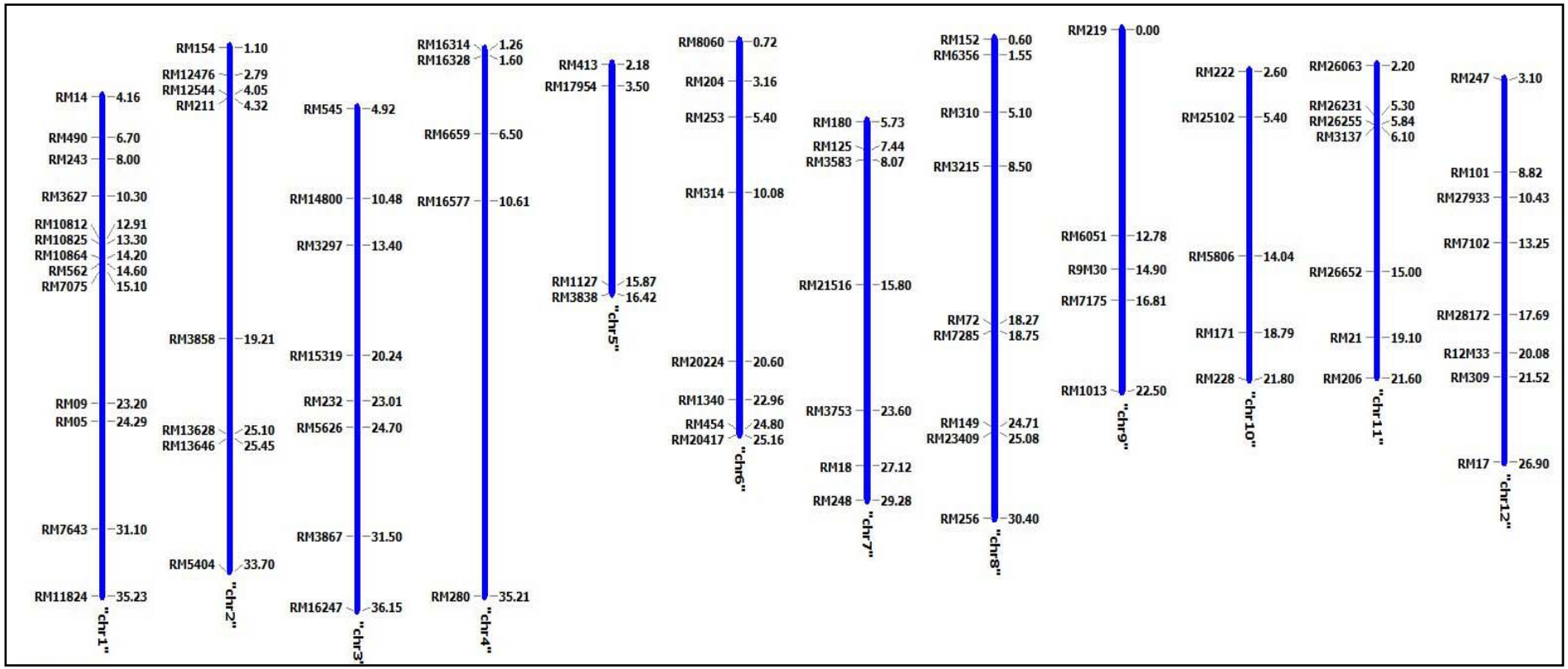


Fig. 4.1.2: Name, physical position and distribution of background polymorphic markers across 12 rice chromosomes used in BR11-*Saltol* MABC event.

4.1.2 Numerical optimization of population sizes in marker-assisted backcross program

To reduce genotyping efforts and cost of a MABC program, calculation of minimum population is a prerequisite of any MABC program. POPMIN a software developed by Hospital and Decoux (2002) was used to determine and plan the backcross population required to obtain at least one double recombinant (probability of success) at the end of the MABC program. This program calculates population size based on the distances of nearest flanking markers used for recombinant selection, on the number of successive backcross (BC) generations (total duration of the breeding program), and on the number of individuals that are genotyped at each generation (population sizes).The genetic distances of the nearest marker in the current MABC program at the telomeric end is 2.4 cM of the recombinant marker RM3627 and at the centromeric end is 4.4 cM of the marker RM10825. Figure 4.1.3 shows the hypothetical *Saltol* QTL with the position and distances of foreground and recombinant markers.

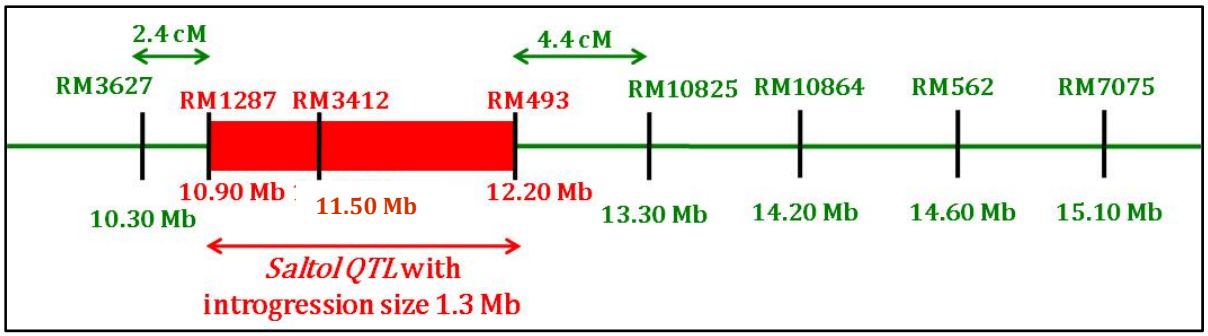


Fig. 4.1.3: Diagram showing the *Saltol* QTL with markers and their positions, including the distances of foreground and nearest recombinant markers on both side of the QTL. Red color denotes the foreground markers and donor introgression and Green color indicates recombinant markers and the recipient background.

The maximum duration of the MABC for BR11 was anticipated for 3 backcross generations. POPMIN software can calculate population sizes by different ways i.e. Constant (fixed population size) and Siman (variable population size). But for ease of generating backcross populations and handling of genotyping in different backcross generations, 'SIMAN' i.e. variable population sizes were computed using the 'simulated annealing' algorithm for this

MABC event. For a three backcross generation, MABC program with 2.4 and 4.4 cM flanking marker distances, the minimum population sizes are 80, 104 and 234 for BC_1, BC_2 and BC_3 respectively, with an average cumulated population size of 228.30 (Table 4.1.2). The probability of success for getting a desired (at least one) double recombinant in each backcross generation was calculated to be 0.03872, 0.736903 and 0.214394 for BC_1, BC_2 and BC_3 respectively, whereas the cumulative probability was 0.990017 at BC_3 (Table 4.1.2).

Table 4.1.2: 'SIMAN' calculation of population sizes for 3 backcross generation MABC with the recombinant marker distances 2.4 and 4.4 cM.

Backcross generation	**Population size**	**Cumulative population size**	**Probability of success**	**Cumulative probability**
BC_1	80	80	0.038720	0.038720
BC_2	104	184	0.736903	0.775623
BC_3	234	418	0.214394	0.990017
Average cumulated population size = 228.30				

4.1.3 MABC for the introgression of Saltol QTL in to BR11

a) Hybridization and F_1 confirmation

For the introgression of *Saltol* QTL into the recipient, BR11 was crossed with FL378 to obtain F_1 where BR11 was used as the mother and FL378 as pollen parent (Fig. 4.1.4). F_1 was confirmed by using a STS-marker (*Methionine Synthetase)* that primarily confirms the presence of *'Saltol'* QTL into the F_1. This STS-marker was designed earlier in the Plant Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka based on the sequence of the *Methionine Synthetase* gene within the *Saltol* QTL region. The marker is co-dominant in nature and the expected product size is 676 bp. So it is very easy to detect heterozygous F_1 by observing double bands. Three agarose gels (4%) for F_1 confirmation is shown in the next page (Fig. 4.1.5-4.1.7).

Fig. 4.1.4: Parents (left) and F_1 (right) growing in the field for crossing and backcrossing.

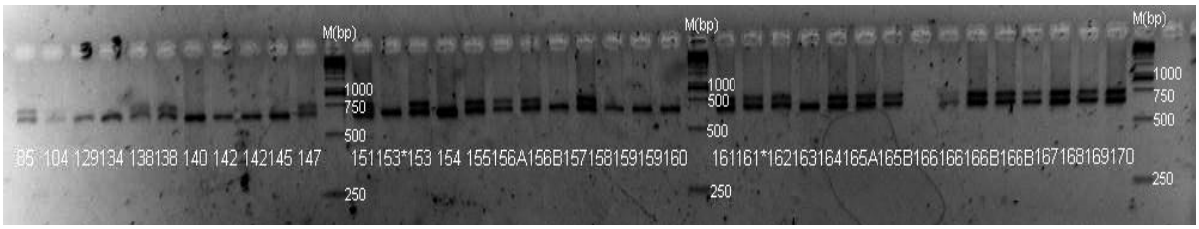


Fig. 4.1.5: Agarose gel (4%) of band patterns of *Methionine synthetase* marker for F_1 confirmation (presence of double band at MW 676 bp confirmed the success of the cross).

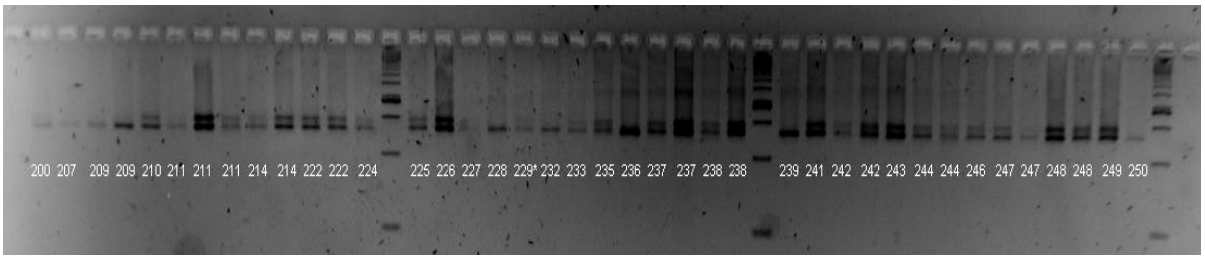


Fig. 4.1.6: Agarose gel (4%) of band patterns of *Methionine synthetase* marker for F_1 confirmation (presence of double band at MW 676 bp confirmed the success of the cross).

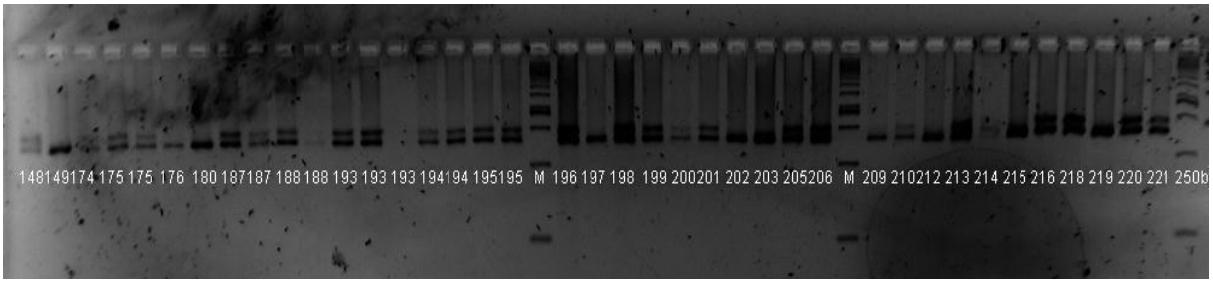


Fig. 4.1.7: Agarose gel (4%) of band patterns of *Methionine synthetase* marker for F_1 confirmation (presence of double band at MW 676 bp confirmed the success of the cross).

b) Genotyping of 1st backcross generation (BC_1):

Confirmed F_1's were backcrossed with recurrent parent BR11 to reconstitute the recurrent genome. In the first backcross generation (BC_1F_1), the target locus was monitored by 3 foreground markers RM1287 (10.90 Mb), RM3412 (11.50 Mb) and RM493 (12.20 Mb) that are tightly linked to the *Saltol* QTL. Individual BC_1F_1progenies were first selected based on the heterozygous forms of the 3 target loci at *Saltol* QTL region. The presence of 3 foreground marker loci in heterozygous form confirms the presence of the *Saltol* QTL as heterozygous (Fig. 4.1.8). The heterozygous form of the QTL allows more recombination for reducing negative linkage drag (removing adjacent additional segment on either side of the QTL possessing undesirable traits). A total of 252 BC_1F_1 were genotyped by 3 foreground markers and 135 progenies were found to have the target *Saltol* QTL as heterozygous.

Recombinant selection is a comparatively new criteria used to transfer target segment more precisely (reducing negative linkage drag). Two markers RM3627 (10.31 Mb) and RM10825 (13.30 Mb) were used to genotype135 progenies that were initially selected by foreground markers (Fig. 4.1.9). In recombinant selection progenies were selected based on recombination of each of the recombinant markers on either side of the QTL, and progenies having recurrent parental band after amplification with recombinant markers were selected. Out of 135 foreground selected progenies, only 8 progenies were selected based on the recombination at the RM3627 locus. So, these selected 8 progenies were subjected to background selection by use of markers on the rest of the chromosomes. These 8 were termed as single recombinants.

A total of 48 microsatellite markers (including recombinant markers and other (non-*saltol*) markers from the donor chromosome) were used for background selection of 8 BC_1F_1 progenies previously selected by foreground and recombinant selection. In the background selection, progenies having recurrent parental band/s for background markers were scored for computing recurrent recovery and progenies with the highest recovery were considered for the next round of backcrossing. The percent of background markers homozygous for the recipient alleles ranged from 32.61 to 54.35% (Fig. 4.1.10). In BC_1F_1, progeny no. 56 had the highest recovery percentage (54.35%) and progeny no. 158 had the lowest recovery (32.61%) (Fig. 4.1.11). The progenies having the highest recovery (more recipient genome) were backcrossed again to reconstitute the recurrent genome.

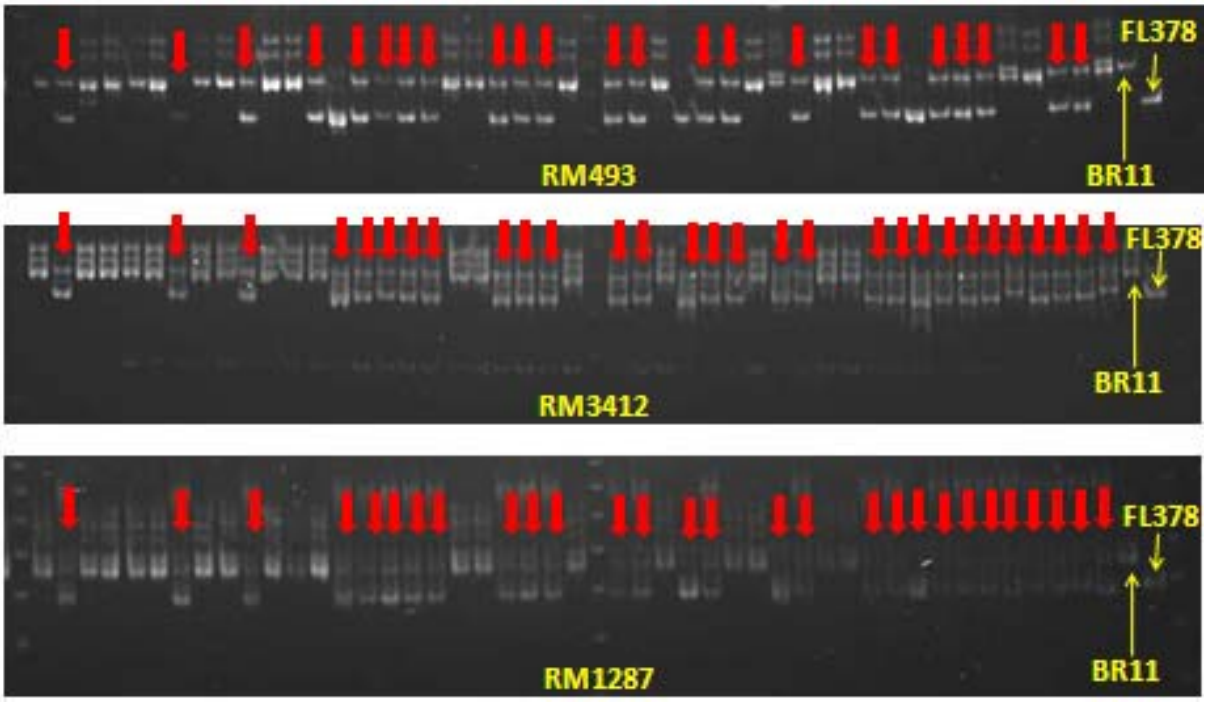


Fig. 4.1.8: PAGE (8%) gels for selection of backcross progenies in foreground selection. Progenies having the bands from both parents (heterozygous) were selected for the next step.

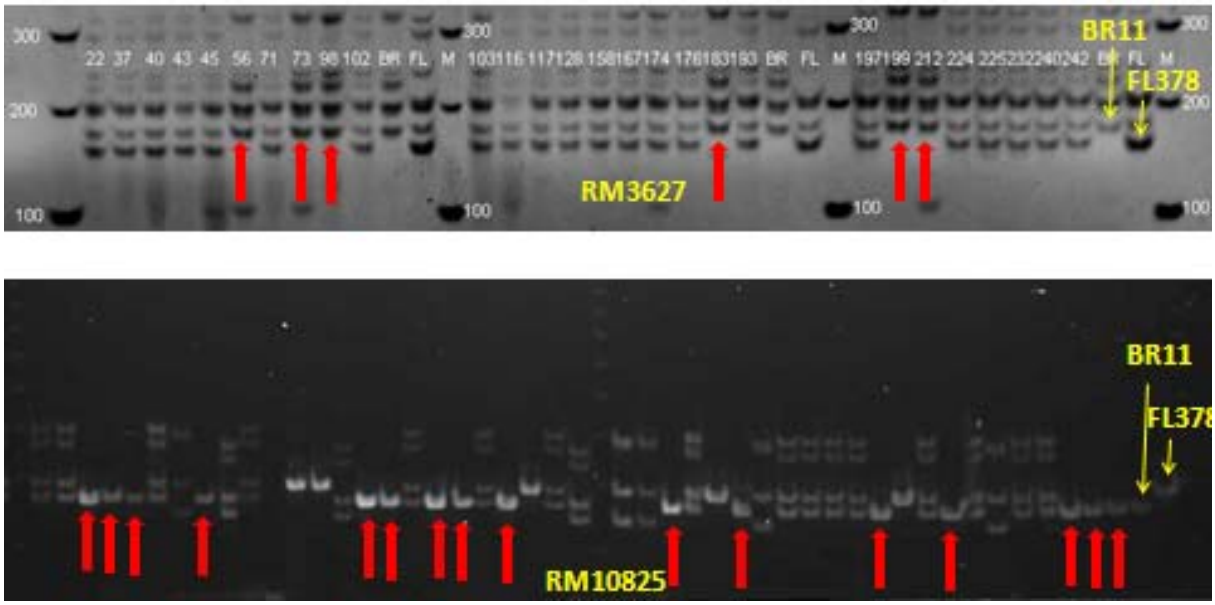


Fig. 4.1.9: PAGE (8%) gels for selection of backcross progenies with recombinant selection. Progenies having the band only for recurrent parent was regarded as having the recombination point (homozygous for recurrent) were selected for next step.

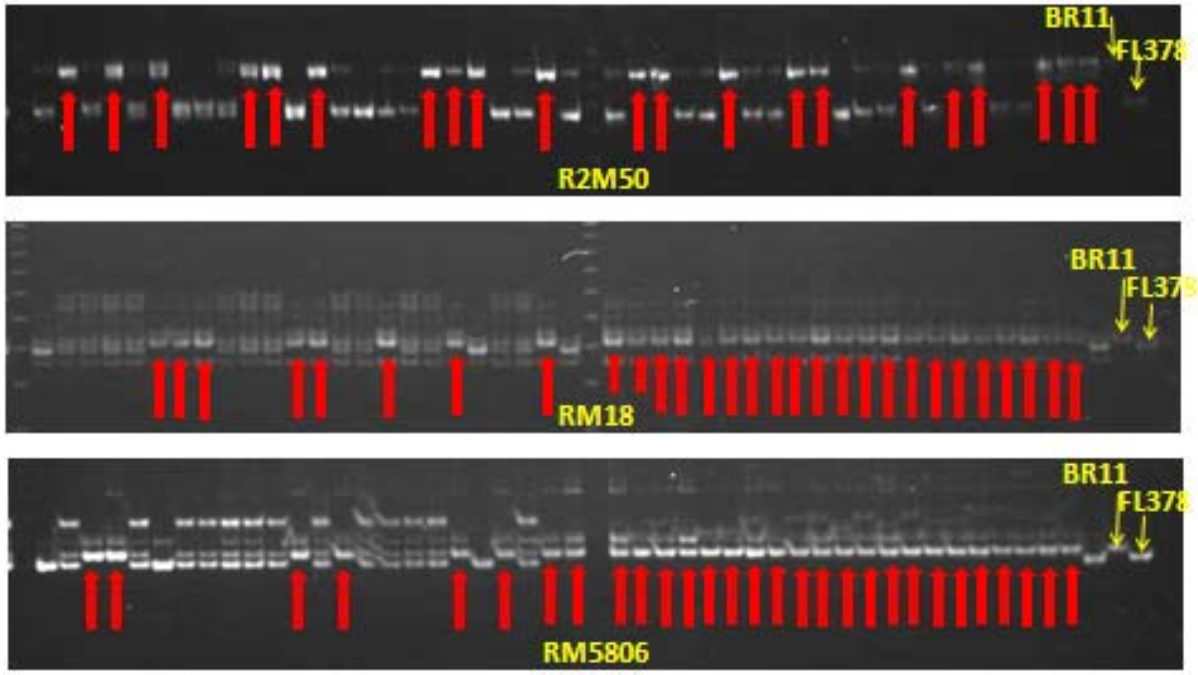


Fig. 4.1.10: PAGE (8%) gels for selection of backcross progenies having the maximal recipient genome. Progenies having the band only for recurrent parent (homozygous for recurrent) were selected for the next step.

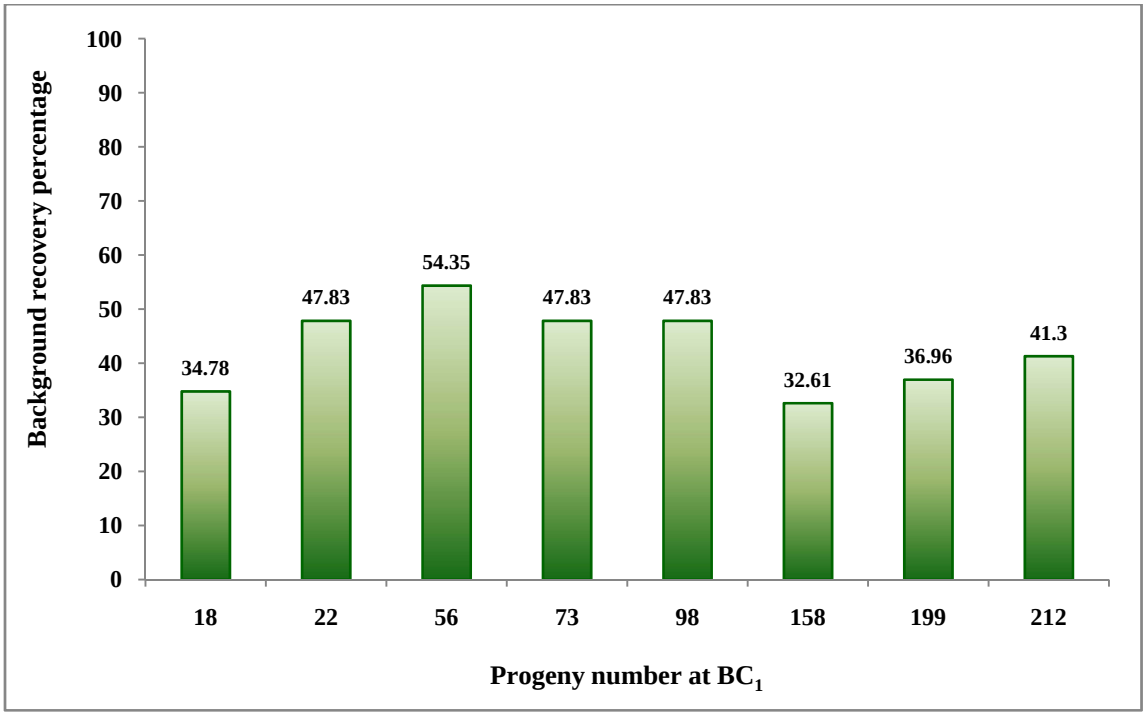


Fig. 4.1.11: Bar graph showing percent recovery of recurrent genome of selected progenies at the first backcross generation (BC_1).

c) Genotyping of the 2nd backcross generation (BC_2):

In the 2nd backcross generation, 342 progenies were genotyped first by the 3 foreground markers (RM1287, RM3412 and RM493) which identified 148 progenies with the QTL as heterozygous. The selected 148 were then genotyped by 2 recombinant markers (RM3627 and RM10825). This analysis identified 2 double recombinants (recombination occurred on both sides of the QTL i.e. with both recombinant markers).For the background selection, 29 more markers were included in addition to the heterozygous markers used in the BC_1. Therefore, a total of 77 markers were used for the background selection in BC_2 and the percent recovery gained was 66.10 to 79.66% (Fig. 4.1.12). Percent recovery of the two double recombinants 73-149 and 18-20 were76.27% and 66.10% respectively (Fig. 4.1.12). The selected two double recombinants were again backcrossed with recurrent parent to obtain a cleaner BR11 background and/or minimum background donor introgression.

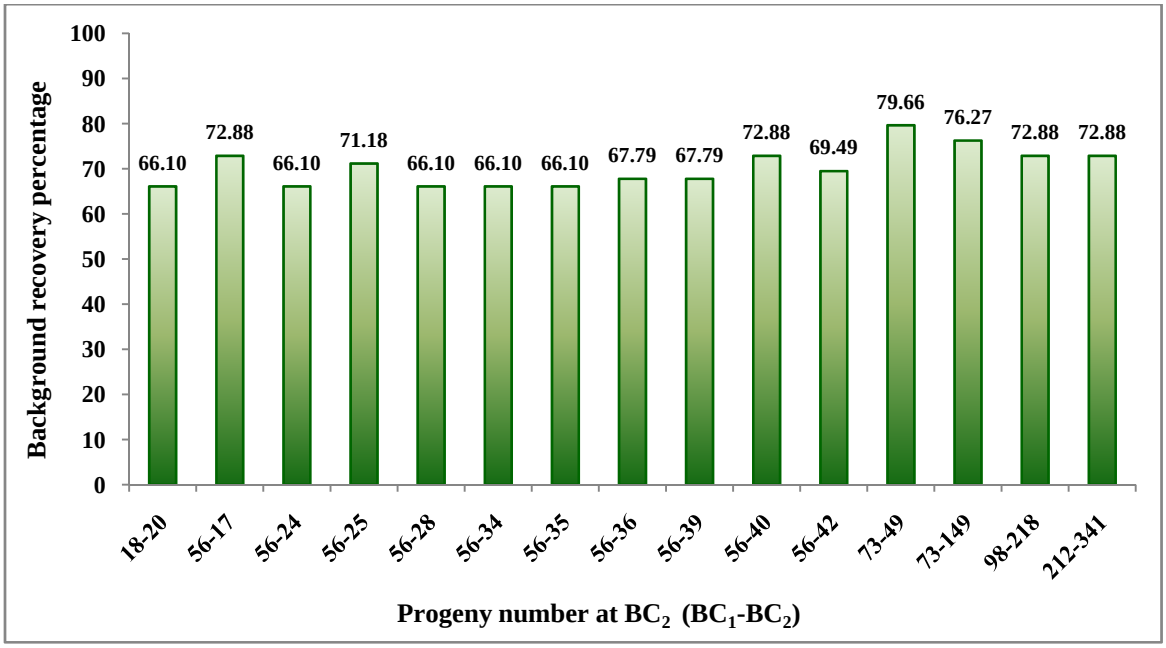


Fig. 4.1.12: Bar graph showing percent recovery of recurrent genomes of selected progenies at BC_2.

d) Genotyping of 3rd backcross generation (BC_3):

At the BC_3 stage, 434 progenies were generated from the 2 double recombinants. Genotyping of 434 progenies by 3 foreground markers and 10 additional background markers in addition to 77 at BC_2 identified two Near Isogenic Lines (NILs) NIL52 and NIL1 i.e. NIL52 in a clean BR11 background and NIL1 with one heterozygous locus in the background. All progenies at BC_3 were double recombinant, so recombinant selection was not carried out at this stage. The selected NILs were selfed and advanced to BC_3F_2.

e) Fixation of Saltol loci at BC_3F_2:

Foreground selection at BC_1 and BC_2 was carried out by selecting heterozygous loci for all 3 foreground markers; allowed more and more recombination for removing or reducing negative linkage drag (removing additional/excessive segments adjacent to the target QTL which may possess undesirable traits). At this stage, 350 progenies from NIL52 and 581 progenies from NIL1 was genotyped by 3 foreground markers. Selfing to BC_3F_2 ensured the

homozygosity at all the 3 foreground loci and hence fixation of *Saltol*. In the case of NIL1 progenies, in addition to fixation of *Saltol*, use of 1 marker which had shown their background to be heterozygous (RM18) in the previous generation was now shown to be homozygous (Fig. 4.1.13, 4.1.14 and 4.1.15). So, at BC_3F_3 stage the percent share of donor and recipient genome is 3.33% and 96.67% respectively in terms of the calculation through SSR/InDel marker genotype for BR11-*Saltol* NILs (Table 4.1.3).

Table 4.1.3: Percent share of marker genotype from donor and final recovery percent of recipient at BC_3F_3 stage of BR11-*Saltol* NILs.

BR11-*Saltol* NILs	**Size of donor introgression at the *Saltol* QTL**	**Foreground markers used to track *Saltol* QTL**	**Percent share of marker genotype from donor at the *Saltol* QTL**	**Recombinant and background markers used to track recurrent loci at the carrier or non-carrier chromosome**	**Percent share of SSR/InDel marker genotype from recurrent genomes**
NIL52	1.3 Mb	3 markers (RM1287, RM3412 and RM493)	3.33	2 (RM3627 and RM10825) and 85 markers*	96.67
NIL1	1.3 Mb	3 markers (RM1287, RM3412 and RM493)	3.33	2 (RM3627 and RM10825) and 85 markers*	96.67

* See BGS marker name and distribution in the figure 4.1.2 above and list in the Appendix-II

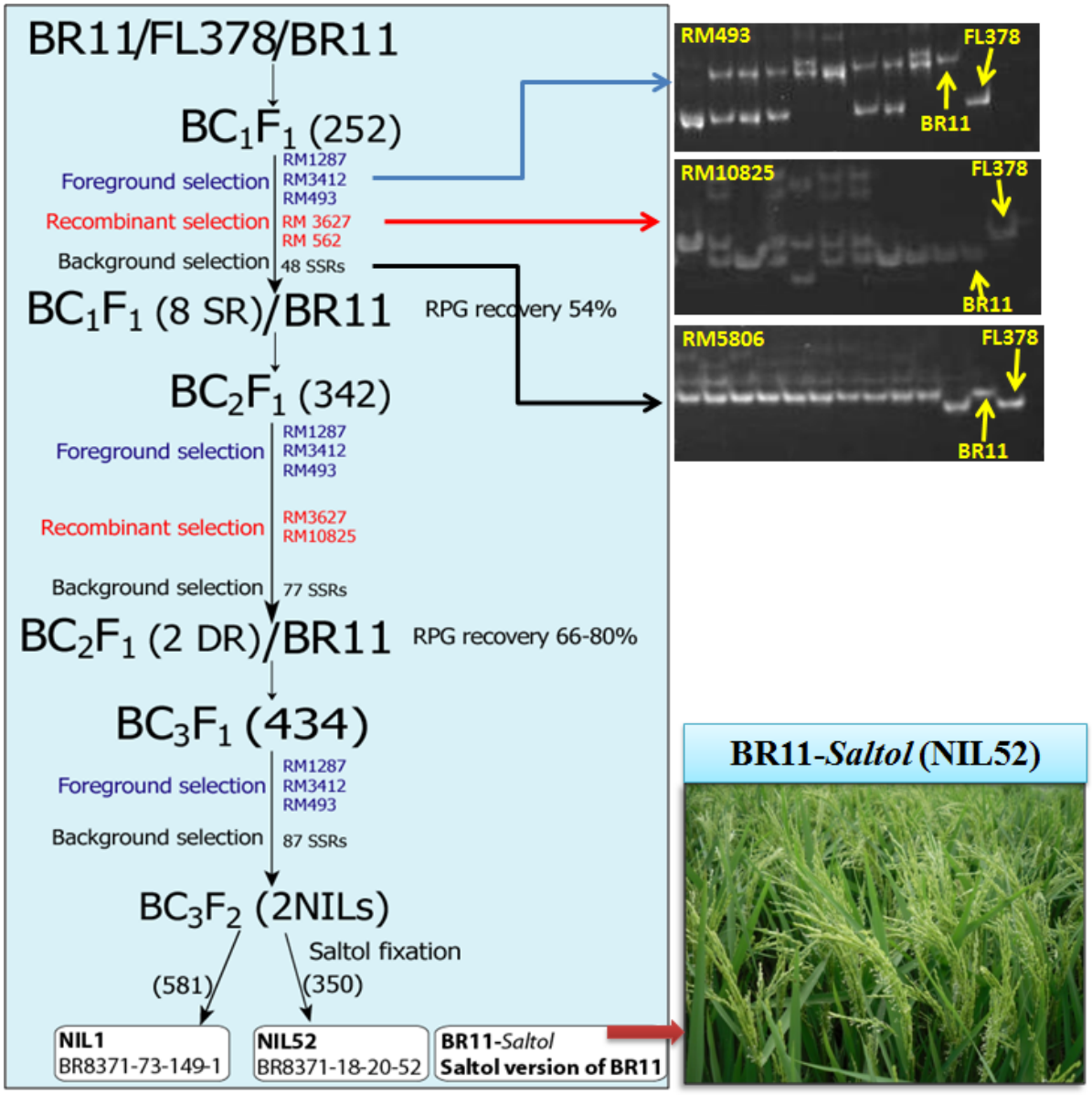


Fig. 4.1.13: MABC scheme for the development of BR11-*Saltol* with markers used for Foreground, Recombinant and Background selection and number of progenies genotyped at different backcross generation (in parentheses) and 3 corresponding PAGE gel photographs at the right, respectively for foreground, recombinant and background selection.

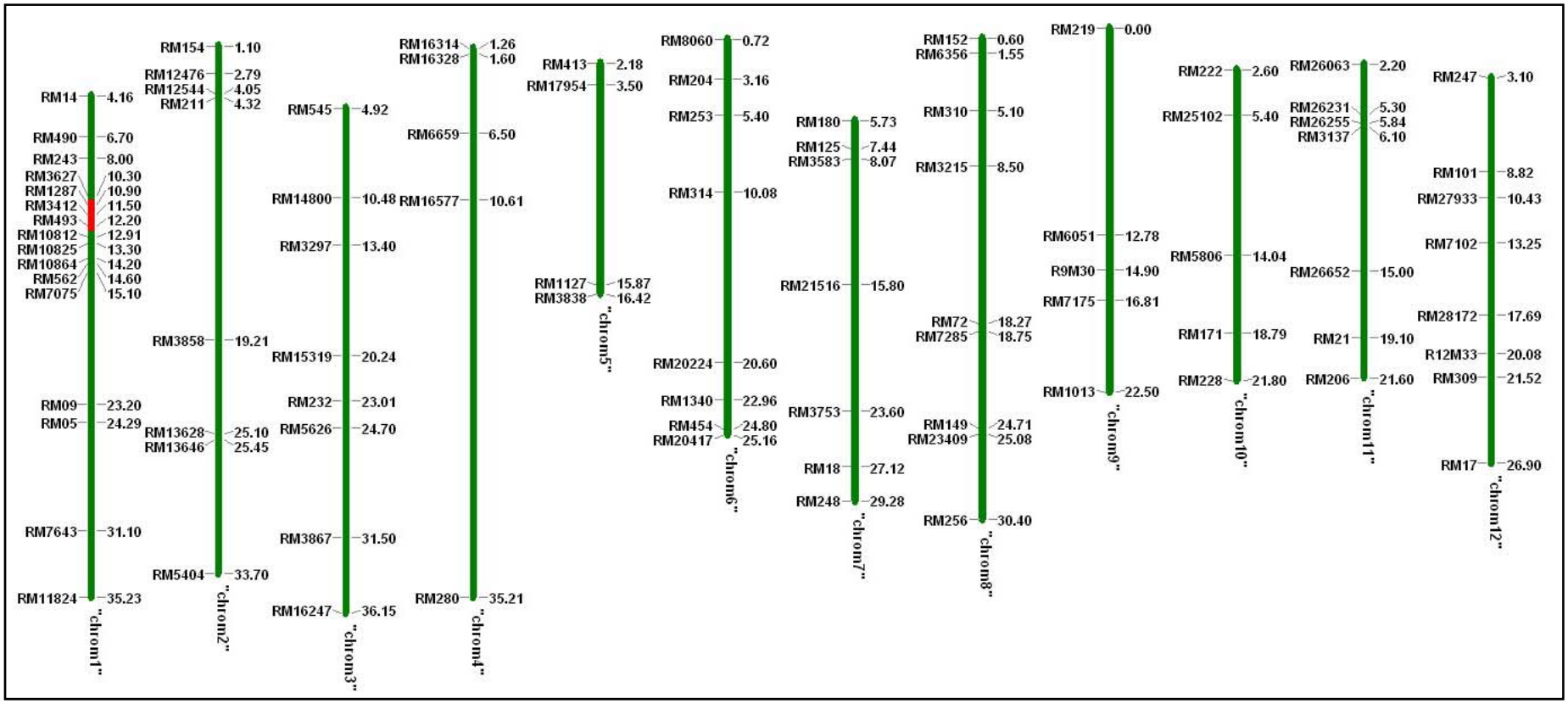


Fig. 4.1.14: Graphical genotype of NIL52 (BR11-*Saltol*) with distribution of markers and corresponding physical position in Mb, green portions are recurrent genome of BR11 and red portion in Chromosome 1 is the targeted introgression from donor FL378.

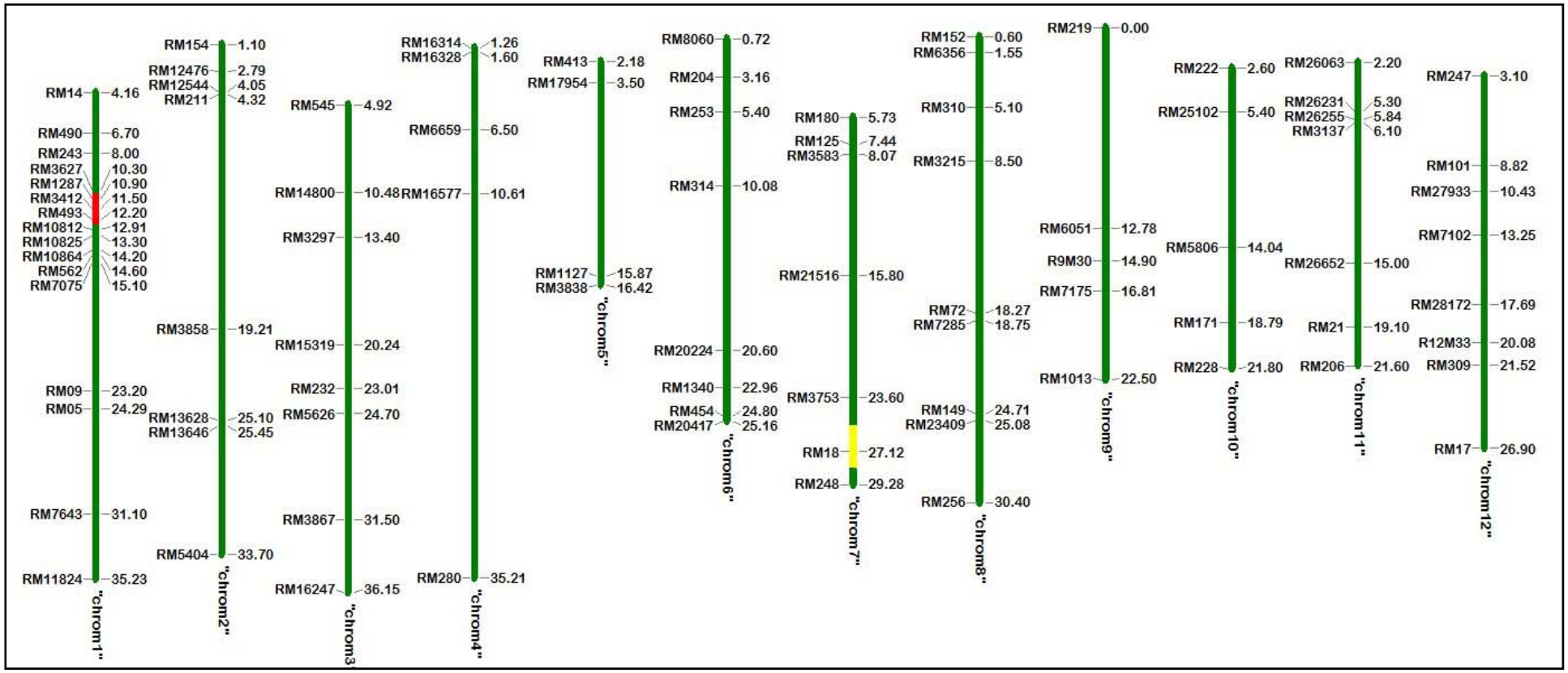


Fig. 4.1.15: Graphical genotype of NIL1 (BR11-*Saltol*) with distribution of markers and corresponding physical position in Mb, green portions are recurrent genome of BR11, red portion in Chromosome 1 is the targeted introgression from donor FL378 and yellow segment is the only heterozygous locus at the background which was fixed at BC_3F_2.

f) SNP genotyping for checking background introgression of developed BR11-Saltol NILs

NILs developed through SSR/STS/InDel/Gene-based markers were further checked by SNP markers for any potential donor introgression at the background. The abundance of SNP markers in rice genome is greater than the SSR/STS/InDel/Gene-based markers. So, there is opportunity to check the background of NILs by SNP markers for any potential donor introgression that was not traced by the SSR/STS/InDel/Gene-based markers. Rice SNP 384-plex chips of Illumina system in IRRI was used to check the background introgression. SNP markers showed similar polymorphisms as found in SSR i.e. 28% for BR11/FL378. Two NILs of BR11-*Saltol* (NIL52 and NIL1) along with two parents (BR11 and FL378) were checked by 384 SNP markers. A single SNP locus () was identified at the targeted *Saltol* QTL region (Fig. 4.1.16). The similarity at SNP level for rest of the background genome was found 93.90% and 79.90% for NIL52 and NIL1, respectively in comparison to the recurrent parent BR11 (Table 4.1.4 and Fig. 4.1.16). In NIL52, one donor background introgression was identified by a SNP marker at chromosome 9, which was found to be homozygous. However, 3 more donor introgression was identified by SNP markers in NIL52 which were in heterozygous condition at chromosome 3 and 4 (Fig. 4.1.17). But in NIL1, 3donor introgressions were identified as homozygous (fixed) in chromosome 2, 3 and 9. Thirteen SNP were found as heterozygous in chromosome 3, 5, 6, 7, 8, 9, 10 and 11 (Fig. 4.1.17). All background donor introgressions identified by SNP markers were those that were not traced by the 87 SSR/STS/InDel/Gene-based markers used for background selection during genotyping of different backcross progenies.

Table 4.1.4: Similarity matrices of SNP alleles between NILs developed through recurrent BR11 and donor FL378. Here, similarities were computed based on the SNP alleles of the recipient parents BR11 through Flapjack software.

	BR11 (recipient)	NIL52	NIL1	FL378 (donor)
BR11 (recipient)	1.000			
NIL52	0.939	1.000		
NIL1	0.799	0.808	1.000	
FL378 (donor)	0.000	0.033	0.098	1.000

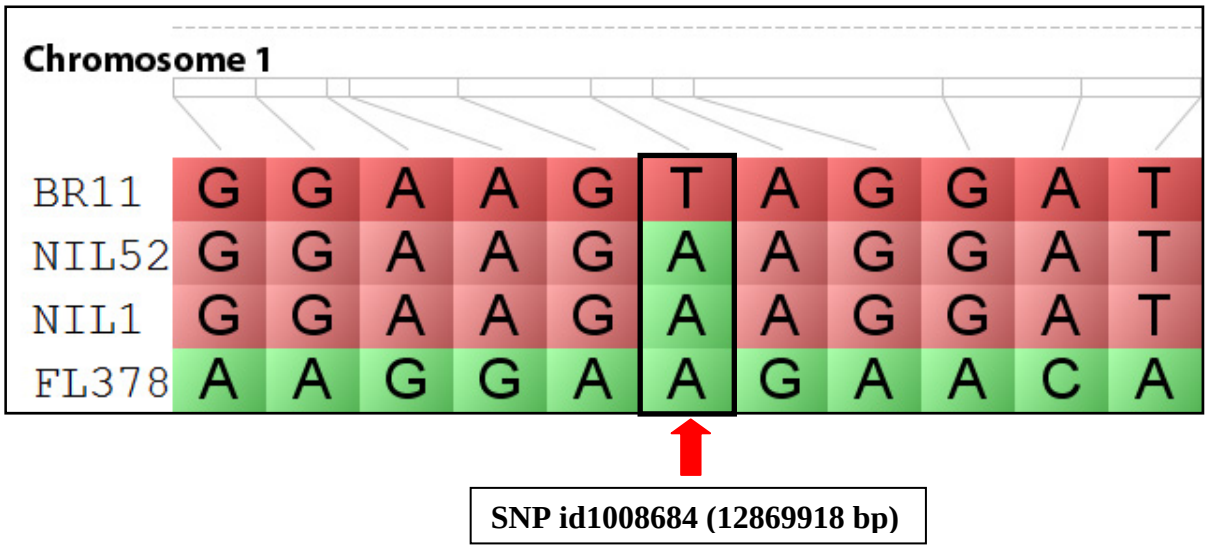
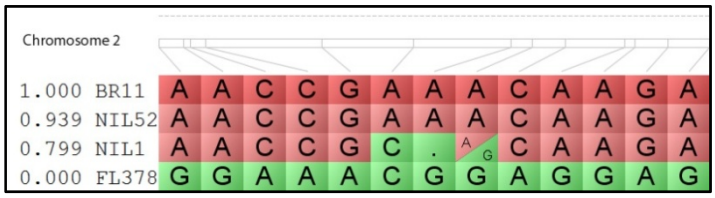
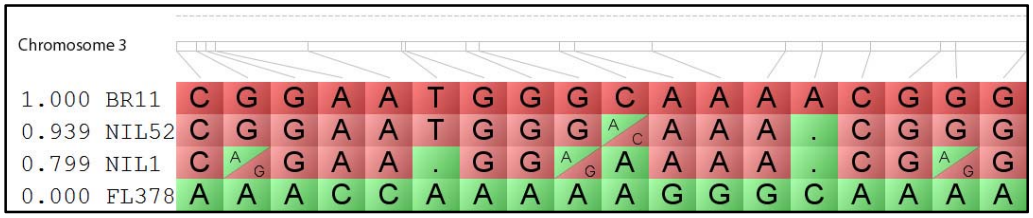
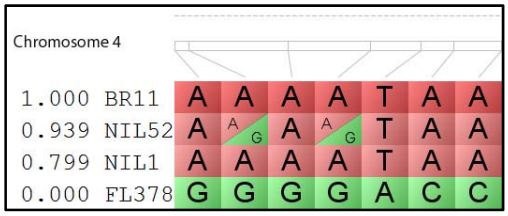
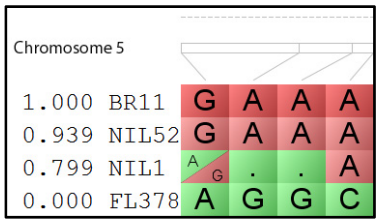


Fig. 4.1.16: SNPs alleles of BR11-*Saltol* NILs with recurrent BR11. SNP panel representing the SNPs on the carrier chromosome 1.Only one SNP i.e. green color 'A'(SNP id1008684) is the only SNP identified within the *Saltol* region introgressed from donor FL378 in the short arm of chromosome 1.









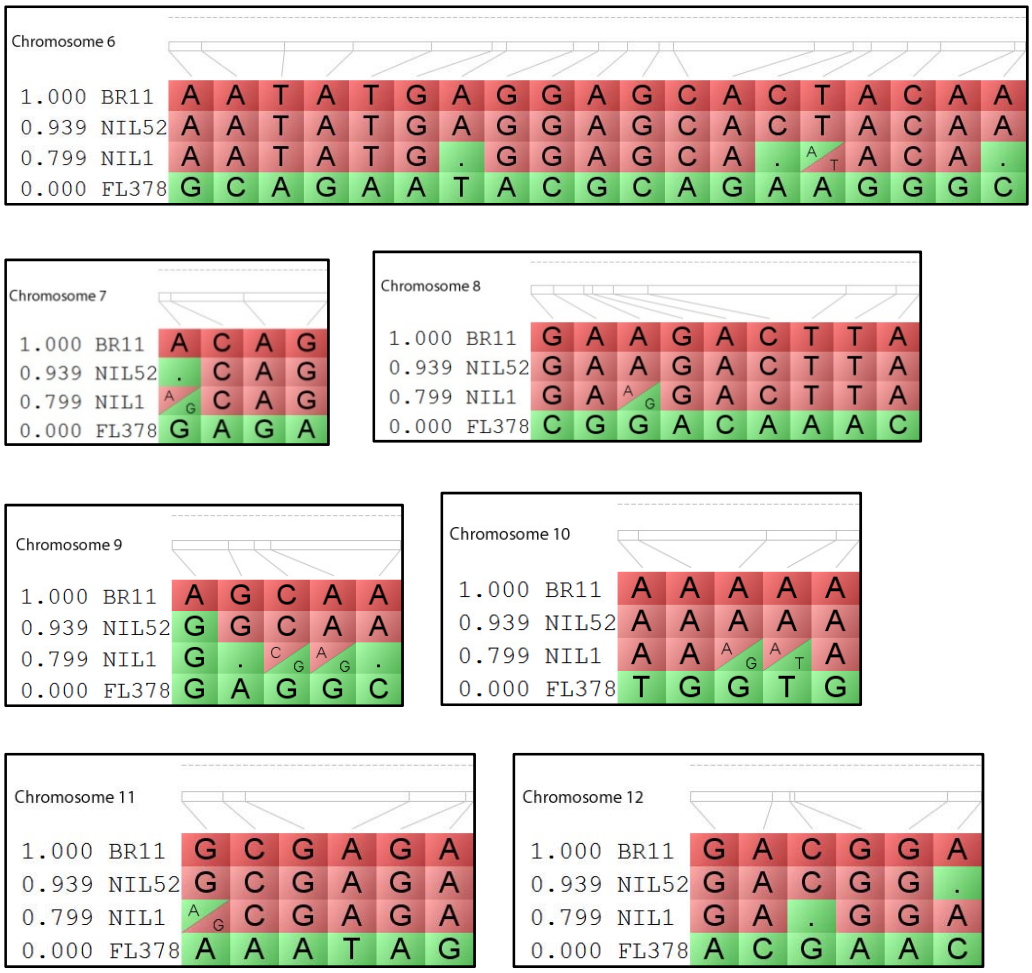


Fig. 4.1.17: Percent similarity of SNPs of BR11-*Saltol* NILs with recurrent BR11 and donor FL378. Pictures representing the SNPs on the rest of the chromosomes (Chr. 2 to Chr. 12).

g) Cross registration and nomenclature of breeding lines for BR11-Saltol MABC event

Crosses are indicated using the female parent and male parent. The genotypes are separated using a backslash. For example, BR11/FL378 indicates a cross with BR11 as the female parent and FL378 as the pollinator. The female parent is always indicated first. Further backcrosses are indicated by more backslashes. For example in the cross BR11/FL378/BR11, the male parent was crossed to BR11 once (to make the F_1 hybrid) and then crossed again to the F_1 to make the BC_1. Therefore, BC_1F_1/BR11 indicates BC_2 and so on. After confirmation, the F_1 generation is given a registration number, for BR11 cross, the registration number is BR8371, where 'BR' is Bangladesh Rice and '8371' is the unique serial number of that cross at Plant Breeding Division of BRRI. After cross registration the pedigree number is written with the progeny number selected in each backcross generation.

For BR11-MABC event two double recombinant progeny was selected, so the pedigrees of these two lines are BR8371-18-20-52 and BR8371-73-149-1. However, to designate these lines in easy and understandable way the lines are called NIL52 and NIL1, here NIL indicates Near-Isogenic Line and 52 or 1 is the last serial number of the pedigree. During phenotypic characterization immediately after the development of NILs the lines are always called by its NIL number as well as BR11-*Saltol*. But later, a phenotypic selection was made due to unexpected segregation of grain size (see details of phenotypic selection in the Materials and Methods section "3.2.5 Field trials and phenotypic selection"). Therefore, based on similarity of grain size with recurrent parent BR11, a total of 12 progeny (4 from NIL52 and 8 fromNIL1) were selected and an additional plant number was added to the pedigree for both NILs (Table 4.1.5). But the pedigree was too long to assign every time therefore a short designation for 12 lines was proposed and used later on every phenotypic and field evaluation, where BR11 indicate recurrent parent and last two pedigree serial number was mentioned for NIL52 and single serial number was added for NIL1 (Table 4.1.5).

Table 4.1.5: Name of the breeding lines, pedigree details and short designation of BR11-*Saltol* lines.

Name of breeding lines and pedigree	Complete pedigree	Short designation
BR8371-18-20-52 (NIL52)	BR8371-18-20-52-55	BR11-52-55
	BR8371-18-20-52-67	BR11-52-67
	BR8371-18-20-52-124	BR11-52-124
	BR8371-18-20-52-145	BR11-52-145
BR8371-73-149-1 (NIL1)	BR8371-73-149-1-7	BR11-7
	BR8371-73-149-1-11	BR11-11
	BR8371-73-149-1-54	BR11-54
	BR8371-73-149-1-65	BR11-65
	BR8371-73-149-1-71	BR11-71
	BR8371-73-149-1-84	BR11-84
	BR8371-73-149-1-97	BR11-97
	BR8371-73-149-1-150	BR11-150

4.1.4 Phenotypic characterization of developed BR11-Saltol introgression lines.

a) Phenotypic characterization at seedling stage under salinity @ 12 dS/m in hydroponics at controlled Net house condition

Two NILs of BR11 background (NIL52 and NIL1) were characterized for salinity tolerance at seedling stage @12 dSm^{-1} in hydroponic culture immediately after fixation of *Saltol* (target qtl region) as well as all background loci at BC_3F_3. Initially only two parameters were recorded (SES score and survivability) to judge the phenotypic gain for salinity tolerance at the seedling stage. These traits are strongly correlated for salinity tolerance at seedling stage in the opposite direction i.e. negatively correlated. Standard Evaluation System for rice i.e. SES score is the scale of rating salinity tolerance at seedling stage. The scale ranges from 1-9, where 1 is highly tolerant and 9 is highly sensitive to salinity and this can be measured visually by leaf damage percentage at seedling stage. SES score gives an overall performance for salinity tolerance of a line, genotype or variety under investigation. However, survivability can give a direct measurement for tolerance by counting the live plants when sensitive checks are almost dead. Table 4.1.6 represents the result of the first experiment for the two measured traits. ANOVA identified highly significant variations among the tested lines, parents and check varieties for the two traits. LSD test for comparing means among the tested genotypes identified Pokkali, the original donor of *Saltol* QTL as significantly superior among the tested genotypes for both SES score and survivability. FL378, the donor used for this introgression was ranked second and showed significantly higher SES value compared to Pokkali but the survivability was statistically similar to Pokkali. NIL52 (BR11-*Saltol*) was ranked 3 i.e. next to FL378 and in between the donor and recipient BR11. NIL52 has some improvements in terms of SES score and survivability but from the statistical point of view these differences are not significant either by comparing with FL378 (donor) or by BR11 (recipient) (Table 4.1.6). But the other introgression line, NIL1 showed poorest performance among all tested genotypes, even poorer than sensitive check IR29. Poor vigor and slow growth at early seedling stage could be the reason for the poor performance of NIL1 at seedling stage. Because early vigorous growth is one of the important traits for tolerance at seedling stage. However, this performance was improved at a later generation (i.e. BC_3F_4). Due to heterogeneity of seed size of both NILs, these were grown in the field and selection was carried out at BC_3F_4. Twelve progenies (8 from NIL1 and 4 from NIL52) were selected and characterization of these progenies at later generation

showed improvement in their performance for tolerance at seedling stage as well as homozygosity and phenotypic similarity with parent BR11 (see details of phenotypic selection in the Materials and Methods section "3.2.5 Field trials and phenotypic selection"). So, the poor performance of NIL1 at BC_3F_3 stage could be due to the presence of a few heterozygous loci which was however removed during selection in subsequent generation using phenotypic selection.

Table 4.1.6: Phenotypic characterization of BR11-*Saltol* NILs (BC_3F_3) at seedling stage in BRRI, 2010.

Lines/varieties	SES score	Survivability (%)
Pokkali (Original *Saltol* donor)	3.97 a	100.00 c
FL378 (Donor used for this MABC)	5.03 b	90.00 c
NIL52 (BR11-*Saltol*)	6.12 bc	73.33 bc
BR11 (Recipient)	6.73 c	66.67 b
IR29 (Sensitive check)	7.15 c	61.67 b
NIL1(BR11-*Saltol*)	7.87 c	30.00 a
Significance	**	**
$LSD_{0.05}$	1.16	16.75
CV (%)	10.30	13.10

*Each value is the mean of 3 replicates; Means followed by a common letter/s within column is not significantly different at 5% level of probability. NIL=Near Isogenic Line.

b) Phenotypic characterization of BR11-Saltol NILs at reproductive stage under salinity @ 6 dSm^{-1} in controlled Net house condition

The two NILs of BR11-*Saltol* which performed better than their BR11 parent were evaluated for tolerance and yield throughout their growth period under salinity stress @ 6 dSm^{-1} in a soil-based system (Gregorio, *et.al.* 1997). Out of 10 parameters measured, 8 showed highly significant variation among the tested genotypes (Table 4.1.7). NIL52 performed significantly better than all tested lines, parents and even better than BRRI dhan47 (a salt tolerant HYV for *Boro* season) in the *T. Aman* season.NIL52 maintained better yield by maintaining higher filled spikelet and subsequent higher biomass production and less sterility (Table 4.1.7). The yield advantage of NIL52 is 1.88 g/plant compared to its parents BR11 in salinity for the whole growth period in a controlled Net house condition. Other traits like days to flower and days to salinization (duration of salinity stress) were found

statistically similar in NIL52 and NIL1 compared to BR11. However, NIL52 maintained significantly better plant height and panicle length compared to NIL1 and BR11 under stress condition. This could indicate its greater tolerance capacity. All of the above traits could help in production of higher numbers of filled grains and thus better yield of NIL52 (Table 4.1.7).

Table 4.1.7: Growth and yield parameters for characterization at whole growth period of BR11-*Saltol* NILs under salinity in soil based system with salinity stress @ 6 dSm^{-1}.

Lines/ Varieties	Days to flowering	Days to salinization	Plant height (cm)	Panicle (no./plant)	Panicle length (cm)	Filled grains (no./plant)	Sterility (%)	Grain weight (gm/plant)	Straw weight (gm/plant)	Harvest Index
NIL52 (BR11-*Saltol*)	112.67 c	73.00 cd	115.00 c	7.50	23.63 c	634.50 c	18.38 a	10.68 b	17.47	0.38 c
NIL1 (BR11-*Saltol*)	113.33 c	74.33 d	103.67 bc	5.67	22.90 bc	284.67 a	47.47 b	5.34 a	17.93	0.23 ab
BR11 (Recipient)	109.00 c	68.50 c	99.75 b	7.50	22.28 bc	462.00 b	32.10 ab	8.80 b	14.01	0.40 c
BR47 (Tolerant ck.)	79.00 a	40.33 a	109.67 c	5.67	24.15 c	436.67 b	21.25 a	9.41 b	17.87	0.35 bc
IR29 (Sensitive ck.)	99.67 b	59.67 b	97.33 b	8.00	21.51 b	223.33 a	68.53 b	4.03 a	18.52	0.17 a
FL378 (Donor)	80.00 a	40.00 a	88.00 a	7.50	16.40 a	128.50 a	59.75 b	4.93 a	10.66	0.32 b
Significance	**	**	**	ns	**	**	**	**	ns	**
LSD0.05	4.69	5.11	8.77	2.39	1.73	189.94	25.99	3.28	6.84	0.13
CV (%)	2.60	4.70	4.70	18.90	4.40	28.90	34.60	25.00	23.40	22.60

**Significance at 1% level, ns=Not significant, Means followed by a common letters within column are not significantly different at 5% level of probability

c) Phenotypic characterization of NIL52 and NIL1 derivative 12 lines at seedling stage under salinity @ 12 dSm^{-1} in hydroponics at controlled Net house condition

Twelve NILs derivative lines were further investigated for tolerance gained at seedling stage after phenotypic selection from NIL52 and NIL1 at BC_3F_5 stage {see details of phenotypic selection in the Materials and Methods section "3.2.5 Field trials and phenotypic selection" and also in the above Results section 4.1.3. (g) and Table 4.1.5}. The experiments were also carried out in hydroponics culture of controlled Net house condition. Here, 4 HYV salt tolerant *T. Aman* varieties (BRRI dhan40, BRRI dhan41, BRRI dhan53 and BRRI dhan54) and the two parents were also included for comparison. Seven different traits including SES score, survivability, Na^+ to K^+ balance in shoots, shoot and root length and dry weights were measured and recorded. Out of the 7 measured traits, first 3 traits (SES score, survivability and Na^+/K^+ ratio in the shoots) are more important for judging the tolerance of lines/genotypes. Correlation coefficients among the first 3 traits showed strong and highly significant but negative relationships between SES and survivability. Also, fair and highly significant, positive relationships between SES and Na^+/K^+ ratio were obtained (Table 4.1.8). Rest of the morphological parameters showed strong, highly significant, negative correlation compared to SES and Na^+/K^+ ratio and positive correlation to survivability (Table 4.1.8). ANOVA identified highly significant variations among the tested lines, parents and HYV checks for all the measured traits. Mean comparison through LSD test showed the donor FL378 ranked first for SES score and survivability. Two HYV check BRRI dhan53 and BRRI dhan40 ranked next to FL378. Out of 12 derivative lines from NIL52 and NIL1, only two lines BR11-52-124 and BR11-150) (see details of short name for all NIL derivative lines in the table 4.1.5) performed superior than recipient BR11 in terms of SES score and survivability. But rest of the lines and HYV check were found poorer than BR11 (Table 4.1.9). However, Na^+ to K^+ balance in the shoot showed a different trend when comparing with SES and survivability. The ratio was found significantly lowest for BRRI dhan53 and then FL378. All four HYV tolerant checks were also maintained lowest ratios (good balance of Na^+/K^+) very near to donor FL378. All 4 NIL52 derivative lines BR11-52-124, BR11-52-145, BR11-67 and BR11-52-55 and one NIL1 derivative lines BR11-150) maintained very similar ratios to the HYV tolerant checks and donor FL378. But rest of the 7 lines/varieties, NIL1 derivative lines maintained significantly higher ratio and performed worse than the other tested lines (Fig. 4.1.18 and Table 4.1.9 and 4.1.10). The above

mentioned 3 traits are the major traits for judging the salinity tolerance for seedling stage (Gregorio, *et. al.* 1997). Rest 4 traits such as seedling height, root length, shoot and root weights were determined to further categorize them under tolerance or sensitivity. *Saltol* QTL explains much of the effect towards maintaining a healthy Na^+/K^+ ratio under salt stress, it does not by itself provide a high degree of salt tolerance, as measured by visual scores (SES) of leaf symptoms (Thomson, *et al.* 2010a). For comparing Na^+/K^+ ratio under salt stress at seedling stage showed a remarkable reduction of 19.35% by a line BR11-52-124, which is very close to the donor FL378 (25.48%) while comparing recipient BR11 (Table 4.1.10). However, out of the 12 tested lines only 5 lines (all 4 from NIL52 and one NIL1 derivative) lines showed a decreased Na^+/K^+ ratio compared to the recipient BR11 (Fig. 4.1.18 and Table 4.1.10). But rest of the lines showed an increased Na^+/K^+ ratio even greater than the recipient BR11.

Table 4.1.8: Correlation (Pearson) coefficients among the traits recorded for judging the salinity tolerance at seedling stage of BR11-*Saltol* lines.

Traits	SES	SUR	Na^+/K^+	SH	RL	SDW	RDW
SES score	1.00						
Survivability	-0.91**	1.00					
Na^+/K^+ ratio	0.58**	-0.56**	1.00				
Seedling height	-0.79**	0.78**	-0.59**	1.00			
Root length	-0.63**	0.55**	-0.43**	0.74**	1.00		
Shoot dry weight	-0.70**	0.60**	-0.26	0.59**	0.47**	1.00	
Root dry weight	-0.76**	0.56**	-0.50**	0.59**	0.47**	0.58**	1.00

**Correlation is significant at the 1% level of probability.

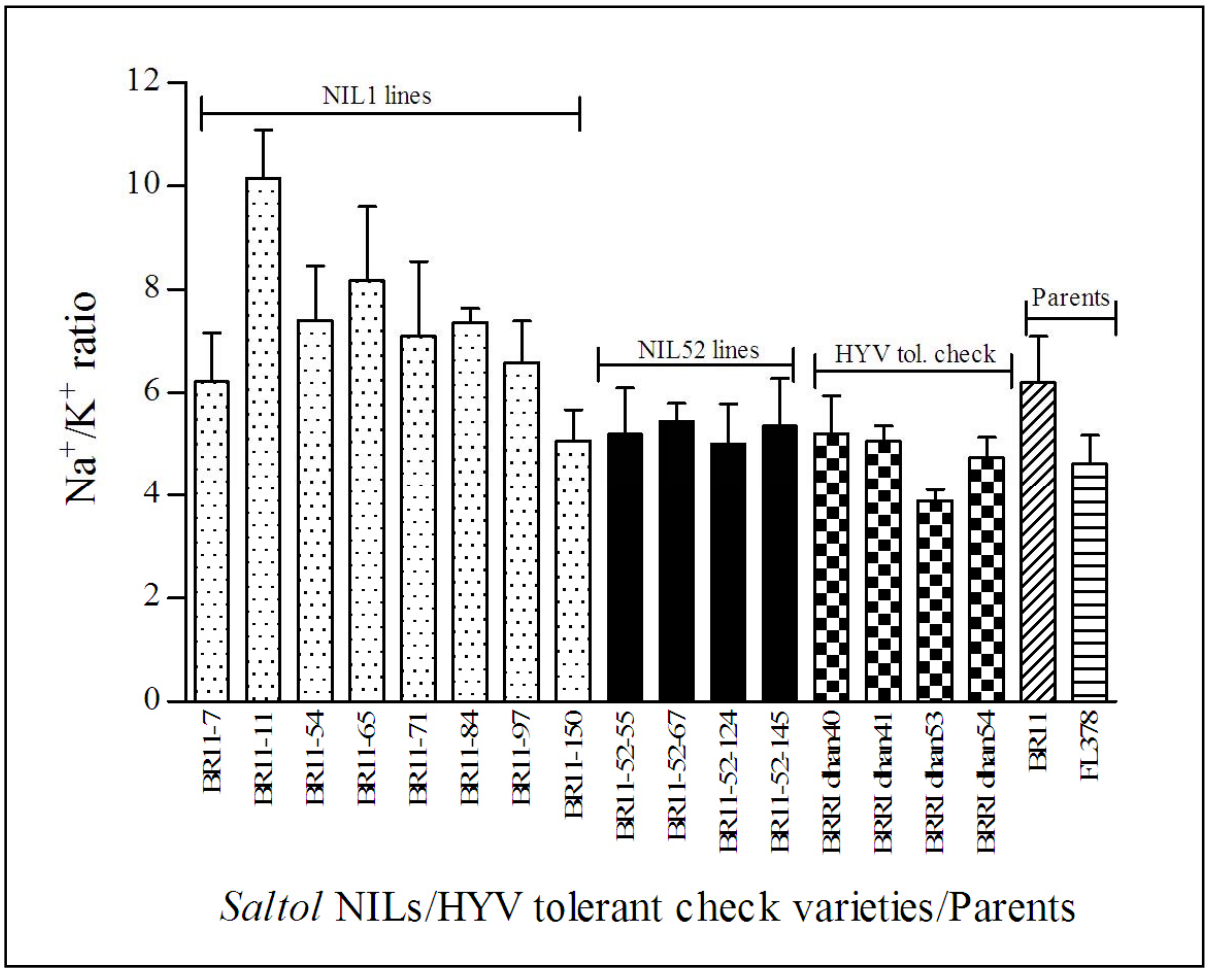


Fig. 4.1.18: Shoot Na^+/K^+ ratio at seedling stage of all selected BR11-*Saltol* lines, HYV tolerant checks and parents characterized under salinity @ 12 dSm^{-1} in controlled Net house condition. Each bar represents means of 3 replicates and error bars SEM (N=5). (in this figure, the first 8 lines are from NIL1 and next 4 lines from NIL52 and then 4 HYV tolerant checks and finally 2 parents).

Table 4.1.9: Phenotypic characterization of BR11-*Saltol* NILs derivative 12 lines at seedling stage with salinity stress @ 12 dSm^{-1} in hydroponics culture at Nethouse in BRRI, 2013

Lines/Varieties	**SES score (leaf damage visual score)**	**Survivability (%)**	**Na^+/K^+ ratio in shoot**	**Seedling height (cm)**	**Root length (cm)**	**Shoot dry weight (mg/seedling)**	**Root dry weight (mg/seedling)**
FL378 (donor)	4.90 a	100.00 d	4.62 ab	38.30. e	16.08 d	201.33 c	26.68 c
BRRI dhan53 (HYV tolerant ck.)	5.22 ab	92.59 cd	3.89 a	37.25 e	17.30 d	180.00 c	29.07 d
BRRI dhan40 (HYV tolerant ck.)	6.09 b	79.63 cd	5.20 ab	34.70. d	14.75 cd	151.33 bc	16.11 b
BR11-52-124	6.28 bc	81.48 cd	5.00 ab	34.88 de	12.95 bc	157.00 bc	16.39 b
BR11-150	6.46 bc	87.03 cd	5.06 ab	34.75 d	15.13 cd	154.33 bc	16.84 b
BR11 (recipient)	6.46 bc	83.33 cd	6.20 b	32.77 cd	13.97 c	145.33 bc	16.25 b
BR11-52-145	6.55 bc	75.93 c	5.36 ab	32.55 cd	13.42 bc	121.33 ab	15.37 ab
BR11-52-67	6.86 c	72.22 c	5.44 ab	33.07 cd	11.20 ab	133.67 b	15.03 ab
BR11-97	7.16 cd	68.52 bc	6.59 bc	33.98 d	10.27 ab	140.00 bc	15.23 ab
BR11-52-55	7.37 cd	55.55 bc	5.19 ab	31.75 c	11.93 b	116.67 ab	14.62 ab
BR11-71	7.49 cd	53.66 bc	7.10 bc	30.62 bc	10.32 ab	107.00 ab	14.12 ab
BR11-54	7.77 d	46.37 bc	7.40 bc	30.33 bc	10.03 ab	122.33 ab	17.62 b
BR11-84	7.79 d	51.70. bc	7.36 bc	29.40 b	10.30 ab	107.00 ab	11.86 a
BRRI dhan41 (HYV tolerant ck.)	7.85 d	50.00 bc	5.06 ab	36.75 e	15.80 cd	117.67 ab	14.46 ab
BR11-65	8.11 de	44.52 bc	8.18 c	29.33 b	9.83 a	110.67 ab	11.80 a
BR11-7	8.18 de	35.18 ab	6.22 b	28.31 ab	9.81 a	128.67 b	16.94 b
BRRI dhan54 (HYV tolerant ck.)	8.36 de	37.04 ab	4.74 ab	29.55 b	12.28 b	80.67 a	12.53 ab
BR11-11	8.73 e	17.12 a	10.16 d	27.06 a	10.12 ab	107.33 ab	12.87 ab
Significance	**	**	**	**	**	**	**
$LSD_{0.05}$	0.75	24.80	1.94	1.91	2.02	43.44	4.10
CV (%)	6.40	23.80	19.40	3.50	9.70	19.80	15.20

*Each value is the mean of 3 replicates; Means followed by a common letter/s within columns are not significantly different at 5% level of probability.

Table 4.1.10: Mean shoot Na^+/K^+ ratio and percent reduction of shoot Na^+/K^+ ratio compared to the recipient BR11.

Lines/Varieties	Mean shoot Na^+/K^+ ratio*	Percent reduction of shoot Na^+/K^+ ratio compared to recipient BR11
BRRI dhan53 (HYV tolerant ck.)	3.89	37.26
FL378 (donor)	4.62	25.48
BRRI dhan54 (HYV tolerant ck.)	4.74	23.55
BR11-52-124	5.00	19.35
BR11-150	5.06	18.39
BRRI dhan41 (HYV tolerant ck.)	5.06	18.39
BR11-52-55	5.19	16.29
BRRI dhan40 (HYV tolerant ck.)	5.20	16.13
BR11-52-145	5.36	13.55
BR11-52-67	5.44	12.26
BR11 (recipient)	**6.20**	**0.00**
BR11-7	6.22	-0.32
BR11-97	6.59	-6.29
BR11-71	7.10	-14.52
BR11-84	7.36	-18.71
BR11-54	7.40	-19.35
BR11-65	8.18	-31.94
BR11-11	10.16	-63.87

* Each value is the mean of 3 replicates.

d) Field evaluation of BR11-Saltol introgression lines (NIL52 and NIL1 derivative 12 lines) in Non-Saline condition at BRRI, Gazipur

The main aim of any MABC conversion process is to maintain the original qualities of the mega variety i.e. the yield and quality of the mega variety in addition to the target trait through introgression of a QTL. Thus for maintaining the qualities of mega varieties, it is important to keep all background loci from the recipient mega varieties. Therefore, after completion of the MABC event it is necessary to compare the developed NILs with its recipient parent for recovery of the yield and quality traits. Therefore, for comparing the yield and quality traits, all developed BR11-*Saltol* NILs were grown in non-saline field condition at BRRI farm Gazipur in respective season (*T. Aman* season, 2012) along with its parents. Significant improvements were observed for most of the yield and yield related traits for most of the lines in comparison with BR11 (Table 4.1.11). Out of 12 lines, 8 showed significantly higher yield than BR11. Three lines showed yields similar to BR11 and only one line (BR8371-73-149-1-11) performed poorer than BR11 (Table 4.1.11). These increased yields were probably due to better panicle number and length, higher filled grains and weights and decreased sterility of the lines compared to BR11 (Table 4.1.11). However, the grain size (1000-grain weight) was reduced significantly among all 12 lines compared to BR11. Though the MABC target was to maintain the original size but it was considered a positive improvement by maintaining higher yield potential. Grain quality parameters also showed improved or similar qualities compared to the recipient BR11. However, the improvement of milling outturn and head rice recovery could help in obtaining higher final yield. The most important improvement was the increase in amylose content by least 1-2% over recipient BR11. Increase amylose content in addition to the improvement in elongation ratio could give positive impact to consumer preference for choosing these lines (Table 4.1.12).

Table 4.1.11: Growth and yield parameters of BR11-*Saltol* NILs, parents and check varieties in non-saline field condition at BRRI, Gazipur, *T.Aman* season 2012.

Lines/Varieties	Plant height (cm)	Panicle (no./hill)	Panicle length (cm)	Filled grains (no./hill)	Filled grains wt. (g/hill)	1000-grains wt. (g)	Sterility (%)	Grain yield (t/ha)	Straw yield (t/ha)	Harvest Index
BR11-52-67	130.17 e	10.00 b	24.47 bc	1195.00 b	27.90 c	23.37 bc	27.21 b	5.74 c	7.22 bc	0.42 b
BR11-7	124.33 d	9.50 b	23.62 b	1100.17 b	25.19 bc	22.92 bc	26.96 b	5.71 c	6.84 bc	0.35 a
BR11-150	126.28 de	9.50 b	23.65 b	1124.33 b	25.37 bc	22.73 b	32.13 bc	5.54 c	7.34 bc	0.39 ab
BR11-52-55	127.00 de	10.17 b	23.13 ab	1190.33 b	28.12 c	23.67 bc	31.97 bc	5.40 c	7.41 bc	0.42 b
BRRI dhan54 (check)	126.78 de	12.00 c	26.71 d	1336.44 b	29.28 c	22.82 bc	16.51 a	5.23 bc	9.92 d	0.48 c
BR11-52-124	120.67 cd	10.00 b	24.03 bc	1065.50 b	25.34 bc	23.68 bc	36.70 cd	5.20 bc	7.26 bc	0.43 b
BR11-97	113.17 b	7.50 a	23.51 ab	858.67 ab	20.36 a	23.71 bc	34.89 c	5.08 bc	5.35 a	0.39 ab
BR11-71	121.00 cd	10.00 b	23.04 ab	1272.00 b	26.67 bc	20.94 a	26.32 b	4.77 b	7.59 bc	0.39 ab
BR11-84	116.50 bc	8.50 a	22.32 a	1153.67 b	25.10 bc	21.75 ab	20.04 ab	4.75 b	6.38 ab	0.39 ab
BR11 (recipient)	115.56 bc	9.59 b	24.68 bc	1112.44 b	27.32 bc	25.54 d	42.23 d	4.63 ab	7.20 bc	0.45 bc
BR11-65	124.25 d	9.17 b	24.22 bc	895.33 ab	21.24 ab	23.71 bc	38.59 cd	4.60 ab	7.05 bc	0.39 ab
BR11-52-145	119.50 c	9.50 b	24.97 c	1225.83 b	28.90 c	23.73 bc	29.21 bc	4.58 ab	7.17 bc	0.43 b
FL378 (donor)	99.00 a	13.67 d	23.13 ab	916.00 ab	26.70 bc	29.25 e	22.81 ab	4.45 ab	5.55 ab	0.45 bc
BR11-54	124.83 d	9.00 b	23.83 bc	922.83 ab	22.11 ab	23.94 c	39.89 cd	4.39 ab	6.90 bc	0.39 ab
BRRI dhan53 (check)	124.22 cd	12.39 c	24.70 bc	728.10 a	24.46 b	21.69 ab	21.57 ab	4.35 ab	7.88 c	0.45 bc
BR11-11	126.83 de	9.33 b	24.01 bc	1022.17 ab	21.23 ab	20.72 a	35.27 c	4.14 a	6.57 b	0.36 a
Significance	**	**	**	**	**	**	**	**	**	**
$LSD_{0.05}$	4.74	1.42	1.28	298.71	3.3	1.2	6.88	0.58	1.12	0.05
CV (%)	2.3	8.5	3.2	16.7	7.8	3.1	13.7	7.1	9.5	7.2

**Significance at 1% level of probability. Each value is the mean of 3 replicates; means followed by common letter/s are not significantly different at 5% level of probability

Table 4.1.12: Grain quality parameters (Physico-chemical and cooking properties) of BR11-*Saltol* lines.

Variety/Line	Milling outturn (%)	Head rice yield (%)	Appearance	Chalkiness	Length (L) mm	Breadth (B) mm	L/B ratio	Size & shape	ER	IR	Cooking time in min.	Amylose (%)	Protein (%)
BR11-7	71.8	95.0	Good	Tr	5.7	2.5	2.3	Medium, Bold	1.3	3.2	18:0	28.0	8.3
BR11-52-55	71.5	91.3	Good	Tr	5.6	2.6	2.2	Medium, Bold	1.5	3.5	18:8	29.0	8.1
BR11-52-124	72.5	92.7	Good	Tr	5.7	2.6	2.2	Medium, Bold	1.4	3.2	18:3	25.0	8.3
BR11-97	70.0	92.5	Good	Tr	5.7	2.5	2.3	Medium, Bold	1.5	4.3	18:0	29.0	7.5
BR11-71	71.8	94.6	V.good	Tr	6.0	2.3	2.6	Medium, Bold	1.3	3.5	18:0	28.0	8.3
BR11-52-145	71.5	90.9	Good	Tr	5.6	2.6	2.2	Medium, Bold	1.5	3.2	18:3	27.0	8.3
BR11 (Recipient parent)	70.8	88.6	Good	Tr	5.7	2.6	2.2	Medium, Bold	1.4	4.6	18:8	27.0	8.2
FL 378 (Donor parent)	69.0	89.4	Good	Tr	6.0	2.6	2.3	Medium, Bold	1.6	3.0	18:3	25.0	8.3

Abbreviations: Tr = Translucent, L/B ratio = Length/Breadth ratio, ER= Elongation ratio, IR=Imbibation ratio; Each value are the means of 3 sub-samples from same lot of paddy, therefore no any analyses were carried out.

e) Field evaluation of BR11-Saltol introgression lines in actual saline condition at BRRI regional station, Satkhira

An on-station secondary yield trial with 12 BR11-*Saltol* lines was carried out in *T. Aman* season 2012 at BRRI Regional Station Satkhira (Salinity Station for BRRI). Only four parameters i.e. growth duration, plant height, grain yield and phenotypic acceptability (PAcp) were recorded and measured for this trial. The salinity level of crop standing water was varied between 1.1-2.2 dSm^{-1} throughout the growing period of the crop. Growth duration and plant height was shown quite similar with BR11 and most of the lines yielded significantly better than original BR11 (Table 4.1.13). Only 2 lines BR11-97 and BR11-150 performed poorer than recipient BR11. Five lines out of 12 scored 3 (phenotypic acceptability) and were selected for the Participatory Variety Selection system (PVS) (Table 4.1.13).

Table 4.1.13: Growth and yield parameters of BR11-*Saltol* NILs on-station trial at BRRI, Satkhira (*T. Aman*, 2012 season).

Sl. No.	Lines/varieties	Growth duration (days)	Plant height (cm)	Grain yield (t/ha)	Phenotypic acceptability
1	BR11-71	138.00 b	117.00 b	4.90 h	3
2	BR11-52-145	136.00 b	115.00 b	4.60 g	3
3	BR11-52-124	139.00 c	111.00 b	4.60 g	3
4	BR11-84	138.00 b	107.50 ab	4.30 f	3
5	BR11-7	137.00 b	113.00 b	4.10 e	3
6	BR11-65	140.00 c	112.00 b	4.10 e	4
7	BR11-52-67	140.00 c	111.00 b	4.10 e	4
8	BR11-54	137.00 b	117.00 b	4.00 de	4
9	BR11-11	140.00 c	109.50 ab	3.90 d	4
10	BR11-52-55	141.00 c	112.00 b	3.90 d	4
11	BR11 (Recipient)	139.00 c	110.50 b	3.70 c	5
12	BR11-97	140.00 c	115.00 b	3.50 b	5
13	BR11-150	138.00 b	112.00 b	3.50 b	5
14	FL478 (Tol. check)	107.00 a	102.00 a	2.20 a	7
	$LSD_{0.05}$	2.50	8.10	0.20	--

Each value is the mean of 3 replicates; means followed by common letter/s are not significantly different at 5% level of probability

4.2 Introgression of Saltol QTL in to a Bangladeshi mega rice variety BRRI dhan28 for Boro season through Marker-assisted Backcrossing.

4.2.1 Polymorphism between parents

Similar to the previous research on MABC described above (see 4.1.1), the same donor FL378 was used for the MABC introgression of *Saltol* in to BRRI dhan28. Both donor and recipient are from the *indica* group and share some common parent, so that a high degree of genetic similarity is expected. Initially, polymorphism survey was carried out with primer stock of the Plant Biotechnology Laboratory of 153 microsatellites, sequence tagged site (STS) and gene-based markers. Out of 153 markers surveyed, 52 were found polymorphic, which represents 33.98% polymorphism. So, genotyping of the 1st backcross generation was done with the 52 polymorphic markers. However during genotyping of the 2nd backcross generation, an additional 17 microsatellite and InDel markers were added to the background marker panel. Later in the HEMMI laboratory of Crop and Environmental Sciences Division (CESD) of IRRI, another 41 additional markers were added to the background panel for genotyping of the 3rd backcross generation. So, finally a total of 110 SSRs, STS and InDels (including FG and RB markers) were used for selecting progenies.

For the introgression of *Saltol* QTL in to BRRI dhan28, the same three highly polymorphic and robust markers [RM1287 (10.90 Mb), RM3412 (11.50 Mb) and RM493 (12.20 Mb)] were used as markers for the foreground selection. For the recombinant selection two highly polymorphic, robust and un-linked marker RM3627 (10.31 Mb) at the telomeric end and RM10825 (13.30 Mb) at the centromeric end were used initially. But later in the 2nd backcross generation an additional 3 recombinant markers [RM10864 (14.20 Mb), RM562 (14.60 Mb), RM7075 (15.10 Mb)] were included at the centromeric ends to increase the introgression size of *Saltol* region. So, at centromeric end a total of 4 recombinant markers (RM10825, RM10864, RM562 and RM7075) were used in a staggered way to increase and check the effect of the size of the donor introgression at the *Saltol* region. The additional donor fragment also allowed the accommodation of candidate genes like *SalT* and *HAK2 homolog* at this end (Fig. 4.2.1). Smaller introgression such as 1.30 Mb (from 10.9 Mb to 12.20 Mb) used in the previous introgression missed these two proven candidate genes responsible for salinity tolerance (Thomson, *et al.* 2007; Singh and Flowers, 2010). A total of 107 markers including recombinant markers were used for the background selection. The distribution of background markers was not equal throughout the genome (see the name,

position and distribution of polymorphic background markers in Fig. 4.2.2 and list in the Appendix-II in Table 8.2.7). A maximum of 16 markers was used in the carrier chromosome, and while the lowest was 5 in Chromosome 11. So, the total coverage of the background markers was 1406.47 cM with an average genetic distance between markers of 13.59 cM (Table 4.2.1).

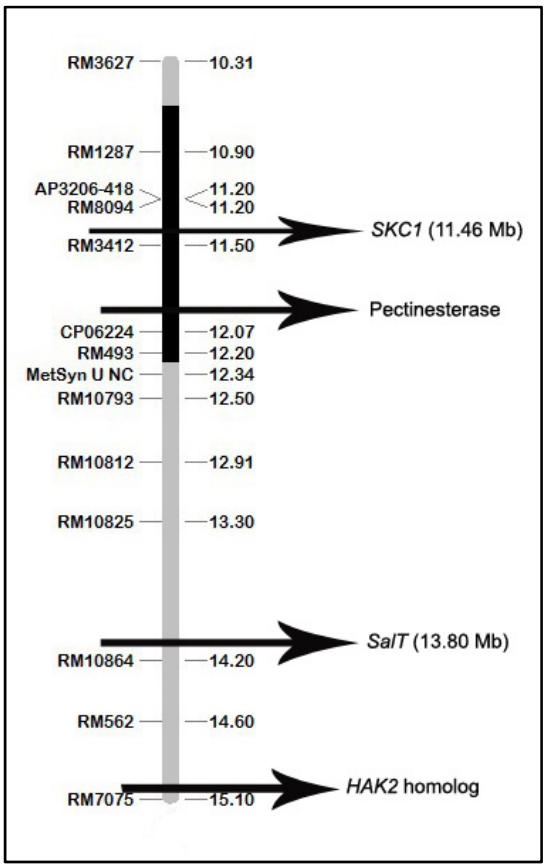


Fig. 4.2.1: *Saltol* QTL region in chromosome 1, markers within the region with physical position in million bases and 4 possible candidate genes within the *Saltol* region (the following 4 candidates were identified and validated in earlier studies, Thomson, *et. al.* 2007).

Table 4.2.1: Number of Markers used in background selection for the recovery of recurrent genome (only background and recombinant markers) in the BRRI dhan28-*Saltol* introgression work.

Sl. no.	Chromosome	No. of markers	Coverage in cM	Average distance between markers in cM
1	Chr1	16	176.97	11.06
2	Chr2	12	146.56	12.21
3	Chr3	11	164.68	14.97
4	Chr4	8	132.46	16.56
5	Chr5	8	74.61	9.33
6	Chr6	10	115.34	11.53
7	Chr7	6	118.82	19.80
8	Chr8	7	121.74	17.30
9	Chr9	8	93.56	11.69
10	Chr10	7	78.63	11.23
11	Chr11	5	79.44	15.89
12	Chr12	9	103.66	11.52
Whole genome		**107**	**1406.47**	**13.59**

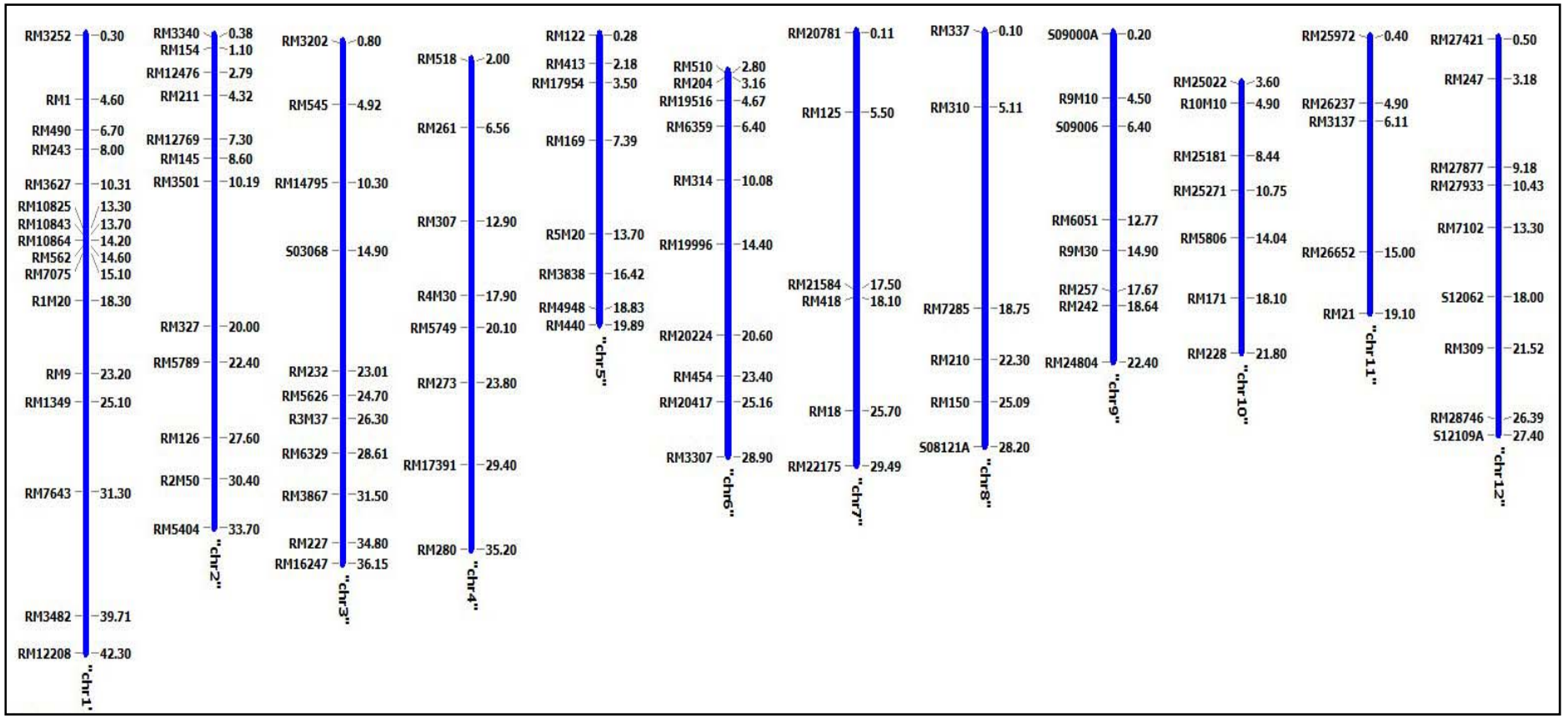


Fig. 4.2.2: Name, physical position and distribution of background polymorphic markers across 12 rice chromosomes used in BRRI dhan28-*Saltol* MABC event.

4.2.2 Numerical optimization of population sizes in marker-assisted backcross program

For the calculation of minimum population size in this MABC program, POPMIN software (same as above) developed by Hospital and Decoux (2002) was used. This MABC program was also set for 3 backcross generations, i.e. maximum duration of backcrossing program and 'SIMAN' i.e. variable population sizes were computed using the 'simulated annealing' algorithm. The genetic distances of recombinant markers from the end of the donor loci used was the same as the previous work above. For the telomeric end, these were (RM3627) 2.4 cM and at the centromeric end (RM10825) 4.4 cM. The difference was the addition of 3 more recombinant markers at the latter end (RM10864, RM562 and RM7075). Here, in this MABC event, the recombinant marker at the telomeric end was fixed by using marker RM3627 at 10.30 Mb but at the centromeric end 3 more recombinant markers were used to increase introgression size. Three more donor segment with differing in size 2.4 Mb, 3.3 Mb and 3.7 Mb was introgressed in addition to previously used size 1.3 Mb for BR11 MABC work (see the size of donor segments, position of recombinant and foreground markers and their position in the carrier chromosome 1 in Fig. 4.2.3). So, by using single recombinant marker at the telomeric end and 4 recombinant markers at the centromeric end, only a single calculation was carried out by using fixed distances at the telomeric end marker distance (2.4 cM) and minimum marker distance at centromeric end (1.6 cM). The reason is that, if there is chance for getting a double recombinant with the calculation of minimum distances then there will be obvious chance for getting double recombinant of larger distances. For a three backcross generation MABC program with 4 different flanking marker distances, the minimum population sizes were found to be 400, 540 and 1170 for BC_1, BC_2 and BC_3 respectively, with an average cumulated population size 703.33 (Table 4.2.2). The probability of success for getting a desired (at least one) double recombinant for 4 different introgression size in each backcross generation was 0.03005625, 0.7317785 and 0.22818625 for BC_1, BC_2 and BC_3 respectively, whereas the cumulative probability was 0.990021 at BC_3 (Table 4.2.2).

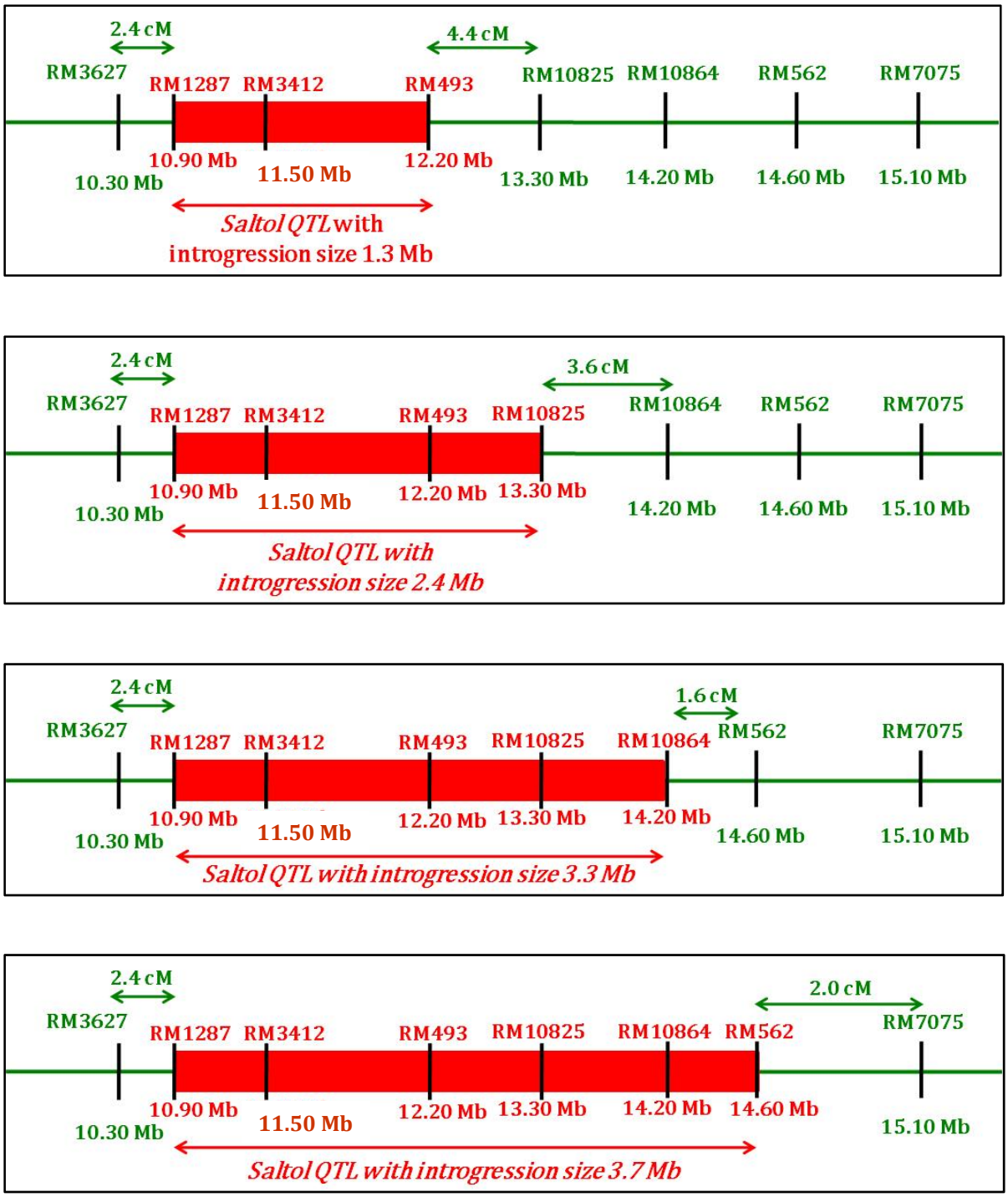


Fig. 4.2.3: Diagram showing the different sized *Saltol* QTL introgressions with marker position and distances of foreground and recombinant markers. Red color denotes the foreground markers and donor introgression and Green color indicates recombinant markers and recipient background.

Table 4.2.2: Results of population sizes calculated through POPMIN software for a 3 backcross generation MABC scheme.

Backcross generation	Population size	Cumulative population size	Probability of success	Cumulative probability
BC_1	400	400	0.03005625	0.03005625
BC_2	540	940	0.73177850	0.76183475
BC_3	1170	2110	0.22818625	0.99002100
Average cumulated population size = 703.3333				

4.2.3 Development of BRRI dhan28-Saltol through MABC

a) Hybridization and F_1 confirmation

For the introgression of the *Saltol* QTL in to the recipient, BRRI dhan28 was crossed with FL378 to obtain F_1 where BRRI dhan28 was considered as mother and FL378 as pollen parent. F_1 was confirmed by using a robust foreground marker RM493 that primarily confirms the presence of '*Saltol*' QTL in hybrids. A total of 240 F_1's were genotyped by RM493 and after removing missing data, 139 F_1 hybrids were confirmed by observing double bands with expected band size at the marker locus.

b) Genotyping of 1st backcross generation (BC_1)

Confirmed F_1s were backcrossed with recurrent parent BR28 to reconstitute the recurrent genome. In the first backcross population at BC_1F_1, the target locus was monitored by 3 foreground markers RM1287 (10.90 Mb), RM3412 (11.50 Mb) and RM493 (12.20 Mb) that are tightly linked to the *Saltol* QTL. Individual BC_1F_1 progenies were first selected based on the heterozygous forms of the 3 target loci at *Saltol* QTL region. A total of 799 BC_1F_1 were genotyped by using 3 foreground markers and 309 was found to have the target *Saltol* QTL in heterozygous form.

Recombinant selection is a comparatively new criteria used to transfer target segment more precisely (reducing linkage drag) (Collard, *et. al.* 2008b; Hospital, 2001). Two markers RM3627 (10.31 Mb) and RM10825 (13.30 Mb) were used to genotype 309 progenies that were initially selected by foreground markers. Out of 309 selected progenies from foreground selection, 16 were again selected based on the recombination at the RM3627 locus. So, the selected16 progenies were single recombinant i.e. recombination occurred at a single recombinant marker locus RM3627 (10.31 Mb) at the telomeric end.

A total of 49 microsatellite markers (including recombinant markers and other markers from the carrier chromosome) were used for the background selection of the 16 progenies selected by foreground and recombinant markers. The percent recovery of recipient alleles ranged from 32.65-69.39% (Fig. 4.2.4).

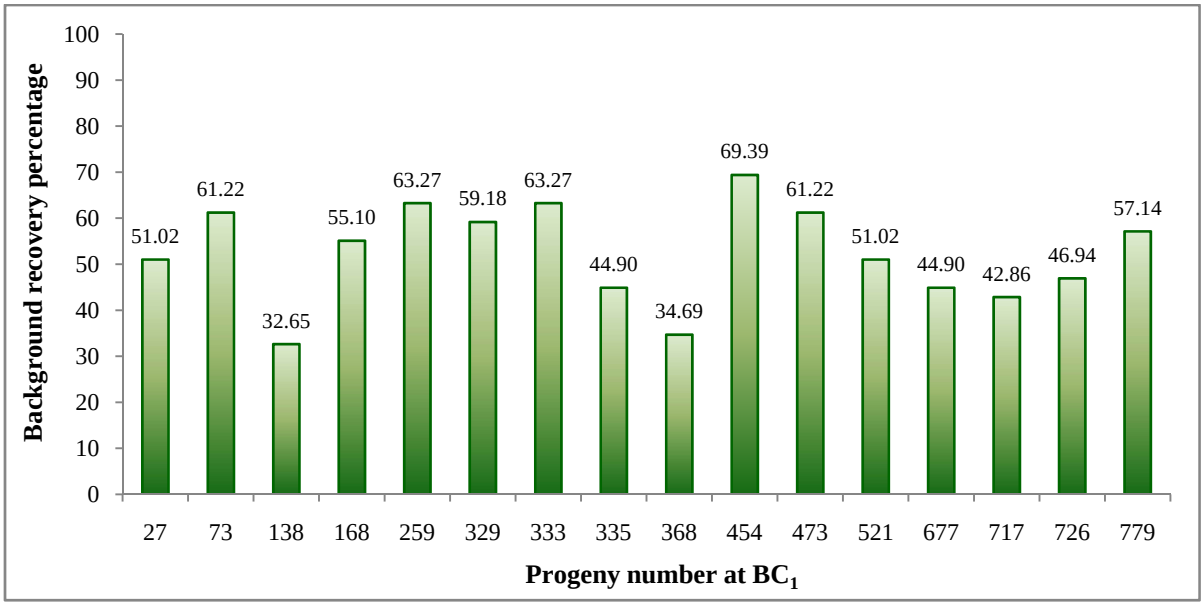


Fig. 4.2.4: Bar graph showing percent recovery of recurrent genomes of selected progenies at first backcross generation (BC_1).

c) Genotyping of the 2nd backcross generation (BC_2)

A different strategy was followed for the genotyping of the 2nd backcross progenies of BRRI dhan28 MABC events for the foreground and recombinant selection. The telomeric end was fixed in the1st backcross generation (BC_1F_1) by selecting the recombinants of flanking marker RM3627. First 3 foreground markers (RM1287, RM3412 and RM493) were used to genotype 779 BC_2 progeny and then 4recombinant markers at the centromeric end were used as foreground markers in staggered way to increase the size of introgression segment. The reason for increasing the introgression size at centromeric end was to include 2 candidates (*SalT and HAK2 homolog*) at this end. So, by using 8 markers in FG and RB selection, 12 double homozygotes (double recombinant) were identified with varying introgression size. The selected 12 double homozygotes were again genotyped by 20 additional background markers and recovery was obtained 78.26-89.86% (Fig. 4.2.5). Thus at the end of 2nd backcross a total of 69 background markers were used for quick recovery of the recurrent genome.

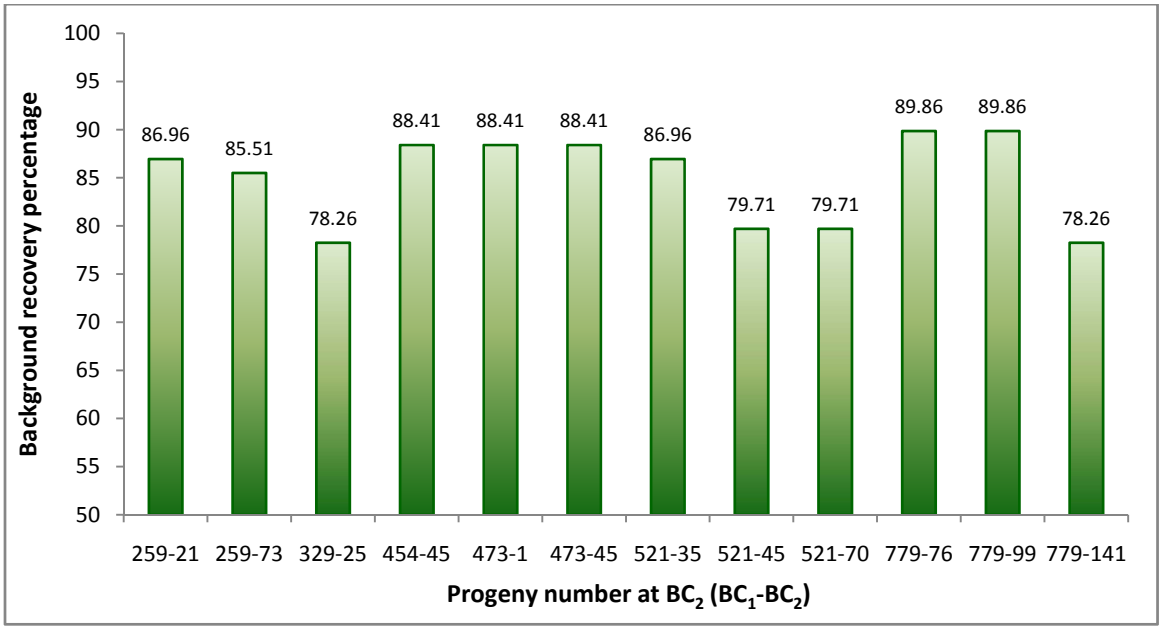


Fig. 4.2.5: Bar graph showing percent recovery of recurrent genomes of selected progenies at second backcross generation (BC_2).

d) Genotyping of 3rd backcross generation (BC_3)

At the 3rd backcross generation (BC_3), a total of 282 progenies were genotyped by 8 foreground and recombinant (RM3627, RM1287, RM3412, RM493, RM10825, RM10864, RM562 and RM7075) and 38 background markers for selection of progenies with maximum background recovery of varying sizes of the donor introgression at the *Saltol* locus. By using different recombinant markers, 12 progenies were selected with 4 different sized donor introgression (1.3, 2.4, 3.3 and 3.7 Mb), with 86.11 to 94.44% background recovery. The selected progenies were selfed and advanced to BC_3F_2 for fixation of *Saltol* and few background markers that were still heterozygous.

e) Fixation of Saltol and heterozygous background loci

Foreground selection in all 3-backcross generations were carried by selecting as heterozygous for all the foreground markers; these allows more recombination adjacent to both ends of the QTL for reducing of excess segments or reduction of negative linkage drag. At BC_3F_2 stage, 846 progenies of 12 lines having4 different sized donor introgressions at *Saltol* were genotyped with 8 foreground and recombinant markers and few background markers that were heterozygous at BC_3. For fixation of the few heterozygous background loci, 215 fixed *Saltol* lines were genotyped by markers which showed heterozygosity in the previous generation (Fig. 4.2.6 illustrates the whole MABC event of BRRI dhan28). In the

smallest segment size (1.3 Mb), 1 progeny with minimum background introgression were selected, where progeny no. 188 with two background donor introgression at RM1 and RM24804 (Fig. 4.2.7) was identified and selected. But for the introgression size 2.4 Mb, 1 progeny was selected with minimum donor introgression at the background, where progeny no. 204 with one background donor introgression at RM12208 (Fig. 4.2.8) was identified and selected. Again for the introgression size 3.3 Mb, two progenies (progeny no. 318 and 341) were selected with minimum donor introgression at the background, where progeny no. 318 has 4 introgressions in the background i.e. RM9, RM1349, RM5806 and RM171 (Fig. 4.2.9). Progeny no. 341 had three background donor introgressions at RM7643, R2M50 and RM171 (Fig. 4.2.10) which was identified and selected. For the largest introgression 3.7 Mb, 3 progenies i.e. progeny no. 412, 434 and 448 having a total of 9 introgressions at the background i.e. RM3307, RM25972 & RM27421 for progeny no. 412; RM3307, RM5789 & R2M50 for progeny no. 434 and RM5789, RM126 & RM310 for progeny no. 448 respectively (Fig. 4.2.11-13). Four progenies (progeny no. 607, 657, 683 and 807) having largest segment 3.7 Mb at the *Saltol* QTL were selected with clean background (Fig. 4.2.14, 4.2.16, 4.2.17, 4.2.18). However, 2 progenies having no *Saltol* QTL (minus QTL) i.e. without introgression at the target region was also selected to compare any possible background effects (progeny no. 618 and 845, Fig. 4.2.15 and 4.2.19. So, at BC_3F_3 stage the percent share of donor and recipient genome ranged from 0 to 5.45% and 91.82 to 100% respectively in terms of the calculation through SSR/InDel marker genotype for BRRI dhan28-*Saltol* NILs (Table 4.2.3).

Table 4.2.3: Percent share of marker genotype from donor and final recovery percent of recipient at BC_3F_3 stage of BRRI dhan28-*Saltol* NILs.

BR28-*Saltol* NILs	**Size of donor introgression at the *Saltol* QTL**	**Foreground markers used to track *Saltol* QTL**	**Percent share of marker genotype from donor at the *Saltol* QTL**	**Recombinant and background markers used to track recurrent loci at the carrier or non-carrier chromosome**	**Percent share of SSR/InDel marker genotype from recurrent genomes**
NIL188	1.3 Mb	3 markers (RM1287, RM3412 and RM493)	2.73	2 (RM3627 and RM10825) and 105 markers, total 107 for BGS*	94.55 (3 background donor introgression RM1, RM490 and RM24804)
NIL204	2.4 Mb	4 markers (RM1287, RM3412, RM493 and RM10825)	3.64	2 (RM3627 and RM562) and 104 markers, total 106 for BGS*	95.45 (1 background donor introgression RM12208)
NIL318	3.3 Mb	5 markers (RM1287, RM3412, RM493, RM10825 and RM562)	4.55	2 (RM3627 and RM10864) and 103 markers, total 105 for BGS*	91.82 (4 background donor introgression RM9, RM1349, RM5806 and RM171)
NIL341	3.3 Mb	5 markers (RM1287, RM3412, RM493, RM10825 and RM562)	4.55	2 (RM3627 and RM10864) and 103 markers, total 105 for BGS*	92.73 (3 background donor introgression RM7643, R2M50 and RM171)
NIL412	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	91.82 (3 background donor introgression RM3307, RM25972 and RM27421)
NIL434	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	91.82 (3 background donor introgression RM5789, R2M50 and RM3307)
NIL448	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	91.82 (3 background donor introgression RM5789, RM126 and RM310)
NIL607	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	94.55 (clean background)
NIL657	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	94.55 (clean background)

NIL683	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	94.55 (clean background)
NIL807	3.7 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	5.45	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	94.55 (clean background)
NIL618	0 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	0.00	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	100.00 (clean background)
NIL845	0 Mb	6 markers (RM1287, RM3412, RM493, RM10825, RM562 and RM10864)	0.00	2 (RM3627 and RM7075) and 102 markers, total 104 for BGS*	100.00 (clean background)

* See BGS marker name and distribution in the figure 4.2.2 above and list in the Appendix-II

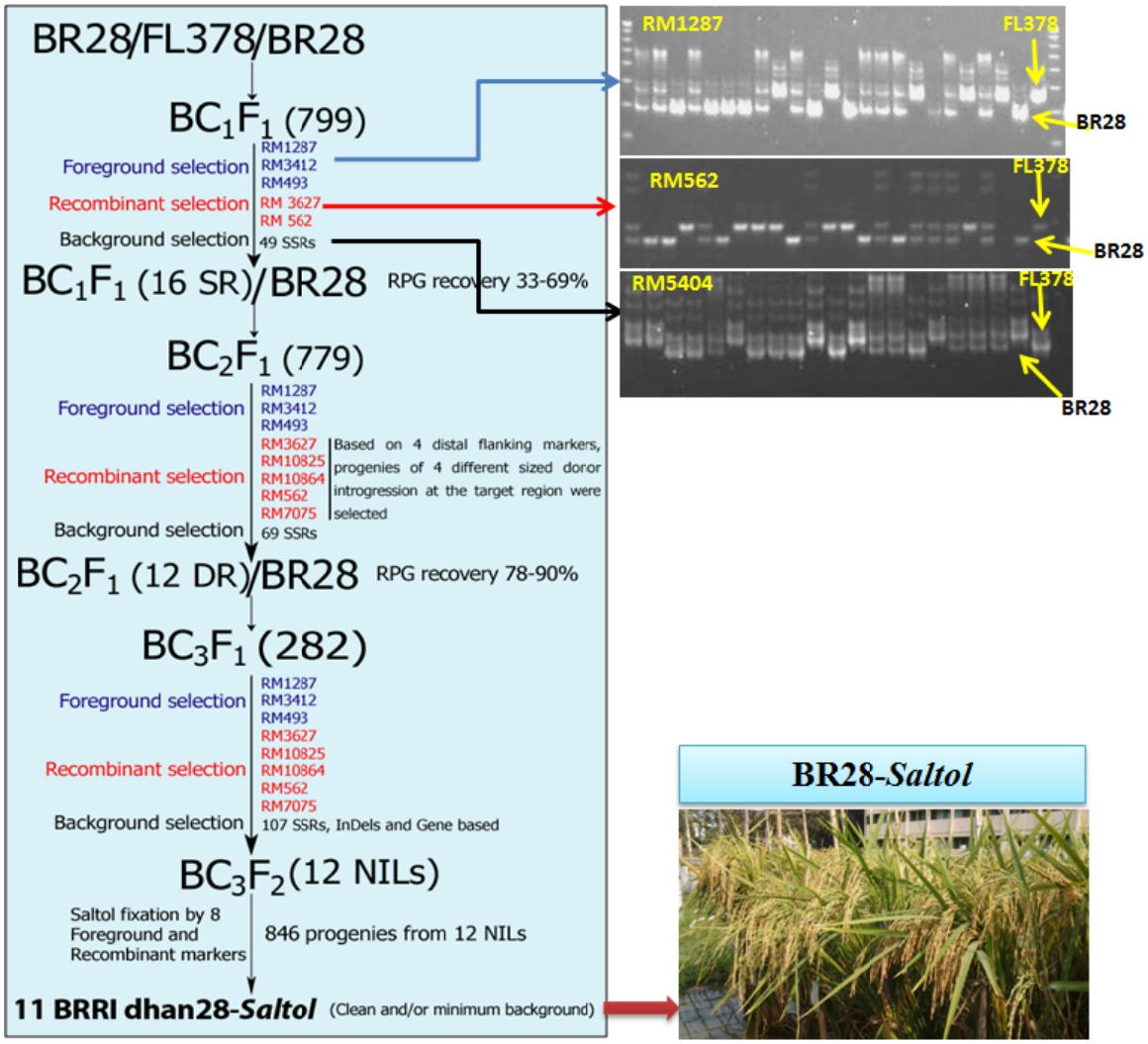


Fig. 4.2.6: Schematic presentation of MABC processes of the development of BRRI dhan28-*Saltol* with the markers used. Number of progenies genotyped and selected at the different backcross generations was shown in parentheses.

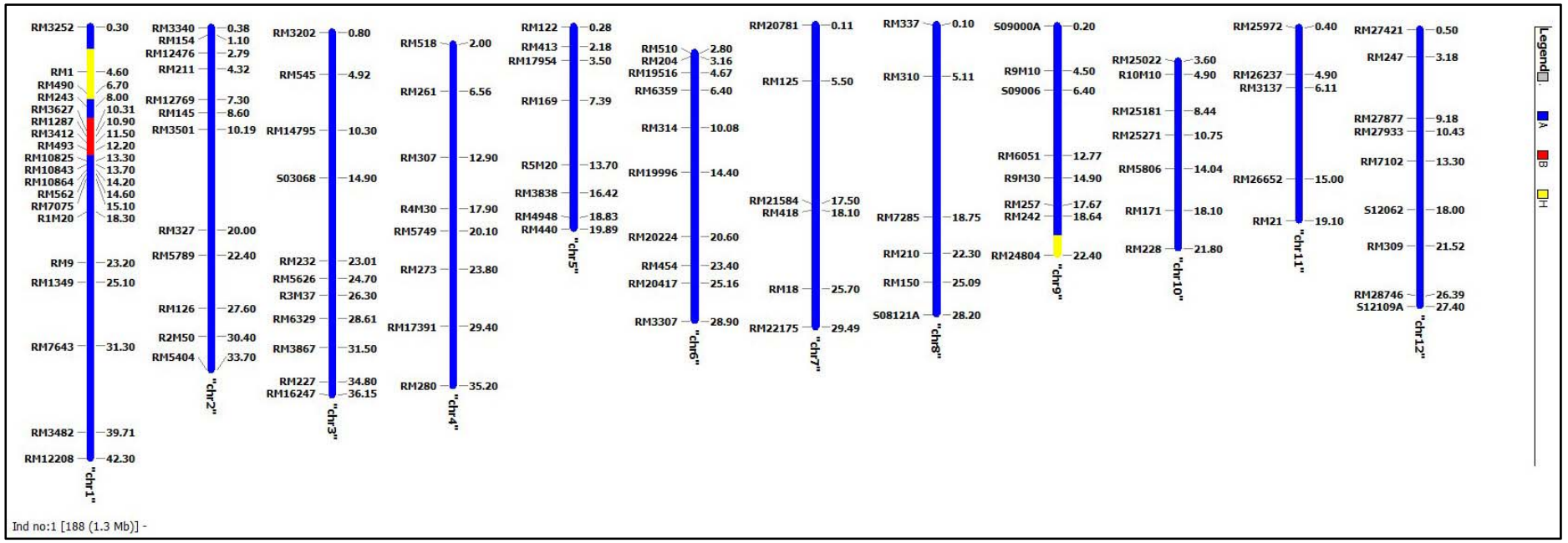


Fig. 4.2.7: Graphical genotype of BRRI dhan28-*Saltol* line, with the distribution of markers and background introgression. Progeny no.188 with 1.3 Mb introgression. Blue, red and yellow segments are recurrent, donor at *Saltol* and background donor introgression, respectively.

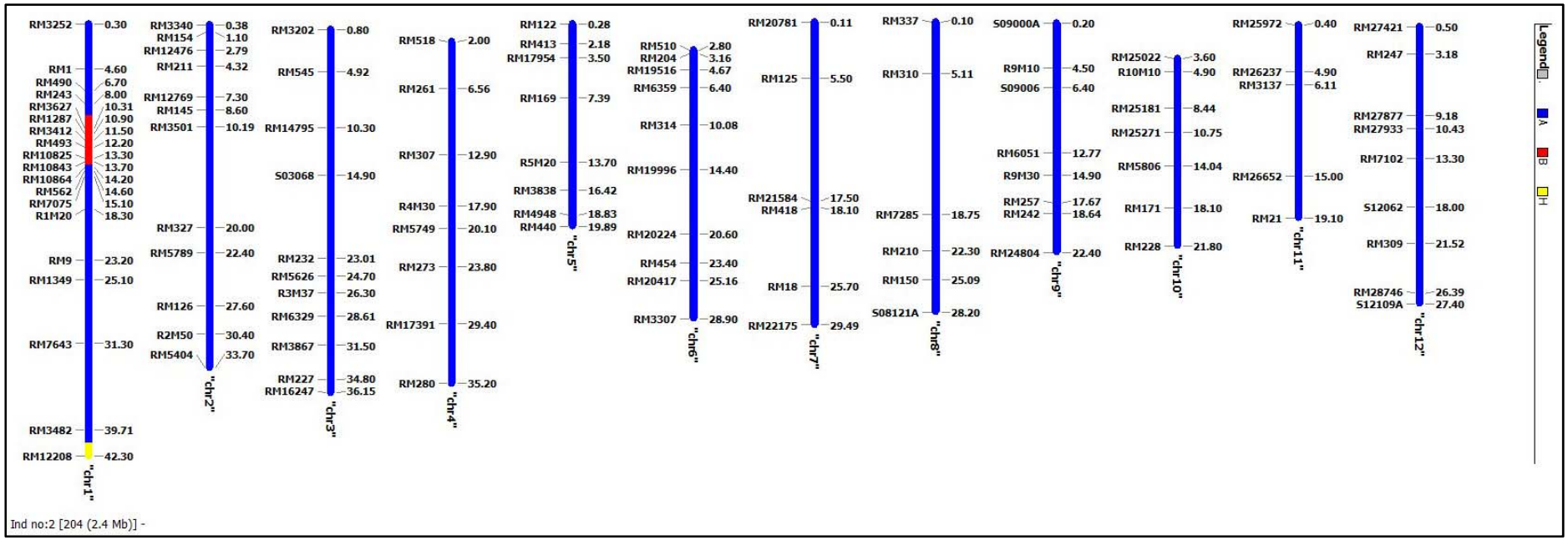


Fig. 4.2.8: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 204 with 2.4 Mb introgression; Blue, red and yellow segments are recurrent, donor at *Saltol* and background donor introgression respectively).

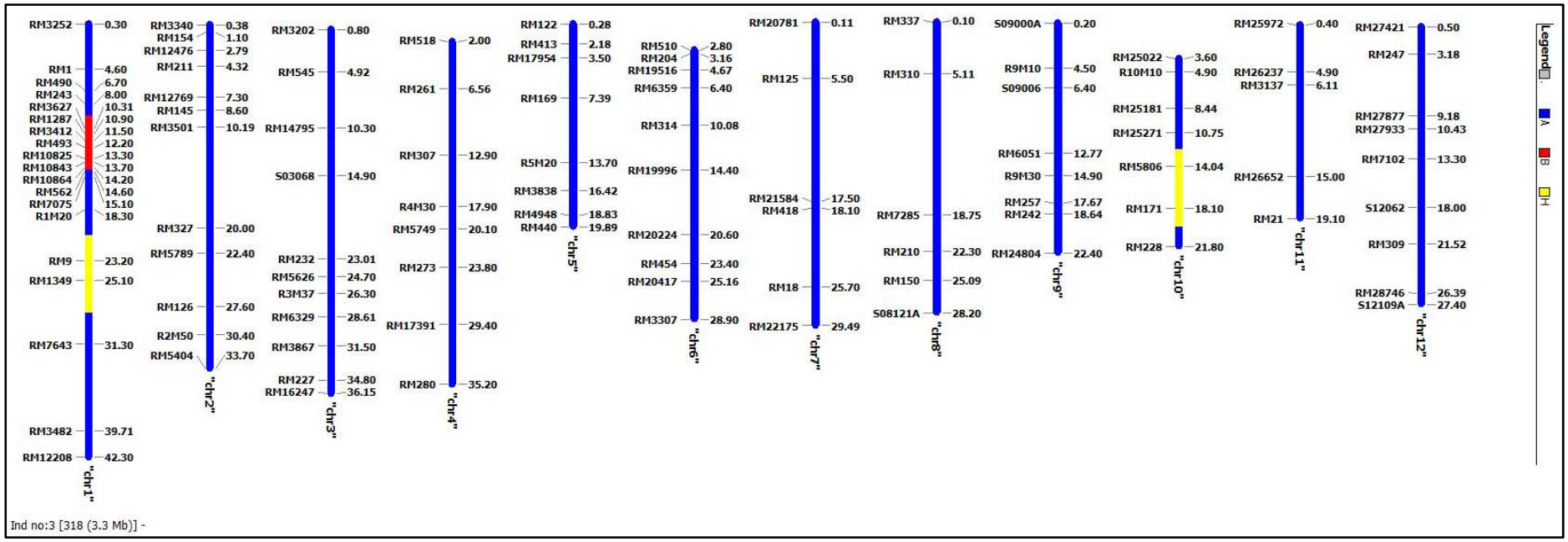


Fig. 4.2.9: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 318 with 3.3 Mb introgression; blue, red and yellow regions are recurrent, donor at *Saltol* and background donor introgression respectively).

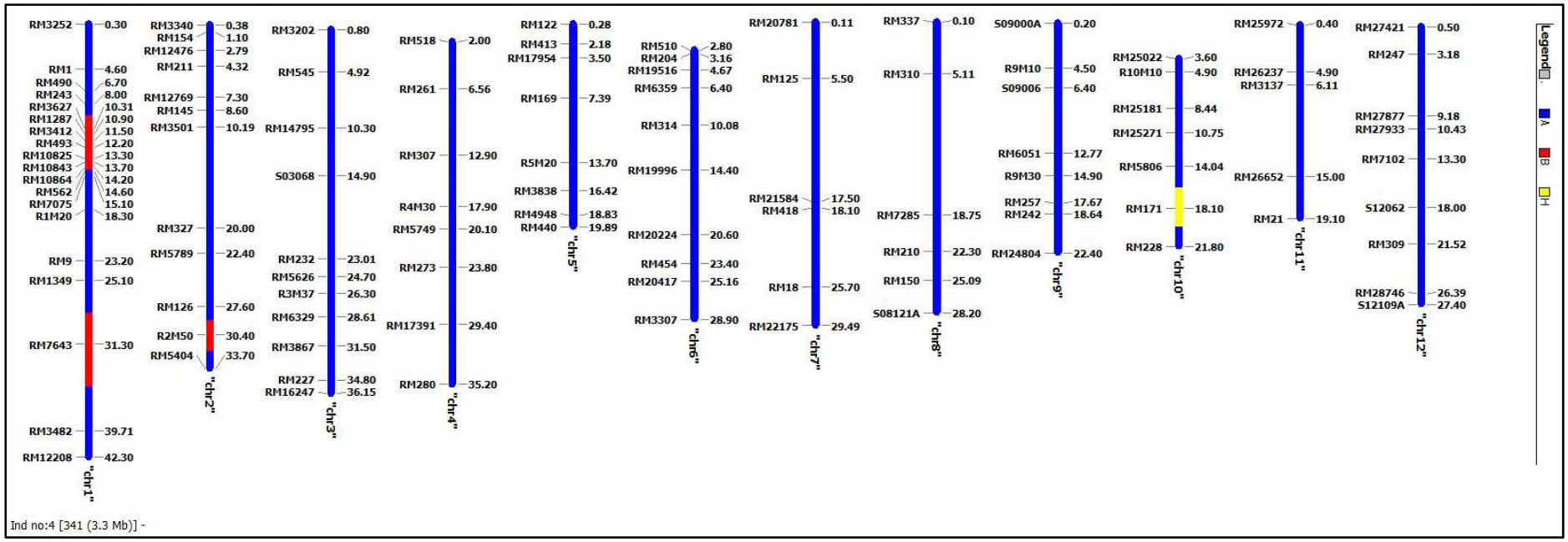


Fig. 4.2.10: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 341 with 3.3 Mb introgression; blue, red and yellow regions are recurrent, donor at *Saltol* and background donor introgression respectively).

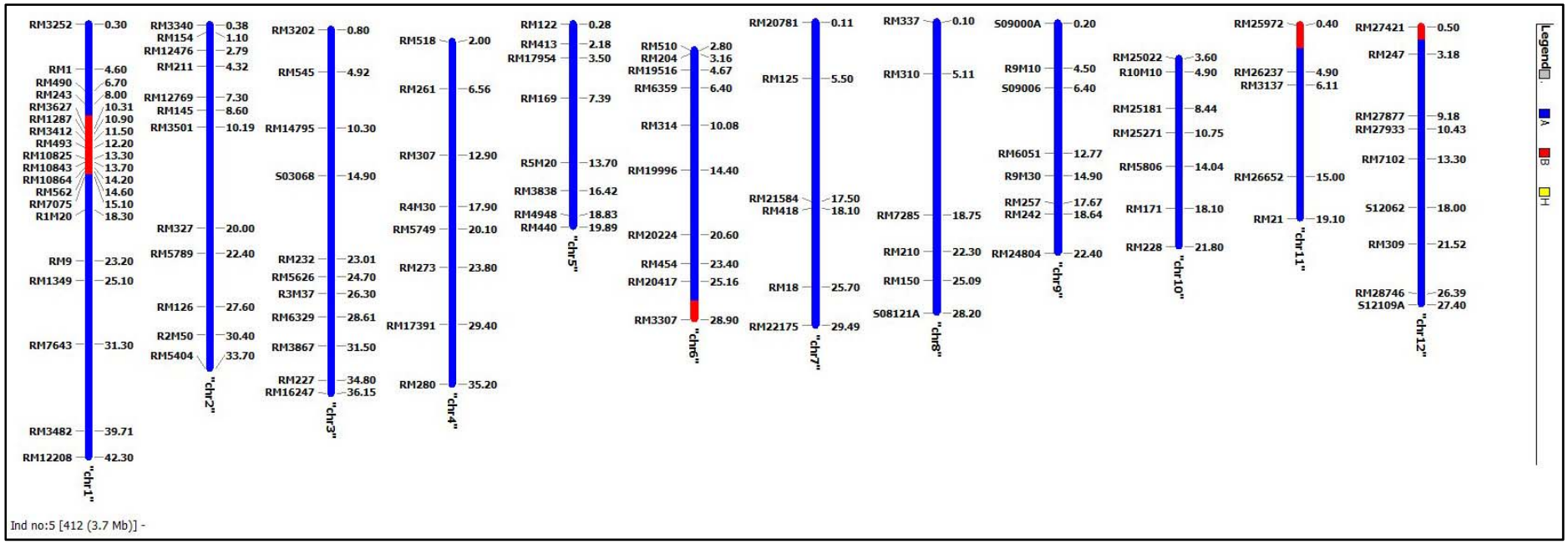


Fig. 4.2.11: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 412 with 3.7 Mb introgression; blue, red and yellow regions are recurrent, donor at *Saltol* and background donor introgression respectively).

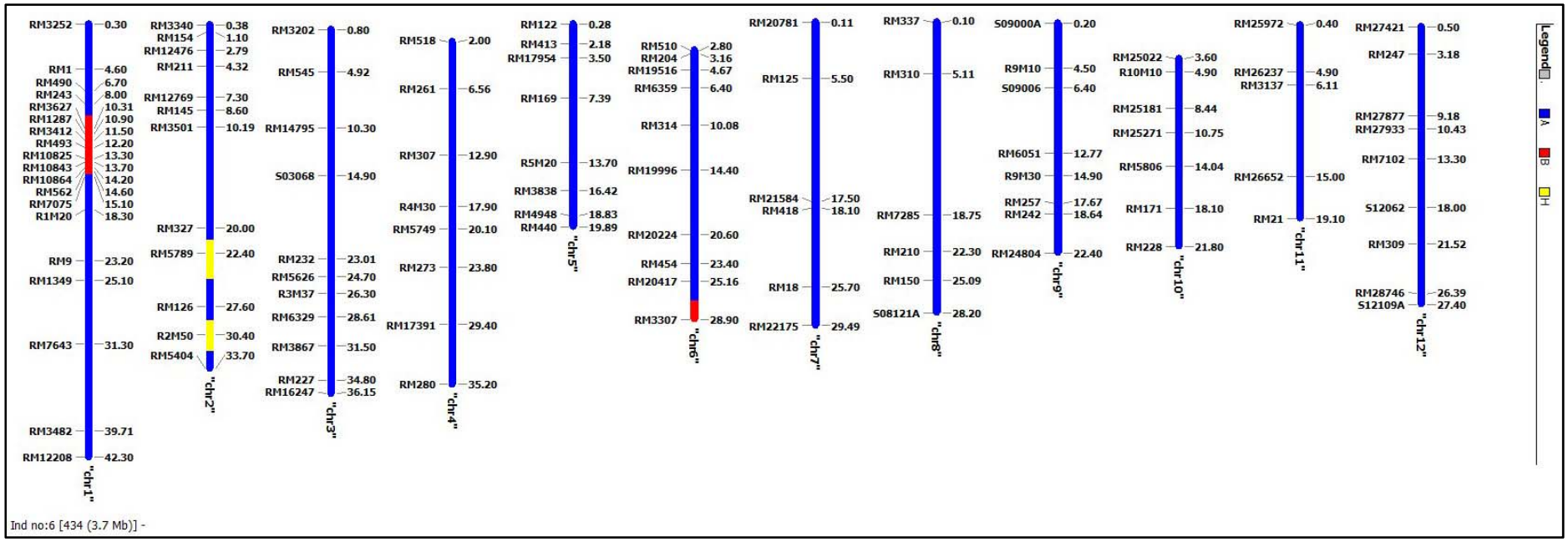


Fig. 4.2.12: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 434 with 3.7 Mb introgression; blue, red and yellow regions are recurrent, donor at *Saltol* and background donor introgression respectively).

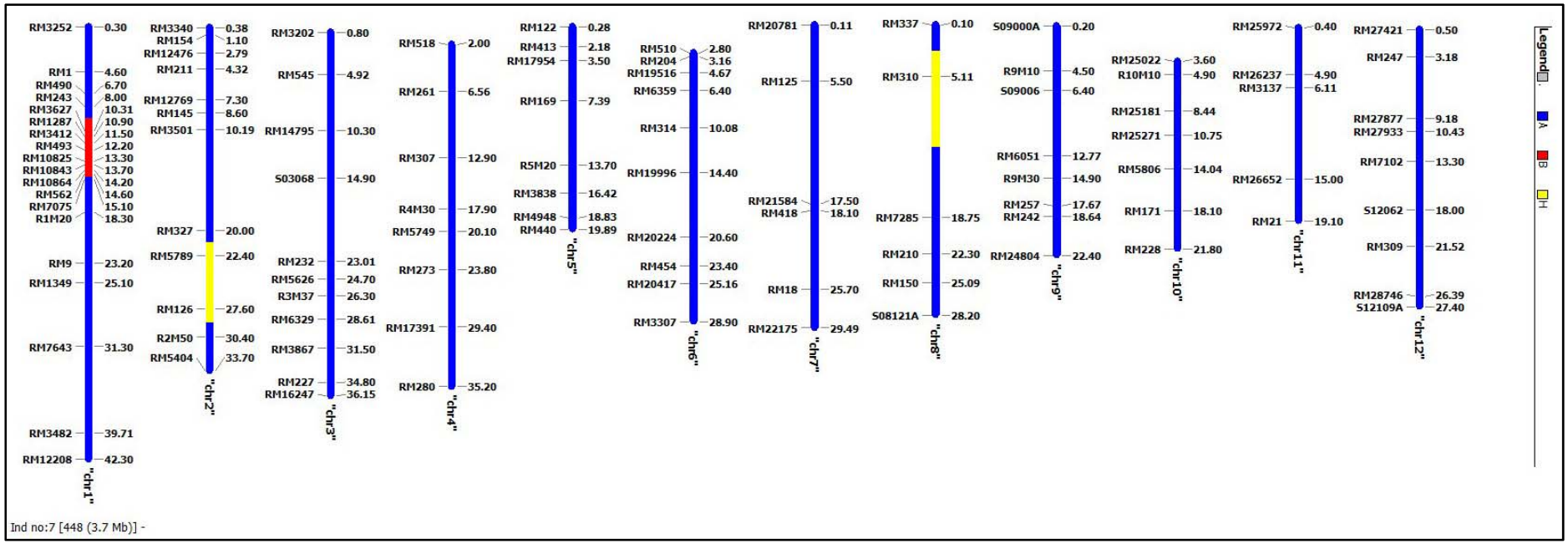


Fig. 4.2.13: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 448 with 3.7 Mb introgression; blue, red and yellow regions are recurrent, donor at *Saltol* and background donor introgression respectively).

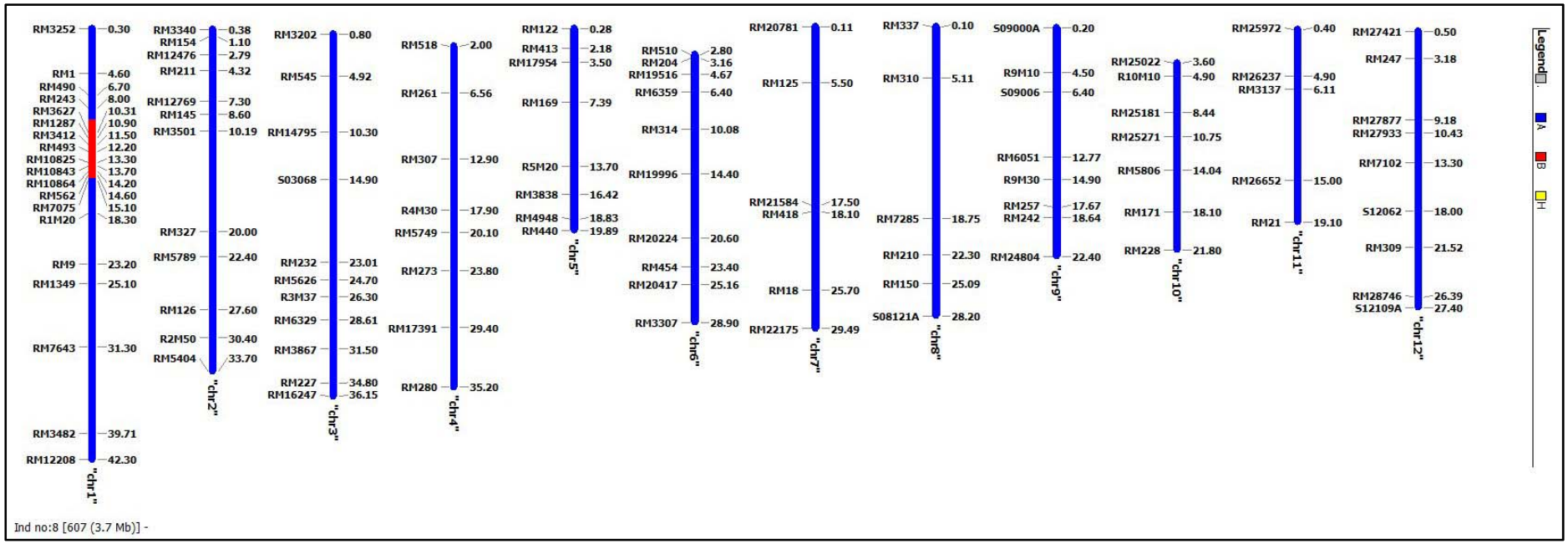


Fig. 4.2.14: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 607 with 3.7 Mb introgression; blue, red and yellow regions are recurrent, donor at *Saltol* and background donor introgression respectively).

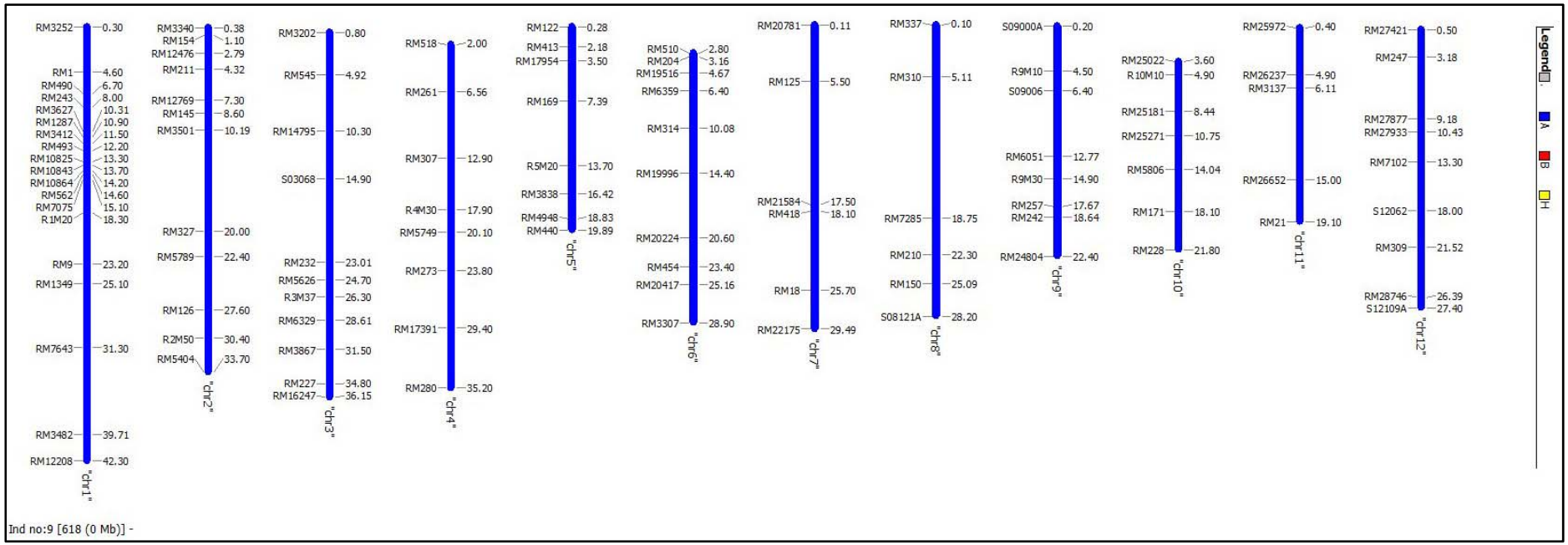


Fig. 4.2.15: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 618 with 0 Mb introgression; blue regions are recurrent genomes).

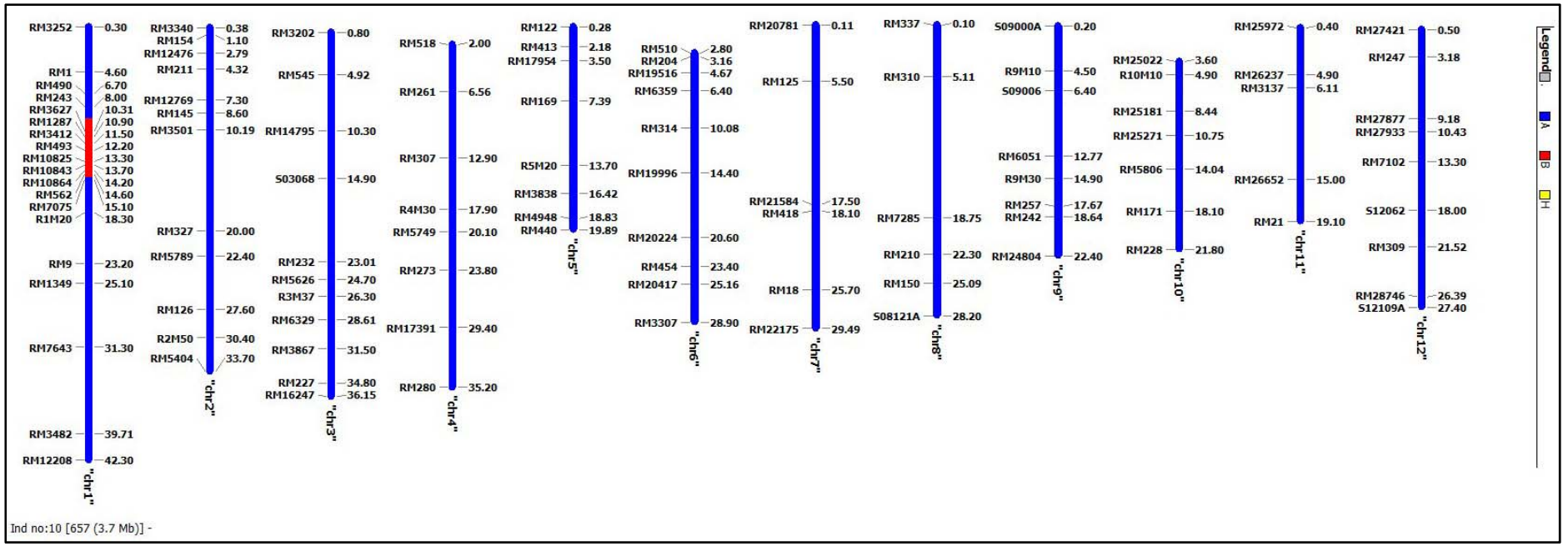


Fig. 4.2.16: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 657 with 3.7 Mb introgression; blue and red regions are recurrent and donor at *Saltol* introgression respectively).

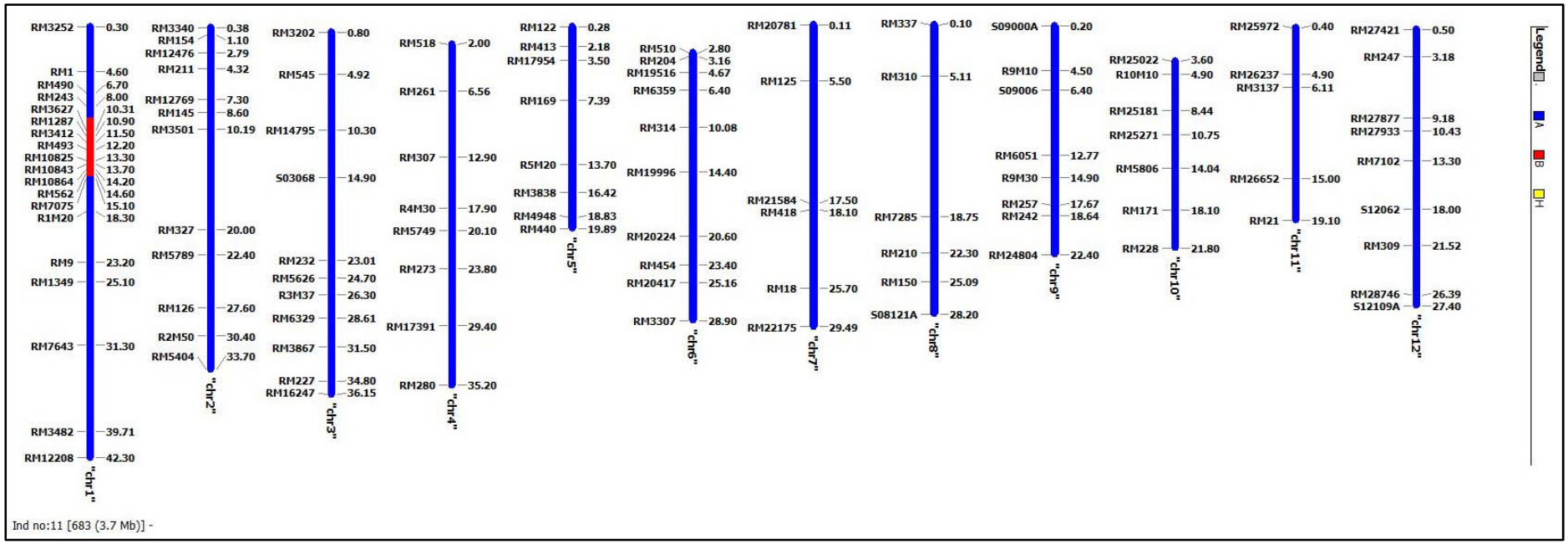


Fig. 4.2.17: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 683 with 3.7 Mb introgression; blue and red regions are recurrent and donor at *Saltol* introgression respectively).

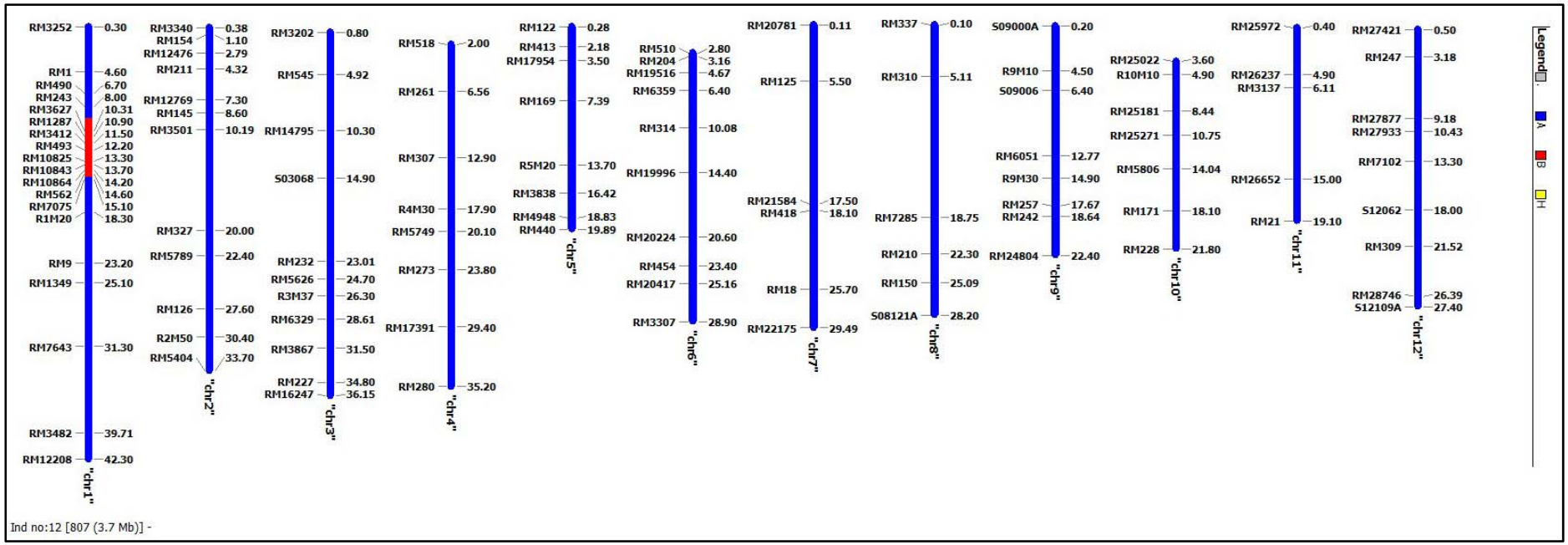


Fig. 4.2.18: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 807 with 3.7 Mb introgression; blue and red regions are recurrent and donor at *Saltol* introgression respectively).

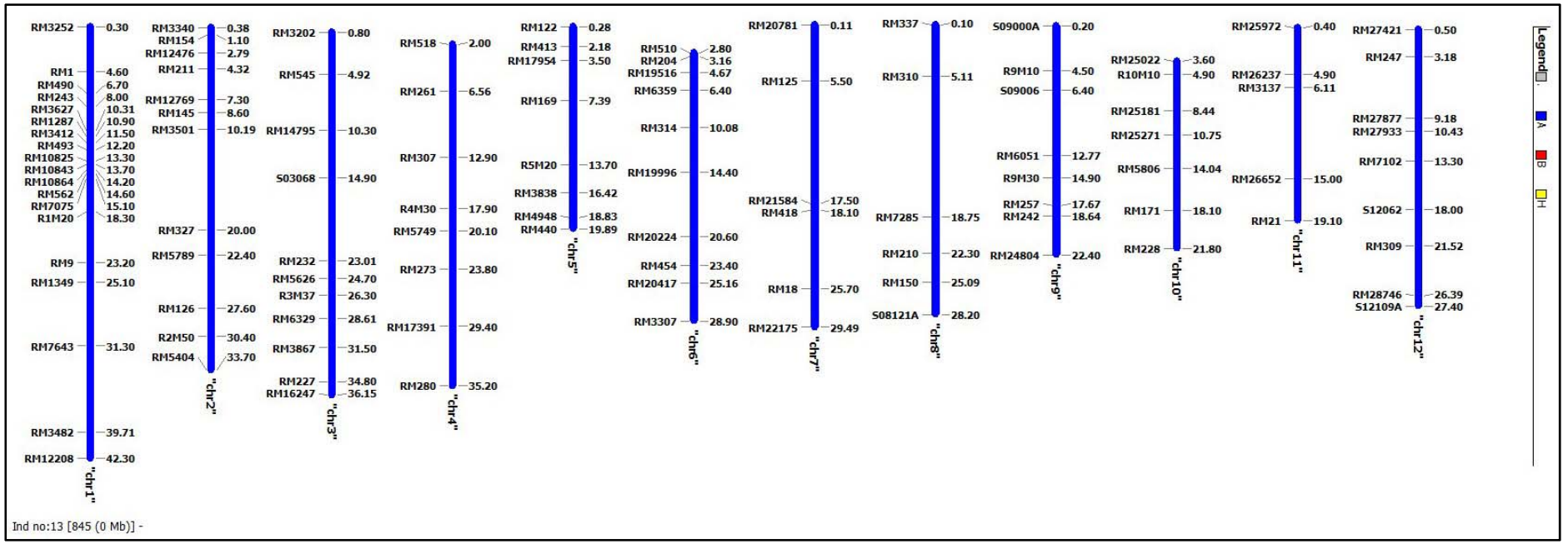


Fig. 4.2.19: Graphical genotypes of BRRI dhan28-*Saltol* line with the distribution of markers and background introgression (progeny no. 845 with 0 Mb introgression; blue regions are recurrent genomes).

f) SNP genotyping of selected BRRI dhan28-Saltol NILs

NILs developed through SSR markers were further checked by SNP markers for any potential background donor introgression. The abundance of SNP markers in rice genome is greater than the other markers. So, there is opportunity to check the background of NILs for any potential introgression which were not traced by markers used for genotyping and selection during different backcross generations. *Oryza* SNP 384-plex chips of Illumina system in IRRI was used to check the background. SNP markers also showed very similar polymorphisms 32% (122/384) for the cross BR28/FL378. Out of 12 NILs selected at BC_3F_2 stage, only eleven selected NILs from BRRI dhan28-*Saltol* with varying sizes donor introgression were checked by SNP markers along with recipient BR28 and donor FL378. Two SNP locus (id1008684 and id1009616) were identified at the targeted *Saltol* QTL region (Fig. 4.2.20). Similarity indexes of BRRI dhan28-*Saltol* NILs by comparing the recipient SNP alleles were ranged from 97.10-87.70% (Table 4.2.4).More number of markers (SNP markers) gives better indication of any potential background introgression (Table 4.2.4). However, a number of background donor introgressions were identified by SNP markers which were not traced by the 107 SSR/STS/InDel/Gene-based markers used for background selection during genotyping of different backcross progenies (Fig. 4.2.20 and 4.2.21).

Table 4.2.4: Similarity indexes of SNP alleles between NILs developed through recurrent BRRI dhan28 and donor FL378. Here, similarities were computed based on the SNP alleles of the recipient parents BRRI dhan28 through Flapjack software.

Genotype/lines	BR28 (Recipient)	188 (1.3 Mb)	204 (2.4 Mb)	618 (0 Mb)	657 (3.7 Mb)	318 (3.3 Mb)	341 (3.3 Mb)	845 (0 Mb)	448 (3.7 Mb)	607 (3.7 Mb)	434 (3.7 Mb)	683 (3.7 Mb)	FL378 (Donor)
BR28 (Recipient)	1.00												
188 (1.3 Mb)	0.97	1.00											
204 (2.4 Mb)	0.97	0.94	1.00										
618 (0 Mb)	0.98	0.95	0.94	1.00									
657 (3.7 Mb)	0.96	0.93	0.95	0.94	1.00								
318 (3.3 Mb)	0.96	0.93	0.94	0.94	0.96	1.00							
341 (3.3 Mb)	0.94	0.91	0.92	0.92	0.94	0.95	1.00						
845 (0 Mb)	0.97	0.94	0.94	0.98	0.96	0.93	0.91	1.00					
448 (3.7 Mb)	0.94	0.91	0.93	0.93	0.97	0.94	0.92	0.94	1.00				
607 (3.7 Mb)	0.93	0.90	0.92	0.93	0.95	0.91	0.90	0.95	0.93	1.00			
434 (3.7 Mb)	0.90	0.87	0.90	0.89	0.93	0.90	0.91	0.90	0.94	0.89	1.00		
683 (3.7 Mb)	0.88	0.85	0.87	0.89	0.89	0.87	0.85	0.90	0.89	0.93	0.88	1.00	
FL378 (Donor)	0.00	0.03	0.03	0.03	0.04	0.05	0.06	0.03	0.06	0.07	0.08	0.07	1.00

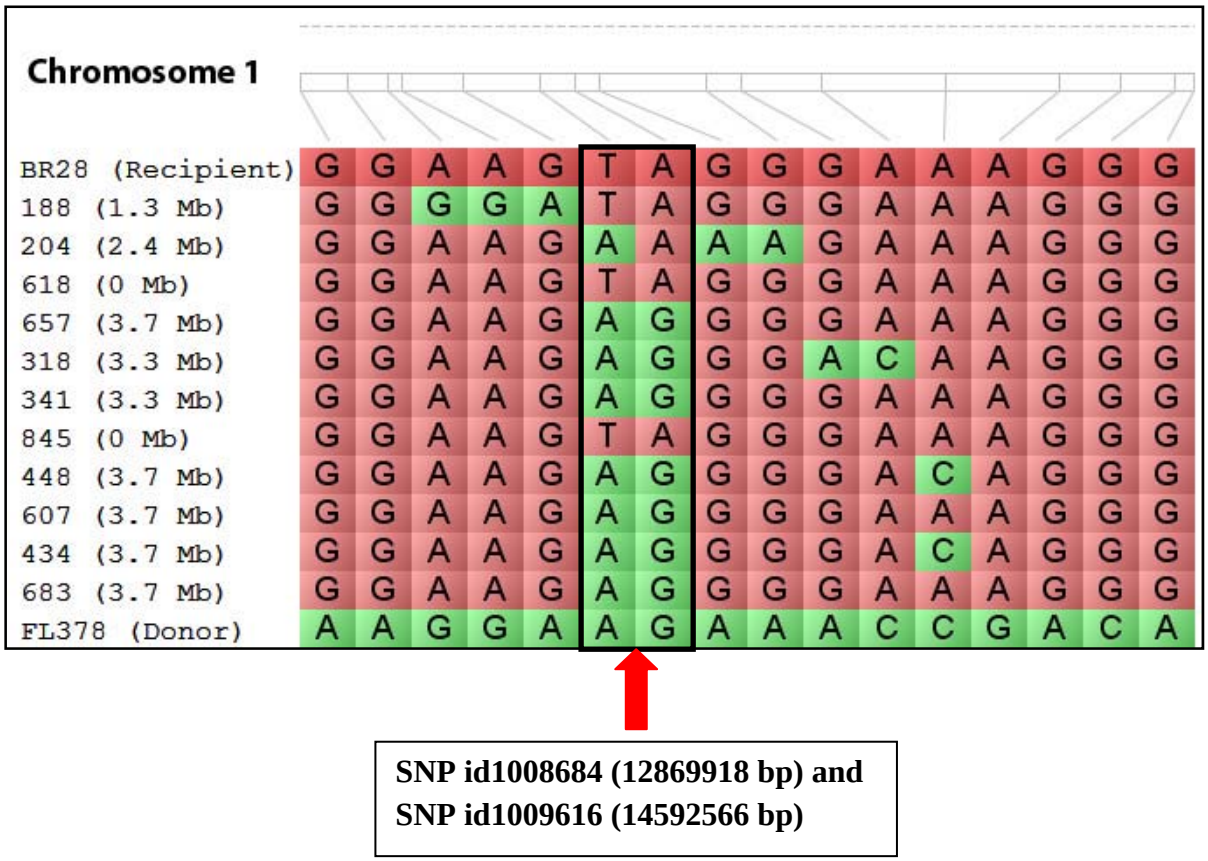
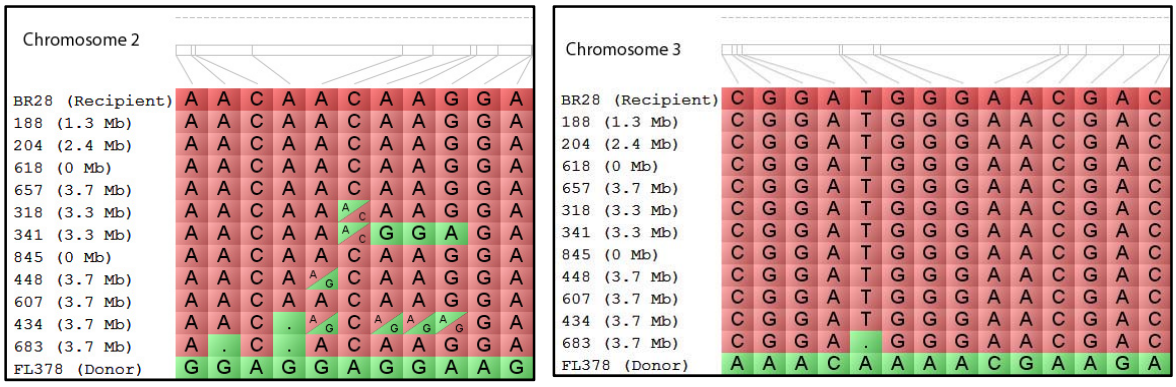
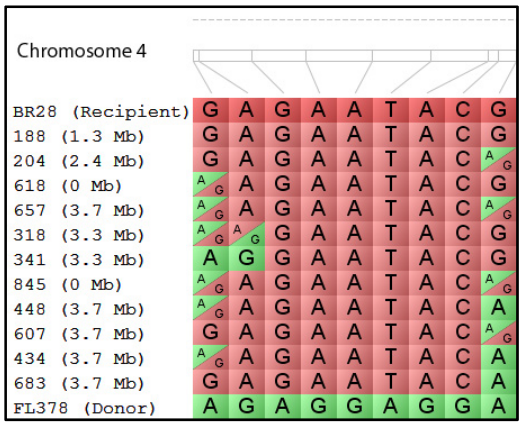
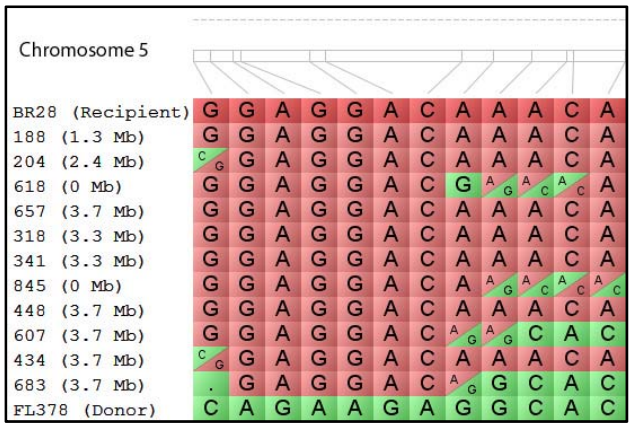
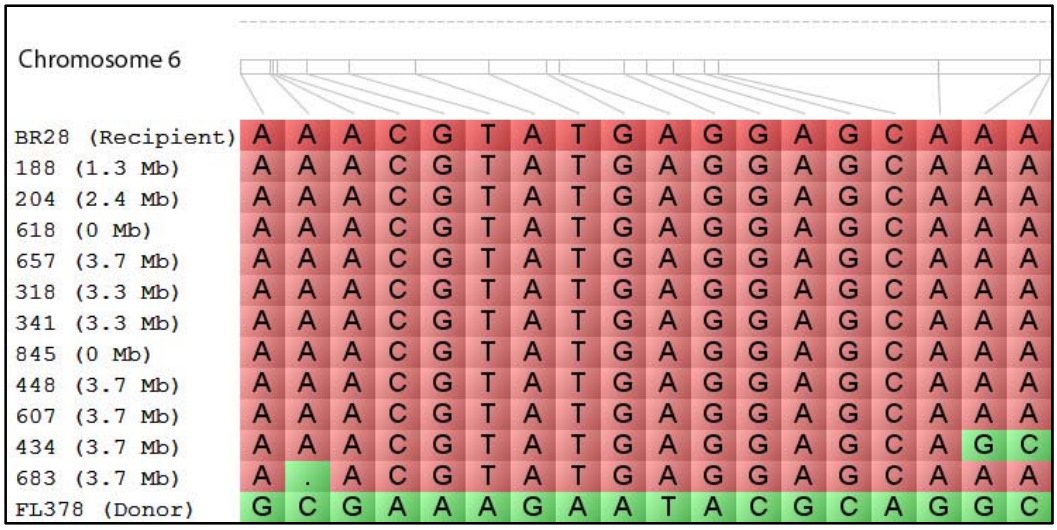


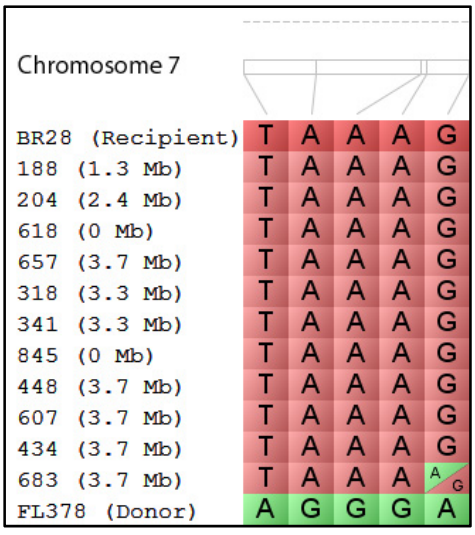
Fig. 4.2.20: SNP alleles of BRRI dhan28-*Saltol* NILs with recurrent BRRI dhan28 and donor FL378. Numbers on the left column are the name of parents and numbers of the progenies of NILs. Reddish nucleotides matched with top row are similar with recurrent BRRI dhan28 and green nucleotides matched with bottom row are similar to donor FL378. Picture represents the SNP markers and SNPs in the carrier chromosome.

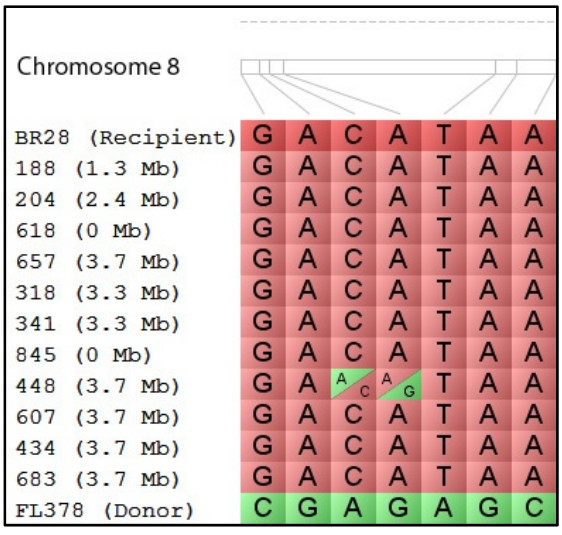



Chromosome 4
BR28 (Recipient)
188 (1.3 Mb)
204 (2.4 Mb)
618 (0 Mb)
657 (3.7 Mb)
318 (3.3 Mb)
341 (3.3 Mb)
845 (0 Mb)
448 (3.7 Mb)
607 (3.7 Mb)
434 (3.7 Mb)
683 (3.7 Mb)
FL378 (Donor)


Chromosome 5
BR28 (Recipient)
188 (1.3 Mb)
204 (2.4 Mb)
618 (0 Mb)
657 (3.7 Mb)
318 (3.3 Mb)
341 (3.3 Mb)
845 (0 Mb)
448 (3.7 Mb)
607 (3.7 Mb)
434 (3.7 Mb)
683 (3.7 Mb)
FL378 (Donor)


Chromosome 6
BR28 (Recipient)
188 (1.3 Mb)
204 (2.4 Mb)
618 (0 Mb)
657 (3.7 Mb)
318 (3.3 Mb)
341 (3.3 Mb)
845 (0 Mb)
448 (3.7 Mb)
607 (3.7 Mb)
434 (3.7 Mb)
683 (3.7 Mb)
FL378 (Donor)


Chromosome 7
BR28 (Recipient)
188 (1.3 Mb)
204 (2.4 Mb)
618 (0 Mb)
657 (3.7 Mb)
318 (3.3 Mb)
341 (3.3 Mb)
845 (0 Mb)
448 (3.7 Mb)
607 (3.7 Mb)
434 (3.7 Mb)
683 (3.7 Mb)
FL378 (Donor)


Chromosome 8
BR28 (Recipient)
188 (1.3 Mb)
204 (2.4 Mb)
618 (0 Mb)
657 (3.7 Mb)
318 (3.3 Mb)
341 (3.3 Mb)
845 (0 Mb)
448 (3.7 Mb)
607 (3.7 Mb)
434 (3.7 Mb)
683 (3.7 Mb)
FL378 (Donor)

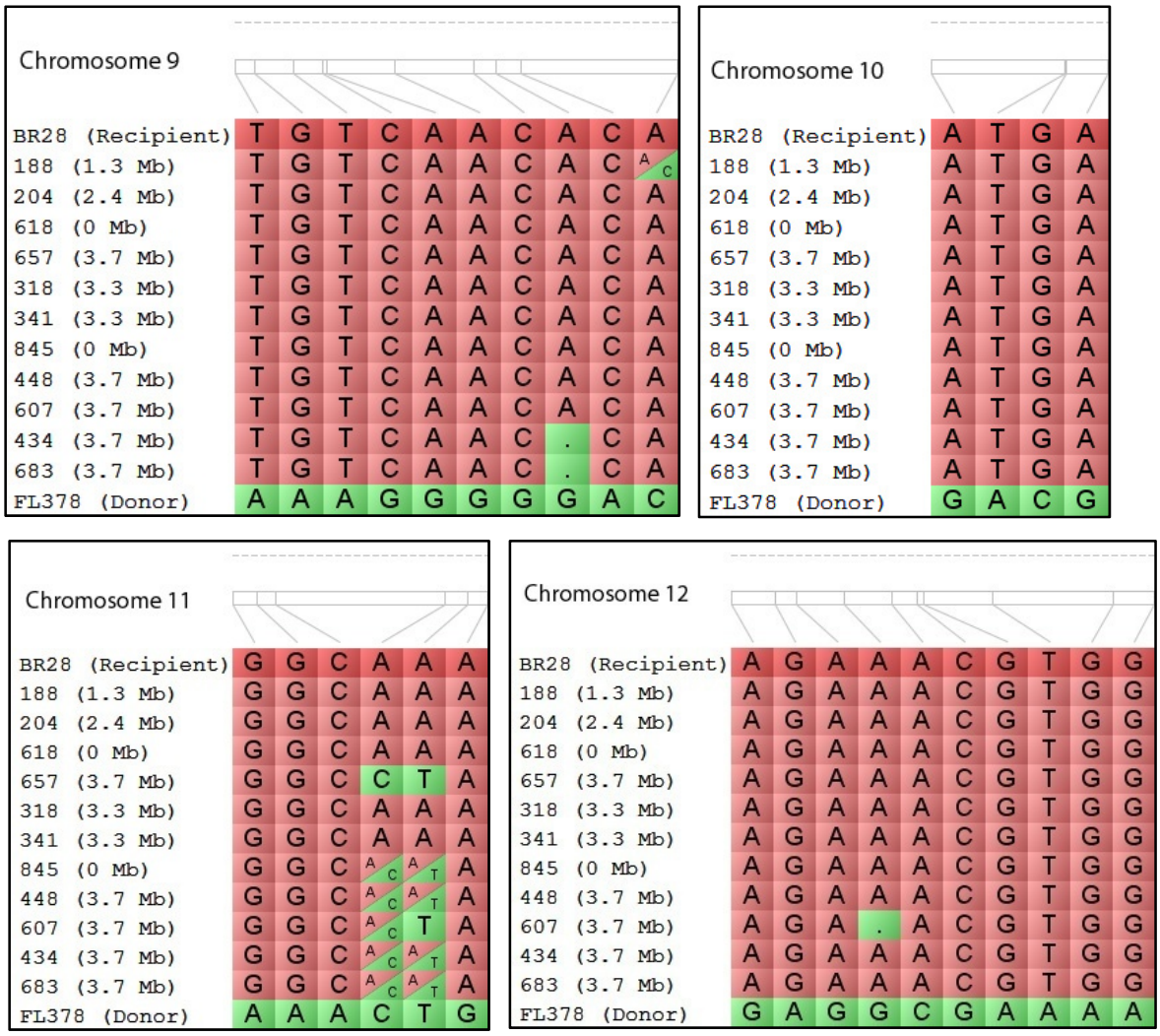


Fig. 4.2.21: SNP alleles of BRRI dhan28-*Saltol* NILs with recurrent BRRI dhan28 and donor FL378. Pictures representing the SNPs on rest of the chromosomes (Chr. 2 to Chr. 12).

g) Cross registration and nomenclature of breeding lines for BRRIdhan28-Saltol MABC event

Similar to the BR11 MABC event, crosses are indicated using the female parent and male parent. The genotypes are separated using a backslash. For example, BRRI dhan28/FL378 indicates a cross with BRRI dhan28 as the female parent and FL378 as the pollinator. The female parent is always indicated first. Further backcrosses are indicated by more backslashes for the parent that was used to cross to the other parent. For example in the cross

BRRI dhan28/FL378/BRRI dhan28, the male parent was crossed to BRRI dhan28 once (to make the F_1 hybrid) and then crossed again to the F_1 to make the BC_1. Therefore, BC_1F_1/BRRI dhan28 indicates BC_2 and so on. After confirmation, the F_1 generation is given a registration number, for BRRI dhan28 cross, the registration number is BR8962, where 'BR' is Bangladesh Rice and '8962' is the unique serial number of that cross at Plant Breeding Division of BRRI. After cross registration number the pedigree was written with the progeny number selected in each backcross generation. For BRRI dhan28-MABC event twelve double recombinant progeny was selected and out of 12 double recombinants 13 progenies were finally selected further advancement. So the pedigrees of these 13 lines are BR8962-779-76-188, BR8962-329-25-204, BR8962-521-45-318, BR8962-521-45-341, BR8962-259-21-412, BR8962-259-21-434, BR8962-259-21-448, BR8962-259-21-607, BR8962-259-21-618, BR8962-259-21-657, BR8962-259-21-683, BR8962-259-21-807 and BR8962-259-21-845. However, to designate these lines in easy and understandable way the lines are called NIL188, NIL204, NIL318, NIL341, NIL412, NIL434, NIL448, NIL607, NIL618, NIL657, NIL683, NIL807 and NIL845, here NIL indicates Near-Isogenic Line and preceding numbers is the last serial number of the pedigree. During phenotypic characterization immediate after the development of NILs the lines are always called by its NIL number. But later, for tracking lines from other NILs and breeding lines an additional name with prefix PHY (means the selection was made at Plant Physiology Division) and suffix an arbitrary number was added (Table 4.2.5). But it is difficult to use long pedigree every time for each lines, therefore a short designation of NIL numbers were always used during phenotyping (Table 4.2.5).

Table 4.2.5: Name of the breeding lines, pedigree details and short designation of BRRI dhan28-*Saltol* lines.

Complete pedigree	Size of donor segment in Mb	Additional notation in the pedigree	Short designation
BR8962-779-76-188	1.3	BR8962-779-76-188–PHY89	NIL188
BR8962-329-25-204	2.4	BR8962-329-25-204-PHY38	NIL204
BR8962-521-45-318	3.3	BR8962-521-45-318-PHY93	NIL318
BR8962-521-45-341	3.3	BR8962-521-45-341-PHY75	NIL341
BR8962-259-21-412	3.7	BR8962-259-21-412-PHY24	NIL412
BR8962-259-21-434	3.7	BR8962-259-21-434-PHY10	NIL434
BR8962-259-21-448	3.7	BR8962-259-21-448-PHY45	NIL448
BR8962-259-21-607	3.7	BR8962-259-21-607-PHY8	NIL607
BR8962-259-21-618	0.0	BR8962-259-21-618-PHY3	NIL618
BR8962-259-21-657	3.7	BR8962-259-21-657-PHY32	NIL657
BR8962-259-21-683	3.7	BR8962-259-21-683-PHY15	NIL683
BR8962-259-21-807	0.0	BR8962-259-21-807-PHY47	NIL807
BR8962-259-21-845	3.7	BR8962-259-21-845-PHY55	NIL845

4.2.4 Phenotypic characterization of BRRI dhan28-Saltol NILs.

a) Phenotypic characterization of BRRI dhan28-Saltol NILs at seedling stage under salinity

To quantify the effects of *Saltol* in the BRRI dhan28 background and to compare the tolerance of different sized introgression segments, phenotyping were carried out at the seedling stage in Yoshida solution (Yoshida, *et al.* 1976) in controlled Green house conditions. Five different traits including SES score, survivability, shoot Na^+/K^+ ratio, shoot and root dry weights were recorded and measured for judging the level of tolerance gained by the *Saltol* lines in comparison with parents and standard checks (Table 4.2.6). Correlation studies among the traits showed a strong and significant but negative association between SES to survivability ($r = -0.49^*$), shoot ($r = -0.56^*$) and root ($r = -0.50^*$) dry weight, but weak and insignificant relation with shoot Na^+/K^+ ratio (r = 0.28). *Saltol* QTL mainly contributed to maintain low Na^+/K^+ ratio in the seedling shoot and subsequently contributed to the tolerance at seedling stage. While the correlation is quite strong and significant for BR11-*Saltol* event (r = 0.58*) because of consistent performance of the developed lines having single and small segment. However, in the BRRI dhan28-*Saltol* event, due to use of 4 different donor segment the developed NILs did not perform consistently (Table 4.2.6). The *Saltol* QTL contributed differently due to different size even differently within same sized segments. Therefore, the correlation between SES and Na^+/K^+ ratio was found weak and insignificant for BRRI dhan28-*Saltol* (r = 0.28). Whereas, shoot Na^+/K^+ ratio showed strong, highly significant and negative association to shoot ($r = -0.65^{**}$) and root ($r = -0.61^{**}$) dry weight (Table 4.2.7). Above relationships revealed that, shoot Na^+/K^+ ratio had definite effects on salinity tolerance at seedling stage reflected through reduction of (negative correlation) shoot and root dry weight but the effect was not visible through SES.

ANOVA showed a highly significant variation among the tested genotypes for 4 traits, while it was significant only for the survivability trait. SES score (the leaf damage score, which is considered overall tolerance for each lines) of all NILs did not follow a definite trend with respect to the introgression size in the target QTL region. According to the tolerance in terms of SES score, the donor FL378 ranked first with least SES score 3.39 and the recipient, BRRI dhan28 ranked last of the table with highest SES score 5.93. However, two lines NIL807 and NIL683 having largest introgression (3.7 Mb) ranked lower than the donor but

had better scores than the original donor Pokkali. However, the SES was found to be statistically similar with the donor, FL378. Pokkali, the original donor of *Saltol* scored significantly poorer than FL378. One *Saltol* line NIL341 with 2nd largest introgression (3.3 Mb) showed very similar tolerance compared to Pokkali. Rest of the NILs and sensitive check IR29 ranked lower and showed statistically similar tolerance in terms of SES score amongst each other but were significantly better than a line NIL845 without *Saltol* and the recipient BRRI dhan28 (Table 4.2.6).

Survivability of the genotypes showed a similar trend as that of the SES score. Donor FL378, Pokkali, 13 NIL lines and IR29 showed significantly better survivability than the recipient BRRI dhan28. Shoot Na^+/K^+ ratio did not follow similar trend as SES and survivability this is due to lack of correlation (r = 0.28) between the traits in this screening study. The donor FL378 was able to maintain a minimum ratio (0.61) in the shoot but which was statistically similar with NIL318 of 2nd largest introgression 3.3 Mb (0.84), Pokkali (0.91) and NIL618 without the *Saltol* QTL (0.98). Among the tested *Saltol* lines, NIL318 (0.84) and NIL618 (0.98) maintained lowest ratios in the shoot and which was significantly better than recipient BR28 and other lines. Decrease of shoot Na^+/K^+ ratio in the NIL318 in comparison to the recipient BR28 at around 31.71% (Table 4.2.8). Six NILs showed a reduction of Na^+/K^+ ratio ranged from 0.81% to 20.33%, but rest of the NILs showed an increased Na^+/K^+ ratio even greater than the sensitive check IR29 (Table 4.2.8). However, few *Saltol* lines, NIL341 (1.16), NIL845 (1.16), NIL807 (1.19), NIL412 (1.20) and NIL434 (1.22) were shown to maintain low ratios in shoots compared to the recipient BR28 but all were statistically similar to the recipient BR28 (1.23) (Table 4.2.6 and Fig. 4.2.22). Shoot and root dry weights showed very similar trends as shoot Na^+/K^+ ratio, where the donor FL378 and Pokkali ranked top with significantly highest shoot and root dry weights and the recipient BR28 was ranked bottom of the table with lowest weights. Some of the NILs ranked intermediate between the FL378 donor and BR28 recipient with respect to these parameters (Table 4.2.6). Due to large variability among the measured traits between tested genotypes and weaker correlations between shoot Na^+/K^+ to SES and survivability therefore no significant association was observed between donor size at the QTL region and tolerance at seedling stage (Table 4.2.6 and 4.2.7).

Table 4.2.6: Phenotypic characterization of BRRI dhan28-*Saltol* NILs at seedling stage with salinity stress @ 12 dSm^{-1} in hydroponics culture at Green house IRRI, 2011.

Varieties/Lines (segment size)	**SES score**	**Survivability (%)**	**Shoot Na^+/K^+ ratio**	**Shoot dry weight (mg/seedling)**	**Root dry weight (mg/seedling)**
FL378 (Donor)	3.39 a	100.00 b	0.61 a	613.06 c	62.36 d
NIL807 (3.7 Mb)	4.00 ab	100.00 b	1.19 bc	352.22 b	28.89 ab
NIL683 (3.7 Mb)	4.20 ab	100.00 b	1.51 bc	365.56 b	33.33bc
Pokkali (Original donor)	4.41 b	99.58 b	0.91 a	659.58 c	55.42 d
NIL341 (3.3 Mb)	4.47 b	96.67 b	1.16 b	408.89 b	35.56bc
NIL607 (3.7 Mb)	4.50bc	100.00 b	1.66 bc	261.11 a	30.00 ab
NIL434 (3.7 Mb)	4.54bc	100.00 b	1.22 bc	313.33 a	20.00 a
NIL204 (2.4 Mb)	4.67bc	100.00 b	1.35 bc	367.78 b	38.89bc
NIL618 (0 Mb)	4.67bc	96.67 b	0.98 a	384.44 b	34.44bc
NIL448 (3.7 Mb)	4.68bc	100.00 b	1.77 c	328.89 a	23.33 ab
NIL318 (3.3 Mb)	4.70bc	100.00 b	0.84 a	445.56 b	33.33bc
NIL657 (3.7 Mb)	4.87bc	96.67 b	1.71 c	292.22 a	31.11 b
NIL188 (1.3 Mb)	4.93bc	96.67 b	1.30 bc	246.67 a	25.56 ab
NIL412 (3.7 Mb)	5.05bc	96.67 b	1.20 bc	416.67 b	32.22bc
IR29 (Sensitive check)	5.14bc	91.67 ab	1.25 bc	472.36bc	42.08 c
NIL845 (0 Mb)	5.32 c	100.00 b	1.16 b	293.33 a	28.89 ab
BRRI dhan28 (Recipient)	5.93 c	91.25 a	1.23 bc	200.28 a	27.92 ab
Significance	**	*	**	**	**
$LSD_{0.05}$	0.83	5.36	0.55	150.38	10.78
CV (%)	10.70	3.30	26.50	23.90	18.90

Each value is the mean of 3 replicates; Means followed by a common letter/s within column is not significantly different at 5% level of probability. NIL=Near Isogenic Line.

Table 4.2.7: Correlation (Pearson) coefficients among the traits recorded for judging the salinity tolerance at seedling stage of BRRI dhan28-*Saltol* lines.

Traits	SES score	Survivability	Shoot Na^+/K^+ ratio	Shoot dry weight	Root dry weight
SES score	1.00				
Survivability	-0.49*	1.00			
Shoot Na^+/K^+ ratio	0.28	0.10	1.00		
Shoot dry weight	-0.56*	0.05	-0.65**	1.00	
Root dry weight	-0.50*	-0.13	-0.61**	0.88**	1.00

*& **Correlation is significant at the 5% & 1% level of probability, respectively.

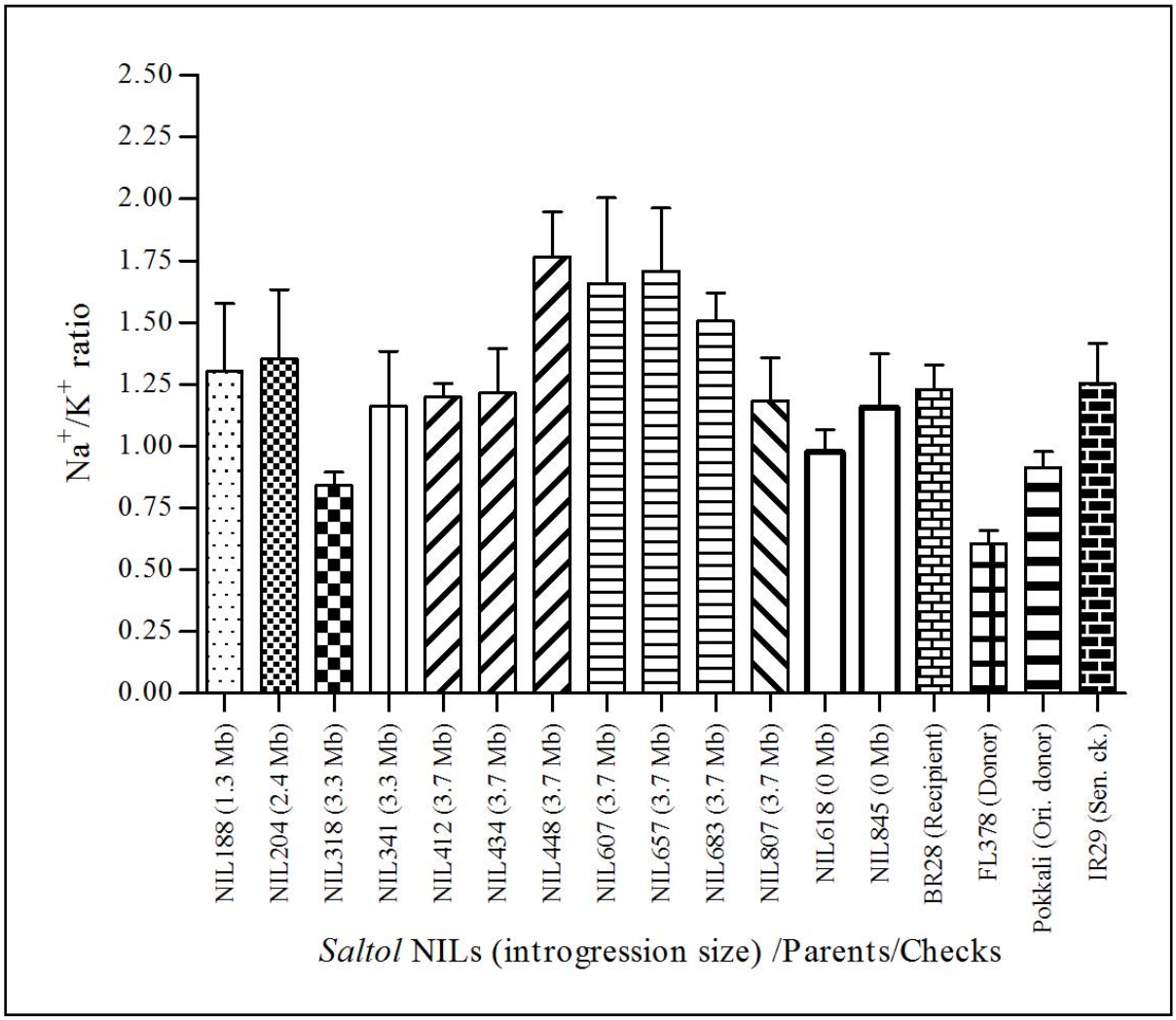


Fig. 4.2.22: Shoot Na^+/K^+ ratio at seedling stage of all selected and tested BRRI dhan28-*Saltol* lines of different introgressed segments with parents and checks characterized under salinity @12 dSm^{-1} in controlled Green house condition. Each bar represents means of 3 replicates and error bars SEM (N=5).

Table 4.2.8: Mean shoot Na^+/K^+ ratio and percent reduction of shoot Na^+/K^+ ratio compared to the recipient BRRI dhan28.

Lines/Varieties	Mean shoot Na^+/K^+ ratio*	Percent reduction of shoot Na^+/K^+ ratio compared to recipient BRRI dhan28
FL378 (donor)	0.61	50.41
NIL318 (3.3 Mb)	0.84	31.71
Pokkali (Original donor)	0.91	26.02
NIL618 (0 Mb)	0.98	20.33
NIL341 (3.3 Mb)	1.16	5.69
NIL845 (0 Mb)	1.16	5.69
NIL807 (3.7 Mb)	1.19	3.25
NIL412 (3.7 Mb)	1.2	2.44
NIL434 (3.7 Mb)	1.22	0.81
BRRI dhan28 (recipient)	**1.23**	**0.00**
IR29 (Sensitive check)	1.25	-1.63
NIL188 (1.3 Mb)	1.3	-5.69
NIL204 (2.4 Mb)	1.35	-9.76
NIL683 (3.7 Mb)	1.51	-22.76
NIL607 (3.7 Mb)	1.66	-34.96
NIL657 (3.7 Mb)	1.71	-39.02
NIL448 (3.7 Mb)	1.77	-43.90

*Each value is the mean of 3 replicates.

b) Phenotypic characterization of BRRI dhan28-Saltol at reproductive stage under salinity @ 8 dSm^{-1} at controlled Net house condition

Out of 13 NILs of BRRI dhan28-*Saltol* event, only 7 NILs having similarity in phenotype with recipient BRRI dhan28 were selected to test similar as the BR11-*Saltol* lines for the whole growth period in a soil-based system at salinity stress of 8 dSm^{-1} under controlled net house conditions. For assessing yield potential of the *Saltol* lines in saline stress condition, recipient BRRI dhan28, HYV tolerant check BRRI dhan47 and donor FL378 were also grown along the lines in respective growing i.e. *Boro* season 2012-13. Physiological behaviors during reproductive development could be a good indicator for judging the tolerance and yield potential of a rice genotype. Therefore, for comparing internal physiological behaviors of the tested genotypes, flag leaf gas exchange parameters i.e. photosynthetic rate, stomatal conductance, internal CO_2 concentration, transpiration and

ratio of photosynthesis to transpiration were measured and recorded at the flowering stage in the flag leaves of all tested entries. Out of the recorded 5 gas exchange parameters, photosynthesis and stomatal conductance were found to vary significantly among the tested genotypes (Table 4.2.9). NIL618 showed significantly highest photosynthesis and stomatal conductance (Fig. 4.2.23). It is interesting to mention here that, NIL618 has no *Saltol* segment (-QTL) line selected mainly to compare *Saltol* lines (+QTL), but this line performed better than all lines even better than parent BRRI dhan28 (Fig. 4.2.23). This superior performance could be due to favorable background introgression that has positive effect on the gas exchange parameters. However, these gas exchange parameters did not contribute much to its tolerance in terms of grain yield (see next paragraph and Table 4.2.10). For rest of the lines, NIL412, 434, 657 and 683 showed better exchanges than BRRI dhan28 and BRRI dhan47 for most of the gas exchange parameters (Fig. 4.2.23). Two *Saltol* lines NIL448 and NIL607 performed worse than BRRI dhan28.This could be due to their poor development at an early stage of their growth. However, NIL683 maintained the highest level of intracellular CO_2 and was found to be the best line which could maintain higher photosynthetic rate with a very low transpiration rate, that indicating its potentiality to tolerate higher stress condition i.e. higher water limiting condition (Table 4.2.9).

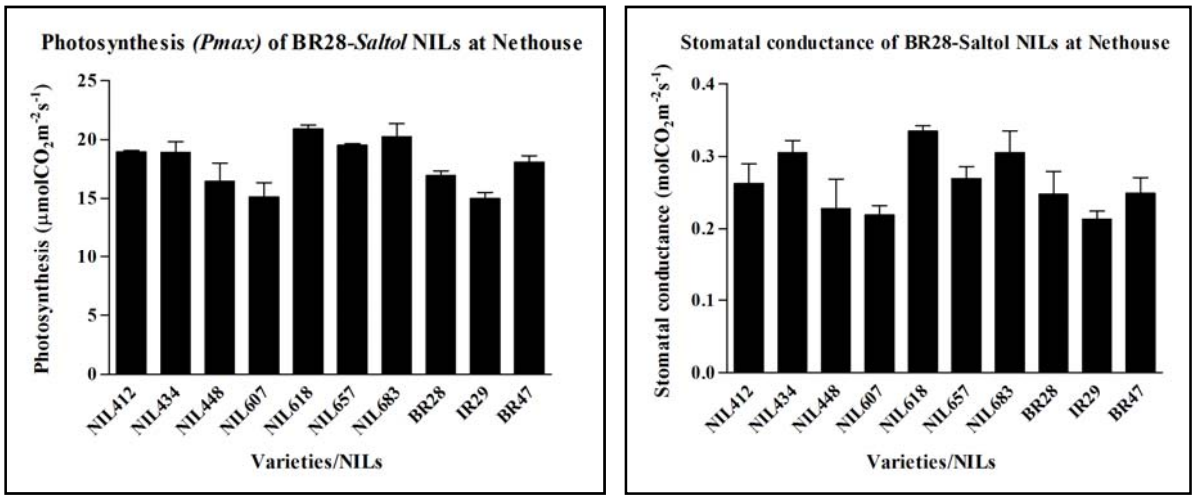


Fig. 4.2.23: Photosynthesis and stomatal conductances of BRRI dhan28-*Saltol* NILs under salinity @ 8 dSm^{-1} for the whole growth period in Net house controlled conditions. Each bar represents means of 3 replicates and error bars represents SEM (N=16).

Eleven parameters were recorded and measured for comparing the improvements and differences of *Saltol* introgressed lines from the recipient BRRI dhan28 and a salt tolerant HYV BRRI dhan47. Out of 11 parameters most of them showed significant variations among the tested genotypes except 4 traits (panicle number, filled grains number, grain weights and harvest index) (Table 4.2.10). The insignificant variations of the 4 important traits was mainly due to high coefficient of variation (>20%), indicating the confounding effects of high experimental errors with small treatment effects. But highly significant differences were observed for most of the traits between BRRI dhan47 and all other lines/varieties tested; this is because of very distinct phenotype of BRRI dhan28 compared to BRRI dhan47. However, for most of the parameters *Saltol* lines of BRRI dhan28 background showed very similar phenotype as the recipient BRRI dhan28.Thi indicates a good recovery of the recipient genome in the *Saltol* lines. Grain yield is the ultimate trait for comparison of tolerance gained by the *Saltol* lines. Though, there were insignificant variations observed between genotypes, 4 lines out 7 produced higher yield compared to the recipient BRRI dhan28 and these were supported by higher filled grains, longer panicles, lower sterility percentage and better conversion of biomass to yield (Table 4.2.10). The highest yield advantage 1.37 g/plant was found in NIL434 under saline stress condition of whole growth period.

Table 4.2.9: Gas exchange parameters of BRRI dhan28-*Saltol* NILs for salinity stress @ 8 dSm^{-1}at whole growth period in control Net house condition.

Genotypes	**Photosynthesis ($\mu molCO_2m^{-2}s^{-1}$)**	**Stomatal conductance ($molCO_2m^{-2}s^{-1}$)**	**Intercellular CO2 (mmol CO_2mol air^{-1})**	**Transpiration (mol H_2O $m^{-2}s^{-1}$)**	**Photosynthesis to transpiration ratio (A/T)**
NIL618	20.91 c	0.34 b	248.56	8.76	2.36
NIL683	20.26 bc	0.31 b	254.56	5.37	2.63
NIL657	19.53 bc	0.27 ab	232.11	8.08	2.44
NIL412	18.98 bc	0.26 ab	233.00	7.28	2.55
NIL434	18.90 bc	0.31 b	251.67	8.44	2.35
BRRI dhan47 (HYV tol. ck.)	18.10 b	0.25 ab	233.44	7.43	2.36
BRRI dhan28 (Recipient)	16.94 a	0.25 ab	238.89	7.10	2.31
NIL448	16.44 a	0.23 a	233.11	6.44	2.43
NIL607	15.13 a	0.22 a	242.56	6.45	2.36
Significance	**	*	ns	Ns	ns
$LSD_{0.05}$	2.57	0.07	--	--	--
CV (%)	8.30	16.30	6.10	18.80	11.20

*&**Significance at 5% & 1% level, ns=Not significant, Means followed by a common letter/s within column are not significantly different at 5% level of probability.

Table 4.2.10: Growth and yield parameters for characterization at whole growth period of BRRI dhan28-*Saltol* NILs under salinity in soil based system with stress @ 8 dSm^{-1}.

NILs/Varieties	Days to heading	Salinity duration (d)	Plant height (cm)	Panicle number (no.)	Panicle length (cm)	Filled grains (no./hill)	Grain weight (g/hill)	Spikelet sterility (%)	Thousand grains weight (g)	Straw weight (g/hill)	Harvest index
BRRI dhan47 (Tolerant ck.)	121.73 d	54.47 d	87.45 c	11.05	23.92 c	526.43	11.00	46.03 b	20.83 c	17.44 c	0.36
NIL434	118.00 c	51.00 c	80.00 b	15.00	18.64 ab	554.00	9.88	36.66 b	17.75 b	14.40 b	0.38
NIL657	116.40bc	49.40bc	78.00 ab	11.60	19.68 b	578.80	9.58	30.67 a	16.49 ab	13.40 b	0.39
NIL683	113.50 ab	46.50 ab	76.90 ab	13.40	19.02 b	548.00	9.08	34.35 a	16.51 ab	13.58 b	0.38
NIL618	115.40 b	48.40 b	75.60 ab	14.00	19.63 b	523.80	8.98	38.61 b	17.29 b	13.82 b	0.37
BRRI dhan28 (Recipient)	116.88bc	49.82bc	77.38 ab	13.93	18.14 ab	517.53	8.41	32.05 a	16.26 a	15.52bc	0.34
NIL412	114.60 ab	47.60 ab	78.60 b	13.40	17.77 a	466.80	7.59	37.28 b	16.20 a	12.29 ab	0.36
NIL607	116.80bc	49.80bc	73.80 ab	12.20	18.50 ab	432.00	7.03	45.88 b	15.80 a	13.13 b	0.30
NIL448	113.00a	46.00 a	73.20 a	9.80	18.60 ab	395.00	6.43	29.08 a	16.36 ab	10.00 a	0.30
Significance	**	**	**	ns	**	ns	ns	**	**	**	ns
$LSD_{0.05}$	2.26	2.35	4.95	4.00	1.17	167.63	2.87	9.79	0.99	2.77	0.09
CV (%)	1.50	3.70	4.90	24.40	4.70	25.80	25.70	20.70	4.50	15.70	20.10

**Significance at 1% level, ns=Not significant, Means followed by a common letter/s within column are not significantly different at 5% level of probability.

c) Field evaluation of BRRI dhan28-Saltol lines in non-saline field condition at BRRI, Gazipur

All seven BRRI dhan28-*Saltol* lines were evaluated in non-saline field condition with recommended cultural managements for comparing the yield and quality phenotypes with recipient BRRI dhan28, HYV tolerant check BRRI dhan47 and donor FL378. For comparing yield potentials, an internal physiological behaviors like-gas exchanges between leaf to air and vis-à-vis were measured and recorded at the flowering stage in the flag leaves of all tested entries. Five gas exchange parameters i.e. photosynthesis, stomatal conductance, internal CO_2 concentration, transpiration and transpiration efficiency (ratio of photosynthesis to transpiration) were considered for this evaluation. None of the 5 measured gas exchange parameters were found to vary significantly among the tested genotypes. Only one *Saltol* line i.e. NIL607 showed higher photosynthetic rate and stomatal conductances than the recipient BRRI dhan28 (Fig. 4.2.24). Insignificant variations among the gas exchange parameters between the *Saltol* lines and the recipient BRRI dhan28 indicating similarity of background of *Saltol* lines could be performing similarly in non-stress environment.

Eleven phenotypic traits were recorded and measured for comparing the phenotype and yield between *Saltol* lines and recipient BRRI dhan28 (Table 4.2.11). Except grain yield all other traits showed significant variation among the tested genotypes. Most of the growth and yield parameters of *Saltol* lines showed very similar or sometimes even better performance than the recipient BRRI dhan28 (Table 4.2.11). However, distinct significant variations were observed between the HYV tolerant BRRI dhan47 and donor FL378 compared to all *Saltol* lines and BRRI dhan28, this is mainly due to wider phenotypic differences among the 3 genotypes. Though the grain yield was insignificant, but two lines (NIL607 and 618) yielded > 5.5 t/ha, that means the following lines has an average yield advantage >0.50 t/ha than recipient parent BRRI dhan28. All lines showed better conversion ability from biomass to grain (harvest index) than BRRI dhan28 (Table 4.2.11).

BRRI dhan28 is not the best yielding variety among the varieties released by BRRI for *Boro* season. However, consumer acceptance and better prices i.e. acceptable grain quality (slender grain) is the main criteria for greater adoption of BRRI dhan28. So, the grain quality comparison is another essential step for any MABC conversion event. There are significant improvements for most the grain physical parameters like- milling outturn, head rice yield,

appearance, chalkiness, grain length and breadth and their ratios, cooking time, elongation ratio and imbibition ratio etc. observed for most of the *Saltol* lines in comparison with the recipient BRRI dhna28. But for comparing grain chemical properties, only two *Saltol* lines (NIL657 and NIL683) showed very similar properties to the recipient BRRI dhan28 in terms of Amylose and protein content. However, rest of the lines showed poorer protein content than the recipient (Table 4.2.12).

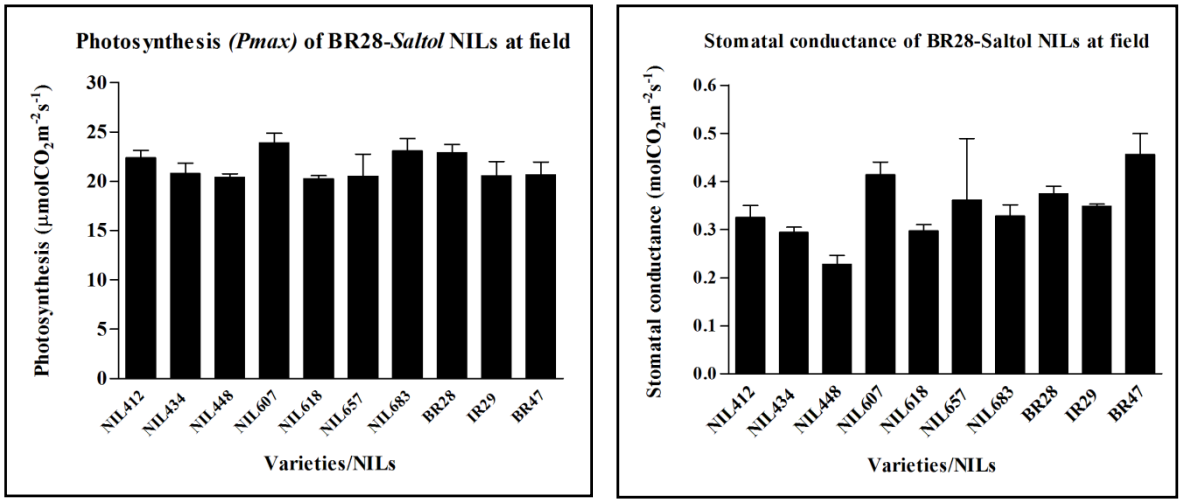


Fig. 4.2.24: Photosynthesis and stomatal conductances of BRRI dhan28-*Saltol* NILs under non-saline field condition. Each bar represents means of 3 replicates and error bars represents SEM (N=16).

Table 4.2.11: Growth and yield parameters of BRRI dhan28-*Saltol* NILs, parents and check varieties in non-saline field condition at BRRI, Gazipur, *Boro* season, 2012-13.

Lines/Varieties	Days to heading	Plant height (cm)	Panicle (no./hill)	Panicle length (cm)	Filled grains (no./hill)	Filled grains wt. (g/hill)	1000-grains wt. (g)	Sterility (%)	Grain yield (t/ha)	Straw yield (g/hill)	Harvest Index
BRRI dhan47 (HYV tol. check)	116.50 c	99.00 b	14.67 ab	25.52 c	1727.67bc	44.80 b	25.96 c	37.27 b	6.19	35.64 b	0.55bc
NIL607	109.50 a	93.67 ab	15.67 ab	23.30 b	1842.33bc	36.71 ab	19.95 ab	16.83 a	5.68	22.10 a	0.62 c
NIL618	110.50 a	96.67 ab	13.33 a	23.52 b	1361.00 ab	30.21 a	22.21 b	17.36 a	5.50	20.69 a	0.59 c
BRRI dhan28 (Recipient parent)	114.50 b	103.00 b	17.33 ab	21.57 ab	1483.67 b	29.43 a	20.06 ab	32.42 b	5.03	25.51 ab	0.54 b
NIL434	111.00 a	102.83 b	24.33 c	23.13 b	2201.00 c	48.47 b	22.06 b	15.29 a	4.92	34.83 b	0.58bc
NIL683	111.00 a	102.67 b	17.00 ab	24.50bc	1720.33bc	35.59 ab	20.67 ab	26.80 ab	4.90	23.46 a	0.60 c
NIL412	109.50 a	90.33 a	17.33 ab	22.10 ab	1912.67bc	36.31 ab	19.03 a	19.90 a	4.51	23.21 a	0.61 c
NIL448	110.50 a	93.33 ab	16.67 ab	21.47 ab	1572.33bc	30.83 ab	19.58 a	14.72 a	4.29	20.85 a	0.60 c
FL378 (Donor parent)	122.50 d	101.00 b	20.67bc	21.33 a	1031.00 a	28.64 a	27.91 c	38.33 b	4.27	31.38 b	0.48 a
NIL657	109.50 a	96.50 ab	18.33 b	22.30 ab	1985.67 c	41.35 b	20.83 ab	16.24 a	4.09	27.23 ab	0.60 c
Significance	**	**	**	**	**	**	**	**	ns	**	**
$LSD_{0.05}$	1.82	6.43	4.25	1.67	429.69	10.59	2.46	12.17	1.36	6.90	0.05
CV (%)	0.90	3.80	14.10	4.30	14.90	17.00	6.60	30.20	16.00	15.20	4.70

**Significance at 1% level of probability, ns= Not significant, Means followed by a common letter/s within column are not significantly different at 5% level of probability.

Table 4.2.12: Grain quality parameters (Physico-chemical and cooking properties) of BRRI dhan28-*Saltol* lines

Variety/Line	Milling outturn (%)	Head rice yield (%)	Appearance	Chalkiness	Length (L) mm	Breadth (B) mm	L/B ratio	Size & Shape	Cooking time min.	ER	IR	Amylose (%)	Protein (%)
NIL412	72.0	95.1	V.good	Tr	6.4	1.9	3.4	Long, Slender	17:0	1.5	4.6	24.0	8.3
NIL434	70.7	96.3	V.good	Tr	6.4	1.9	3.4	Long, Slender	17:0	1.4	4.3	23.0	8.6
NIL448	71.1	96.3	V.good	Tr	6.4	1.9	3.4	Long, Slender	17:0	1.5	4.3	24.0	8.7
NIL607	71.2	94.5	V.good	Tr	6.5	1.9	3.4	Long, Slender	17:0	1.4	4.6	24.0	8.9
NIL618	71.5	93.3	V.good	Tr	6.5	1.9	3.4	Long, Slender	17:0	1.4	4.6	24.0	8.9
NIL657	69.8	90.9	V.good	Tr	6.6	1.8	3.7	Long, Slender	17:3	1.4	4.3	27.0	9.0
NIL683	72.0	94.5	V.good	Tr	6.2	1.9	3.3	Long, Slender	17:0	1.5	4.0	27.0	9.0
BRRI dhan28 (recipient parent)	68.7	95.3	V.good	Tr	6.3	1.9	3.3	Long, Slender	17:3	1.5	3.2	27.0	9.2
FL 378 (donor parent)	69.0	89.4	Good	Tr	6.0	2.6	2.3	Medium , Bold	18:3	1.6	3.0	25.0	8.3

Abbreviations: Tr = Translucent, L/B ratio = Length/Breadth ratio, ER= Elongation ratio, IR=Imbibation ratio; Each value are the means of 3 sub-samples from same lot of paddy, therefore no analysis was carried out.

d) Field evaluation of BRRI dhan28-Saltol introgression lines in actual saline condition at BRRI regional station, Satkhira

An on-station preliminary yield trial with 6 BRRI dhan28-*Saltol* lines (one line NIL448 was not included due to poor phenotypic performance in earlier trials) along with recipient BRRI dhan28, standard *Boro* check BRRI dhan29 and HYV tolerant check BRRI dhan47 was carried out in *Boro* season 2012-13 at BRRI Regional Station Satkhira (Salinity Station for BRRI). Six different parameters i.e. growth duration, plant height, filled grains, spikelet sterility, grain yield and phenotypic acceptability (PAcp) were recorded and measured for this trial. The salinity level of crop standing water was varied between 2.0-3.2 dSm^{-1} throughout the growing period of the crop. All parameters varied significantly among the tested genotypes (Table 4.2.13). Most of the growth and yield parameters showed significant differences between *Saltol* lines and BRRI dhan28 compared to BRRI dhan29 and BRRI dhan47. This is because of distinct phenotypic differences of these two varieties (i.e. BRRI dhan28 and BRRI dan29). In general, *Saltol* lines were shown to be quite similar to the recipient BRRI dhan28 and all lines except NIL657 and NIL618 yielded higher than recipient (Table 4.2.13). However, only 2 lines NIL434 and NIL607 showed superior performance and yielded significantly better than any other tested genotypes and varieties (Table 4.2.13). The following two lines scored 4 for the phenotypic acceptability scoring system and were selected for next season evaluation under Participatory Variety Selection system (PVS) (Table 4.2.13).

Table 4.2.13: Growth and yield parameters of BRRI dhan28-*Saltol* NILs on-station preliminary yield trial at BRRI, Satkhira (*Boro*, 2012-13 season).

Sl. No.	Lines/genotypes /varieties	Duration (Days)	Plant height (cm)	Filled Grains (no./panicle)	Sterility (%)	Yield (tha^{-1})	PAcp
1	NIL412	132.00 ab	96.00 ab	123.00 ab	10.20 a	5.80 ab	5
2	NIL434	131.00 a	100.00 b	127.00 ab	8.60 a	6.90 c	4
3	NIL607	134.00 ab	94.00 a	134.00 b	9.10a	6.60 c	4
4	NIL618	137.00 b	95.00 ab	139.00 b	9.70 a	5.70 ab	7
5	NIL657	136.00 b	96.00 ab	110.00 a	17.50 ab	5.30 a	5
6	NIL683	135.00 ab	95.00 ab	113.00 a	22.00 b	6.00 b	6
7	BRRI dhan28 (Recipient)	133.00 ab	101.00 b	139.00 b	9.80 a	5.80 ab	6
8	BRRI dhan29 (Standard check)	146.00 c	101.00 b	127.00 ab	17.50 ab	6.10bc	6
9	BRRI dhan47 (HYV tolerant check)	136.00 b	104.00 b	131.00 b	10.20 a	6.50bc	4
$LSD_{0.05}$		**4.20**	**5.90**	**17.10**	**9.00**	**0.60**	**--**
CV(%)		**1.50**	**2.80**	**6.30**	**33.30**	**4.30**	**--**

Each value is the mean of 3 replicates; means followed by common letter/s are not differed significantly at 5% level of probability

e) Mean performances of BRRI dhan28-Saltol lines over two locations

Out of 13 NILs developed in BRRI dhan28 background, 6 NILs were selected and tested in the *Boro* season of 2012-2013 in two different locations i.e. non-saline, Gazipur and saline, Satkhira to observe performances in the field condition along with recipient parent and standard salt tolerant HYV (Table 4.2.11 and 4.2.13). Average grain yield of two locations of all tested NILs, parent and check showed BRRI dhan47 performed better compared to all lines and parent (Table 4.2.14). The reason of this superiority is mainly due to different plant type and because its yield potential and growth duration is normally higher than the recipient parent BRRI dhan28. However, the main problem of BRRI dhan47 variety is the bold grain and grain shattering problems during its maturity, while the targeted recipient was chosen basically for its superior grain quality and shorter growth duration. Out of 6 lines 4 of them i.e. NIL607, NIL434, NIL618 and NIL683 performed better than the recipient BRRI dhan28 (Table 4.2.14). However, mean performance of two lines NIL412 and NIL657 was slightly less than the recipient but this is due their poor performance at non-saline Gazipur, while

grain yield of these two lines were very similar to recipient and phenotypic acceptability was better than BRRI dhan28 (Table 4.2.14). Based on overall performances i.e. grain yield and phenotypic acceptability two lines, NIL607 and NIL434, which were ranked next to BRRI dhan47 were selected for further evaluation and release (Table 4.2.14).

Table 4.2.14: Mean performances of BRRI dhan28-*Saltol* NILs over two locations in the *Boro* season 2012-2013 in the non-saline Gazipur and saline environment Satkhira.

Lines/Varieties	Grain yield (t/ha) at Gazipur 2012-13	Grain yield (t/ha) at Satkhira 2012-13	Average Grain yield (t/ha)	PAcp* (Breeder's visual selection)
BRRI dhan47 (HYV tol. check)	6.19	6.50	6.35	4
NIL607	5.68	6.60	6.14	4
NIL434	4.92	6.90	5.91	4
NIL618	5.50	5.70	5.60	7
NIL683	4.90	6.00	5.45	6
BRRI dhan28 (Recipient parent)	5.03	5.80	5.42	6
NIL412	4.51	5.80	5.16	5
NIL657	4.09	5.30	4.70	5

*PAcp means Phenotypic acceptibility

4.3 Genotype by Environment interaction (G × E) and stability analysis of BR11-Saltol introgression lines for multi-environment trials

The BR11-*Saltol* NIL derivative 12 lines were evaluated in 3 different locations in the saline and non-saline environments of Koyra, Satkhira and Gazipur respectively. However, the non-saline trial at Gazipur was repeated in two different seasons i.e. *T Aman* and *Boro* season. The trials were carried out from *Boro* 2011-2012 to *T. Aman* 2013-2014. All the trials were conducted in RCB design with 3 homogenous blocks. A number of traits were measured and recorded from first two trials but only grain yield was recorded for later two trials. Therefore, G by E interactions and stability analyses were done only for the grain yield trait. Data from the individual trial was analyzed first for checking the quality of trials and plotting the residuals in Q-Q plot for testing normalcy and distribution and then computation of adjusted means (BLUEs) for G by E analysis. In the second step of analysis, the computed adjusted means were subjected to G by E interactions and stability analyses through Breeding View software (www.integratedbreeding.net). The four environments and seasons were designated as Gazipur *Boro* 2011-2012 as (Gaz B11), Gazipur *T. Aman* season 2012-2013 as (Gaz TA12), Satkhira *T. Aman* season 2012-2013 as (Sat TA12) and Koyra *T. Aman* season 2013-2014 as (Koy TA13). Based on mean performance of grain yield, Gaz B11 and Sat TA12 were found to be the best performing and least performing environments, respectively (Table 4.3.1). However, Gaz B11 is the highly variable environment as expected but Gaz TA 12 is the least variable environments without any out-layered data (Table 4.3.1 and Fig. 4.3.1). In Bangladesh, *Boro* season (dry winter) is the most productive season for growing rice. *Boro* season is characterized with irrigated and low and high temperature at the beginning and at the end of the season respectively. Therefore, due to low initial temperature rice plant gets more time for growth and development at vegetative stage coupled with capture of more radiation and proper nutrient uptake ultimately reflecting on higher yield. BR11 (recipient) and the BR11-*Saltol* NIL derivative lines are recommended for *T. Aman* season, while evaluating these lines across season during *Boro* 2011-2012 in non-saline Gazipur environment, all lines as well as the parent gave greater yields . Mean grain yield of all tested genotypes is 7.64 tha^{-1} (Table 4.3.1). However, all genotypes when tested in next *T.Aman* season 2012-2013 in the same non-saline Gazipur then mean grain yield drops to 4.93 tha^{-1} (Table 4.3.1). Large differences of grain yield around 2.5 tha^{-1} between *Boro* and *T. Aman* season of all genotypes including parent could be probably due to seasonal effects. Because *Boro* season is the most favorable for growing rice for maximizing yield with

favorable weather conditions. Correlations among the environments showed strong association between Gaz B11 to Sat TA12 and very weak correlations between Gaz TA12 to Sat TA12 (Fig. 4.3.2). Correlation between environments gives indication of similarity and dissimilarity between environments and also amount of G by E interactions. Here, strong association between Gaz B11 to Sat TA12 indicated similarity between environments as well as strong G by E interaction while there was an opposite environment and poor G by E interaction between Gaz TA12 and Sat TA12 (Fig. 4.3.2).

ANOVA of Finlay and Wilkinson modified joint regression analysis showed highly significant variations for environment and genotypic main effects but was insignificant for the sensitivity (i.e. GEI), at the probability value of 0.062 (Table 4.3.2). Though there was insignificant GEI according to Finaly and Wilkinson ANOVA, but some cross-over interaction existed between the genotypes and the environments shown in the Finlay and Wilkinson regression analysis (Fig. 4.3.3). In Fig. 4.3.3 Finlay and Wilkinson regression line showed all BR11-*Saltol* lines perform significantly better than recipient BR11 in the best (Gaz B11) and average (Gaz TA12 & Koy TA13) performing environments while only five lines perform poorer than recipient BR11 in the least performing (Sat TA12) environment (Fig. 4.3.3).

Based on the indication about cross-over interaction identified in Finlay and Wilkinson regression analysis, an AMMI analysis was performed. ANOVA of AMMI analysis showed highly significant variations for environment and genotypic main effects and an improvement was observed for G by E interaction, where interaction for first Principal Component (IPCA1) was found significant (Table 4.3.3). The reason is that, in the AMMI model, principal components analysis on the GEI (Gabriel, 1978; Gauch, 1988) maximized the variation explained by the products of the genotypic and environmental scores. The first product term (IPCA1) explained most of the variation (63.80%) and the second term (IPCA2) explained only 22.55%. However, IPCA1 is found significant (5% level of probability) whereas IPCA2 is insignificant (Fig. 4.3.4 and Table 4.3.3). Therefore, AMMI can be regarded as suitable model to explain GEI for these multi location trials. In Fig. 4.3.4 of AMMI bi-plot, the first significant component (PC1) efficiently separates the Gaz B11 environment compared to the others 3 ones, since this is the high performing environment. Moreover, it has also separated genotypes depending on their performance in each of the

environments based on GEI. Out of 12 *Saltol* lines 9 were grouped in to the high performing environment Gaz B11 and rest 3 *Saltol* lines (BR11-71, BR11-52-55 and BR11-150) and 2 parents remained in the poor and average performing environments (Fig. 4.3.4).

The GGE (Genotypic main effects and Genotype by Environment interaction) model can explain total genetic variation through a powerful bi-plot with environmental scaling for identification of the mega-environment. In the Fig. 4.3.5 GGE bi-plot, the first component (PC1) can explain the total genetic variation as high as 70.91% and the second component (PC2) as 13.96%. These increase or decrease of the 1st and 2nd components is due to inclusion of genotypic main effects to the GEI (Fig. 4.3.5). By the inclusion of genotypic main effects to the GEI, only one genotype (BR11-71) can be moved from poor environments to the high performing environment, where all 4 environments groups remain in the same axis. Two mega-environments were identified by GGE bi-plot, one mega-environment was comprised by Gaz B11 and Sat TA12 and other was Gaz TA12 and Koy TA13. In the first mega-environment, the vertex genotype is BR11-52-124, which indicates this line could give best performance in the first mega-environment or similar environments, whereas there are no such vertex genotypes for the 2nd mega-environments (Fig. 4.3.5). However, the genotypes close to the origin i.e. BR11-65, BR11-97 and BR11-52-145 could give average performance across the environments.

Table 4.3.1: Summary statistics of grain yield of BR11-*Saltol* NIL derivative lines tested in to 4 different environments.

Environments	Mean	Median	Min	Max	Lower quartile	Upper quartile	Variance
Gaz B11	7.64	8.03	5.00	9.28	7.23	8.45	1.44
Gaz TA12	4.93	4.76	4.14	5.74	4.58	5.40	0.27
Koy TA13	4.66	4.95	2.60	5.00	4.80	5.00	0.48
Sat TA12	3.96	4.05	2.20	4.90	3.70	4.30	0.42

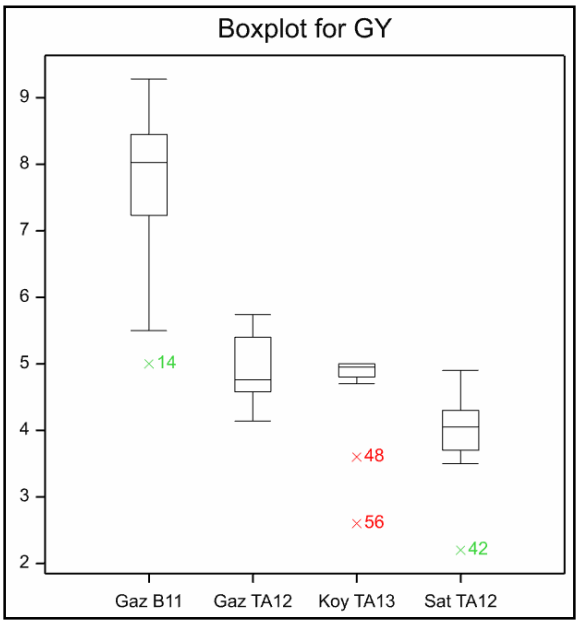


Fig. 4.3.1: Boxplot for grain yield of 4 environments

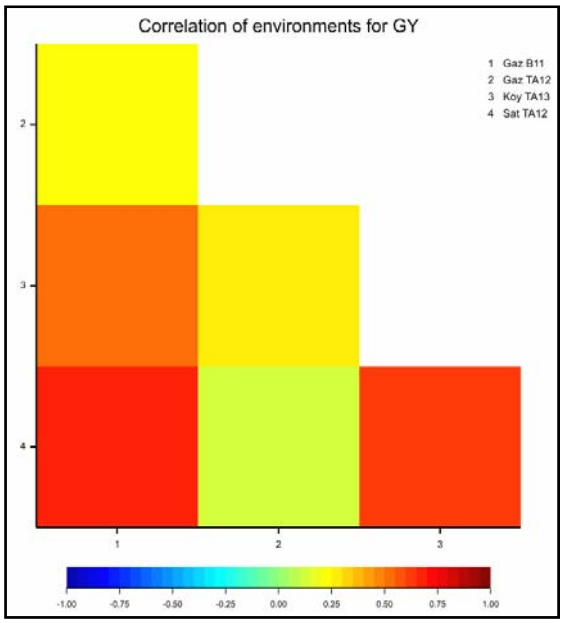


Fig. 4.3.2: Correlations among the 4 environ.

Table 4.3.2: ANOVA for Finlay and Wilkinson modified joint regression analysis

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Genotypes	13	18.11	1.39	4.58	<0.001
Environments	3	109.42	36.47	119.96	<0.001
Sensitivities	13	7.97	0.61	2.02	**0.062**
Residual	26	7.91	0.30		
Total	55	143.41	2.61		

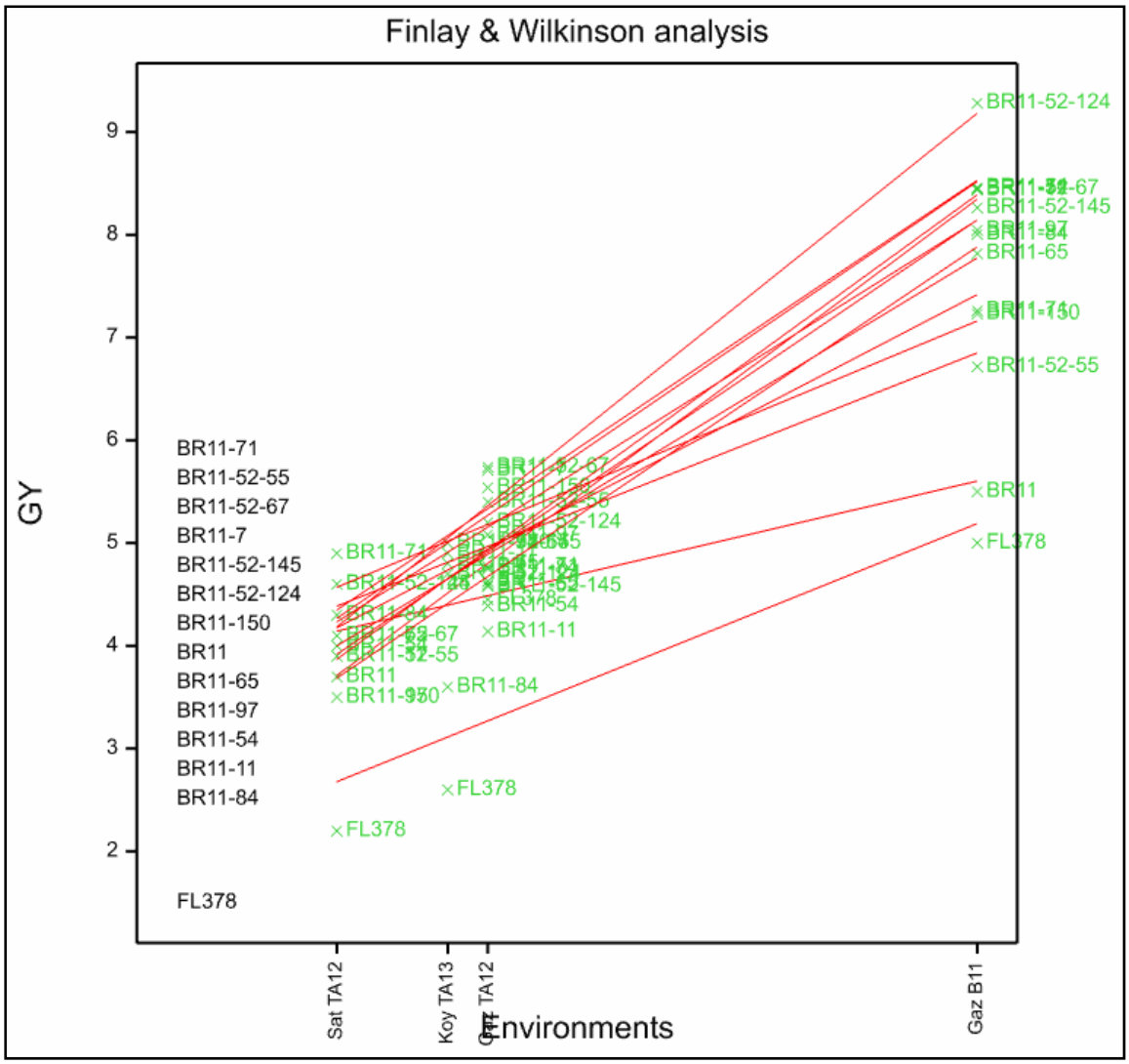


Fig. 4.3.3: Finlay-Wilkinson regression lines for grain yield.

Table 4.3.3: ANOVA for AMMI model

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr**
Genotypes	13	18.11	1.39	3.42	**0.0014**
Environments	3	109.42	36.474	89.61	**<0.001**
Interactions	39	15.87	0.41		
IPCA 1	15	10.13	0.68	3.43	**0.0225**
IPCA 2	13	3.58	0.28	1.40	**0.2926**
Residuals	11	2.17	0.20		

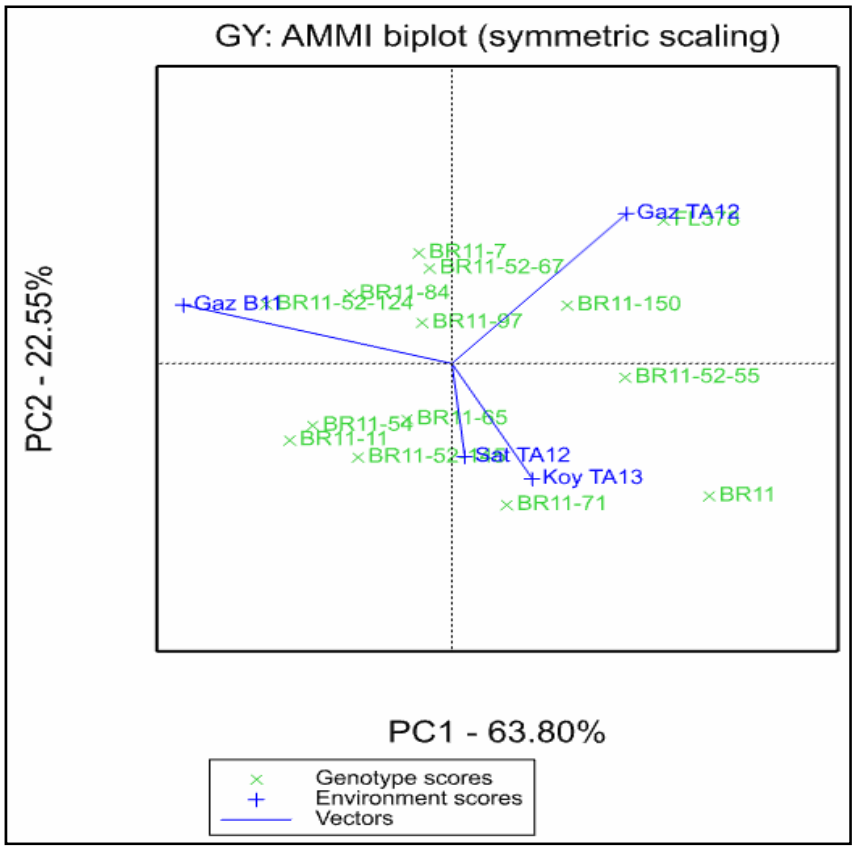


Fig. 4.3.4: AMMI bi-plot (IPCA1 and IPCA2)

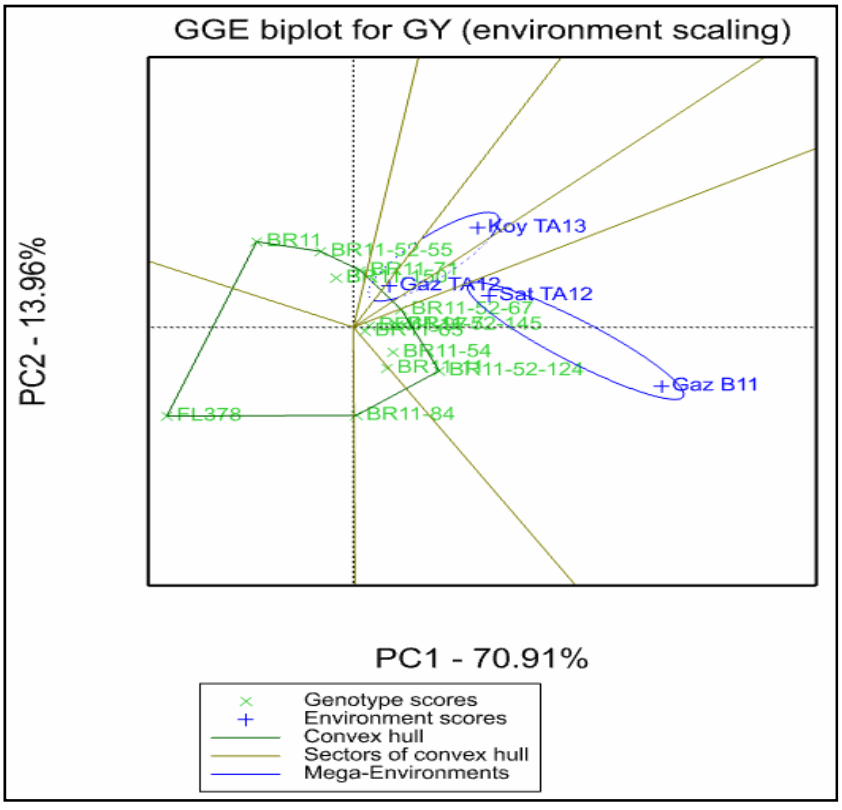


Fig. 4.3.5: GGE bi-plot for mega-environment

Another perspective to study the performance of genotypes in a range of environments (i.e. G×E analysis) is that of stability. The concept of stability is very closely related to the concept of consistency of performance and therefore with the concept of variation around some expected performance. Stable genotypes are those that are able to give a consistent response. A number of stability parameters were analyzed for the identification of superior and stable genotypes across environments. According to mean performance of genotypes, all the BR11-*Saltol* lines produced mean grain yield >5.0 tha^{-1} which is significantly superior to both recipient BR11 and donor FL378. But the highest and lowest mean yield was observed in BR11-52-124 (5.95 tha^{-1}) and FL378 (3.56 tha^{-1}) respectively (Table 4.3.4). Based on sensitivity estimates from Finlay-Wilkinson regression lines, 9 out of 12 lines showed superior performance with the sensitivity being above average ranging from 1.37 to 1.04. Only 3 genotypes perform below the average with sensitivity ranges from 0.89 to 0.68. However, the average performance was shown by two genotypes BR11-65 (1.04) and BR11-52-145 (1.07), when the sensitivity values ranged close to 1.00 (Table 4.3.4).

Mean square deviation is another estimate of stability which is actually the deviation from Finlay-Wilkinson regression and is popularly known as Eberhart-Russel stability measurement. Based on mean square deviation, BR11-65 was the most stable among the tested genotypes and FL378 was the least stable across environments (Table 4.3.4). Static stability is a type 1 parameter which estimates this based on mean performance of a variety across environments. According to static stability BR11-52-124 and BR11 were the best and poor performing genotypes across environments. Wricke's ecovalence is a type 2 stability parameter which considers both mean performance and variances for stability estimation of a genotype. Based on Wricke's ecovalence, BR11-65 and BR11 were the most and least stable genotypes across environments (Table 4.3.4).

Alternatively, cultivar superiority measurement could give a better idea for selecting superior and stable genotypes, which combine both performance and stability in a single measure. One of such measure is the Lin and Binns (1988) superiority measure (cultivar superiority). Lower values of cultivar superiority coefficient indicate better performance very close to the top genotypes in each of the environments. Based on cultivar superiority measurement, all 12 BR11-*Saltol* lines perform in a superior manner (close to the top performing genotypes in all environments) than the recipient BR11 (2.13) and the donor FL378 (4.13) (Table 4.3.4). However, the best and poor genotypes were BR11-52-124 and FL378 respectively.

Table 4.3.4: Mean Performance, Sensitivity, Stability and Superiority measures of BR11-*Saltol* lines tested in 4 different environments.

Sl. No.	Genotypes	Means	Sensitivity	Mean square deviation	Static stability	Wricke'sec ovalence	Cultivar superiority
1	BR11-52-124	5.95	1.37	0.17	5.02	1.50	0.06
2	BR11-52-67	5.82	1.15	0.12	3.51	0.34	0.17
3	BR11-7	5.77	1.17	0.13	3.64	0.43	0.17
4	BR11-52-145	5.61	1.07	0.24	3.16	0.64	0.31
5	BR11-71	5.48	0.71	0.14	1.42	1.03	0.63
6	BR11-54	5.46	1.24	0.22	4.16	0.97	0.41
7	BR11-97	5.41	1.16	0.17	3.62	0.47	0.49
8	BR11-11	5.34	1.27	0.32	4.43	1.31	0.54
9	BR11-65	5.33	1.04	0.06	2.84	0.18	0.52
10	BR11-150	5.32	0.89	0.46	2.37	0.86	0.78
11	BR11-52-55	5.25	0.68	0.24	1.35	1.19	0.96
12	BR11-84	5.16	1.15	0.53	3.81	1.33	0.62
13	BR11	4.66	0.40	0.20	0.55	3.12	2.13
14	FL378	3.56	0.69	0.96	1.88	2.50	4.13
Average		**5.29**	**1.00**	**0.28**	**2.98**	**1.13**	**0.85**

Conclusions of G by E and stability analyses: Based on the above analyses and discussion, 3 BR11-*Saltol* lines (BR11-52-124, BR11-52-67 and BR11-7) could be recommended for release and cultivation in high performing environments, whereas BR11-65 could be recommended for release and cultivation all over the country because of its average performance and stability of different environments.

Chapter 5: Discussion

Chapter 5
Discussion

Development of salt-tolerant crop varieties using selective breeding techniques has not proven successful (Ashraf, 2010; Yamaguchi & Blumwald, 2005), and developing salt tolerant plants through modern biotechnology has been considered a high research priority (Tester and Langridge, 2010; Todorovska, *et al.* 2010). Several reviews suggested the use of advances in biotechnology to make rice breeding more efficient. They mentioned that better understanding of the function and regulation of the responsive genes and their association to QTL regions will allow a more structured design to breeding for salinity tolerance (Negrao, *et al*. 2011). The rapid advances made in plant physiology and biochemistry, molecular genetics, and structural and functional genomics demonstrated the ability to identify complex mechanisms of salt tolerance and use them to isolate genes and identify major QTLs for salt tolerance. Genetic dissection of the QTLs and their incorporation into high-yielding varieties will significantly enhance and stabilize rice productivity in the problem soils (Ismail, *et al.* 2007).The use of genetic modifications or transgenic approaches has also been suggested but the marker-assisted selection (MAS) techniques are free of the political issues that have plagued the application of GM technologies. MAS involves using variation at the DNA level to track and monitor specific regions of the genomes during crossing and selection (Moose and Mumm, 2008). The greatest benefit of MAS occurs where the target traits are of low heritability, are recessive in nature, and involve difficult and costly phenotyping, and where pyramiding of genes is desired for results such as disease and pest resistance. In these cases, MAS is likely to be more reliable, more convenient, or cheaper than phenotype based selection, and MAS currently provides the only viable method for gene pyramiding (Tester and Langridge, 2010).

Recent advances in understanding molecular and physiological mechanisms of abiotic stress responses, along with breakthroughs in molecular marker technologies, have enabled the dissection of the complex traits underlying stress tolerance in crop plants. Quantitative trait loci (QTLs) controlling different abiotic stress traits form the basis for a precise marker-assisted backcrossing (MABC) strategy to rapidly transfer tolerance loci into high-yielding, but stress-sensitive varieties (Thomson, *et al.* 2010b). The advances in genomics have paved the way for clear and reliable methods for MAS in plants: from QTL identification, NIL

development and fine-mapping to transferring the QTL into popular varieties using a precise marker assisted backcrossing (MABC) strategy (Mackill, 2006; Collard, *et al.* 2005; Collard and Mackill, 2008; Collard, *et al.* 2008b). MABC involves the manipulation of genomic regions involved in the expression of particular traits of interest through DNA markers, and combines the power of a conventional backcrossing program with the ability to differentiate parental chromosomal segments. The efficiency of a MABC program depends on a number of factors, including the size and reliability of the target QTL effect, the precision of the target gene/QTL fine-map, the rate of polymorphism when identifying background markers, as well as the cost, speed, and failure rate of the markers employed in each customized MABC system. So far, the greatest success in MABC for improving tolerance of biotic and abiotic stresses has been achieved with QTLs proven to provide high levels of tolerance in many different genetic backgrounds and environments (Collard and Mackill, 2008a; Collard, *et al.* 2008b). A good example in cereals is the introgression of *SUB1*, the major QTL for submergence tolerance, into several popular rice varieties (Xu, *et al.* 2006; Neeraja, *et al.* 2007; Iftekharauddaula, *et al.* 2011). The effectiveness of MAS in salinity tolerance can be observed in the newly developed durum wheat lines with Tm*HKT1;5-A* (a gene conferring Na^+ exclusion) introgressed by MAS, which showed a yield increase of 25% when compared to control wheat, at least when grown in saline fields (Munns, *et al.* 2012; James, *et al.* 2012). Moreover the successful introgression of *SUB1* QTL in to several mega varieties through MABC within shorter time such IR64-*Sub1*, BR11-*Sub1*, Samba Mahsuri-*Sub1* and Ciherang-*Sub1* created great impact of the submergence prone areas of India, Bangladesh, Nepal and some African nations (Xu, *et al.* 2006; Neeraja, *et al.* 2007; Iftekharauddaula, *et al.* 2011; Septiningsih, *et al.* 2009, 2015). It is now considered as second green revolution of the submergence affected countries. Because all these are mega varieties for the respective countries therefore the varieties are easily adopted by the farmers considering their wider adaptability and retaining all important traits (Septiningsih, *et al.* 2009 and 2015; Bailey-Serres, *et al.* 2010).

To date '*Saltol'* is the only major effect QTL for salinity tolerance, mapped in a RIL population between the highly tolerant landrace Pokkali and sensitive IR29. The designated *Saltol*, on chromosome 1, accounts for seedling shoot Na^+/K^+ ratio with phenotypic variance 64% reported first by Gregorio (1997). A follow-up study confirmed the effect of the *Saltol* to the shoot Na^+/K^+ ratio with a reduced phenotypic variance up to 43% using RILs populations (Bonilla, *et al.* 2002). Several other studies such as Niones (2004); Elahi, *et. al.*

(2004); Islam (2006a); Alam, *et. al.* (2011); Mohammadi-Nejad, *et. al.* (2008); Aliyu, *et. al.* (2011), Islam, *et al.* (2012) used either Pokkali and IR29 derived RIL or NIL populations reported the presence of *Saltol* for the trait shoot Na^+/K^+ ratio. While neither of these studies presented the percent variation explained for visual SES tolerance scores or survival, it was assumed that by controlling the key mechanism of Na^+/K^+ homeostasis under stress, *Saltol* is a major contributor to seedling stage tolerance. However, in a recent study by Thomson, *et al.* (2010a) confirmed that *Saltol* contributes to Na^+/K^+ homeostasis with phenotypic variance 27% and a decrease of 30% in the shoot Na^+/K^+ ratio in the IR29/Pokkali backcross lines, while the *Saltol* effect on SES scores in the QTL population and backcross lines was much smaller. The fact that *Saltol* affected the Na^+/K^+ ratio more than other traits supports the possibility that the sodium transporter *SKC1* (Os*HKT1;5* as in Platten, *et al.* 2006) may be the causal gene underlying the *Saltol* QTL. *SKC1* was found to encode a sodium transporter that helps control Na^+/K^+ homeostasis through unloading of Na^+ from the xylem identified from another tolerant *Indica* landrace Nona Bokra (Ren, *et al.* 2005), which has been suggested to function primarily in roots to reduce the amount of Na^+ ions that are transported to the leaves (Hauser and Horie, 2010). Recent studies showed variations in the *SKC1* sequence, which may result in changes in protein function affecting or enhancing its Na^+ total accumulation (Negrao, *et al*. 2013).

Considerable progress was made in mapping the major QTL *Saltol* thus providing opportunities to fast track the introgression of *Saltol* QTL in to popular rice varieties. Therefore, the present research focuses the possibility of using MABC for the introgression of *Saltol* in to two Bangladeshi mega rice varieties BR11 and BRRI dhan28 for *T. Aman* and *Boro* season respectively. Based on in-depth studies about *Saltol* by Thomson, *et al.* (2007, 2010a) the current MABC program was designed and carried out. The targeted donor introgression at the '*Saltol'* QTL region was initially assumed to be 1.30 Mb covering only large QTL peak region. However, later during the introgression in to BRRI dhan28, in addition to 1.3 Mb QTL peak, 2.4, 3.3 and 3.7 Mb sized donor segments were targeted for introgression of whole *Saltol* QTL regions which covers two annotated genes *SalT* and *HAK2* homolog downstream to the *Saltol* at the centromeric end. The following additional work was taken mainly to maximize the effects of *Saltol* and with the advantage of the presence of common Pokkali allele across whole *Saltol* QTL interval in the donor FL378 (Thomson, *et al.* 2007 and 2010a). Considering the importance of the flanking markers for recombinant selection in the reduction of the linkage drag, SSR markers bordering the QTL

but unlinked to the QTL were selected and used, so that the transfer of the donor segment was precisely monitored. In both MABC events the targeted lines having *Saltol* QTL with clean and/or minimum background donor introgression selected at third backcross generation and each of the backcross generation sufficient number of backcross progenies were genotyped by using several markers (an average of 7.25 and 8.92 markers for BR11 and BRRI dhan28 respectively) in the carrier and non-carrier chromosomes. Therefore excellent background recoveries (recurrent genome recovery to retain all recipient mega varietal traits) were obtained for both of the MABC events confirmed by SNP markers (see details for both MABC events in the result section).

Several researchers from Bangladesh (Rahman, 2010; Sarker, 2012) and Vietnam (Huyen, *et al.* 2012; Linh, *et al.* 2012 and Vu, *et al.* 2012) also started *Saltol* introgression work at IRRI by using a F_8 RIL FL478. This RIL lines is a sister line of FL378 used in the current work but had different Pokkali alleles with very small introgression confirmed by Thomson, *et al.* (2010a). All these 5 work reported a successful introgression of *Saltol* from FL478 in at least 3 backcrossing scheme except a report of Rahman (2010) of BR11, where introgression was completed by 2 backcrossing. Most of the reported work used G11 (9.3 Mb) or RM1287 (10.9 Mb) or RM10694 (11.0 Mb) as flanking at telomeric end and always used RM7075 (15.1 Mb) as flanking marker at centromeric end (Rahman, 2010; Huyen, *et al.* 2012; Linh, *et al.* 2012; Vu, *et al.* 2012; Sarker, 2012). The reported work used substantial numbers of backcross progenies at each backcross generation but none of them used POPMIN to calculate expected progenies to genotype at each backcross generation. While, this is recommended for use to get success in obtaining double recombinants of a defined or fixed backcross program within the certain level of probability of success (Collard, *et al.* 2008a). Again the reported work also showed successful recoveries of the background as much as 86-100% (Rahman, 2010; Huyen, *et al.* 2012; Linh, *et al.* 2012; Vu, *et al.* 2012; Sarker, 2012). But only two reports (Rahman, 2010; Sarker, 2012) used SNP markers to check potential background introgression and showed 96-99% SNP recovery.

The developed *Saltol* lines (BR11-*Saltol* and BRRI dhan28-*Saltol*) in the current work from both MABC events were evaluated for gain of tolerance at seedling stage in controlled stress condition. Two NILs (NIL52 and NIL1) and 12 phenotypically selected NIL derivative lines from BR11 background were shown to have increased tolerance over recipient BR11 in terms of SES score (leaf injury score) and survivability in several studies, but this

improvement was statistically insignificant (Table 4.1.6). NIL52 and its derivative lines performed equally well and better than NIL1 and its derivative lines in all the seedling tolerance studies. Therefore, an in-depth study with all NIL derivative lines was carried out at later generation. A notable reduction of seedling shoot Na^+/K^+ ratios was observed for all 4 lines from NIL52 compared to the recipient BR11 (around 19.35%). On the other hand only one line (BR11-150) from NIL1 showed a significant reduction of seedling shoot Na^+/K^+ ratios compared to BR11 (about 18.39%) (Fig. 4.1.18 and Table 4.1.10). Based on seedling shoot Na^+/K^+ ratios, the following five lines with reduced seedling shoot Na^+/K^+ ratios were found similar and very close to the standard high-yielding checks and donor for *T. Aman* season, respectively. For the BRRI dhan28-*Saltol* lines, significant variations were observed between different sized donor introgression lines and recipient for SES, survivability and shoot dry weights but insignificant variations were found for shoot Na^+/K^+ ratio and root dry weights (Table 4.2.6). Larger introgression (3.3-3.7 Mb) at the *Saltol* showed significantly better tolerance in terms of SES. Two NIL with 3.7 Mb (NIL807 and NIL683) and one NIL with3.3 Mb (NIL341) donor introgression showed significantly higher tolerance similar to the original donor Pokkali (Table 4.2.6). However, the result is not consistent when comparing other NILs consisting of similar sized introgression. For example several other NILs like NIL607, 434, 448, 657, 412 having 3.7 Mb, NIL318 having 3.3 Mb and NIL204 having 2.4 Mb showed improved tolerance but this was statistically insignificant (Table 4.2.6). The variability of tolerance among the tested lines of different sized donor introgressions is therefore mainly due to the background genotypic effects. The percent recurrent genome recovery of the tested lines varied between 86-97% indicating that the lines do not have a clean background (Table 4.2.3 and 4.2.4). NIL318 having the donor introgression 3.3 Mb showed significantly reduced Na^+/K^+ ratio compared to the recipient BRRI dhan28 (Fig. 4.2.22 and Table 4.2.8). The reduction of Na^+/K^+ ratio in the NIL318 was 31.71%. However, the reduced Na^+/K^+ ratio in negative QTL lines could also be due to the background genotype effects.

Results for seedling stage salinity tolerance from both MABC events revealed that, *Saltol* could be contributing to Na^+/K^+ homeostasis in seedling shoots rather than overall tolerance measured through SES or survivability. None of the preliminary mapping considered SES and survivability for the seedling stage tolerance, all were based on Na^+/K^+ homeostasis in seedling shoot as the key mechanism for controlling seedling tolerance (Gregorio, 1997; Bonilla, *et.al.* 2002; Niones, 2004). Thomson, *et. al.* (2010a) confirmed the smaller effect of

Saltol towards tolerance, when mapping *Saltol* again in 140 RIL populations with LOD 7.6 and R^2 27% encompasses 30% decrease in seedling shoot Na^+/K^+. Again Thomson, *et al.* (2010a) also showed that, *Saltol* explains much of the effect towards maintaining a healthy Na^+/K^+ ratio under salt stress and it does not by itself provide a high degree of salt tolerance, as measured by visual scores of leaf symptoms. The results from the present study with smaller donor introgression 1.3 Mb in the *Saltol* within a clean background (maximum RP recovery) from both background (4 lines from NIL52 and 1 line from NIL1 of BR11 background and NIL318 from BRRI dhan28) confirmed the fact that *Saltol* affected the Na^+/K^+ ratio more than other traits. The *Saltol* region is very important for salinity tolerance of rice; several studies frequently identify QTLs within this region, for example *SKC1* was found to encode a sodium transporter that helps control Na^+/K^+ homeostasis through unloading of Na^+ from xylem (Ren, *et.al.* 2005) but this was detected in another Indian salt tolerant rice, Nona Bokra. Again a recent study identified a QTL for maintaining Na^+/K^+ ratio on the same region of *Saltol* (11.10-14.20 Mb) from an upland japonica variety Moroborekan (Haq, *et. al.* 2010) suggesting that the *Saltol* region may have functional significance for salt tolerance across rice germplasms. Realizing the contribution of sodium transporter for sodium exclusion and the similarity with wheat *HKT1;5* (Platten, *et. al.* 2006), a diversity study of *HKT1;5* was carried out in *Oryza sativa* L. and *O. glaberrima* Steud. and a strong association between leaf Na^+ concentration and *HKT1;5* allele was identified (Platten, *et.al.* 2013).

Salt tolerance indices, such as the visual score of damage given by the SES and the ST given as a proxy for biomass reduction under salt stress, are often used to assess plant salinity tolerance. However, these indices are not strongly correlated with each other (Pires, *et al.* 2015). This was not unexpected since ST at seedling stage only accounts for the effect of salt stress on biomass, while SES considers survival/death of plants, level of leaf chlorosis and curving, and visual difference in biomass between plants in control and salt stress conditions (Gregorio, *et al.* 1997). Kanawapee, *et al.* (2012) observed a decrease in K^+ content combined with an increase in Na^+ content with growth in saline conditions. They suggested that the K^+/Na^+ ratio is the most important mechanism controlling salinity tolerance in rice. Pires, *et al.* (2015) observed that K^+ content was not significantly affected by salt stress. Garcia, *et al.* (1997a) concluded that in rice K^+/Na^+ is less relevant as a trait than the individual content of Na^+ and K^+, contrary to what might happen in wheat. Their conclusions are based on the fact that in rice Na^+ uptake is mechanistically different from K^+ uptake

(Garcia, *et al.* 1997a). This observation is particularly important because several studies assume that a low K^+/Na^+ ratio is the most important goal in terms of ion concentrations in rice salinity tolerance and emphasize this value (Theerakulpisut, *et al.* 2011, Kanawapee, *et al.* 2012). Also, the content of Na^+ and K^+, independently assessed, is less affected by measurement errors, since a ratio always combines the errors associated with the measurement of the variable in the denominator and with the measurement of the variable in the numerator (Pires, *et al.* 2015). An increase in Na^+ content in the plant accompanied by growth reduction has been recurrently observed (see Parida and Das, 2005), and it is known that growth reduction occurs both as consequence of the deleterious effect of Na^+ in the cells and as a general stress response (see Roy, *et al.* 2014). Additionally, the Na^+ content that negatively affects one genotype may be different from that affecting another genotype. In fact, Yeo and Flowers (1983) showed that the Na^+ content that resulted in a chlorophyll content loss of 50% differed between nine rice genotypes, and that leaf chlorosis occurred at different levels of Na^+ content in leaves. Pires, *et al.* (2015) observed a high variability of physiologic response to salt stress between genotypes with similar levels of Na^+ accumulated in leaves, and this is probably caused by the existence of other mechanisms for salt tolerance (Munns and Tester, 2008; Wang, *et al.* 2012). This suggests that, rice exhibits all three mechanisms of response to salinity stress previously described by Munns and Tester (2008), and no one mechanism is preferentially used, therefore different genotypes may be needed to study each of the different mechanisms of plant salinity tolerance. A good example of this observable fact is *Saltol,* which explains much of the effect towards maintaining a healthy Na^+/K^+ ratio under salt stress and it does not by itself provide a high degree of salt tolerance, as measured by visual scores of leaf symptoms.

Interestingly when the donor introgression at the *Saltol* was increased from 1.3>2.4>3.3>3.7 Mb in the BRRI dhan28 background, the level of tolerance for larger introgressions in terms of SES was significantly improved (Table 4.2.6). Eleven NILs having *Saltol* region with 4 different size (3.7, 3.3, 2.4 and 1.3 Mb) showed significantly better SES and survivability compared to the recipient BRRI dhan28 (Table 4.2.6). However, while comparing Na^+/K^+ ratio of all NILs having *Saltol* region with 4 different sizes segment then the scenario was changed remarkably. Only NIL318 having introgression 3.3 Mb showed significantly low Na^+/K^+ ratio compared to the recipient (Table 4.2.6). Therefore, correlation between Na^+/K^+ ratio and SES or survivability was found weak and insignificant (Table 4.2.7). This is in contrast to the results obtained in BR11-*Saltol* indicating additional determinants could

restrain the sole effects of *Saltol* in terms of Na^+/K^+ homeostasis but improve overall tolerance in terms of SES due to increase in size at the *Saltol.* Significant improvement of tolerance in terms of SES in the larger introgressions could be due to the effects of the downstream genes i.e. *SalT* and *HAK2* homolog (personal communication M.J. Thomson and J.D. Platten). Os*Sal*T is possibly associated with the production of compatible solutes (Negrão, *et al.* 2013). Two negative *Saltol* (without *Saltol*) lines NIL618 and NIL845 were also tested and showed a higher level of tolerance and a significantly lower level of Na^+/K^+ ratio in the shoots of the NIL618 (Table 4.2.6). The tolerance of NIL618 could be due to the effects of introgressed background loci in the carrier and non-carrier chromosomes, since the donor is a F_8 RIL from IR29/Pokkali, so literally the RIL has similar percentage of genome shared from both parents and the tolerance of the donor FL378 could have arisen not only due to *Saltol* loci but the combinations of genes throughout the genome. Several NILs without a Pokkali allele at *Saltol* still exhibited higher tolerance than the IR29 check (Thomson, *et. al.* 2010a), which suggests that additional Pokkali-derived QTLs are involved in providing tolerance. Similar results were also seen in a recent study using IR29/Pokkali backcross lines, where non-*Saltol* lines showed an averaged SES score of 6.3 compared to IR29 at SES 9 under salt stress of 18 dSm^{-1} (Alam, *et. al.* 2011).

Tolerance during seedling stage seems to correlate poorly with tolerance during reproduction, suggesting different sets of traits are probably involved at each stage (Moradi, *et al.* 2003). Reproductive stage is another developmental stage when rice is sensitive to salinity stress, more specifically this stage is the booting stage (7-10 days before and after booting stage) (Singh and Flowers, 2010). The reproductive stage is crucial as it ultimately determines grain yield, but the importance of the seedling stage cannot be underestimated as it determines crop establishment. There are few studies that address the effects of salinity on yield. Most research has been limited to the seedling or early vegetative stages or only reports parameters such as fresh or dry weight although the ultimate aim has been to increase grain yield with limited resources (Lee, *et al.* 2006 and 2007; Morsy, *et al.* 2007; Tajbakhsh, *et al.* 2006; Chen, *et al.* 2007; Moradi and Ismail, 2007; Singh, *et al.* 2007a; Cheng, *et al.* 2008; Jain, *et al.* 2008; Kanneganti and Gupta, 2008; Zang, *et al.* 2008). Hence, to know the response of the rice plant to salinity as a whole, it is imperative that the effects be observed in all the various stages of its development, that is at early seedling, vegetative and reproductive stages (Gregorio, *et al.* 1997). *Saltol* shows its affect through seedling stage

salinity tolerance by maintaining good Na^+/K^+ ratio in the shoots. However the developed lines were also tested at reproductive stage for their tolerance and yield in pursuance of further advancement of the lines for release as high yielding salinity tolerant variety. Under a moderate stress situation (EC 6 dSm^{-1}) for the whole growth period, NIL52 was able to maintain good yield even better than tolerant check BRRI dhan47 in *T. Aman* season, whereas NIL1 performed poorer than the recipient BR11 (Table 4.1.7). The *Saltol* lines of BRRI dhan28 background were also tested in moderate salinity stress (EC 8 dSm^{-1}) under controlled condition for whole growth period. Out of 7 lines tested, 4 line showed increased yield compared to BRRI dhan28 but 3 lines perform poorer than recipient (Table 4.2.10). Out of the best performing 4 lines, 3 of them showed significantly higher photosynthesis rate in the flag leaf and were able to maintain good stomatal conductance at reproductive stage (Table 4.2.9 and Fig. 4.2.23). From both MABC event, the NILs perform better at seedling stage salinity tolerance also perform superiorly for yield at reproductive stage indicating possibility of the effects of *Saltol* could be extended for reproductive stage i.e. the *Saltol* loci could affect pleiotropically. Kumar, *et al.* (2015) recently reported an association between *Saltol* and reproductive stage tolerance through Na^+/K^+ while studying 220 rice accessions in a GWAS study.

Yield and quality traits are the main criteria for wider adoption of any mega variety, so retaining some agronomic characteristics in addition to the added traits could be an advantage in any marker assisted backcross conversion program. For the current MABC conversion of salt tolerant BR11 and BRRI dhan28, all *Saltol* introgression lines were evaluated in non-saline field condition for yield and quality traits. Out of 12 lines from BR11 background, 8 lines yielded better than the recipient with an average yield advantage 0.64 tha^{-1}, ranging from 0.12 to 1.11 tha^{-1} (Table 4.1.11). But 4 lines (BR11-52-67, BR11-7, BR11-150 and BR11-52-55) out-yielded (with an average yield advantage ~1.0 tha^{-1}) another high-yielding salt tolerant variety BRRI dhan54 (Table 4.1.11). Grain quality traits of the *Saltol* lines from BR11 genotype were successfully retained and in some cases, there were improvement for most of the lines especially with respect to amylose and protein content, L/B ratio, milling outturn and head rice yield. All these improvements could be regarded as positive introgression from the donor (Table 4.1.12). In the BRRI dhan28 MABC work, 7 selected NILs were evaluated in non-saline field condition for yield and quality traits. Only 2 lines (NIL607 and NIL618) out of 7 yielded better than the recipient with yield advantage ranging 0.47 to 0.65 tha^{-1} but rest of the NILs performed same as the

recipient or better than donor FL378 (Table 4.2.11). Flag leaf gas exchange parameters showed variation among the tested *Saltol* lines and varieties, but this variation was statistically insignificant (Fig. 4.2.24). For grain quality traits, all the lines showed similar qualities compared to the recipient BRRI dhan28, except for amylose and protein content, Only 2 NILs (NIL657 and NIL683) could retain the original qualities (Table 4.2.12).

Saltol lines developed from both MABC events were again evaluated in actual saline field condition for yield performance and phenotypic acceptability in respective growing seasons. The salinity level of crop standing water ranged from 1.1-2.2 dSm^{-1} throughout the growing season indicating a low salinity level. Significant yield advantage was observed for 10 lines out of 12 BR11-*Saltol* lines tested. The yield advantage ranged from 0.2-1.2 tha^{-1} compared to the recipient BR11 with an average 0.55 tha^{-1} (Table 4.1.13). Phenotypic acceptability among 10 best yielded lines ranged from 3-4 indicating better phenotypic appearance and acceptable qualities for suitability of release as a variety. However, growth duration and plant height of all *Saltol* lines were very similar to the recipient BR11 (Table 4.1.13). Six BRRI dhan28-*Saltol* lines were also evaluated in saline field condition for observing yield performance and phenotypic acceptability. The salinity level of crop standing water was slightly higher than previous trial ranging from 2.0-3.2 dSm^{-1}. Significant variation for grain yield was observed among the tested lines and genotypes. Two lines (NIL607 and NIL434) yielded significantly higher than recipient BRRI dhan28 and even higher than salt tolerant HYV check BRRI dhan47 with similar phenotypic acceptability score 4 (Table 4.2.13). The following lines (NIL607 and NIL434) were found moderately tolerant at seedling stage having SES 4.50 and 4.54 respectively (Table 4.2.6). However, the NIL434 also perform superiorly at reproductive stage under a moderate stress level (8 dSm^{-1}) in controlled Net house condition compared to rest of the lines and parents (Table 4.2.10). Other traits like-growth duration and plant heights of all the lines were similar to the recipient BRRI dhan28. There appeared to be some differences among the tested BR11 and BRRI dhan28 derived *Saltol* lines due to variable amount of introgression from the donor parent identified by SNP markers throughout the genomes. Consistency of performances for seedling stage salinity tolerance and superior yield potentials of all NIL52 and few NIL1 lines from BR11 background and few NILs (NIL607, NIL683 and NIL434) of largest donor introgression from BRRI dhan28 background might be due to some fixed donor alleles at the background in addition to the *Saltol*. Iftekharuddaula, *et. al.* (2011) reported that, a fixed donor allele at

the background might contribute to consistent and excellent submergence tolerance and yield of a precision inbred line (PIL).

Multi-environments testing of genotypes provides an opportunity to the plant breeders to identify the adaptability of a genotype to a particular environment and also stability of the genotype over different environments. Stability in grain yield is one of the most desirable traits of a genotype to be released as a variety for commercial cultivation. The developed varieties should adapt to a wide range of target environments. This is the eventual goal of plant breeders. Hence, pattern of responses of genotypes is studied by the plant breeders for testing genotypes in different environments to assess the genotype by environment (G × E) interactions. To estimate the level of interaction of genotypes to environments and to eliminate the unexplainable and extraneous variability contained in the data, several statistical models have been developed to describe G × E interaction and to measure the stability of genotypes. *Saltol* introgression in to BR11 was started first and completed well in advance than BRRI dhan28. So, there was enough time to test the BR11-*Saltol* lines in different environments. All 12 BR11-*Saltol* lines were evaluated in 3 different locations (2 saline and 1 non-saline) across growing seasons (3 for *T. Aman* and 1 for *Boro*) comprising a total of 4 environments. Considering the importance of G × E interaction and stability analysis, the grain yield data of BR11-*Saltol* lines obtained from multi-environment trials were subjected to analyses by a series of models to measure G× E interactions and stability.

Grain yield data of BR11-*Saltol* lines obtained from multi-environment trials were first analyzed by a series of descriptive and explorative models i.e. Finlay–Wilkinson modified joint regression model (Finlay and Wilkinson, 1963), Additive Main Effects and Multiplicative Interaction (AMMI) model (Gollob, 1968; Mandel, 1969; Gabriel, 1978; Gauch, 1988; van Eeuwijk, 1995), Genotypic main effects and GEI model (GGE model) (Yan, *et al.* 2000). Significant G × E interaction was identified by the AMMI model through a principal components analysis on the GEI (Table 4.3.3), while Finlay-Wilkinson model only able to detect significant variation for G and E main-effect (Table 4.3.2). Therefore, AMMI model was found suitable to explain the G × E interactions for this multi-environment trial data. ANOVA for AMMI model showed that the grain yield was significantly affected by G, E and GEI. However, for GEI only the first axis (IPCA1) was found significant and explained most of the variations (63.80%) (Table 4.3.3 and Fig. 4.3.4). In AMMI biplot, the first axis (PC1) clearly separated the 4 environments in to two groups

where the 3 *T. Aman* environments grouped together and *Boro* environment made a separate group. But Gazipur was identified as high performing environment for both *T. Aman* and *Boro* season. By combining genotypic main effects to the GEI described by GGE model more variations were explained through the first axis (70.91%). In the GGE biplot, 2 mega-environments were identified, where Gazipur-*Boro* and Satkhira-*T. Aman* grouped together and Koyra-*T. Aman* grouped with Gazipur-*T. Aman* (Fig. 4.3.5). All *Saltol* lines concentrated around origin indicating their stable performance across environments compared to the recipient BR11 and donor FL378. Consistency of performance of the genotypes across environments is the stability of a genotype. Based on mean performances and a number of stability parameters (Table 4.3.4) three BR11-*Saltol* lines (BR11-52-124, BR11-52-67 and BR11-7) could be selected and recommended for release and cultivation in high performing environments/good quality environments. Whereas BR11-65 could be selected and recommended for release and cultivation throughout the country because of its average performances and stability of different environments. The selection of above 4 lines is solely based on their average performance (average grain yield) over 4 environments not for level of tolerances at seedling or reproductive stages respectively. The selection of first 3 lines (BR11-52-124, BR11-52-67 and BR11-7) for high performing environments or good quality environment is mainly due to their consistent better performance in good quality environments, while performance of these lines in poor environments could be poorer than average performance, therefore these lines only recommends for good quality or high performing environments. Again, the line BR11-65, consistently perform close to average over all types of environments i.e. the performance (yield) is not much varied across good quality and poor environments, therefore due to its stable and consistent performance the line recommends for throughout the country.

With the success of *SUB1A* introgressionin to several mega varieties and its benefits to the farmers of Asian and African regions, a comprehensive effort was taken at IRRI for introgression of major salinity tolerance QTL *Saltol* in to several mega varieties of Bangladesh and Vietnam (Ismail, A.M. personal communication). Three mega varieties were taken from Bangladesh i.e. BR11 and BRRI dhan28 (Rahman, 2010) and BRRI dhan29 (Sarker, 2012) and 3 varieties from Vietnam i.e. BT7 (Linh, *et al.* 2012), Bachthom7 (Vu, *et al.* 2012) and AS996 (Huyen, *et al.* 2012). All these works have been reported to be successfully completed and the improved lines are now under field trial. Most of the report showed a full recovery of elite background at the 3rd backcross generation in terms of

background recovery percentage and important agronomic traits (Rahman, 2010; Sarker, 2012; Linh, *et al.* 2012; Vu, *et al.* 2012; Huyen, *et al.* 2012). However, only 3 reports (Rahman, 2010; Sarker, 2012; Huyen, *et al.* 2012) showed a significant improvement of salinity tolerance only in terms of SES but not for Na^+/K^+ ratio at seedling stage. The current work however shows improvement of lines in terms of lower Na^+/K^+ under saline stress. The reasons for these workers getting significant tolerance at seedling stage could be due to use of FL478 as donor. The reason for using FL478 as donor for most of the MABC events is mainly to its better tolerance than FL378; however donor line possesses a different *Saltol* allele (Thomson, *et al.* 2010a).

The present work clearly demonstrated the entire procedures of a typical marker-assisted conversion of mega varieties in to its salt tolerant version with added traits. This includes parental selection, polymorphism survey, validation of linked and un-linked markers, marker-assisted introgression of QTL, background recovery, checking potential background introgression, phenotypic gain at seedling and at reproductive stage for target trait, phenotypic performance for yield and similarity of grain quality traits, multi-environment testing for genotype × environment interactions and recommendation for release of top rank lines for farmers use of getting ultimate benefits.

In conclusion the key points of this study are: *Saltol* QTL was successfully introgressed in to BR11 and BRRI dhan28 background with 97-99% SSR/InDel marker and 94-97% SNP marker of respective recipient genome recovery through a three successive backcross breeding program. Introgressed *Saltol* QTL with a size of 1.3 Mb and 3.3 Mb could reduce seedling shoot Na^+/K^+ ratio up to about 20% and 32% in to BR11 and BRRI dhan28 background respectively. Large segment at the *Saltol* QTL region up to 3.7 Mb could enhance overall salinity tolerance in terms of SES probably due to inclusion of *SalT* and *HAK2* homolog gene at the downstream of *Saltol*. Out of 12 BR11-*Saltol* lines only five lines i.e. BR11-52-124, BR11-52-55, BR11-52-145, BR11-52-67 and BR11-150 was selected based on better reduction of seedling shoot Na^+/K^+ ratio. Out of 13 BRRI dhan28-*Saltol* NILs 12 lines i.e. NIL807, NIL683, NIL341, NIL607, NIL434, NIL204, NIL618, NIL448, NIL318, NIL657, NIL188 and NIL412 was selected based on better tolerance in terms of SES. Finally a total of 13 lines/NILs (7 from BR11-*Saltol* and 6 from BRRI dhan28-*Saltol*) were selected based on tolerance at seedling stage and also for yield at field condition. Agronomic parameters and grain physico-chemical properties for most of the

developed lines/NILs from both MABC events showed successful recovery of the BR11 and BRRI dhan28 traits with an improvement of yield potential in both saline and non-saline field conditions. NIL52 of BR11-*Saltol* and 3 NILs (NIL434, NIL657, and NIL683) of BRRI dhan28-*Saltol* performed superiorly in a moderate salinity stress (6-8 dSm^{-1}) condition during reproductive stage in controlled Net house condition. G by E interactions and stability analyses identified 3 BR11-*Saltol* lines such as BR11-52-124, BR11-52-67 and BR11-7 performed superiorly in best performing environments; therefore these could be recommended for cultivation in better environments, whereas a line BR11-65 could be recommended for cultivation all over the country due to its stable and average performances across different environment. Three NILs (NIL434, NIL607 and NIL683) of BRRI dhan28-*Saltol* yielded superiorly compared to the recipient while evaluated in saline and non-saline environments could be recommended for further evaluation.

The MABC approaches described in the present investigation adapted to successfully introgress *Saltol* in to BR11 and BRRI dhan28 could be utilized for introgression in the other mega varieties with a minimal segment and within a short time frame. The improved lines have desirable grain physico-chemical and cooking quality characteristics, with the added salt tolerance traits and improved yield at par with the mega variety BR11 and BRRI dhan28. BR11-*Saltol* lines have already been evaluated in different environments and 4 lines were selected and recommended for cultivation and considered for variety testing trials for further processing towards release. BRRI dhan28-*Saltol* lines will now be evaluated under multi-location trials for evaluating their stability and performance across locations for possible entry in to the variety testing and release process. Moreover, all the lines from both events were also considered for evaluation in favorable environments due to their superior yield potentials over existing recipients' i.e. BR11 and BRRI dhan28.

Chapter 6:

Conclusions and Recommendations

Chapter 6
Conclusions and Recommendations

The aim of this study was to develop salt tolerant versions of two Bangladeshi mega rice varieties for suitable cultivation in the saline coastal environments of Bangladesh. The target of this process was to retain all favorable and important traits of these mega varieties while adding the major salinity tolerance QTL *Saltol* through the molecular breeding approach i.e. Marker Assisted Backcrossing (MABC). Considering above mentioned aims and targets, the present study clearly demonstrated the whole process of production through characterizing a series of backcross breeding lines by foreground, recombinant and background markers. This was followed by obtaining the desired recovery of recipient genomes. The selected fixed lines were also characterized phenotypically for recovery of mega variety traits in addition to salinity tolerance at the seedling stage as well as yield potential at field condition. Based on the completed research, the following conclusions can be drawn:

1) I successfully introgressed *Saltol* QTL in to BR11 and BRRI dhan28 with 95-97% SSR/InDel marker and 94-97% SNP marker recovery of the respective recipient genome through a three successive backcross breeding program.
2) Around 20% and 32% reduction of Na^+/K^+ ratio was achieved due to the introgression of *Saltol* with a size of 1.3 Mb for BR11 and 3.3 Mb for BRRI dhan28 background respectively.
3) Larger introgression (3.7 Mb) at the *Saltol* QTL could enhance overall salinity tolerance in terms of SES, probably due to inclusion of *SalT* and *HAK2* homolog gene at the downstream of *Saltol*.
4) A total of 13 lines/NILs (seven BR11-*Saltol* i.e. BR11-52-124, BR11-52-55, BR11-52-145, BR11-52-67, BR11-150, BR11-65, BR11-7 and six BRRI dhan28-*Saltol* i.e. NIL434, NIL412, NIL607, NIL618, NIL657, NIL683) finally selected based on tolerance at seedling stage and also for yield at field condition.
5) Agronomic parameters and grain physico-chemical properties for most of the developed lines/NILs from both MABC events showed successful recovery of the BR11 and BRRI dhan28 traits with an improvement of yield potential in both saline and non-saline field conditions.

6) NIL52 of BR11-*Saltol* and NIL434, NIL657, NIL683 of BRRI dhan28-*Saltol* showed superior performance at moderate salinity stress (6-8 dSm^{-1}) condition during reproductive stage in controlled Net house condition.
7) G by E interactions and stability analyses identified 3 BR11-*Saltol* lines such as BR11-52-124, BR11-52-67 and BR11-7 whose performance was superior in best performing environments; therefore these could be recommended for cultivation in better environments, whereas a line BR11-65 could be recommended for cultivation all over the country due to its stable and average performances across different environment.
8) Three NILs (NIL434, NIL607 and NIL683) of BRRI dhan28-*Saltol* showed superior yields compared to the recipient when evaluated in saline and non-saline environments and could be recommended for further evaluation.

Future challenges and recommendations

The need for increased food production is more acute now than ever before due to the rapidly increasing population and the high pressure being exerted on limited natural resources. Rice, being the most important food for consumption, needs to see a dramatic increase in production. As expanding the land area is not an option for increased rice production in most areas, new options should be explored to help increase rice yield potential. There is a need to improve the genetic yield potential for both low and high input cultivation environments.

The complex mechanism underlying salt tolerance as well as the complex nature of salt stress itself and the wide range of plant responses make the trait inexplicable. Identification of molecular markers associated with salt stress tolerance genes or QTL conferring tolerance to high salinity has been demonstrated. Significant breakthroughs have been made on the mechanism and control of salinity stress tolerance in rice, but large gaps about our understanding in this field remained to be explored. Thus, further investigations are needed to sufficiently explain the underlying mechanisms of protection of rice under salt stress condition. Identification of the role of different components providing salt stress and the cross talks between these components will be a future challenge to disentangle the complete genome network of rice providing salinity tolerance. An emerging scope to identify novel

cis-acting elements and elements acting in tandem may possibly lead to unraveling the complex web pattern for salinity signaling. Development of plants with improved tolerance to salt remains a big challenge despite the significant progress made in the genomics of salt tolerance in rice. Rice exhibits cellular ion homeostasis and enormous genetic variability in its sensitivity to salt stress. The *indica* varieties such as Pokkali and Nona Bokra have higher endogenous ABA level during osmotic shock and are classified as highly salt tolerant ecotypes. To maximize the productivity of rice under saline soils there is an urgent need to look for sources of genetic variation that can be used for developing new cultivars with greater yield potential and stability over seasons and eco-geographic locations.

The recent advances in genomics, the development of highly polymorphic and informative molecular markers such as single-nucleotide polymorphisms (SNPs), and high-throughput genotyping capabilities such as genotyping by sequencing (GBS), it is expected that the use of markers in large breeding populations will be streamlined which in turn will facilitate employing MAS for crop breeding for improved salinity tolerance. Moreover, as more molecular markers and saturated maps are becoming available and larger numbers of major and minor QTLs are identified for salinity tolerance, it may be more effective to improve crop salinity tolerance via genomic selection that is selection solely based on the genotypes of all markers associated with salinity tolerance.

Chapter 7: References

Chapter 7
References

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Chapter 8: Appendices

Chapter 8
Appendix-I

Preparation of Yoshida culture solution for screening at seedling stage

8.1.1 Preparation of nutrient solution

According to Yoshida, *et al.* (1976), the nutrient solution was made in two separate steps i.e. preparation of stock solution and preparation of culture/working solution. First all the listed chemicals required for nutrient solution (Table 8.1.1) was dissolved with distilled water as 2 L volume (the volume of stock solution 2 L is entirely depends how much solution required for that experiment) then the culture solution was prepared by mixing specific amount of all the macro and micro stock solution by getting required concentration of all the elements.

8.1.2 Preparation of stock solutions (Macronutrient and Micronutrient) for using in the nutrient solution

Nutrient solution is essential to avoid nutrient deficiency of the growing seedlings in controlled condition. Fresh stock solution was prepared in every two weeks. Table 8.1.1 gives the name, grade and quantity of macro and micro nutrients for preparing 2 liters stock solution. For the macronutrient stock solution, the required amount of reagents were weighed and transferred to a 1000-ml beaker and mixed with 750 ml distilled water. The mixture was poured to the 2 liters volumetric flask and the volume was made to 2 liters by adding distilled water. The mixture was stirred for 15 min using a magnetic stirrer then transferred to stock solution bottle. Using the same procedure all 5 (N, K, P, Ca and Mg) macro nutrient solution were prepared and stored in separate bottled in dry, cool and dark condition.

The preparation of micro nutrient solution was quite different where all the reagents first weighed and then dissolved in separate beaker with minimum amount of distilled water and then all were mixed in the same bottled and stored in the condition like macronutrient. Each reagent of the micronutrient was dissolved separately in 50 ml distilled water except Ferric chloride. Ferric chloride was dissolved in 100 ml distilled water. All the solutions were mixed together and make volume up to 1 liter distilled water and poured in a 2 liters volumetric flask. The ferric chloride solution was added to the mixture just before the

addition of Citric acid and was stirred the mixture for 15 min using a magnetic stirrer. Finally 100 ml sulfuric acid was added to the mixture and made the volume to 2 liters by adding distilled water. This was stirred for another 10 min and stored in a dark glass bottle. The final color of this solution was yellowish brown.

Table 8.1.1: Macro and micro nutrients, grade and quantity for the preparation of stock solution

Elements	**Reagent (Analytical Reagent grade)**	**Preparation (g/2L soln.)**
	Macronutrient	
N	Ammonium Nitrate (NH_4NO_3)	182.80
P	Sodium Phosphate, monobasic monohydrate ($NaH_2PO_4.H_2O$)	80.60
K	Potassium sulphate (K_2SO_4)	142.80
Ca	Calcium chloride ($CaCl_2.2H_2O$)	177.20
Mg	Magnesium sulphate ($MgSO_4.7H_2O$)	648.00
	Micronutrient	
Mn	Manganous chloride, 4-hydrate ($MnCl_3.4H_2O$)	3.000
Mo	Ammonium molybdate,4-hydrate [$(NH_4)6Mo_7O_{24}.4H_2O$]	0.148
Zn	Zinc sulphate.7-hydrate($ZnSO_4.7H_2O$)	0.070
B	Boric acid (H_3BO_3)	1.868
Cu	Cupric sulphate,5-hydrate ($CuSO_4.7H_2O$)	0.062
Fe	Ferric chloride,6-hydrate ($FeCl_3.6H_2O$)	15.40
	Citric acid, monohydrate ($C_6H_8O_7.H_2O$)	23.80

All mix in volumetric flask then add 100 ml conc. H_2SO_4 and then make volume with distilled H_2O

Source: adapted from Yoshida, *et al.* (1976)

8.1.3 Preparation of culture solution

Nutrient culture solution was made in a big plastic container. For preparing 360 liters culture solution, 450 ml of each macronutrient and 450 ml of micronutrient from the stock solution were mixed with 360 liters distilled water (Table 8.1.2). The pH meter was used to measure pH of the solution. The desired pH range was 5.0-5.5.

Table 8.1.2: Element composition of nutrient solution and concentration of each element

Elements	Reagent	Stock solution (ml/360L)	Concentration of element in culture solution (ppm)
	Macronutrients		
N	NH_4NO_3	450	40
P	$NaH_2PO_4.2H_2O$	450	10
K	K_2SO_4	450	40
Ca	$CaCl_2.2H_2O$	450	40
Mg	$MgSO_4.7H_2O$	450	40
	Micronutrients		
Mn	$MnCl_2.4H_2O$		0.50
Mo	$(NH_4)_6.Mo_7O_{24}.4H_2O$		0.05
Zn	$ZnSO_4.7H_2O$	450	0.01
B	H_3BO_3		0.20
Cu	$CuSO_4.5H_2O$		0.01
Fe	$FeCl_3.6H_2O$		2.00

Source: adapted from Yoshida, *et al.* (1976)

Appendix-II

List of SSR, STS, Gene-based and InDel markers used for marker assisted introgression of Saltol QTL in to BR11 and BRRI dhan28

Table 8.2.1: List of validated markers across *Saltol* region (Adapted from Ferdousi, 2008).

Sl. No.	Marker name	Marker type	Position (Mb)	F-ratio	P-value
1	RM3627	SSR	10.31	2.05	0.13
2	RM1287	SSR	10.90	4.37	0.01
3	AP3206-418	BAC-Clone	11.23	1.25	0.28
4	CP06224	EST	12.07	3.68	0.05
5	RM493	SSR	12.20	5.31	0.00
6	MetSyn U NC	STS	12.34	0.02	0.98
7	RM10793	SSR	12.50	3.27	0.03

Table 8.2.2: List of foreground selection markers used in the MABC of BR11-*Saltol.*

Sl. No.	Marker name	Marker type	Chromosome	Position (Mb)
1	RM1287	SSR	1	10.90
2	RM3412	SSR	1	11.50
3	RM493	SSR	1	12.20

Table 8.2.3: List of recombinant selection markers used in the MABC of BR11-*Saltol.*

Sl. No.	Marker name	Marker type	Chromosome	Position (Mb)
1	RM3627	SSR	1	10.31
2	RM10825	SSR	1	13.30
3	RM10864	SSR	1	14.20
4	RM562	SSR	1	14.60
5	RM7075	SSR	1	15.10

Table 8.2.4: List of recombinant and background selection markers used in the MABC of BR11-*Saltol.*

Sl. No.	Marker Name	Marker type	Chromosome	Physical Position (Mb)	Used in selection
1	RM14	SSR	1	4.16	BGS
2	RM490	SSR	1	6.70	BGS
3	RM243	SSR	1	8.00	BGS
4	RM3627	SSR	1	10.30	RBS & BGS
5	RM10812	SSR	1	12.91	RBS & BGS

6	RM10825	SSR	1	13.30	RBS & BGS
7	RM10864	SSR	1	14.20	BGS
8	RM562	SSR	1	14.60	BGS
9	RM7075	SSR	1	15.10	BGS
10	RM09	SSR	1	23.20	BGS
11	RM05	SSR	1	24.29	BGS
12	RM7643	SSR	1	31.10	BGS
13	RM11824	SSR	1	35.23	BGS
14	RM154	SSR	2	1.10	BGS
15	RM12476	SSR	2	2.79	BGS
16	RM12544	SSR	2	4.05	BGS
17	RM211	SSR	2	4.32	BGS
18	RM3858	SSR	2	19.21	BGS
19	RM13628	SSR	2	25.10	BGS
20	RM13646	SSR	2	25.45	BGS
21	RM5404	SSR	2	33.70	BGS
22	RM545	SSR	3	4.92	BGS
23	RM14800	SSR	3	10.48	BGS
24	RM3297	SSR	3	13.40	BGS
25	RM15319	SSR	3	20.24	BGS
26	RM232	SSR	3	23.01	BGS
27	RM5626	SSR	3	24.70	BGS
28	RM3867	SSR	3	31.50	BGS
29	RM16247	SSR	3	36.15	BGS
30	RM16314	SSR	4	1.26	BGS
31	RM16328	SSR	4	1.60	BGS
32	RM6659	SSR	4	6.50	BGS
33	RM16577	SSR	4	10.61	BGS
34	RM280	SSR	4	35.21	BGS
35	RM413	SSR	5	2.18	BGS
36	RM17954	SSR	5	3.50	BGS
37	RM1127	SSR	5	15.87	BGS
38	RM3838	SSR	5	16.42	BGS
39	RM8060	SSR	6	0.72	BGS
40	RM204	SSR	6	3.16	BGS
41	RM253	SSR	6	5.40	BGS
42	RM314	SSR	6	10.08	BGS
43	RM20224	SSR	6	20.60	BGS
44	RM1340	SSR	6	22.96	BGS
45	RM454	SSR	6	24.80	BGS
46	RM20417	SSR	6	25.16	BGS
47	RM180	SSR	7	5.73	BGS

48	RM125	SSR	7	7.44	BGS
49	RM3583	SSR	7	8.07	BGS
50	RM21516	SSR	7	15.80	BGS
51	RM3753	SSR	7	23.60	BGS
52	RM18	SSR	7	27.12	BGS
53	RM248	SSR	7	29.28	BGS
54	RM152	SSR	8	0.60	BGS
55	RM6356	SSR	8	1.55	BGS
56	RM310	SSR	8	5.10	BGS
57	RM3215	SSR	8	8.50	BGS
58	RM72	SSR	8	18.27	BGS
59	RM7285	SSR	8	18.75	BGS
60	RM149	SSR	8	24.71	BGS
61	RM23409	SSR	8	25.08	BGS
62	RM256	SSR	8	30.40	BGS
63	RM219	SSR	9	7.80	BGS
64	RM6051	SSR	9	12.78	BGS
65	R9M30	InDel	9	14.90	BGS
66	RM7175	SSR	9	16.81	BGS
67	RM1013	SSR	9	22.50	BGS
68	RM222	SSR	10	2.60	BGS
69	RM25102	SSR	10	5.40	BGS
70	RM5806	SSR	10	14.04	BGS
71	RM171	SSR	10	18.79	BGS
72	RM228	SSR	10	21.80	BGS
73	RM26063	SSR	11	2.20	BGS
74	RM26231	SSR	11	5.30	BGS
75	RM26255	SSR	11	5.84	BGS
76	RM3137	SSR	11	6.10	BGS
77	RM26652	SSR	11	15.00	BGS
78	RM21	SSR	11	19.10	BGS
79	RM206	SSR	11	21.60	BGS
80	RM247	SSR	12	3.10	BGS
81	RM101	SSR	12	8.82	BGS
82	RM27933	SSR	12	10.43	BGS
83	RM7102	SSR	12	13.25	BGS
84	RM28172	SSR	12	17.69	BGS
85	R12M33	InDel	12	20.08	BGS
86	RM309	SSR	12	21.52	BGS
87	RM17	SSR	12	26.90	BGS

Table 8.2.5: List of foreground selection markers used in the MABC of BR28-*Saltol.*

Sl. No.	Marker name	Marker type	Chromosome	Position (Mb)
1	RM1287	SSR	1	10.90
2	RM3412	SSR	1	11.50
3	RM493	SSR	1	12.20

Table 8.2.6: List of recombinant selection markers used in the MABC of BR28-*Saltol.*

Sl. No.	Marker name	Marker type	Chromosome	Position (Mb)
1	RM3627	SSR	1	10.31
2	RM10825	SSR	1	13.30
3	RM10864	SSR	1	14.20
4	RM562	SSR	1	14.60
5	RM7075	SSR	1	15.10

Table 8.2.7: List of recombinant selection (RBS) and background selection (BGS) markers used in the MABC of BR28-*Saltol.*

Sl No.	Marker Name	Marker type	Chromosome	Physical position (Mb)	Used in selection
1	RM3252	SSR	1	0.30	BGS
2	RM1	SSR	1	4.60	BGS
3	RM490	SSR	1	6.70	BGS
4	RM243	SSR	1	8.00	BGS
5	RM3627	SSR	1	10.31	RBS & BGS
6	RM10825	SSR	1	13.30	RBS & BGS
7	RM10843	SSR	1	13.70	RBS & BGS
8	RM10864	SSR	1	14.20	RBS & BGS
9	RM562	SSR	1	14.60	RBS & BGS
10	RM7075	SSR	1	15.10	RBS & BGS
11	R1M20	InDel	1	18.30	BGS
12	RM9	SSR	1	23.20	BGS
13	RM1349	SSR	1	25.10	BGS
14	RM7643	SSR	1	31.30	BGS
15	RM3482	SSR	1	39.71	BGS
16	RM12208	SSR	1	42.30	BGS
17	RM3340	SSR	2	0.38	BGS
18	RM154	SSR	2	1.10	BGS
19	RM12476	SSR	2	2.79	BGS
20	RM211	SSR	2	4.32	BGS
21	RM12769	SSR	2	7.30	BGS
22	RM145	SSR	2	8.60	BGS
23	RM3501	SSR	2	10.19	BGS
24	RM327	SSR	2	20.00	BGS
25	RM5789	SSR	2	22.40	BGS
26	RM126	SSR	2	27.60	BGS

27	R2M50	InDel	2	30.40	BGS
28	RM5404	SSR	2	33.70	BGS
29	RM3202	SSR	3	0.80	BGS
30	RM545	SSR	3	4.92	BGS
31	RM14795	SSR	3	10.30	BGS
32	S03068	InDel	3	14.90	BGS
33	RM232	SSR	3	23.01	BGS
34	RM5626	SSR	3	24.70	BGS
35	R3M37	InDel	3	26.30	BGS
36	RM6329	SSR	3	28.61	BGS
37	RM3867	SSR	3	31.50	BGS
38	RM227	SSR	3	34.80	BGS
39	RM16247	SSR	3	36.15	BGS
40	RM518	SSR	4	2.00	BGS
41	RM261	SSR	4	6.56	BGS
42	RM307	SSR	4	12.90	BGS
43	R4M30	InDel	4	17.90	BGS
44	RM5749	SSR	4	20.10	BGS
45	RM273	SSR	4	23.80	BGS
46	RM17391	SSR	4	29.40	BGS
47	RM280	SSR	4	35.20	BGS
48	RM122	SSR	5	0.28	BGS
49	RM413	SSR	5	2.18	BGS
50	RM17954	SSR	5	3.50	BGS
51	RM169	SSR	5	7.39	BGS
52	R5M20	InDel	5	13.70	BGS
53	RM3838	SSR	5	16.42	BGS
54	RM4948	SSR	5	18.83	BGS
55	RM440	SSR	5	19.89	BGS
56	RM510	SSR	6	2.80	BGS
57	RM204	SSR	6	3.16	BGS
58	RM19516	SSR	6	4.67	BGS
59	RM6359	SSR	6	6.40	BGS
60	RM314	SSR	6	10.08	BGS
61	RM19996	SSR	6	14.40	BGS
62	RM20224	SSR	6	20.60	BGS
63	RM454	SSR	6	23.40	BGS
64	RM20417	SSR	6	25.16	BGS
65	RM3307	SSR	6	28.90	BGS
66	RM20781	SSR	7	0.11	BGS
67	RM125	SSR	7	5.50	BGS
68	RM21584	SSR	7	17.50	BGS
69	RM418	SSR	7	18.10	BGS
70	RM18	SSR	7	25.70	BGS
71	RM22175	SSR	7	29.49	BGS
72	RM337	SSR	8	0.10	BGS
73	RM310	SSR	8	5.11	BGS
74	RM7285	SSR	8	18.75	BGS

75	RM210	SSR	8	22.30	BGS
76	RM150	SSR	8	25.09	BGS
77	RM256	SSR	8	24.14	BGS
78	S08121A	InDel	8	28.20	BGS
79	S09000A	InDel	9	0.20	BGS
80	R9M10	InDel	9	4.50	BGS
81	S09006	InDel	9	6.40	BGS
82	RM6051	SSR	9	12.77	BGS
83	R9M30	InDel	9	14.90	BGS
84	RM257	SSR	9	17.67	BGS
85	RM242	SSR	9	18.64	BGS
86	RM24804	SSR	9	22.40	BGS
87	RM25022	SSR	10	3.60	BGS
88	R10M10	InDel	10	4.90	BGS
89	RM25181	SSR	10	8.44	BGS
90	RM25271	SSR	10	10.75	BGS
91	RM5806	SSR	10	14.04	BGS
92	RM171	SSR	10	18.10	BGS
93	RM228	SSR	10	21.80	BGS
94	RM25972	SSR	11	0.40	BGS
95	RM26237	SSR	11	4.90	BGS
96	RM3137	SSR	11	6.11	BGS
97	RM26652	SSR	11	15.00	BGS
98	RM21	SSR	11	19.10	BGS
99	RM27421	SSR	12	0.50	BGS
100	RM247	SSR	12	3.18	BGS
101	RM27877	SSR	12	9.18	BGS
102	RM27933	SSR	12	10.43	BGS
103	RM7102	SSR	12	13.30	BGS
104	S12062	InDel	12	18.00	BGS
105	RM309	SSR	12	21.52	BGS
106	RM28746	SSR	12	26.39	BGS
107	S12109A	InDel	12	27.40	BGS

Appendix-III

Preparation and maintenance of reagent for CTAB method.

8.3.1. Isolation of plant DNA by CTAB method (Doyle and Doyle, 1990)

The primary objective of the isolation process is to recover the maximum yield of high molecular weight DNA devoted of protein and other restriction enzymes (Sambrook and Russell, 2001).

8.3.1.1. Reagents and Materials

Liquid nitrogen

1M Tris-HCl, pH8.0

0.5M EDTA, pH8.0

Buffer saturated phenol.

Chloroform: Isoamyl alcohol (24:1v/v)

TE buffer, pH8.0

3M Na-acetate, pH5.2

RNase solution 10μg/ml (DNase free)

8.3.1.2: CTAB isolation buffer.

Ethanol

Isopropanol

5M NaCl.

All solutions were made with de-ionized, sterile and autoclaved water (except phenol).

8.3.1.3: Preparation of stock solutions

Liquid Nitrogen: Liquid nitrogen was collected from Bangladesh Oxygen Company (BOC)

1M Tris- HCl, pH8.0 (Stock solution): To prepare 100 ml 1M Tris-HCl (MW-121.11) solution, 12.11g of Tris- base was dissolved in 80 ml of double distilled water with the help of magnetic stirrer, pH was adjusted to 8.0 by concentrated HCl. Final volume was made 100 ml and sterilized by autoclaving.

0.5M EDTA, pH 8.0: To prepare 100 ml, 0.5M EDTA (disodium ethylene diamine tetra acetate, MW 372.2.) solution, 18.61g of EDTA was dissolved in ~80 ml distilled water and then stirred vigorously with a magnetic stirrer. pH8.0 was adjusted with NaOH (~2g pellets). Final volume (100ml) was made by distilled water. Then the solution was autoclaved and stored at room temperature.

Buffer saturated phenol: Before use phenol must be equilibrated to a pH of >7.8 because the DNA partitions into the organic phase at acidic pH. The phenol was equilibrated according to the following protocol modified by Sambrook and Russell, 2001.

Reagents and Materials:

Distilled phenol

Hydroxyquinoline

0.5M Tris-HCl (pH 8.0)

0.1M Tris-HCl (pH 8.0)

β-Mercaptoethanol

pH paper.

Procedure:

1) Crystalline phenol was redistilled at 160 ^{0}C to remove oxidation products. Such as quinons etc.
2) Liquefied phenol was stored at -20 ^{0}C
3) The phenol was removed from the freezer, allowed it to room temperature and then it was melted at 68 ^{0}C
4) Hydroxy-quinoline (antioxidant) was added to a final concentration of 0.1%.
5) An equal volume of buffer (0.5M Tris-Cl, pH 8.0) was added to the melted phenol at room temperature.
6) The mixture was stirred on a magnetic stirrer for 15 minutes.

7) The mixture was turned off and when the two phases had separated, as much as possible of upper (aqueous) phase was aspirated using a glass pipette attached to a vacuum equipped with appropriate traps.
8) An equal volume of 0.1M Tris-Cl (pH 8.0) was added to the phenol.
9) The mixture was stirred on magnetic stirrer for 15 minutes.
10) The stirrer was turn off and the upper aqueous phase was removed
11) The extraction was repeated until the pH of the phenolic phase was >7.8 as measured with pH paper.
12) After the phenol was equilibrated and the final aqueous phase had been removed, 0.1 β-mercapto ethanol was added.
13) The phenol solution was stored in a light-tight bottle at 4 ^{0}C for periods of up to 1 month.

Chloroform: Isoamylalcohol (24:1 v/v).

TE buffer,

i. 10 mM TrisHCl (pH 8.0)

ii 0.1 mM EDTA (pH 8.0)

3M Na-acetate (pH5.2): For 50 ml solution, 12.3045g of Na-acetate was weighed and dissolved in 35 ml of distilled water; pH was adjusted to 5.2 using glacial acetic acid. Then the final volume (50ml) was made with distilled water.

RNase solution 10μg/ml (DNase free): For stock solution (10 mg/ml) pancreatic RNase (RNase A) was dissolved at a concentration of 10mg/ml in 0.01M Na-acetate (pH5.2). It was allowed to cool slowly at room temperature after heating to 100 ^{0}C for 15 minutes. The pH was adjusted by adding 0.1 volume of 1.0M Tris-HCl (pH7.4). This RNase solution was dispended into aliquots and stored at -20 ^{0}C.

Table 8.3.1: CTAB isolation buffer (per 100ml)

Component	Amount
CTAB	2.0 g
5M NaCl	28.0 ml
0.5M EDTA	4.0 ml
1M Tris-HCl	10.0 ml
β-Mercaptoethanol	0.2 ml

All the materials were added in a conical flask and heated at 60 ^{0}C in the water bath until all the CTAB melted. Then the volume was adjusted to 100ml with autoclaved water and stored at room temperature.

Ethanol:

I. 70% (Seventy percent)

ii. 99% (Ninety nine percent)

Isopropanol:

5M NaCl: 29.22g of NaCl were dissolved in 40 ml of ddH_2O. The Volume was then made 100ml by ddH_2O and the solution was autoclaved.

8.3.2. Preparation and maintenance of reagent for Rice DNA Extraction IRRI Miniprep Protocol:

Table 8.2.2: DNA extraction buffers

Sl. No.	Components	Stock concentration	Final concentration	5 ml	10 ml
1	Tris (pH 8.0)	1.0 M	50 mM	0.25 ml	0.50 ml
2	EDTA (pH 8.0)	0.5 M	25 mM	0.25 ml	0.50 ml
3	NaCl	5.0 M	300 mM	0.30 ml	0.60 ml
4	SDS	10.0%	1%	0.50 ml	1.00 ml
5	H_2O			3.7 ml	7.40 ml

Table 8.3.3: TE buffer (pH 8.0)

Sl. No.	Components	Stock concentration	Final concentration	50 ml	100 ml
1	Tris (pH 8.0)	1.0 M	10 mM	0.50 ml	1.00 ml
2	EDTA (pH 8.0)	0.5 M	1 mM	0.10 ml	0.20 ml
3	H_2O	--	--	49.40 ml	98.80 ml

8.3.2.1. Preparation of stock solution

1M Tris-HCL (pH 8.0): Trizma base (Sigma, FW =121.10) @ 30.28 g was dissolved in 200 ml distilled water. The pH of the solution was adjusted to 8.0 with concentrated HCl. The volume was adjusted to 250 ml with distilled water after cooling in to room temperature. Finally the solution was sterilized by autoclaving.

0.5 M EDTA (pH 8.0): Di-hydrated EDTA (Sigma, FW = 372.2) @ 46.53 g was dissolved to 200 ml distilled water. The solution was stirred vigorously with 4 g of NaOH pellets by a magnetic stirrer. Volume of the solution was adjusted to 250 ml with distilled water. Sterilized the solution by autoclaving.

5 M NaCl: Extrapure NaCl (Sigma, FW = 58.44) @ 73.05 g was dissolved to 200 ml distilled water. The volume was adjusted to 250 ml with distilled water. Sterilized the solution by autoclaving.

10% SDS: Sodium dodecyl sulfate (SDS) (Sigma, FW = 288.4) @ 288.40 g was dissolved in 200 ml sterile distilled water. The solution was heated to 65 ^{0}C for ease to dissolve. The volume was adjusted to 250 ml by sterile distilled water.

Appendix-IV

Composition and preparation for Polymerase Chain Reaction (PCR).

8.4.1. Composition of PCR components

1) 10× PCR reaction buffer: 500 mM KCl, 100 mM Tris-HCl (pH8.3), 0.1% gelation
2) 50mM $MgCl_2$ in water for PCR.
3) Deoxynucleotide (dNTP) mixture.
   Na-salt of deoxyadenosine-5′-triphosphate (100mM aqueous solution, pH 7.35)
   Na-salt of deoxythymine- 5-′ triphosphate (100mM aqueous solution, pH7.35)
   Na-salt of deoxyguanosine -5′-triphosphate (100mM aqueous solution, pH 7.35)
   Na-salt of deoxycytidine -5′-triphosphate (100mM aqueous solution, pH7.35)
4) Taq DNA polymerase. (The Taq DNA polymerase used in this work was isolated from *Escherichia coli* strain taq-2 transformed with *pTaq* plasmid containing *Taq* gene expressed under control of taq promoter.
5) Two oligonucleotide primers (SSR/InDel/gene specific).
6) Template DNA
7) 20% DMSO (dimethyl sulfoxide) (20% DMSO was prepared by mixing as a composition of 200μl of DMSO in 800μl of deionized sterile water and was stored at -20 ^{0}C)
8) Autoclaved ultra pure water.
9) TE solution for the dilution of the template DNA.
10) 10mM Tris –HCl (pH 8.0)
11) 0.1mM EDTA (pH 8.0)

8.4.2. Preparation of PCR components

Preparation of dNTPs mixture: 10 μl of dATP, dGTP, dCTP and dTTP (concentration of each being 100 mM) were mixed in fresh, autoclaved eppendorf tube and the final volume was made 1000 μl by adding 960 μl of autoclaved ultra pure water and dispensed as aliquots in tubes and stored at –20 ^{0}C. The concentration of each of the nucleotide in the above mixture was 1.0 mM.

8.4.3. Dilution of the template DNA

The DNA isolated from traditional rice verities was highly concentrated and unsuitable for using in PCR reaction; for this, it was diluted with TE solution (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0) before use so that, the working concentration of the template DNA was 50 ng/µl.

8.4.4. Dilution of primers:

Primers were diluted by adding equal volume of TE to the tube (provided by the manufacture) as its molecular weight. As for example 12 µl of the primer was added to 188 µl of TE. So, the final concentration of the primer was 60 ng/µl (~10 µM).

8.4.5. Preparation of the master mixture

Generally master mixture for any PCR contains buffer, dNTPs, Mg^{2+}, specific primer pair and Taq polymerase. PCR components should pipette for one more reactions (to avoid shortage of the master mixture, due to pipetting losses). For example, pipette for 51 reactions for 50 reactions in sterile 0.5 ml microfuge tube:

Table 8.4.1: Components for preparing master mix

Components	1 reaction (µl)	51 reaction (µl)
PCR buffer (10×)	1.5	76.5
DNTPs (1mM)	1.5	76.5
Mg^{2+}(50 mM)	0.5	25.5
Primer forward (100 ng/ml)	0.5	25.5
Primer reverse (100 ng/µl)	0.5	25.5
Taq polymerase (15× diluted)	0.5	25.5
Total	**5.0**	**255.0**

N.B. In each reaction, the volume of the PCR buffer used was one-tenth of the total reaction volume.

Appendix-V

Composition, Preparation and Maintenance of Agarose gel

8.5.1. Preparation of 0.8% Agarose gel (100 ml)

1) 0.8 g of Agarose powder was weighed in a conical flask.
2) 2 ml of 50× TAE buffer was taken in a measuring cylinder and the volume was made up to 100 ml with ddH_2O and was poured in the flask containing agarose and then melted in micro oven at 60 ^{0}C for 2 minutes.

8.5.2. Reagents and Materials

1) Ultra pure Agarose (typing grade)
2) Electrophoresis buffer (TAE)

Table 8.5.1: Components of electrophoresis buffer (TAE buffer)

Stock solution (50×)	Per Liter	Working solution (1×)	Concentration
Tris base	242 g	Tris acetate	40 mM
Glacial acetic acid	57.1 ml	EDTA	1 mM
0.5 M EDTA (pH 8.0)	100 ml		

To prepare stock solution, all components were dissolved and then final volume was adjusted to 1000 ml with ddH_2O. Finally it was sterilized by autoclaving.

1 L or 1000 ml of 1× TAE working electrophoresis buffer was prepared by mixing 20 ml of 50× TAE stock solution and 980 ml of ddH_2O.

3) Gel loading buffer (6×)

Table 8.5.2: Components of gel loading buffer

Components	Quantity/Percentage
Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol, in H_2O	30.00%

This buffer was stored of at 4 ^{0}C.

4) Composition of DNA dye

Table 8.5.3: Components of DNA dye

Components	**Quantity/Percentage**
Bromophenol blue	0.05%
Sucrose	40.0%
EDTA	0.1M
SDS	0.5%

5) Ethidium bromide:Ethidium bromide was prepared as a stock solution of 10 mg/ml in H_2O, which was stored at room temperature in dark bottles.
6) DNA size standards/ DNA marker:A stock solution of size standards was prepared by dilution with a gel-loading buffer and then used as needed in individual electrophoresis experiments.
7) Equipments for agarose gel electrophoresis:
    i. Clean dry horizontal electrophoresis apparatus with chamber
    ii. Clean dry glass/ plastic plates with appropriate comb.
    iii. Gel-sealing tape
    iv. Power supply device.

8.5.3. Procedure

1) The open ends of a clean, dry plastic tray was sealed with tape and placed on a horizontal section of the bench.
2) Sufficient electrophoresis buffer (usually 1× TAE or 0.5× TBE) was prepared to fill the electrophoresis tank and to cast the gel.
3) A solution of agarose in electrophoresis buffer was prepared in an Erlenmeyer flask at a concentration appropriate for separating the particular size fragments expected in the DNA sample.
4) The flask was placed in the microwave oven on high temperature (i.e. 60 ^{0}C-80 ^{0}C) for 2-3 minutes or until the agarose dissolves.
5) Using insulated gloves; the flask was transferred into a water bath at 55 ^{0}C. The gel solution was mixed thoroughly by gentle swirling when the molted gel had cooled.

6) The cooled gel solution was poured into the gel tray. An appropriate comb was placed previously for forming the sample slots in the gel and assuring that there were no bubbles around the combs. (A pipette tip was used to remove if there was any bubble.)
7) The gel was allowed to set completely (30-45 minutes at room temperature), then poured a small amount electrophoresis buffer on the top of the gel, and the comb was removed carefully. The electrophoresis buffer was poured off and the tape was removed carefully. The gel was mounted in the electrophoresis tank.
8) Electrophoresis buffer (1× TAE) was adjusted sufficiently to cover the gel to a depth of ~ 1 mm.
9) The samples of DNA was mixed with 0.20 volume of the desired 6× gel-loading buffer and loaded slowly into the slots of the submerged gel using a disposable micropipette. Size standard that will depend on the type of marker being analyzed was loaded into slots on both the right and left sides of the gel.
10) The lid of the gel tank was closed and the electrical leads were attached to the power supply device so that the DNA will migrate toward the positive anode (red lead).
11) A voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes) was applied.
12) The electric current was turn off when the DNA samples or dyes had migrated a sufficient distance through the gel and the leads and lid from the gel tank was removed.
13) The gel was stained by immersing it in electrophoresis buffer or H_2O containing ethidium bromide (0.5 μg/ml) for 30-45 minutes at room temperature.
14) Photograph of the gel was taken under UV illumination.

Appendix-VI

Composition, Preparation and Maintenance of PAGE

8.6.1. Compositions

1. Acrylamide/ bis-acrylamide (40%)

Table 8.6.1: Components of 40% acrylamide solution

Components	Quantity
Acrylamide	190 g
N, N′-Methylene bisacrylamide	10 g
ddH_2O, volume to	500 ml

The solution was heated to dissolve the chemicals. After preparing 40% acrylamide/bis-acrylamide solution, it was filtered through Whatman filter paper and was stored in a dark bottle at 4 ^{0}C.

2. Ammonium per sulfate (APS) (10%)

Table 8.6.2: Components of 10% APS

Components	Quantity
Ammonium per sulfate	1 g
ddH2O, volume to	10 ml

After aliquoting in eppendorf tubes, the ammonium per sulfate (250 µl in each) was stored at –20 ^{0}C.

3. TBE Buffer (stock solution) (5×)

Table 8.6.3: Components of TBE buffer

Components	Quantity
Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH8.0)	20.0 ml
ddH_2O volume to	1000.0 ml

N.B. The pH of the concentrated stock buffer should be ~8.3.

4. TBE buffer, used in gel electrophoresis (1×)

Table 8.6.4: Components for preparing 1× TBE

Components	**Quantity**
5× TBE	100 ml
ddH_2O, volume to	500 ml

N.B. concentrated stock buffer was diluted just before use.

5. Gel loading dye

Table 8.6.5: Components of gel loading dye

Components	**Quantity**
Bromophenol blue	0.05%
Sucrose	40%
EDTA	0.1M
SDS	0.5%

6. Preparation of polyacrylamide gel (for 2 gels) (8%)

Table 8.6.6: Components of 8% polyacrylamide gel

Components	**Volume**
40% acrylamide	10 ml
5× TBE	10 ml
ddH_2O volume up to	50 ml
10% APS	500 µl
TEMED	42.5 µl

7. Ethidium bromide (10 mg/ml)

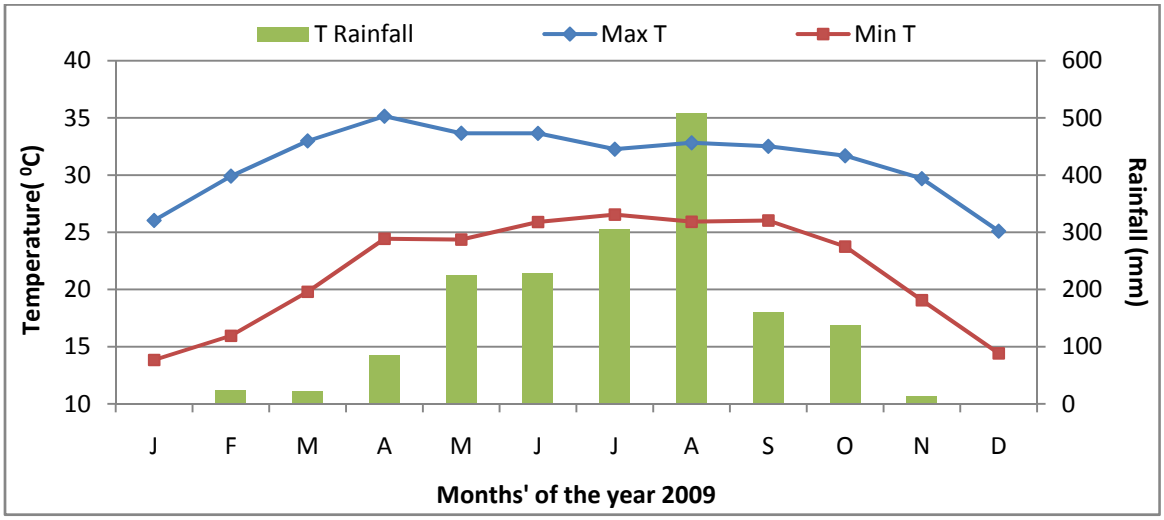
PAGE preparation procedure:

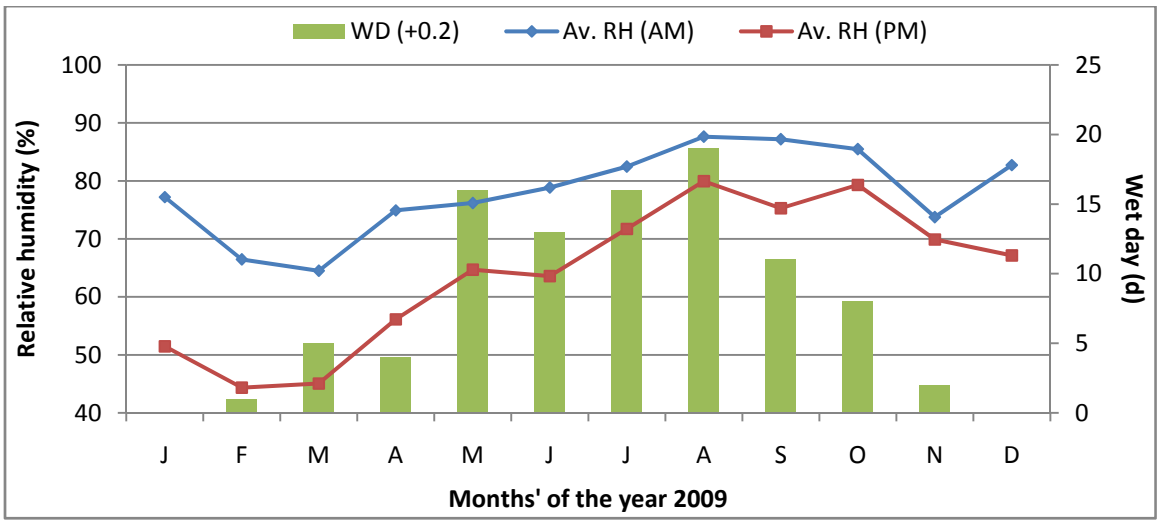
1) Both glass plates were cleaned carefully by 99% ethanol and were assembled, placing both cleaned surface inside, with spacers (~1.5 mm thick) and elastic rubber as a sealer surrounding the edges of glass plate.
2) The assembly was leveled and checked for leakage with ddH_2O.

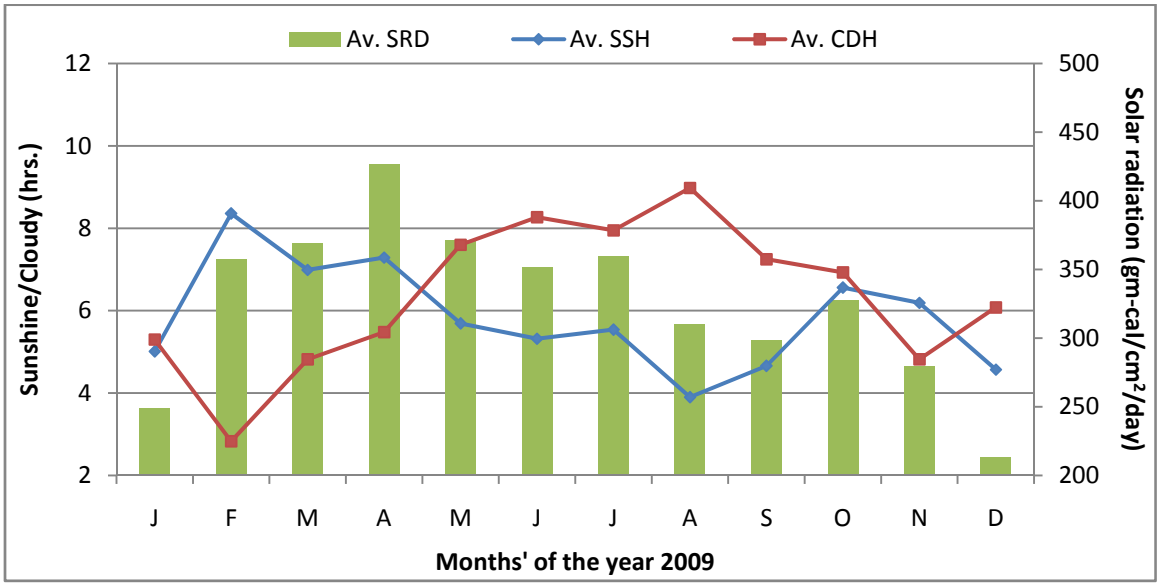
3) The gel was poured in the gel case and the comb was assembled for wells formation. The gel was allowed to solidify for ~ 1 hr
4) The combs were removed and sandwich glass plate/gel was attached with the electrophoresis apparatus.
5) The PCR product was mixed with appropriate volume of loading buffer and denatured by incubation for 5 minutes, in 95 ^{0}C.
6) 2-4 μl of the mix was generally loaded onto 6% denaturing polyacrylamide gel very carefully to prevent cross contamination.
7) Electrophoresis was carried out in 1× TBE buffer at 100W for 2 hours or up to the time when the bromophenol blue traveled a satisfactory distance.
8) The electric current was turn off and both glass plates were disassembled. Elastic rubber and spacers were removed.
9) The gel was stained by immersing it in electrophoresis buffer or ddH_2O containing ethidium bromide (0.5 μg/ml) for 30-45 minutes at room temperature.
10) Photograph of the gel was taken under UV illumination.

Appendix-VII

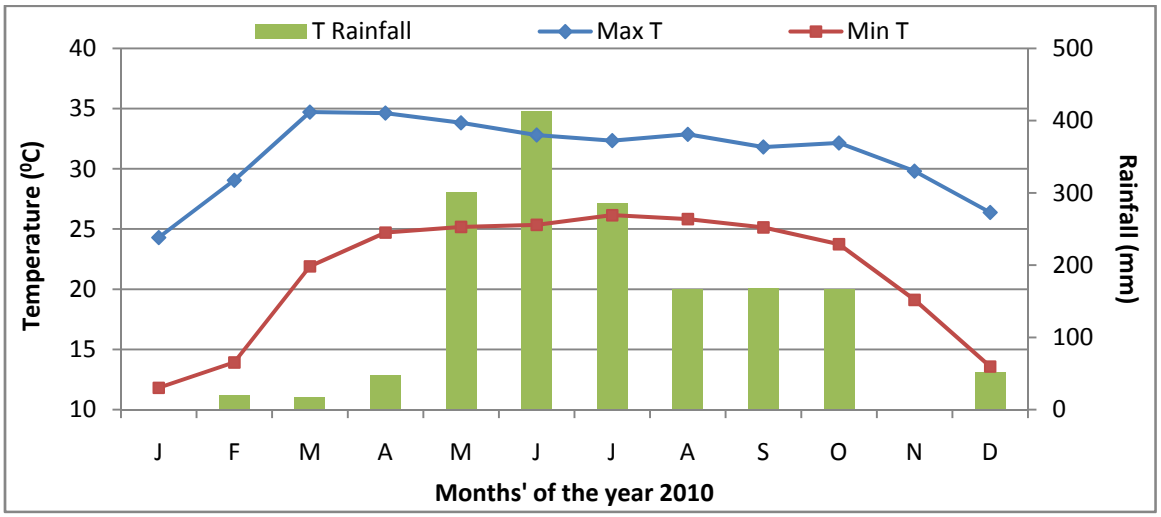
Weather conditions during the experimental periods of the year 2009:

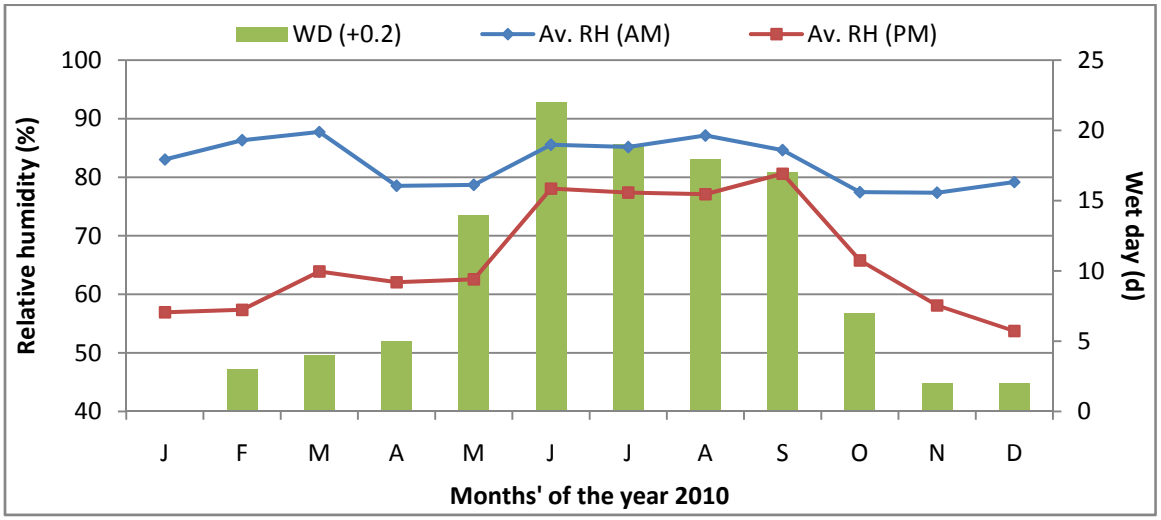


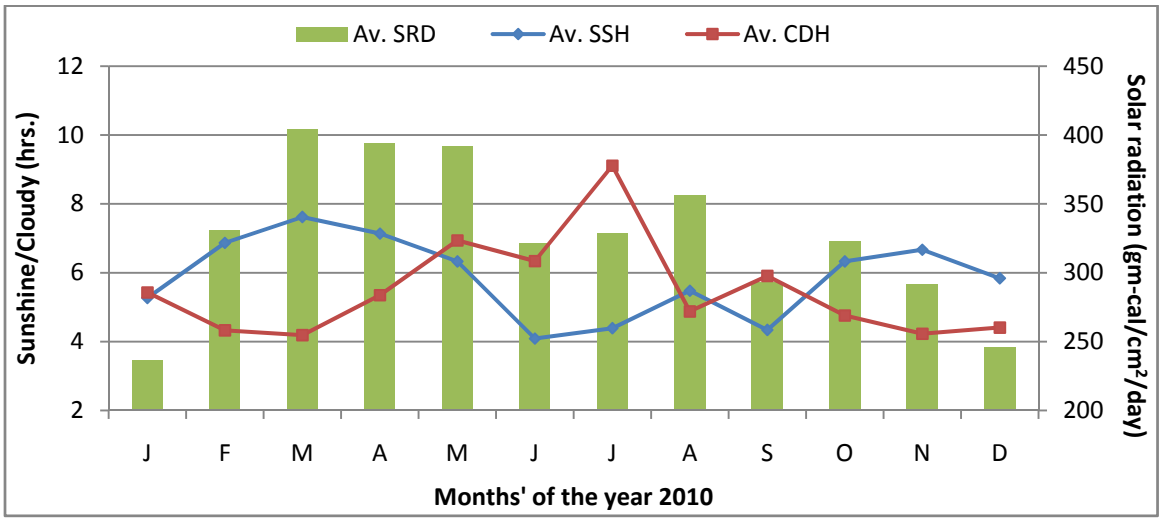




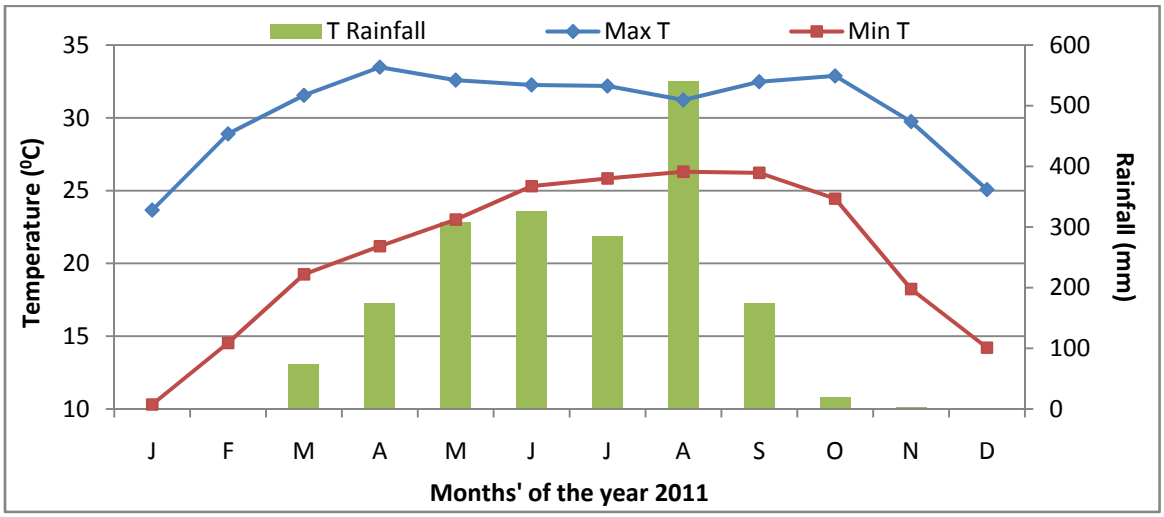
Weather conditions during the experimental periods of the year 2010:

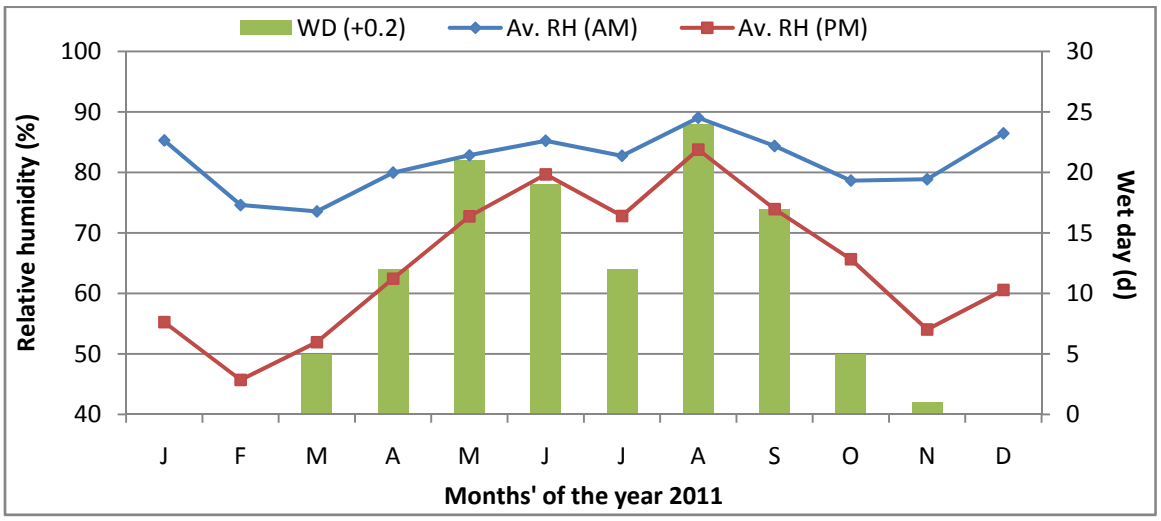


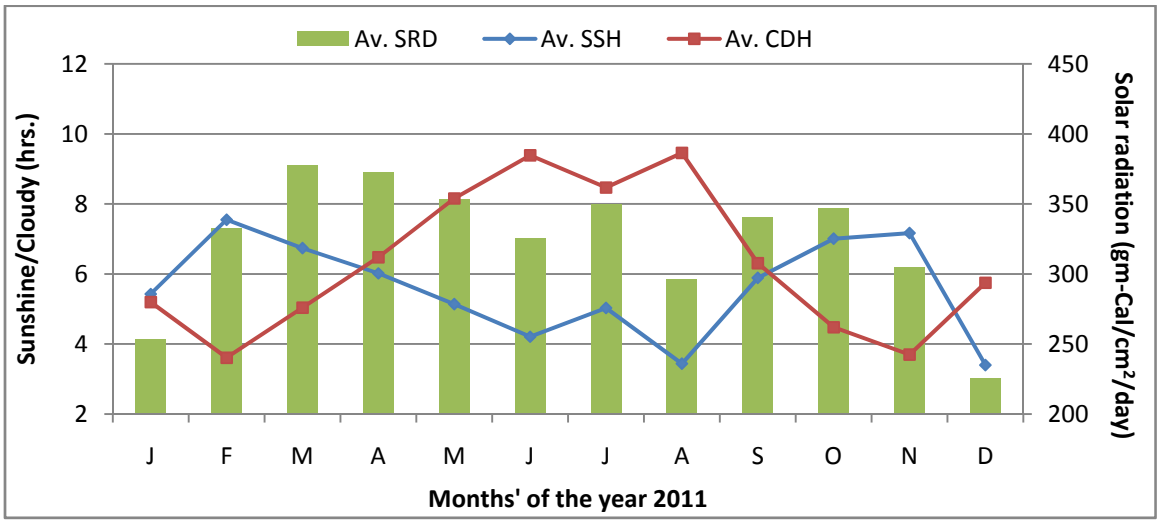




Weather conditions during the experimental periods of the year 2011:







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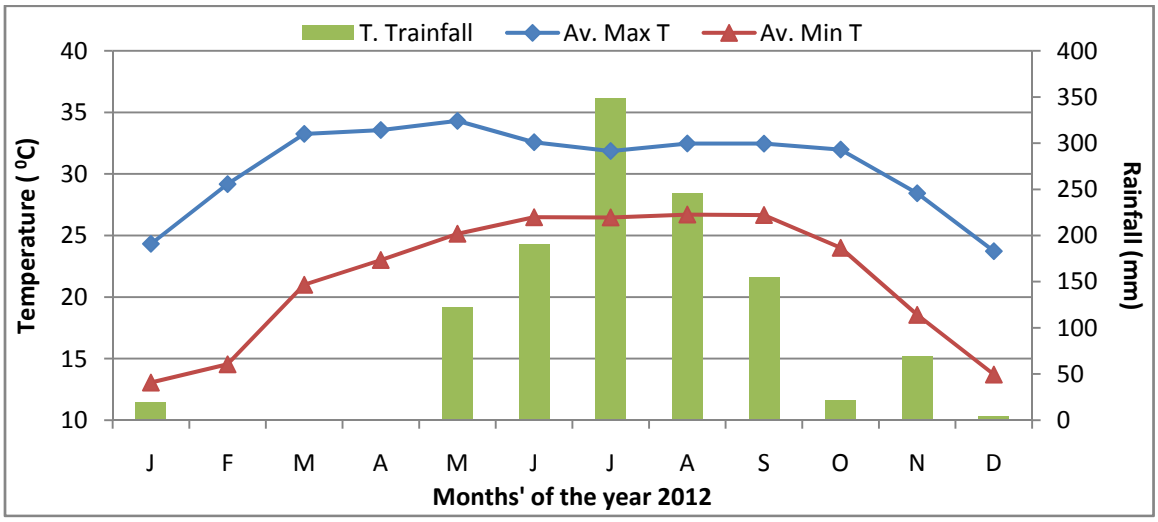

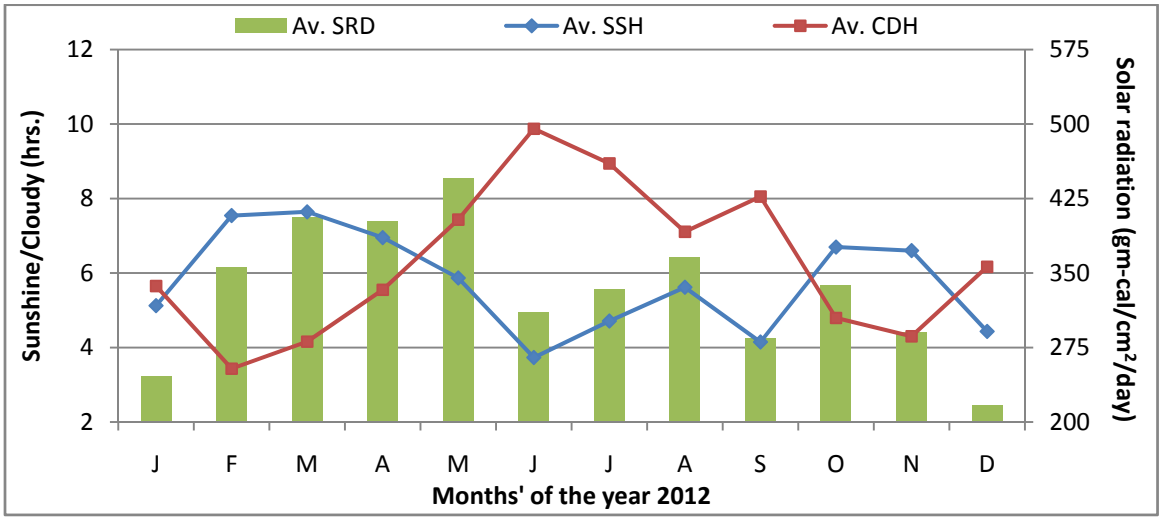

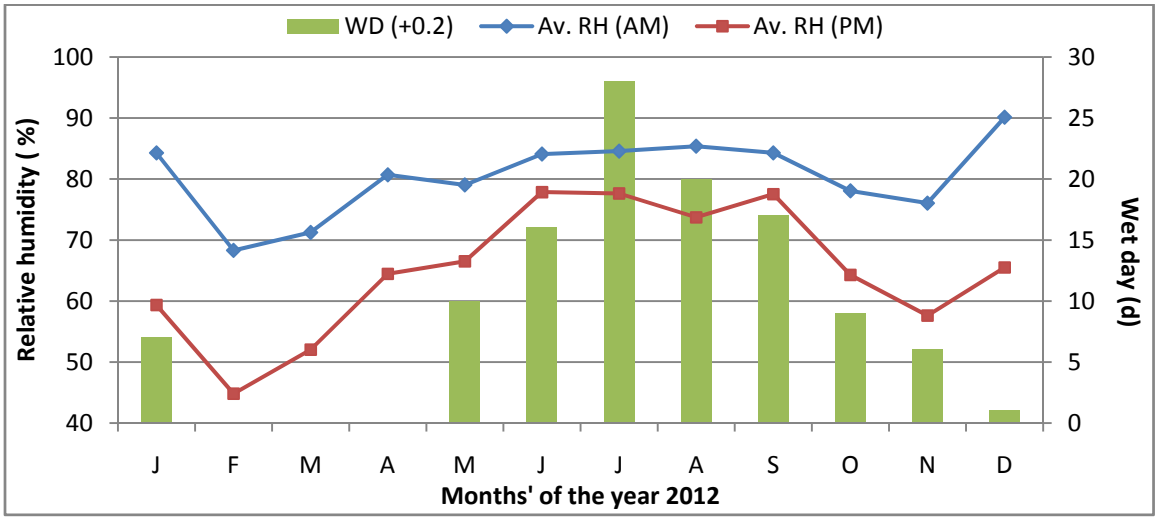

Weather conditions during the experimental periods of the year 2013: