Characterization of a Bangladeshi rice landrace *Horkuch* as a suitable donor for salinity tolerance traits for development of new salt tolerant rice cultivars

Ph.D. Thesis

Sumaiya Farah Khan

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
FACULTY OF BIOLOGICAL SCIENCES
UNIVERSITY OF DHAKA
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Characterization of a Bangladeshi rice landrace *Horkuch* as a suitable donor for salinity tolerance traits for development of new salt tolerant rice cultivars

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Sumaiya Farah Khan

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Faculty of Biological Sciences,
Department of Biochemistry and Molecular Biology,
University of Dhaka,
Dhaka, Bangladesh.

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Certificate

This is to certify that Sumaiya Farah Khan has conducted her thesis work entitled, "Characterization of a Bangladeshi Rice Landrace Horkuch as a Suitable Donor for Salinity Tolerance Traits for the Development of New Salt Tolerant Rice Cultivars" under my supervision for the fulfillment of the degree of 'Doctor of Philosophy in Biochemistry and Molecular Biology' from the University of Dhaka. Furthermore, her work is also co-supervised by Dr. Rumena Yasmeen, CSO (A. C.) & Head, Plant Physiology Division, Bangladesh Rice Research Institute (BRRI). I would like to acknowledge with gratitude Dr. Adam Price, Professor, Plant Molecular Genetics, Institute of Biological and Environmental Sciences, University of Aberdeen for hosting Sumaiya for parts of her research work and providing his valuable guidance. The work or any part of the thesis has not been submitted anywhere for any other degree.

Supervisor

.....

Date: 20/12/2017

Zeba Islam Seraj, Ph. D.

Professor,
Department of Biochemistry &
Molecular Biology
University of Dhaka.
Ph: +880-2-9661920x7647
zebai@du.ac.bd

Co-Supervisor

11/12/17

.....

Date:

Rumena Yasmeen, Ph. D.

CSO (A. C.) & Head
Plant Physiology Division
Bangladesh Rice Research Institute
Gazipur.
Ph: +880-2-9257517
r_yasmeen67@yahoo.com

Dedication

To my parents

Dr. Nurur Rahman Khan

Mrs. Fikriyah Begum

The reason of what I become today.

Thanks for your great support and contimuous care.

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Abstract

Bangladesh is a low-lying developing country adversely affected by climate change with its coastal area under constant threat of hazards like floods, cyclones, high temperature, sea level rise, salinity intrusion, etc. The rice landrace *Horkuch (Oryza sativa*) is adapted to the saline conditions in the southern coast of Bangladesh and maintains efficient photosynthesis and detoxification under salt stress, but is low yielding. Therefore, this study was aimed to find the QTLs responsible for the salinity tolerance of *Horkuch*. A cross was made from salt tolerant *Horkuch* and high yielding but sensitive *IR29*. A set of the $F_{2:3}$ population containing 200 individuals was genotyped using DArTseq™ for discovering over 4000 polymorphic SNP markers. 200 F_3 progenies were phenotyped under salt stress at seedling stage and 100 selected high and low tails from the seedling stage screening were phenotyped at reproductive stage. A linkage map was generated from the polymorphic SNPs identified**.** The total length of linkage map was 2257 cM, with each chromosome varying from 128.8 cM to 241 cM. Average distance between adjacent markers on each chromosome was 8.11 cM. Linkage map construction was followed by identification of multiple novel QTLs**.** QTLs for root length, total chlorophyll, potassium content and sodium content at seedling stage as well as spikelet fertility, days to flowering, thousand grain weight, plant height and yield, all under stress, were identified at the reproductive stage. A robust QTL associated with total chlorophyll was detected at position 157.47 cM on chromosome 7, with a LOD score of 5.44. It explained 14.25% of the total phenotypic variance. The donor of the allele was *Horkuch*, the salt tolerant parent. Six QTLs were also detected for root length, of which three were major and three were putative QTLs. Two QTLs were identified for stomatal conductance on chromosome 4 and 5, showing a large effect on stomatal conductance explaining 13.1% and 20.5% of the total phenotypic variance, respectively. A robust QTL associated with primary branching was detected at position 114.36 cM on chromosome 1, with a LOD score of 7.08. It explained 37.2% of the total phenotypic variance. Two QTLs for yield were mapped on chromosomes 1 and 10.

The one on chromosome 1 had a LOD score of 3.78 and explained 11.83% of the total phenotypic variance.

Selected plants from the population, containing several QTLs from both seedling and reproductive stage were identified. The population was further advanced up to the F_5 generation. Presence of QTLs was confirmed by molecular and phenotypic validation at both developmental stages at the F_5 generation. QTLs for total chlorophyll, stomatal conductance, root length and third leaf length were confirmed by using SSR markers. Plants with a combination of several QTLs were identified at F_5 that also showed tolerance to salinity at seedling stage and had better yield properties under salt stress at the reproductive stage. These plants can therefore be used as donors for introgression and pyramiding of multiple tolerance loci into commercial genotypes to develop salt tolerant variety with high yields. Seedling and reproductive QTLs have never been combined and tested before to produce more tolerance, which is unique to this study. Markers linked to the important QTLs have also been identified and can be used to introgress multiple QTLs into elite varieties using marker assisted backcrossing.

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List of Abbreviations

ABA	Abscisic acid
AEZ	Agro-ecological zones
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
APS	Ammonium per sulfate
BARC	Bangladesh Agricultural Research Council
BAS	Bangladesh Academy of Science
BINA	Bangladesh Institute of Nuclear Agriculture
Bp	base pair
BRRI	Bangladesh Rice Research Institute
C	Celsius
Ca^{2+}	Calcium ion
CGIAR	Consultative Group on International Agriculture Research
ChlA	Chlorophyll A
ChlB	Chlorophyll B
Cl^-	Chloride ion
cm	Centimeter
cM	Centi Morgan
Conc.	Concentration
CRD	Completely Randomized Design
CSR	Central Salinity Rice
CSSRI	Central Soil Salinity Research Institute
CTAB	Cetyl Trimethyl Ammonium Bromide
D	Day(s)
DArT	Diversity Array Technology
ddH_2O	distilled deionized water
ddRAD	Double digested restriction associated DNA sequencing
DF	Days to flowering
DM	Days to maturity
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
EDTA	Ethylene diamine tetra acetic acid
ERF	Ethylene Response Factor
ESTs	Expressed sequence tags
et al.	and others
ET	Effective tiller
EtBr	Ethidium bromide
etc.	Et cetera
FAO	Food and Agriculture Organization of United Nations
FGN	Filled grain number
FGW	Filled grain weight
FLL	Flag leaf length
FLW	Flag leaf weight

G by E	Genotype by Environment
g or gm	Gram
GGT	Graphical genotyping
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*
LW	Lower leaf weight
M	Molar
MABC	Marker assisted backcrossing
MAS	Marker assisted selection
Mb	Megabase
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
Mt	Million tons
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
PD	Panicle damage
PB	Primary branching
PF	Percent fertility
PH	Plant height
pH	Negative logarithm of hydrogen ion concentration
PL	Panicle length
ppm	parts per million
QTL	Quantitative trait loci
RDW	Root dry weight
RIL	Recombinant Inbred Line
RL	Root Length
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RRWC	Root relative water content
RW	Rachis weight
SA	Salicylic Acid
SB	Secondary branching
SC	Stomatal conductance
SDS	Sodium dodecyl sulfate
SES	Standard Evaluation System
SeB	Seed breadth
SeL	Seed length
SL	Shoot length
SNP	Single Nucleotide Polymorphism
SF	Spikelet fertility
SRWC	Shoot relative water content
SSR	Simple sequence repeat
SW	Straw weight
TChl	Total chlorophyll
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
TGW	Total grain weight
ThGW	Thousand grain weight
TLL	Third leaf length
TLW	Third leaf weight
TT	Total tiller
TW	Total weight
UFGN	Unfilled grain number
UFGW	Unfilled grain weight
USDA	United States Department of Agriculture

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CHAPTER 1 INTRODUCTION

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1.1 Rice origin & cultivation

Rice is world's most important staple food crop. For more than half of humanity, rice is life. It is the grain that has played a part to build civilizations, shape societies and feed a growing world (Callaway 2014). Approximately three billion people or more than half of the world's population relies on rice as the major daily source of calories and protein (FAO, 2004). Hence, rice is fundamental for food security for them.

Asia produces more than 90% of the total rice produced in the world every year (Elert 2014). However, 5 countries in Asia, such as China, India, Bangladesh, Vietnam and Thailand are the major producers, consumers and exporters (Table 1.1). Global nutrition mostly relies on 3 cereals such as rice, wheat and maize. In Bangladesh, the dependence of daily calorie from rice is much higher and tops the world consumption at 70% (Elert 2014).

Table 1.1: Average annual production and consumption of the top 5 Asian country 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	12.0	2.3
Bangladesh	31.80	26.0	5.9	--
Vietnam	26.10	12.80	13.40	3.7
Thailand	22.10	7.60	14.50	6.1
Total	307.80	238.60	69.40	12.30
% of world's total	67	67	69	76

Rice belongs to the family Gramineae, genus *Oryza,* which has two cultivated species and 22 wild species (Wang and Leung 1998; Brar and Khush 2002). One of the two cultivated species, *Oryza sativa* is the main cultivated rice and was first cultivated in India and China 15,000 years ago (Normile 1997). The other species, *O. glaberrima* is African rice and is limited to West Africa. The rice crop is well adapted to high temperatures and flooded soils (Bonman, Khush et al. 1992), although it can be grown in four major ecosystems: irrigated, upland, rainfed lowland and flood-prone (Irri 2002).

1.2 Rice production: Bangladesh scenario

Rice is grown in Bangladesh in three seasons namely *Aus* (March/April – July), *Aman* (July – December) and *Boro* (November – May). The total rice area is about 11.42 million ha over 3 seasons and total production is 33.83 million tons (clean rice) in 2013 (Table 1.2). 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. 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.

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.

While Bangladesh has made significant achievements over the last 40 years, it still faces considerable challenges like shrinking arable lands, increasing population growth, effects of climate change, persistent poverty and one of the highest malnutrition rates in the world (Rayhan and Khan 2006).

Table 1.2: Rice statistics of Bangladesh.

Seasons	Area		Production		Yield (t/ha)	
	Million ha	%	Million ton	%	Clean Rice	Paddy
Aus	105	9.22	2.16	6.38	2.05	3.10
Aman	561	49.11	12.89	38.12	2.29	3.48
Boro	476	41.67	18.78	55.5	2.95	5.98
Total	1142	100	3383	100	Av = 2.76	Av = 4.19

Source: BBS, 2013

1.3 Climate change and its impact on agriculture

The earth climate has been changing rapidly for the last few decades. Different kinds of climatic changes from global warming have been identified, such as an imbalance in rainfall pattern, melting glaciers that cause sea level rise, rise of temperature in the ocean and so on. Moreover, it has been observed that from 1993 to 2003 the polar ice caps had been melting because of global warming and it had been causing 3.1 mm rise of sea level per year (Lemke, Ren et al. 2007; Pender 2008). Although delta areas cover only 1% of the world's land, 7% of the global population lives in these delta areas (Szabo, Hossain et al. 2016). Hence, climate change becomes a threat to these people, especially in the coastal areas of developing countries.

Bangladesh is considered as one of the developing countries in the world which is adversely affected by climate change. The country is the most disaster prone due to its geophysical location (Ahmed and Glaser 2016). In the year 2012, the global climate risk index ranked Bangladesh in the first position and in 2016 it ranked the country in sixth position in the list as one of the most vulnerable climate change affected countries in the world (Harmeling and Eckstein 2012; Kreft,

Eckstein et al. 2014; Ahmed and Glaser 2016). The coastal area of this country is under constant threat of climatic hazards and climate change impacts regarding floods, cyclones, droughts, excessive rainfall, high temperature, sea level rise, salinity intrusion etc.

Agriculture is the dominant economic sector in Bangladesh (Khan, Bryceson et al. 2015) and the primary source of livelihood for the people of the country. The Ministry of Agriculture of Bangladesh reported that due to climate change impact, the country lost around 80,000 ha of arable land (Habiba, Ali et al. 2015). In this context, the coastal regions of Bangladesh are suffering greatly due to climate change in terms of sea level rise, saline water intrusion and salinity increase in the soil (Sinha and Singh 1976; Nagarajan, Singh et al. 2014). According to the IPCC fourth assessment report, global warming caused saline water intrusion in groundwater (DMB GoB, 2010) and by the year 2050 the production of rice and wheat is expected to decrease by 8% and 32%, respectively.

1.4 Traditional coastal rice landraces

This increasing demand of rice production has to be met from our limited and shrinking land resources. The most logical way to raise production is to target high yields with a higher cropping intensity. The only way with available technology in order to meet the demand is to extend production to unfavorable ecosystem i.e. flood-prone area, saline zone, haor area etc. Higher sensitivity of modern rice to the unfavorable conditions make the farmers grow their traditional landraces which have low yields.

Despite the introduction of modern varieties (MVs) in most of the districts of Bangladesh, which give the farmers a yield advantage, low yielding traditional cultivars are still very popular. These popular rice landraces (LRs) are well adapted to the prevailing soil stresses, including soil salinity. The traditional cultivars possess acquired traits enabling them to survive in sub-optimal conditions. At least 700 LRs are still grown and some of these are among the top 20 cultivars grown by farmers (Bose, Isa et al. 2001). In the saline coast in the T.

Aman and Aus seasons, LRs account for more than 50 and 60% of the total rice cultivation area respectively.

1.5 Saline coastal belt of Bangladesh

The total cultivable area of Bangladesh is 9 million hectares, in which 1 million hectares is affected by varying degrees of salinity. Salinization is the accumulation of water soluble salts in the soil to a level that affects agricultural production. A soil is considered saline if the electrical conductivity of its saturation extract (ECe) is above 4dS/m (USDA-ARS, 2008), which is equivalent to approximately 40mM NaCl and generates an osmotic pressure of approximately 0.2 MPa. Large fluctuations in salinity levels over time is observed at almost all areas in these regions. The salinity is highest in the dry season. Increasing from November, it peaks in March until the start of the annual rains, from April to October. The Sundarbans forest area covers one-third of the southern portion of 3 districts in Bangladesh and is known to be highly saline. From west to east, that is, from Satkhira to Khulna and Bagerhat, salinity level declines gradually; Satkhira being highly saline with 70% of the area having soil salinity levels of 4-16 dS/m (Karim and Iqbal 2001). Further eastwards, there are the coastal regions of Pirojpur, Barisal, Barguna and Patuakhali. These areas are slightly saline (2-4 dS/m) with some pockets being non-saline. The coastland extends north-eastwards, where there are the districts of Lakshmipur, Noakhali and Feni. The salinity level in Noakhali is moderate (4-8 dS/m) and extends to larger areas compared to Lakshmipur and Feni. The coastline moves southwards into Chittagong and Cox's Bazar where there are pockets of high salinity as well as mild levels.

Soil Resource Development Institute (SRDI) has temporal and spatial data on regular monitoring of the salinity level of soil and water 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. Figure 1.1 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. Moderately to strongly

saline soil (S3 and S4: 8.1-16 dS/m) has 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). 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).

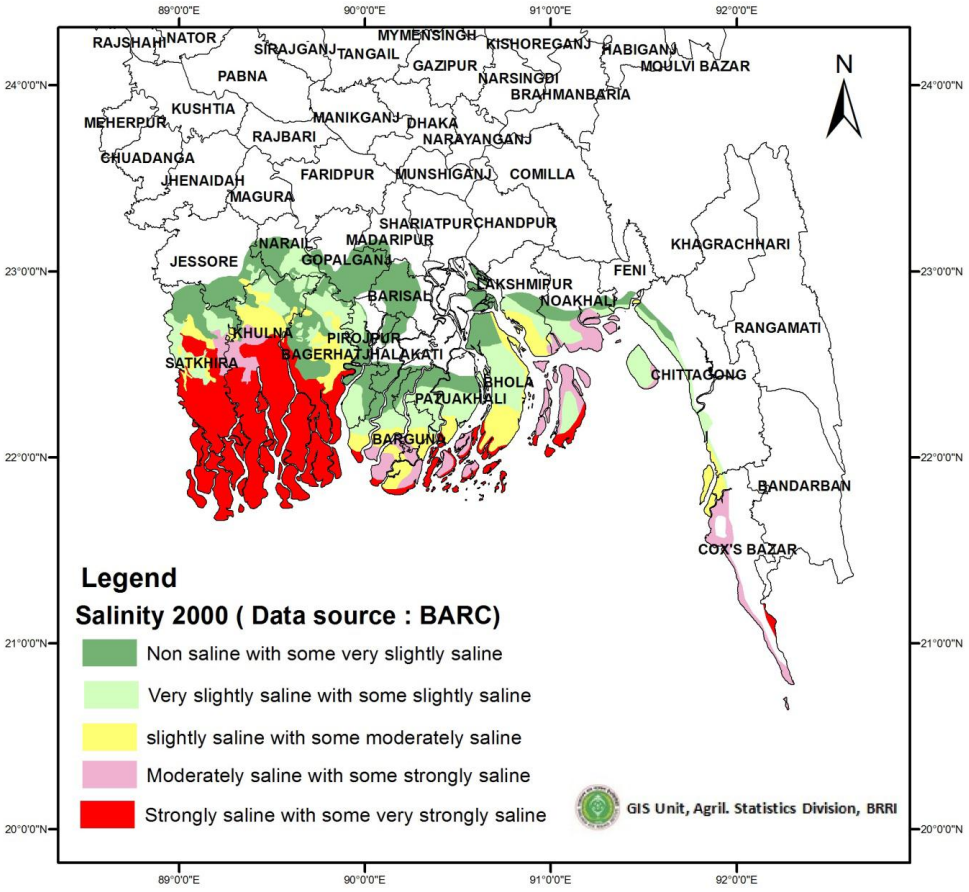

Figure 1.1: Soil salinity map of Bangladesh, data source BARC 2000 (map redrawn by GIS Unit, Agricultural Statistics Division, BRRI).

Soil salinity, at any time, is maximum in the surface layers (0-15 cm). Subsoil salinity is usually much lower than topsoil salinity. Moderately to strongly saline underground water is found within 1-2 meters below the soil surface at all locations in the dry season. The spatial and temporal variations in soil salinity indicate the need for separate plans for crop production for different locations in the coastal areas. No generalization can be made in this regard (Panaullah 1993).

1.6 Rice production under salinity stress

Soil salinity is the most widespread soil toxicity problem in rice growing countries. Rice is usually considered as a salt sensitive crop, especially when soil salinity is more than 3 dS/m (deci Siemens per meter), causing a significant reduction in yield at a rate of 12% per deci Siemens (Maas and Hoffman 1977). Rice shows a wide range of variability in salinity tolerance (Flowers and Yeo 1981; Lisa, Seraj et al. 2004; Mahmood, Turner et al. 2004; Munns, James et al. 2006).

The pattern of rice responses to salinity also varies in different growth stages. In most cases, rice at young seedling stage is very sensitive to salinity (Pearson and Bernstein 1959; Kaddah 1963; Flowers and Yeo 1981; Heenan, Lewin et al. 1988; Lutts, Kinet et al. 1995). Panicle length, spikelet number per panicle, and grain yield were significantly reduced when salt treatments were applied at panicle initiation stage (Sajjad 1984; Heenan, Lewin et al. 1988; Cui, Takeoka et al. 1995; Khatun, Rizzo et al. 1995; Maas and Grattan 1999). Seedling and flowering stages of rice are more likely to be affected by salinity, with reduction in seedling growth and yield (Rood 2000). Traditionally tolerant varieties can produce relatively good yields (35-40% reduction, relative to non-saline condition) even at 12.5 dS/m, while other varieties fail (Rana 1985).

1.7 Current status of conventional breeding for rice salt tolerance: Genetics and Breeding strategies

About 20% of cultivated lands are affected (~45 million ha) by increasing salinity worldwide (Gupta and Huang 2014). So, the development of salt tolerant rice varieties is a priority in rice breeding programs (Lin, Zhu et al. 2004) (Senavirathne, Jayatilake et al. 2017). It is also necessary to discover and understand the multiple metabolic pathways coping with tolerance, so that complementary traits can be pyramided for a higher level of tolerance than present in currently bred cultivars.

Breeding for salt stress tolerance in rice has been moderately successful (Mishra, Singh et al. 2001; Senadhira, Zapata-Arias et al. 2002). Conventional breeding practices are lengthy and require 10-15 years in order to stabilize a new variety. Only a few salt-tolerant rice donors have been identified for breeding purposes so far. Among the donors identified, the cultivars Pokkali, Nona Bokra, Cheriveruuppu, SR26B, Damodar and Getu are reported to possess poor agronomic characteristics (Lisa, Elias et al. 2011). The highly salt tolerant landraces like Nona Bokra and Pokkali are among the most extensively used in breeding. However, some of their negative characters commonly found in traditional genotypes as well as the complexity of traits involved in salinity tolerance have hampered progress in their use in conventional breeding.

Selection under stress for grain yield has been reported as the best approach to breed stress-tolerant rice varieties (Mishra and Tyagi 1986; Munns, James et al. 2006; Venuprasad, Lafitte et al. 2007; Kumar, Shriram et al. 2008). Selection for the component traits to breed a stress-tolerant rice variety have also been proposed (Yeo, Yeo et al. 1990) and have long been used (Singh, Singh et al. 2004; Singh, Gregorio et al. 2008; Serraj, Kumar et al. 2009). Morphological traits such as the ratio of filled/unfilled grains, grains per panicle, spikelet fertility, plant height, fertile tillers, and flowering are good indicators associated with salt tolerance in rice. However, grain yield has been negatively correlated with physiological traits like Na^+ concentration and Na^+/K^+ ratio, whereas it showed a positive correlation with K^+ concentration under both alkaline and saline soil conditions (Rao, Mishra et al. 2008).

Among the NARES institutes and IRRI, 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). Most of the initial salt tolerant rice varieties like Damodar (CSR1), Dasal (CSR2), Getu (CSR3), Pokkali, Vytilla1, Vytilla2, Vytilla3, Vytilla4, Vytilla5 etc. had been developed through pure line selection from the local traditional cultivars prevailing in the Sundarbans areas in West Bengal, India.

Later, other salt tolerant varietal series like CSR10, CSR13, CSR23, CSR27, CSR30, CSR36**,** CR Dhan402, CR Dhan 403, TRY1, TRY2, TRY3, White ponni, CO43 etc. had been developed through recombination breeding methods like Pedigree method and Modified bulk pedigree method.

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 pests and most importantly low yield in high stress condition. BRRI dhan61 and dhan67 are relatively new releases and so, their level of acceptability is unknown. In view of the predicted increase in salinity levels, more tolerant varieties are needed, preferentially with a wider genetic base. Characterization of farmer popular landraces, still endemic to the Southern coast of Bangladesh for their stress-coping mechanism will help in identifying quantitative trait loci (QTLs) or genes that could be introduced into modern varieties for their improvement.

1.8 Molecular markers and QTLs for rice salinity tolerance

Salinity tolerance is a complex trait and is a combination of different mechanisms, mostly governed by polygenes (Moeljopawiro and Ikehashi 1981; Mishra and Tyagi 1986; Mishra, Singh et al. 1998; Mishra, Singh et al. 2001). A number of

mapping studies have attempted to identify QTLs located on different chromosomes which are implicated for salinity tolerance in rice (Zhang, Guo et al. 1995; Gong, He et al. 1999; Prasad, Bagali et al. 2000; Singh, Gregorio et al. 2007; Haq, Gorham et al. 2010; Pandit, Rai et al. 2010) but there is limited information on salt tolerance at reproductive stage (STRS). The reproductive stage is the most crucial as it ultimately determines grain yield. Therefore, STRS is an important trait for stable rice production in salt affected areas. Only a limited number of QTLs for STRS have been mapped (Mohammadi, Mendioro et al. 2013; Hossain, Rahman et al. 2015). 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, Ahmadi et al. 2009). Discovering the genetic control of each salt tolerance mechanism will open up the opportunity to develop varieties with better salinity tolerance by precisely transferring underlying QTLs into popular varieties and pyramiding multiple loci for a particular stress-prone environment (Ashikari and Matsuoka 2006).

For the seedling stage, a major QTL for salinity tolerance, *Saltol*, explaining >50% of the phenotypic variance responsible for selective ion uptake has been mapped on chromosome 1 (Gregorio 1997; Bonilla, Dvorak et al. 2002). Other researchers have also detected the same QTL in some other rice cultivars (Takehisa, Shimodate et al. 2004; Ren, Gao et al. 2005; Islam and Gregorio 2013). There are reports of introgression of this *Saltol* QTL into rice varieties like BR11, BRRIDhan28, Q5DB, IR64, AS996 and PB1121 (Luu TN, Luu M et al. 2012; Gregorio, Islam et al. 2013; Huyen, Cuc et al. 2013; GUO and YE 2014; Hasan, Rafii et al. 2015; Babu, Krishnan et al. 2017). The reported gain in tolerance of these lines due to introgression of *Saltol* was moderate to strong depending on the genetic background. However, since the advantage gained was mainly at the seedling stage, considerable loss in grain yield under saline stress was also observed.

DNA markers are the most widely used markers predominantly due to their abundance and they are not affected by the environment as well as the development stages of the plant (Winter and Kahl 1995). A study reported that it

arises from different classes of DNA mutations, such as substitution mutations, rearrangements or errors in replication of tandemly repeated DNA (Paterson 1996). In this study, a new approach based on traditional DArT and next-generation sequencing technique, called as DArTseq™ (Raman, Cowley et al. 2014), was used to analyze the genome of the rice population. DArTseq is a genotyping technology that detects all types of DNA variation (SNP, indel, CNV, methylation). The traditional method called Diversity Arrays Technology (DArT) is a microarray-based DNA marker technique for genome-wide discovery and genotyping of genetic variation. Simultaneous scoring of hundreds of restriction site based polymorphisms between genotypes is possible in DArT and it does not require DNA sequence information or site specific oligonucleotides. DArT can detect DNA polymorphisms by scoring the presence or absence of specific DNA sequences in a defined genomic representation (i.e., a representative subset of genomic fragments) through hybridization to microarrays (Jaccoud, Peng et al. 2001; Wenzl, Carling et al. 2004). At present, genotyping by DArT has become sequencing based. The DArTseq method deploys sequencing of the representations on the Next Generation Sequencing (NGS) platforms. QTL mapping using DArTseq technology has been done in other crops like winter rye, wheat, sugarcane etc (Balsalobre, da Silva Pereira et al. 2017; Hackauf, Haffke et al. 2017; Yu, Liu et al. 2017).

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

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 (Abler, Edwards et al. 1991) loci as a substitute for or to assist in 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 only rather than their phenotype. Marker-assisted selection may greatly increase the efficiency and effectiveness of breeding compared to conventional breeding.

The recent advances in genomics have made the way easier 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 (Collard, Jahufer et al. 2005; MacKill 2006; Collard and Mackill 2008; Thomson, Ismail et al. 2009). 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. And multiple and complementary loci are introgressed by pyramiding these using markers. By using MAB, one can ensure that the background yield which is also controlled by multiple genes stays intact. So far, success in MABC for improving biotic and abiotic stress tolerance has been achieved with QTLs that provide high levels of tolerance in many different genetic backgrounds and environments (MacKill 2006; Collard and Mackill 2008; Thomson, Ismail et al. 2009). A good example in rice is the introgression of *SUB1*, the major QTL for submergence tolerance, into several popular rice varieties (Xu, Xu et al. 2006; Neeraja, Maghirang-Rodriguez et al. 2007; Iftekharuddaula, Newaz 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, Maghirang-Rodriguez 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 flanking 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). 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 fight with the adverse effects of climate change.

1.10 Objectives of the study

This current study focused on mapping QTLs of a tolerant, low yielding Bangladeshi rice landrace *Horkuch*. The study plant *Horkuch* is a farmer-popular salt tolerant rice (*Oryza sativa* L.) variety from the south-west coast of Bangladesh. *Horkuch* grows in a number of places of Khulna division such as Shoronkhola, Dumuria, Dakop, Paikgacha, Kaliganj, Shyamnagar and Fakirhat ("Deshi dhaan er jat", a Bengali book on Bangladeshi local landraces, published by BRRI, 1982). In a previous study (Lisa, Elias et al. 2011), *Horkuch* was characterized up to maturity under NaCl stress, together with a modern variety (BRRI dhan41), a sensitive control (BRRI dhan29) and Pokkali, the salt-tolerant benchmark for rice. *Horkuch* was tested for its level of tolerance at both seedling and reproductive stages where it was found to be equivalent to Pokkali. The gene expression pattern of *Horkuch* seedling in 150mM was analyzed and attempts were made to explain its superior physiological performance under salt stress. The upregulated genes included transcription factors, signal transducers, metabolic enzymes, reactive oxygen species (ROS) scavengers, osmoprotectants and some specific salt-induced transcripts. An increase in expression of photosynthesis-related genes as well as ROS scavengers suggested that this could be the reason for the better yield performance of *Horkuch*. It can be concluded from the combination of physiological and gene expression data that *Horkuch* could be a potentially useful donor for future programs in breeding or engineering salt-tolerant rice.

In order to identify the tolerance loci in *Horkuch*, a mapping population derived from *Horkuch* and high-yielding *IR29* was characterized. All phenotypic analysis were performed at the two developmental stages-seedling and reproductive. According to the $F_{2:3}$ design, the population was genotyped in the F_2 population using DArT based SNP genotyping and phenotyped in the F_3 population. The QTLs were validated by molecular and physiological analysis at F_5 generation. The existing varieties which are mostly produced by traditional breeding do not grow

beyond 8dS/m and that too with 50% loss in yield**.** Salinity is a trait controlled by multiple traits and a single marker cannot give that much tolerance (Thomson, de Ocampo et al. 2010). By finding markers linked to multiple traits, we wanted to pave the way for pyramiding several of the traits together in one individual using marker-assisted backcrossing.

The specific objectives of the study are:

1. SNP genotyping of the segregating F_2 population for generating large number of molecular markers across the whole genome using DArT-based markers to produce linkage map.
2. Phenotyping of F_3 population in both seedling and reproductive stages for quantitative estimation of specific traits.
3. Mapping QTLs combining genotyping and phenotyping information.
4. Genotypic validation of QTLs in recombinant inbred lines (RILs, F_5 advanced generation) using SSR markers.
5. Phenotypic validation of the recombinant inbred lines (RILs, F_5 advanced generation).
6. Identifying progeny plants with a combination of several QTLs that can act as salt tolerance donors in breeding lines.
7. Combining seedling and reproductive QTLs in a single line for complementation of salt tolerance traits in order to get higher level of tolerance.
8. Identifying RILs which can act as donor of multiple salt tolerance traits into high-yielding mega varieties by marker assisted breeding.

CHAPTER 2 *REVIEW OF LITERATURE*

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2.1 Diversity of salinity tolerance of cereals and rice

Variation of salt tolerance exists among plant species (Munns and Tester 2008). The salinity tolerance responses of different plant species are shown in figure 2.1, where rice (*Oryza sativa*) is the most sensitive, bread wheat (*Triticum aestivum*) is moderately tolerant and barley (*Hordeum vulgare*) is the most tolerant among the cereals. 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 of salinity tolerance in dicotyledonous species is greater than that of monocotyledonous species. Some legumes are even more sensitive than rice; alfalfa or lucerne (*Medicago sativa*) is very tolerant, and halophytes such as saltbush (*Atriplex* spp.) are able to grow well at salinities greater than seawater (Munns and Tester 2008).

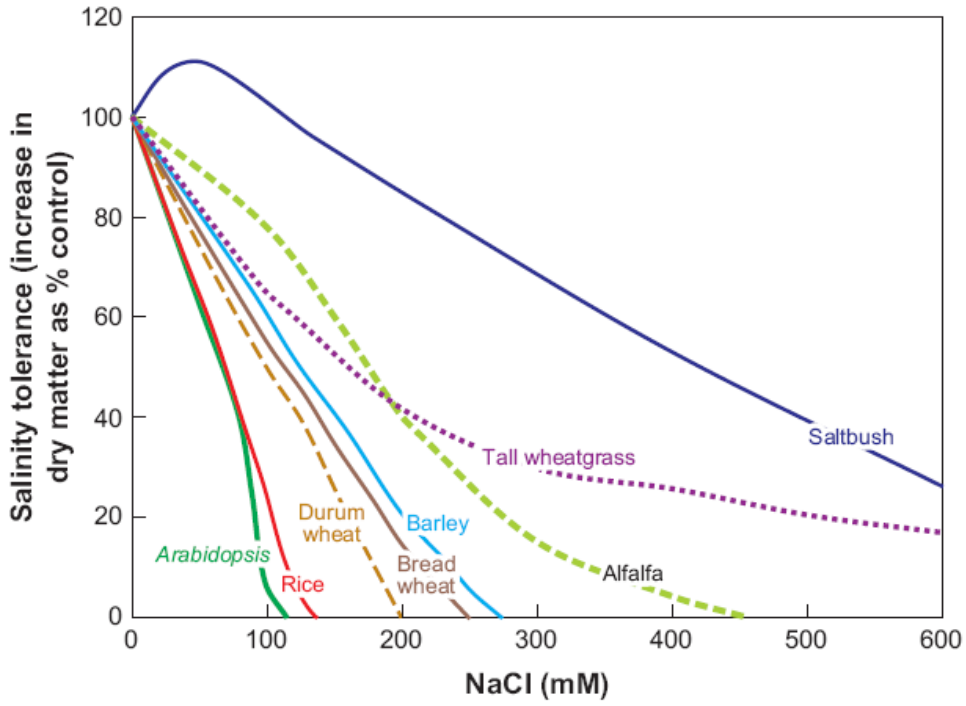

Figure 2.1: Diversity in the salt tolerance of various species, shown as increases in shoot dry matter after growth in solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl (Munns and Tester 2008).

Among the important crops in the world, wheat is one of the most salt-tolerant crop species, and can tolerate salt upto 15 dS/m (approximately 150 mM NaCl). Rice is more salt-sensitive, and many cultivars face a 50% growth reduction at half this concentration of salts. In terms of salt sensitivity, maize falls between these two species (Munns and Tester 2008).

Rice shows a large variation for tolerance to salinity (Flowers and Yeo 1981; Lisa, Seraj et al. 2004; Mahmood, Turner et al. 2004; Zeng, Kwon et al. 2004; Munns, James et al. 2006). Several rice landraces from India and Bangladesh were identified as tolerant and were used in mapping and breeding for salinity tolerance. Some of these tolerant species are Pokkali, Nona Bokra, Cheriviruppu, Kalarata, Kuti Patnai, *Horkuch*, Jamai Nadu, Ashfal, Ashfal balam, Capsule etc. (Gregorio, Senadhira et al. 1997; Lisa, Seraj et al. 2004; Singh and Flowers 2010; Hossain, Rahman et al. 2015; Rahman, Thomson et al. 2016).

The tolerance or sensitivity of rice to salt not only varies between genotypes but between stages of plant development. The germination stage is relatively tolerant, but rice 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, Ayers et al. 1966; Khatun, Rizzo et al. 1995; Lutts, Kinet et al. 1995; Makihara, Tsuda et al. 1999; Singh, Singh et al. 2004; Shereen, Mumtaz et al. 2005; Singh, Gregorio et al. 2008). Singh, Gregorio *et al.* 2008 has depicted the changes of tolerance and sensitivity according to different developmental stages (figure 2.2).

The seedling stage is very sensitive to salinity and there is a large genetic variation among rice in case of sensitivity, where many are relatively tolerant. Traditional land races such as Pokkali and Nona Bokra have been used as donors of salt tolerance in breeding programs as well as other landraces and improved genotypes are found to be tolerant to a salinity of 12 dS/m at the seedling stage (Singh and Flowers 2010).

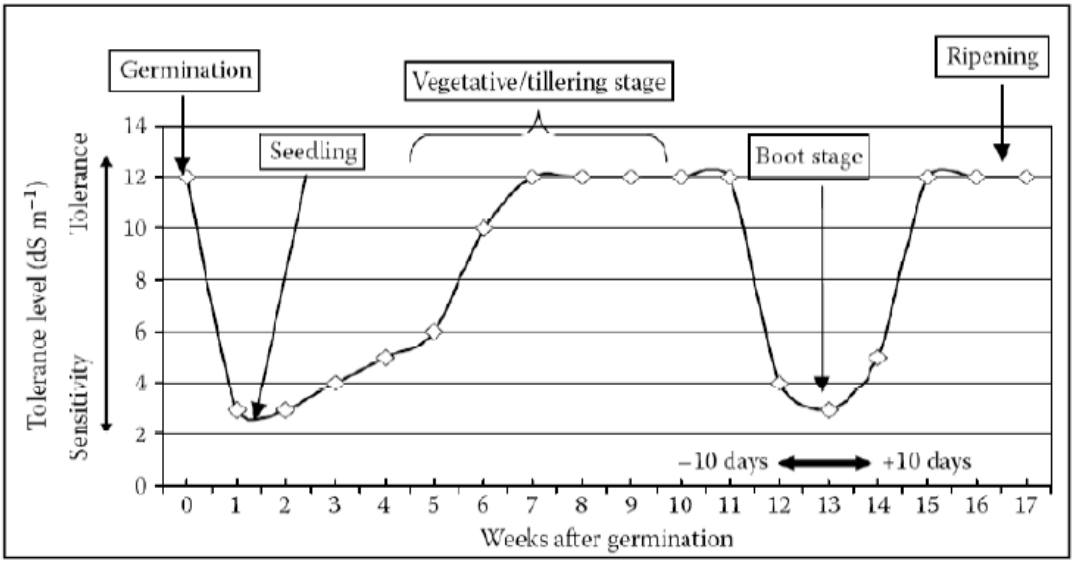

Figure 2.2: Variation in the sensitivity of rice to salinity during its ontogeny. (Adapted from Singh, Gregorio *et al.* 2008)

Reproductive stage is another phase when rice is sensitive to salinity stress, specifically at the booting stage (7-10 days before and after booting stage). The reproductive stage is crucial as it ultimately determines grain yield. There are few studies that report the effects of salinity on yield. A sharp decline of seed set with increasing salinity was observed in a rice variety called IR36 (Khatun and Flowers 1995).

In another study, it was found that under varying salinity stress, rice showed delayed flowering, reduced number of productive tillers, less number of fertile florets per panicle, reduced weight per grain and subsequently poor yield (Khatun, Rizzo et al. 1995). In another study, an average yield decline by 30% was reported compared to normal soils under salinity (10 dS/m) using six rice varieties (Mahmood, Quereshi et al. 1999). Another study (Rao, Mishra 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 of 8 dS/m. Hence, to know the response of the rice plant to salinity as a whole, the effects should be observed in all the various stages of its development, that is at early seedling, vegetative and reproductive stages (Gregorio, Senadhira et al. 1997).

2.2 Physiological responses of rice to salinity stress

The physiological responses of rice to salt stress are different in different stages of development. To improve the salinity tolerance of rice it is necessary to understand the entire responses from single cell 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 (Negrão, Courtois et al. 2011).

2.2.1 Short-term responses

There are two phases in short term response: a rapid response to the increased external osmotic pressure, and a slower response caused by accumulation of Na^+ in leaves (Munns and Tester 2008).

2.2.1.1 Osmotic stress or Ion independent stress

The 'osmotic stress' or shoot-ion independent stress is the early response immediately after salt stress. This fast response is related to Na^+ sensing and signaling in the root resulting in shoot growth reduction and stomatal closure under saline condition (Moradi and Ismail, 2007**,** Munns and Tester 2008). The "osmotic stress" caused by salt reduces leaf and root growth and decreases stomatal conductance and thereby photosynthesis (Munns 1993). However, this phase is relatively quick in rice; growth recovers (not necessarily to the original rate) within 24 h, so any osmotic effect is transient (Yeo, Lee et al. 1991; Roshandel and Flowers 2009). There are genotypic differences in this fast response, where the reduction of stomatal conductance rate is relatively slower in salt sensitive genotypes than the salt-tolerant ones (Moradi and Ismail 2007).

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 protein kinases, such as two component histidine-kinases (Urao, Yakubov et al. 1999; Marin, Suzuki et al. 2003;

Kacperska 2004) and wall-associated kinases (Kohorn 2001). According to another study (Yeo, Lee et al. 1991), these perception mechanisms seem to be activated not only by NaCl but also by KCl or mannitol and it was concluded that the initial growth reduction was due to a shortage of water supply caused by variation in osmotic pressure. It was showed in a study (Termaat, Passioura et al. 1985) that NaCl itself is enough to promote growth reduction. Recently, it was found in *Arabidopsis thaliana* that NaCl around the roots elicits a calcium (Ca^{2+}) wave signal that propagates throughout the plant (Choi, Toyota et al. 2014). It might be responsible for the initial and fast plant responses to salinity. This study also showed that exposure of *Arabidopsis* root tips to sorbitol, an osmotic control for NaCl, did not elicit similar long-distance Ca^{2+} signal as that of NaCl. From these results, it was hypothesized that the growth reduction is probably a specific signaling and sensing response to NaCl stress which is independent of osmotic pressure and other agents like KCl or mannitol (Pires, Negrão et al. 2015).

2.2.1.2 Ionic stress

Ionic stress develops over time and is caused by a combination of ion accumulation in the shoot and an inability to tolerate the accumulated ions. In this phase, salinity response starts when salt concentration in the old leaves reaches toxic levels (old leaves no longer expands and as a result no longer dilutes the salt coming inside as young growing leaves do), leading to leaf death. If the rate of leaf death is greater than the rate of emergence of new leaves, the photosynthetic capacity of the plant will be unable to supply the required carbohydrate to the young leaves, which further reduces their growth rate. Ionic stress affects growth much later, and the effect is less severe than the osmotic stress, especially at low to moderate salinity levels. Only at high salinity levels, or in sensitive species that are unable to control Na^+ transport, the ionic effect dominates the osmotic effect (Munns and Tester 2008).

In rice, Na^+ ions make their way into roots through apoplastic and symplastic pathways. In symplastic transport, movement through plasma membrane is

required before off-loading to the xylem. In apoplastic transport, also called bypass flow, ions move through cell walls and other extracellular spaces to the xylem. In several studies, it has been shown that in rice roots, apoplastic transport has been a significant part of sodium entry under saline conditions (Yeo, Yeo et al. 1987; Yeo 1992; Yadav, Flowers et al. 1996; Yeo 1998; Ochiai and Matoh 2002; Anil, Krishnamurthy et al. 2005; Gong, Randall et al. 2006; Krishnamurthy, Ranathunge et al. 2009). The magnitude of apoplastic transport in rice is thought to depend on the anatomical and morphological developments of the roots.

In symplastic transport, the uptake of Na^+ involves a transporter. There are several proteins capable of transporting Na^+ and K^+ (Tester and Davenport 2003; Maathuis 2007; Zhang, Flowers et al. 2010). In low affinity uptake, the transporters that usually play roles are nonselective cation channels (NSCCs), high-affinity K^+ transporters (*HKTs*), K^+ uptake permeases/high-affinity Na^+/K^+ transporters (*KUP/HAK/KT*), cation-chloride co-transporters (*CCCs*) and probably members of the Shaker family of K^+ transporter (*AKT*) (Flowers and Colmer 2008; Zhang, Flowers et al. 2010).

Different *HKT* members may have different transport properties. The founding member of the *HKT* family, *TaHKT1*, mediates K^+/Na^+ symport and Na^+ transport (Genet, Lowman et al. 1995; Gassmann, Rubio et al. 1996), whereas *AtHKT1* selectively transports Na^+ (Berthomieu, Conéjéro et al. 2003). In rice, *OsHKT1*, like AtHKT1, transports Na^+ only (Horie, Yoshida et al. 2001; Garciadeblás, Senn et al. 2003) and *OsHKT2* mediates *TaHKT1*-like K^+/Na^+ symport (Horie, Yoshida et al. 2001). A rice QTL, *SKC1*, was previously mapped that maintained K^+ homeostasis in the salt-tolerant variety under salt stress (Lin, Zhu et al. 2004), consistent with the earlier finding that K^+ homeostasis is important in salt tolerance (Maathuis and Amtmann 1999). In another study (Ren, Gao et al. 2005), it was found that it encoded a member of *HKT*-type transporters. *SKC1* is preferentially expressed in the parenchyma cells surrounding the xylem vessels. Voltage-clamp analysis showed that *SKC1* protein functions as a Na^+ selective transporter. Physiological analysis suggested that *SKC1* is involved in regulating K^+/Na^+ homeostasis under

salt stress, providing a potential tool for improving salt tolerance in crops. In another study, a gene in the *Nax2* locus, *TmHKT1;5-A*, encodes a Na^+ selective transporter located on the plasma membrane of root cells surrounding xylem vessels, which is therefore ideally localized to withdraw Na^+ from the xylem and reduce transport of Na^+ to leaves. Field trials on saline soils demonstrated that the presence of *TmHKT1;5-A* significantly reduced leaf [Na^+] and increased durum wheat grain yield by 25% compared to near-isogenic lines without the *Nax2* locus.

A *Salt Overly Sensitive (SOS)* stress signaling pathway is involved in ion homeostasis and salt tolerance (Shi, Quintero et al. 2002). The tonoplast-localized *NHX1 (*Blumwald and Poole 1985) and plasma membrane-localized *SALT OVERLY SENSITIVE 1* (*SOS1*, also known as *NHX7*) Na^+/H^+ antiporters (Qiu, Guo et al. 2002; Yamaguchi, Hamamoto et al. 2013) are the two major factors that maintain low cytoplasmic Na^+ concentrations in plant cells. Though most *NHXs* are essential for Na^+ detoxification via Na^+ sequestration 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 (Zhu 2002; Chinnusamy, Schumaker et al. 2004). The *SOS* signaling pathway involves three major proteins, *SOS1, SOS2,* and *SOS3*. Though *SOS1* is expressed in various tissues, it is particularly expressed 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 observed that is supposed to be conducted by the *salt overly sensitive (SOS)* pathway. A plasma membrane Na^+/H^+ antiporter is encoded by *SOS1* and the antiporter is essential in regulating Na^+ efflux at cellular level. It also enhances long distance transport of Na^+ from root to shoot. Overexpression of this antiporter confers salt tolerance in plants (Shi, Ishitani et al. 2000; Shi, Quintero et al. 2002). *SOS2* gene encodes a serine/threonine kinase and is activated by salt stress elicited Ca^{2+} signals. Finally, the *SOS3* protein 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, Liu et al. 2000). Salt stress elicits a cytosolic calcium signal, a major second-messenger signaling molecule. A

myristoylated-calcineurin B-like protein (*CBL4*), originally designated by *SOS3*, senses the salt-elicited calcium signal. *SOS3* undergoes dimerization after binding to Ca^{2+} 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 bound to *SOS3 (CBL4)*, which enables the phosphorylation and thus the activation of the membrane-bound Na^+/H^+ antiporter *SOS1* (Zhu 2002; Martínez-Atienza, Jiang et al. 2007; Munns and Tester 2008). The rice orthologs of *SOS1, SOS2,* and *SOS3* were identified and it was demonstrated that the *SOS* pathway of Na^+ control is structurally highly conserved in rice (Martínez-Atienza, Jiang et al. 2007). 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. The *SOS3* protein then interacts with 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 (Martínez-Atienza, Jiang et al. 2007).

2.2.2 Long-Term Responses

Long-term responses can be explained by increased osmotic and tissue tolerance leading to an increased ability to maintain growth for a given accumulation of Na^+ in the leaf tissue. According to a study (Negrão, Courtois et al. 2011) the long-term responses has been explained by osmotic adjustment, Na^+ exclusion and K^+ homeostasis and regulation of antioxidants.

2.2.2.1 Osmotic adjustment

During high salinity stress, this is the first tolerance response to occur. Immediately after plant senses Na^+ in the soil or apoplastic solution, mechanisms that prevent cell dehydration, protein denaturation and destabilization of cellular structures are activated (Yancey, Clark et al. 1982). Nontoxic metabolites, known as “compatible solutes” accumulate in the cytoplasm to balance osmotic potential between the cytosol and apoplastic solution. These compatible solutes include

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) (Bohnert, Nelson et al. 1995; Bohnert and Shen 1998; Flowers and Colmer 2008). Trehalose is a nonreducing disaccharide and trehalose-6-phosphate can act as a signal of sucrose status and as a regulatory molecule especially in sugar influx and metabolism (Paul, Primavesi et al. 2008; Iturriaga, Suárez et al. 2009). Besides accumulation of certain compatible solutes in the cytosol under salinity/osmotic stress, ion accumulations in the cytosol (mainly K^+) and in the vacuole (Na^+, especially in salt tolerant cultivars/species) also play important roles for the osmotic adjustment of plant cells (Gorham, Jones et al. 1985). Compatible solutes also appear 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 1998).

Two genes in rice encode the enzyme betaine aldehyde dehydrogenase, which catalyzes betaine aldehyde to glycine betaine (GB), a compatible solute. However, rice is unable to produce GB due to the lack of an upstream enzyme, choline monooxidase (CMO), which converts 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 and Murata 1998; Shirasawa, Takabe et al. 2006). However, relatively small amount of GB accumulation and a small increase in salt tolerance of transgenic rice plants were observed in some conditions tested, probably due to low activities and/or mislocalization of the introduced enzymes (Shirasawa, Takabe et al. 2006). Transgenic rice with BADH from barley and fed exogenous glycine betaine shows increased salt tolerance (Kishitani, Takanami et al. 2000). In other studies, an enhancement of salt and drought tolerance has been observed upon exposure to exogenous glycine betaine (Harinasut, Tsutsui et al. 1996; Demiral and Türkan 2004; Farooq, Basra et al. 2008). There is evidence that glycine betaine is involved in protecting major enzymes and membrane structures as well as they play a vital role in maintaining the activities of ROS scavenging

enzymes (Raza, Athar et al. 2007; Chen and Murata 2011; Guinn, Pegram et al. 2011).

In rice, the compatible solute that is produced in significant amount under salt stress is proline. In tolerant rice genotypes, there is a fast increase in proline accumulation rate under salt stress. It is a confirmation of its protective role against hyperosmotic stress (Demiral and Türkan 2006). They also help balance the ion osmotic pressure in the vacuole, where Na^+ and Cl^- are sequestered. It is found from molecular analyses that salt stress stimulates proline synthesis whereas its catabolism is enhanced during recovery (Székely, Ábrahám et al. 2008; Sharma and Verslues 2010). Though it was presumed that proline plays a crucial role in osmotic adjustment, alternative suggestions are that it acts as a reactive oxygen scavenger, redox buffer, or molecular chaperone, stabilizing proteins and membrane structures under stress conditions (Verbruggen and Hermans 2008; Ashraf and Foolad 2013).

OtsA and *OtsB* (*E. coli* trehalose-6-phosphate synthase) genes were introduced in rice and an increase in salt, drought, and cold tolerance was observed in the transgenic plants (Garg, Kim et al. 2002). The trehalose-6-phosphate synthase gene (*OsTPS1*) overexpressing rice lines showed improved tolerance to cold, high salinity and drought treatments without any morphological changes (Li, Zang et al. 2011). However, it was found that osmoprotectants might have roles in osmotic adjustment but has minimum effect on yield enhancement (Serraj and Sinclair 2002). This mechanism is therefore of low practical use since the main concern is salinity stress tolerance which results in significant yield gains.

2.2.2.2 Na^+ exclusion and K^+ homeostasis

During salinity stress, over-accumulation of Na^+ in the cytoplasm creates a toxic environment that disturbs protein synthesis, enzyme activity and photosynthesis (Yeo and Flowers 1986; Glenn, Brown et al. 1999; Tsugane, Kobayashi et al. 1999; Blaha, Stelzl et al. 2000). Eventually, salinity stress leads to Na^+ over-accumulation

in shoots, especially in old leaves. According to several studies, restricting Na^+ accumulation in shoots under salinity stress is associated with salt tolerance of wheat and barley (Jeschke 1984; Gorham, Jones et al. 1990; Munns and James 2003; Garthwaite, von Bothmer et al. 2005) as well as survival of rice plants under salinity stress (Yeo, Yeo et al. 1990). Stress symptoms such as chlorosis and necrosis triggered by ionic stress are observed in older leaves leading to premature senescence (Munns, Husain et al. 2002; Munns, James et al. 2006), which causes a significant reduction of growth and productivity of cereals. Therefore, to survive in salinity stress effective strategies for glycophytes are to keep cytosolic as well as shoot Na^+ levels low. The influx of salt into roots activates perception and signaling mechanisms that tend to (i) inhibit the entry of further Na^+ into the roots, (ii) reduce long-distance Na^+ transport from root to shoot and (iii) restore leaf ion homeostasis.

Acquisition and maintenance of K^+ had also been found to have a significant impact on plant salt tolerance (Wu, Ding et al. 1996; Zhu, Liu et al. 1998). To maintain a high cytosolic K^+/Na^+ ratio especially in shoots have been crucial for the survival of glycophyte plants under salinity stress (Gorham, Hardy et al. 1987; Gorham, Jones et al. 1990; Blumwald 2000; Horie, Motoda et al. 2005; Ren, Gao et al. 2005; Yamaguchi and Blumwald 2005; Hauser and Horie 2010). Plants have three mechanisms mainly to achieve a high cytosolic K^+/Na^+ ratio: exclusion of Na^+ ions at entry, excluding Na^+ ions out of the cells and vacuolar compartmentation of Na^+ ions. At whole plant level, exclusion of Na^+ ions takes place in different ways usually: 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^+ in the cells lining the transpiration stream (Negrão, Courtois et al. 2011). Exclusion capacity and salt tolerance are highly correlated according to a couple of studies (Lee, Choi et al. 2003; Zhu, Kinet et al. 2004). Despite of not being a good excluder, rice excludes at least 94% of the soil Na^+ from the transpiration stream (Munns 2005). Even when 99% of Na^+ arriving inside is successfully sequestered into the expanded rice leaves, the apoplastic Na^+ concentration can rise upto 500 mM within 7 days

of salinity stress, which would lead to severe cell dehydration and stomatal closure (Yeo and Flowers 1986). Shoot apoplastic Na^+ concentrations showed negative correlation with the survival of rice varieties including the salt tolerant cultivar Pokkali (Krishnamurthy, Ranathunge et al. 2009; Krishnamurthy, Ranathunge et al. 2011). Therefore, reduction of Na^+ transport to the shoots via apoplastic bypass flow is fundamental in order to enhance salinity tolerance of rice plants. At tissue or organelle level, exclusion might take place through intracellular ion compartmentation, an adaptive mechanism present in most species and is called tissue tolerance. Tissue tolerance probably requires 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 (Flowers and Colmer 2008; Munns and Tester 2008). Three main mechanisms responsible for shoot tissue tolerance have been targeted: Na^+ accumulation in the vacuole, synthesis of compatible solutes and producing enzymes that catalyze the 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 success in improving crop salinity tolerance. Though approaches to improve the tissue tolerance of crops through increased production of compatible solutes and enzymes involved in ROS metabolism appear to have been successful, there are several reports of poor performance by the transgenic lines in low stress environments (Karakas, Ozias-Akins et al. 1997; Romero, Bellés et al. 1997; Sheveleva, Chmara et al. 1997; Cortina and Culiáñez-Macià 2005; Suárez, Calderón et al. 2009). These negative effects can be avoided by the use of tightly regulated stress-inducible promoters (Sheveleva, Chmara et al. 1997; Garg, Kim et al. 2002; Vendruscolo, Schuster et al. 2007).

2.2.2.3 Regulation of antioxidants

Under salt stress, harmful oxygen radicals are produced as the reduced rate of photosynthesis activates Mehler Reaction (Hsu and Kao 2003). Different cellular

components including membrane lipids, proteins and nucleic acids are damaged by these cytotoxic reactive oxygen species (ROS) (Halliwell and Gutteridge 1986; McCord 2000). According to a study (Ismail, Seo et al. 2014), the two response modes, adaptation versus cell death, depend on the relative timing of two signal chains: one triggered by Ca^{2+} and the other triggered by oxidative burst in the apoplast. A delay in generation and dissipation of a salinity-triggered Ca^{2+} dependent signal relative to a signal conveyed by ROS will lead to the unconstrained activation of jasmonate (JA) signaling culminating in cell death. On the other hand, the same molecular signal carrier (Ca^{2+}), if properly timed, can start adaptive processes such as sequestration and extrusion of Na^{+} and induce efficient constraint of JA signaling through the activation of abscisic acid (ABA) signaling.

Plants need to strictly maintain ROS homeostasis to alleviate the toxicity of ROS. Therefore, different scavenging machinery exist in plants 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), guaiacol peroxidase (GOPX), and glutathione *S*-transferase (GST). The most effective intracellular enzymatic antioxidant is the metalloenzyme SOD that acts by dismutating superoxide to H_2O_2, which in turn can be detoxified by APX, GPX, and CAT. The non-enzymatic antioxidants include ascorbic acid (ASH), glutathione (GSH), phenolic compounds, alkaloids, non-proteinogenic amino acids, and α-tocopherols (Apel and Hirt 2004; Gill and Tuteja 2010). ROS accumulation can also be checked by other signals, such as NO. NO is able to neutralize Fenton-type oxidative damage by scavenging superoxide that prevent the formation of oxidants (such as O_2^{-}, H_2O_2, and alkyl peroxides).

Antioxidant capacity and salinity tolerance shows correlation in several plant species including rice (Dionisio-Sese and Tobita 1998; Demiral and Türkan 2004; Demiral and Türkan 2005). In the tolerant genotypes of rice, the activities of ROS

scavenging enzymes such as ascorbate peroxidase and peroxide dismutase, was either constitutively high or up-regulated by salt stress (Moradi and Ismail 2007). Recently, another study (Bose, Rodrigo-Moreno et al. 2014) argued that, truly salt-tolerant species possess efficient mechanisms of Na^+ exclusion from the cytosol and therefore, may not require a high level of antioxidant activity, as excessive ROS is not allowed to produce in the first instance. Finally, Bose, Rodrigo-Moreno et al. 2014 emphasized the importance of non-enzymatic antioxidants as the only effective way to prevent harmful effects of hydroxyl radicals on cellular structures. Hence, increased antioxidant activity should be treated as a damage control mechanism rather than a trait that directly confers salinity tolerance.

2.2.3 Whole plant responses

Rice genotypes showed different types of adaptations under salt stress based on inherent tolerance or sensitivity over a period of acclimation through short-term and long-term responses. The tolerant genotypes had more responsive stomata that tend to close faster during the first few hours of stress, followed by partial recovery after a short period of acclimation (Moradi and Ismail 2007). In the sensitive types, stomatal conductances (gs) continued to decrease for longer period with no recovery afterward. This finding is an indication of growth responses at the whole plant level. For better understanding of the responses at the whole plant level, the regulation of this response at both vegetative and reproductive stage is discussed below.

2.2.3.1 Regulation of growth and development at vegetative stage

Osmotic or water-deficit effect of salinity may lead to overall growth reduction because of reduced uptake of water. Again, excessive salt entering the transpiration stream of the plant will be injurious to cells in the transpiring leaves causing further growth reductions. This is called the salt-specific or ion-excess effect of salinity (Greenway and Munns 1980). Cells regain their original volume and turgor within hours due to osmotic adjustment, but still cell elongation rates

are reduced (Yeo, Lee et al. 1991; Passioura and Munns 2000; Cramer 2002). Over days, reductions in cell elongation and division causes slower leaf appearance and smaller, thicker leaf (Munns and Tester 2008). At high level of salt stress and/or if the tolerance mechanism is not sufficient to effectively exclude salt from the transpiration stream, leaves that have been transpiring for a long time will accumulate salt to toxic levels, finally causing their death. As new leaf growth is supported by the export of carbon from the mature leaves, the fate of the plant depends on the balance between the rate of mature leaf death and young leaf production. Plant may die if the rate of leaf death overtakes the rate of new leaf initiation and surface expansion, since leaves supply the photosynthates 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 CO2 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 (Munns and Tester 2008; Chaves and Davies 2010). Apparently, under salt stress, shoot growth is usually more affected than root growth.

2.2.3.2 Regulation of growth and development at reproductive stage

Under severe salt stress condition (NaCl >100 mM), rice plants die before maturity. Under less severe conditions (NaCl <50 mM), salt stress causes delayed panicle initiation and flowering (Grattan, Zeng et al. 2002) and 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 also depends on ionic toxicity. Improved Na^+ partitioning between older tissues or leaves may protect the developing panicles from excessive Na^+ accumulation (Mohammadi-Nejad, Singh et al. 2012). Tolerant genotypes actually tend to exclude salt from flag leaves,

crucial organs for carbohydrate synthesis at the reproductive stage and panicles (Yeo and Flowers 1986; Moradi, Ismail et al. 2003; Lisa, Elias et al. 2011).

2.3 Mechanisms of salinity tolerance in rice

In rice, salinity tolerance may take place at three levels, that is, whole plant (Munns, Greenway et al. 1983; Jeschke and Hartung 2000; Munns and Tester 2008), cellular (Munns, Greenway et al. 1983; Munns and Tester 2008), and molecular levels (Blumwald 2000; Munns, Husain et al. 2002). It includes physiological mechanisms:

(1) Salt exclusion: Plants do not take up excess salt by selective absorption;

(2) Salt reabsorption: Though tolerant varieties absorb excess salt, but it is reabsorbed from the xylem so that Na^+ is not translocated to the shoot;

(3) Root-shoot translocation: Salinity tolerance is associated with high electrolyte content in the root and low content in the shoot;

(4) Salt translocation: Tolerant plants are able to translocate lower amount of Na^+ to the shoot;

(5) Salt compartmentation: Excess salt is transported from younger to older leaves;

(6) Tissue tolerance: Absorbed salt by plant are properly compartmentalized in leaf vacuoles to lessen the deleterious effects on plant growth; and

(7) Salt dilution: Salt taken up by plants is diluted by fast growth rate and high water content in the shoot (Yeo and Flowers 1984).

2.4 Physiological traits associated with salinity tolerance in rice: Current understanding

Salinity damage, and consequently adaptation to salinity, is a complicated process. It is difficult to correctly determine the target traits for the improvement of salinity tolerance because there is no strong evidence that any traits would be sufficient to confer salt tolerance to rice. No single process can be responsible for the variation of plant's response to salinity. For example, the uptake of salt is as

important as the subsequent distribution of salt within the plant (Yeo and Flowers 1984; Yeo and Flowers 1986; Yeo and Flowers 1989). In a recent study (Pires, Negrão et al. 2015) phenotypic data of 56 rice genotypes were analyzed. It was found that none of the three salinity tolerance mechanisms, that is, shoot-ion independent tolerance or osmotic tolerance, ion exclusion, and tissue tolerance was predominant among rice genotypes. It was also revealed that, the K^+ concentration in rice had not been significantly affected by salt stress. In a previous study (Kanawapee, Sanitchon et al. 2012), it was observed that in saline conditions, a decrease in K^+ content was accompanied by a subsequent increase in Na^+ content with plant growth. Consequently, the authors suggested that maintaining a low K^+/ Na^+ ratio is the most important mechanism of salinity tolerance in rice. Many other studies support the notion that a low K^+/Na^+ ratio is the most important goal in terms of ion concentrations in rice salinity tolerance (Kanawapee, Sanitchon et al. 2011; Kanawapee, Sanitchon et al. 2012). In a study with 49 rice genotypes, significant genotypic variations and correlations were observed among the traits such as salt injury score (SIS), ion leakage, chlorophyll reduction, shoot length reduction, shoot K^+ concentration, and shoot Na^+/K^+ ratio (De Leon, Linscombe et al. 2015). 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 should be taken into consideration for assessing salinity tolerance. They also emphasized on using traits like high shoot K^+ concentration and low Na^+/K^+ ratio from the donors like FL378, Damodar, Hasawi, Ketumbar, PSBRC50, Cheriviruppu and IR2706-11-2 to improve salinity tolerance. Another study (Pires, Negrão et al. 2015) found that growth reduction under salt stress can be the same in plants with different biomass, but in general, the plant with more biomass will be more able to tolerate salt. It was also discovered that, higher SES score is accompanied by a higher Na^+ level in the 3rd leaf (Platten, Egdane et al. 2013) and genotypes with similar levels of Na^+ in leaves can be different in their physiological responses to salt stress. From the analysis of different groups of genotypes, Pires, Negrão et al. 2015 recommended some common and important aspects, for example, proper controls should be used; when the function of a certain gene under study is known,

experiments should be designed to dissect the specific salinity tolerance mechanism; quantitative data on the trait of interest should be presented etc. In most breeding strategies, the simple visual salt injury scoring (Gregorio 1997) is widely used for characterization because it reflects the overall plant's response to salt stress. Other studies suggest the use of Na-Ca selectivity (Zeng, Poss et al. 2003), tiller number and Na^+- K^+ selectivity (Zeng 2005), and proline concentration (Kanawapee, Sanitchon et al. 2012) as parameters for classification of rice varieties based on salt tolerance. Though there is no particular morphological marker for salt tolerance or sensitivity in rice, but a combination of traits give a good indication toward the salt response of crop plants. Hence, a combination of several parameters are used for the effective and reproducible results for salt tolerance and sensitivity.

2.5 Breeding strategies to improve salinity tolerance

2.5.1 Molecular markers, linkage mapping and quantitative trait loci (QTL) identification

In genetics, a molecular marker (identified as genetic marker) is a fragment of DNA that is associated with a certain location within the genome. Molecular markers are used in molecular biology and biotechnology to identify a particular sequence of DNA in a pool of unknown DNA. Markers provide signs or flags through which a species can be recognized or identified for a particular trait of interest. There are three major types of genetic markers in crop plants: (1) morphological (classical or visible), (2) biochemical (allelic variants of enzymes called isozymes) and (3) DNA markers, which reveal sites of variation in DNA (Winter and Kahl 1995; Jones, Ougham 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, Jahufer et al. 2005). The major disadvantages of morphological and biochemical markers

are that they 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 because they are abundant and they remain unaffected by the environment as well as the development stages of the plant (Winter and Kahl, 1995). DNA markers 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, Ougham et al. 1997; Gupta, Varshney et al. 1999; Joshi, Ranjekar et al. 1999). In breeding, readily used DNA markers are RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), RAPD (Randomly Amplified Polymorphic DNA) and SSR (Microsatellites/Simple Sequence Repeats). Recently, the discovery and use of SNPs (Single Nucleotide Polymorphisms) would become very popular to the breeders. Latest advances in genotyping single nucleotide polymorphism (SNP) markers have introduced more efficient and cost-effective marker systems. SNPs are the most abundant polymorphism in plant genomes. Recent studies identify approximately five SNPs/kb across 20 *Arabidopsis* accessions and nine SNPs/kb across 517 resequenced rice varieties (Clark, Schweikert et al. 2007; Huang, Sang et al. 2010). Hence, SNP markers are ideal for high resolution genotyping for association studies, rapid genome-wide scans for genomic selection and routine genetic diversity analysis, linkage mapping, and marker-assisted selection (MAS) (McCouch, Zhao et al. 2010; Tung, Zhao et al. 2010).

2.5.1.1 DArTseq™ markers

Diversity Arrays Technology (DArT) is a technology used in molecular genetics to develop sequence markers for genotyping and other genetic analysis. Efforts of crop improvement in polyploid species are hampered by the complexity of the genome and the difficulties to develop high-throughput genotyping platforms.

Diversity Arrays Technology (DArT) offers an inexpensive and high throughput whole-genome genotyping technique as initially shown for rice (Jaccoud, Peng et al. 2001). The efficacy of DArT markers in the analysis of genetic diversity, population structure, association mapping and construction of linkage maps has been demonstrated for a variety of species, specially for plants (http://www.diversityarrays.com/dart-resources-papers). Furthermore, DArT has been applied successfully to species with large genomes such as barley (Wenzl, Carling et al. 2004) and with complex or/and polyploid genomes such as the decaploid sugarcane (Heller-Uszynska, Uszynski et al. 2011), hexaploid wheat and oat (Akbari, Wenzl et al. 2006; Tinker, Kilian et al. 2009) or the paleoploid apple (Schouten, van de Weg et al. 2012). The DArT method allows for simultaneous detection of several thousand DNA polymorphisms (depending on the species) arising from single base changes and small insertions and deletions (InDels) by scoring the presence or absence of DNA fragments in genomic representations generated from genomic DNA samples through a process of complexity reduction (Jaccoud, Peng et al. 2001). Contrary to other existing SNP genotyping platforms, DArT platforms does not rely on previous sequence information. With the development of next generation sequencing (NGS), DArT technology faced a new development by combining the complexity reduction of the DArT method with NGS. This new technology named DArTseq™ represents a new implementation of sequencing of complexity reduced representations (Altshuler, Pollara et al. 2000) and more recent applications of this concept on the next generation sequencing platforms (Baird, Etter et al. 2008; Elshire, Glaubitz et al. 2011). DArTseq™ is rapidly gaining popularity as a preferred method of genotyping by sequencing (Kilian, Wenzl et al. 2012; Courtois, Audebert et al. 2013; Raman, Raman et al. 2014). Similarly to DArT methods based on hybridizations, the technology is optimized for each organism and application by selecting the most appropriate complexity reduction method (both the size of the representation and the fraction of a genome selected for assays). QTL mapping of disease resistance had been conducted in pearl millet, hop, wheat (Zwart, Thompson et al. 2010; Henning, Townsend et al. 2011; Prat, Buerstmayr et al. 2014; Ambawat, Senthilvel et al. 2016). Drought resistance QTLs in soybean (Vu,

James et al. 2015), QTL mapping for fruit quality has recently been done in *Citrus* using DArTseq markers (Curtolo, Cristofani-Yaly et al. 2017). Diversity analysis using DarT markers has been performed in strawberry, wheat, sorghum etc. (Jing, Bayon et al. 2009; Sánchez-Sevilla, Horvath et al. 2015; Sohail, Manickavelu et al. 2015).

DArT™ and medicine: The application of DArT to the human genome is an interesting frontier. The technology is currently being improved for use in the mouse genome (http://www.cambia.org). The complexity and redundancy of the genome may create challenges, but these are far outstripped by the potential discoveries and human health improvements that may result.

Another major potential application of DArT in improving human health is the identification of pathogens in a wide variety of settings. For example, DArT might be usable to determine the presence of pathogenic intestinal bacteria, leading to more rapid treatment for illnesses of the digestive system.

DArT™ and Biodiversity: DArT has been used to examine the genetic diversity of a protected otter population in England. Preliminary evidence suggested that DArT could be used to identify potentially polymorphic representatives within the otter population. This information may provide further insight into the population's genetic diversity, mating patterns, and other behavioral traits (http://www.cambia.org).

Linkage mapping: Linkage maps indicate the positions and relative genetic distances between markers along the chromosomes (figure 2.3). Use for linkage maps is to identify chromosomal locations containing genes and QTLs associated with the traits of interest. Linkage maps are constructed from the analysis of many segregating markers. The greater the distance between markers, the greater the chance of recombination occurring during meiosis. Distance along a linkage map is measured in terms of the frequency of recombination between genetic markers (Paterson 1996). Mapping functions are required to convert recombination

fractions into centiMorgans (cM) because recombination frequency and the frequency of crossing-over are not linearly related (Kearsey and Pooni 1998; Hartl 2001). When map distances are small (<10 cM), the map distance equals the recombination frequency. However, this relationship does not apply for map distances that are greater than 10 cM (Hartl 2001). Two commonly used mapping functions are the Kosambi mapping function, which assumes that recombination events influence the occurrence of adjacent recombination events, and the Haldane mapping function, which assumes no interference between crossover events (Kearsey and Pooni 1998; Hartl 2001).

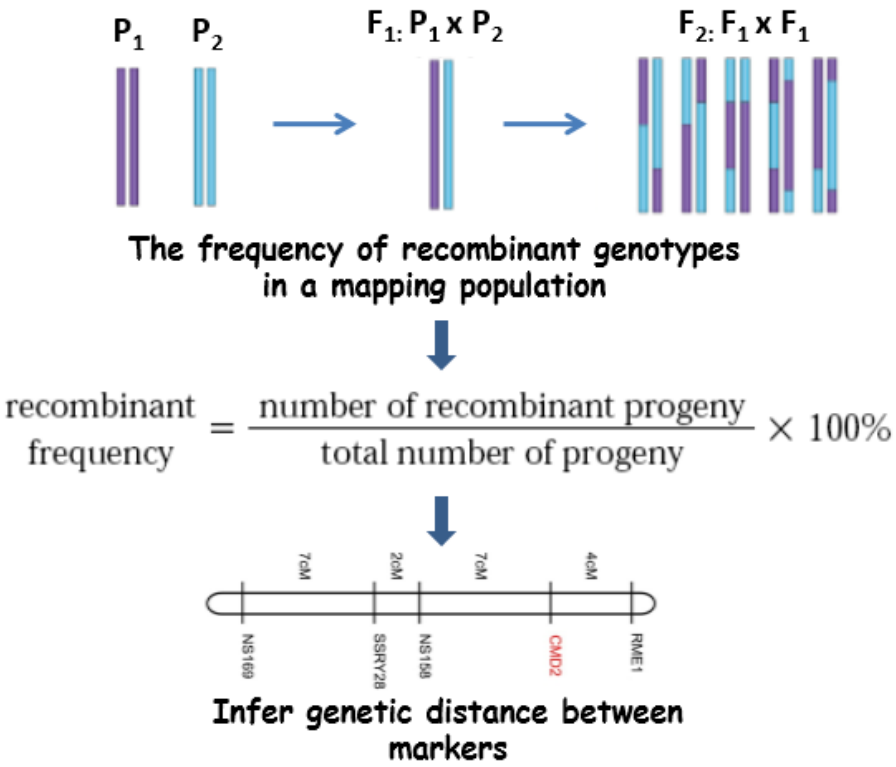

Figure 2.3: Steps of linkage map construction.

Linkage map can only be used to detect the gene or QTL controlling the trait of interest. Linked markers are grouped together into 'linkage groups', which represent chromosomal segments or entire chromosomes.

Quantitative trait loci (QTL) identification: A quantitative trait locus (QTL) is a section of DNA (the locus) which correlates with variation in a phenotype (the

quantitative trait). Usually the QTL is linked to, or contains, the genes which control that phenotype. 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 status of linkage with other genes, but can easily be done with molecular markers. QTL mapping is the process of locating genes with effects on quantitative traits using molecular markers (McCough and Doerge 1995; Paterson 1996; Mohan, Nair et al. 1997). Its aims are to detect which regions (QTL) of the genome affect the trait and to describe the effect of the QTL on the trait. That is, how much of the variation of the trait is caused by the QTL and what is the gene action associated with the QTL (additive/dominant effect). A 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, Jakkula et al. 2001; Lindhout 2002; Pilet-Nayel, Muehlbauer et al. 2002).

QTL mapping is based on the principle that genes and markers segregate via chromosome recombination (cross-over) during meiosis. Genes or markers that are close together or tightly-linked will be transmitted together from parent to progeny more frequently than genes or markers that are located further apart.

QTL analysis detects 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). A significant difference between phenotypic means of the groups, depending on the marker system and type of population, indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait.

Three popular methods for detecting QTLs are single-marker analysis, simple interval mapping and composite interval mapping (Tanksley 1993; Liu 1997). Single-marker analysis is the simplest method for detecting QTLs associated with single markers. The simple interval mapping (SIM) method makes use of linkage maps and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander and Botstein 1989). More recently, composite interval mapping (CIM) has become popular for mapping QTLs. This method combines 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; Jansen and Stam 1994; Zeng 1994). The main advantage of CIM is that it is more precise and effective at mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved. Many researchers have used QTL Cartographer (Basten 1994; Basten, Weir et al. 2002), MapManager QTX (Manly, Cudmore Jr et al. 2001) and PLABQTL (Utz and Melchinger 1996) to perform CIM.

Few studies have been conducted on salinity tolerance in rice (Flowers, Koyama et al. 2000; Prasad, Bagali et al. 2000; Koyama, Levesley et al. 2001; Bonilla, Dvorak et al. 2002; Lin, Zhu et al. 2004; Takehisa, Shimodate et al. 2004; Ammar, Singh et al. 2007; Lee, Ahn et al. 2007; Sabouri, Rezai et al. 2009; Haq, Gorham et al. 2010; Alam, Sazzadur Rahman et al. 2011). A list of QTLs linked to salinity tolerance in rice can be found in Gramene (http://www.gramene.org). And a detailed information on these QTLs has been compiled in the Rice module of the TropGene database (http://tropgenedb.cirad.fr). A number of mapping studies have attempted to identify QTLs located on different chromosomes which are implicated for salinity tolerance in rice (Zhang, Guo et al. 1995; Gong, He et al. 1999; Prasad, Bagali et al. 2000; Singh, Gregorio et al. 2007; Haq, Gorham et al. 2010; Pandit, Rai et al. 2010) but there is limited information on salt tolerance at reproductive stage (STRS). The reproductive stage is the most crucial as it ultimately determines grain yield. Therefore, STRS is an important trait for stable

rice production in salt affected areas. Only a limited number of QTLs for STRS have been mapped (Mohammadi, Mendioro et al. 2013; Hossain, Rahman et al. 2015).

For the seedling stage, a major QTL for salinity tolerance, Saltol, explaining >50% of the phenotypic variance responsible for selective ion uptake has been mapped on chromosome 1 (Gregorio 1997; Bonilla, Dvorak et al. 2002). Other researchers have also detected the same QTL in some other rice cultivars (Takehisa, Shimodate et al. 2004; Ren, Gao et al. 2005; Islam and Gregorio 2013). There are reports of introgression of this *Saltol* QTL into rice varieties like BR11, BRRIDhan28, Q5DB, IR64, AS996 and PB1121 (Luu TN, Luu M et al. 2012; Gregorio, Islam et al. 2013; Huyen, Cuc et al. 2013; GUO and YE 2014; Hasan, Rafii et al. 2015; Babu, Krishnan et al. 2017) The reported gain in tolerance of these lines due to introgression of *Saltol* was moderate to strong depending on the genetic background. However since the advantage gained was mainly at the seedling stage, considerable loss in grain yield under salt stress was also observed.

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). Salinity is still considered as a single character in many of these studies mentioned. 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 noted that most of these studies have been conducted under hydroponic conditions and only one in field condition (Takehisa, Shimodate et al. 2004). None of the QTLs described as the most significant under field conditions co-localized with QTLs detected in hydroponics. The QTL studies also suggested that different *loci* were involved in the different plant responses under short *versus* long term salt stress (Haq, Gorham et al. 2010).

2.5.2 Molecular breeding approaches: Marker Assisted Selection (MAS)/Marker Assisted Backcrossing (MABC)

Marker assisted selection (MAS) refer to the use of DNA markers that are tightly-linked to target loci as a substitute for or to assist in phenotypic screening. The advantages of MAS compared to conventional phenotypic selection are: (1) Simpler compared to phenotypic screening, (2) Selection may be carried out at seedling stage, and (3) Single plants may be selected with high reliability. Furthermore, selection based on DNA markers may be more reliable due to the influence of environmental factors on field trials. Sometimes, use of DNA markers may be more cost effective than the screening for the target trait. Another benefit of MAS is that the total number of test lines might be reduced. Moreover, 'background' markers may also be used to accelerate the recovery of recurrent parents during marker-assisted backcrossing (Collard and Mackill 2008).

The basis of marker assisted backcrossing (MAB) is to transfer a specific allele of the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome. MAB has previously been used in rice breeding to incorporate a number of traits, e.g. bacterial blight resistance gene *Xa21* (Chen, Xu et al. 2001), waxy gene (Zhou, Tan et al. 2003), root trait QTLs (Steele, Price et al. 2006) and submergence tolerance *Sub1* QTL was incorporated into several mega rice varieties like Swarna, IR64, BR11, Samba Mahsuri, Ciherang (Neeraja, Maghirang-Rodriguez et al. 2007; Iftekharuddaula, Newaz et al. 2011). Various other successful experiments reported the manipulation of known genes with linked markers, including pyramiding of several major resistance genes in rice (Huang, Angeles et al. 1997; Hittalmani, Parco et al. 2000; Sanchez, Brar et al. 2000; Singh, Sidhu et al. 2001; Jiang, Feng et al. 2012) wheat (Anli, Huagang et al. 2005) and tomato (Barone, Ercolano et al. 2005; Yang and Francis 2005).

The advantages of using MAS in plant breeding have been well documented (Xu and Crouch 2008). For example, *Saltol is* a favorable QTL identified in rice responsible for the bulk of genetic variation in ion uptake under saline conditions

(Gregorio, Senadhira et al. 2002; Ashraf and Foolad 2013). Considerable progress had been made in mapping major QTLs such as *Saltol* thus providing opportunities to fast track the introgression of *Saltol* QTL to popular varieties in Bangladesh, India, Vietnam and Philippines (Thomson, de Ocampo et al. 2010; Gregorio, Islam et al. 2013). Yeo, *et al.* (1990) emphasized on the importance of combining all favorable and complementary physiological traits in to single elite background, rather than considering salinity tolerance as a single trait. 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. It has been proposed that it is more practical to enhance plant stress tolerance by manipulating only one or a few main components of the regulatory gene network instead of engineering several molecular mechanisms (Golldack, Lüking et al. 2011). Besides conventional breeding methods, combining 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.

SNPs have become the marker system of choice for genetic mapping, diversity, and association analysis and for use in marker-assisted breeding because of their polymorphic abundance and the availability of rapid, high-throughput platforms for genotyping (Ganal, Altmann et al. 2009). For example, the Illumina GoldenGate 384-plex SNP genotyping assay has been successfully used to carry out genome-wide diversity studies and to assess allelic diversity at major loci such as *Saltol*, in addition to its use in marker-assisted breeding (Thomson, Zhao et al. 2012). In-depth studies of allelic diversity at key loci for traits underpinning salt tolerance will determine whether new accessions have better and novel alleles. Overall, these approaches can enable marker-guided precision breeding based on multiple genes. The availability of SNP platforms has made it possible to track several genes associated with salt tolerance while selecting for traits of agronomic importance, such as yield and quality.

Genome wide association study* (GWAS)*: Genome wide association study (GWAS) can offer a valuable insight into trait architecture as well as candidate loci for subsequent validation (Korte and Farlow 2013). QTL mapping has two fundamental limitations: Firstly, only allelic diversity that segregates between the parents of the particular F_2 cross or within the RIL population can be assayed and secondly, the amount of recombination that occurs during the creation of the RIL population limits the mapping resolution. If an advanced intercross RIL is used, this resolution can be dramatically improved by several generation of intercrossing. Also there are population e.g. Multi-parent Advanced Generation Inter-Cross (MAGIC) and *Arabidopsis* multi-parent RIL (AMPRIL) where the allelic diversity is increased within a mapping population by intercrossing multiple genetically diverse accession before establishing RIL (Kover, Valdar et al. 2009). GWAS overcomes the two main limitations of QTL analysis mentioned above, but introduces several other drawbacks. However, GWAS is gaining popularity among plant scientists as sequencing and reseqeuncing efforts now provide SNPs and InDels at the right density for GWAS study (Delseny, Han et al. 2010). Generally, after identifying a phenotype of interest, GWAS can serve as a foundation experiment to explore the genetic architecture of the trait thus allowing an informed choice of parents for QTL analysis and suggesting candidates for transgenic and mutagenesis. Thus, GWAS are often complementary to QTL mapping and, when conducted together, they mitigate each other's limitations.

CHAPTER 3 *METHODS & MATERIALS*

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3.1 QTL mapping of Horkuch with DArTseq markers

QTL analysis detects an association between phenotype and the genotype of markers. QTL mapping can be divided into two parts:

(i) Linkage map construction by genotyping through markers

(ii) Phenotypic screening of the population

For QTL mapping, the *Horkuch* and *IR29* cross was advanced upto F_3, where $F_{2:3}$ mapping strategy was followed to generate separate QTL maps for seedling and reproductive stage. Section 3.1.3 and 3.1.4 will deal with genotyping and linkage mapping, section 3.1.5 with phenotyping and section 3.1.6 will deal with QTL mapping, respectively.

3.1.1 Confirmation of heterozygosity of F_1 plants using SSR marker

3.1.1.1 Plant materials

Rice cultivar *Horkuch* (IRGC 31804) and *IR29* (IRGC 30412) were collected from IRRI Genebank (collection site/origin of *Horkuch* was mentioned as Bangladesh). Here, *IR29* was used as the mother parent. The collected seeds were sown in IRRI crossing block during wet season of 2011 (June-July) after breaking dormancy at 70˚C for 5 days in an oven. A cross was made between *Horkuch* and *IR29* during October to November, 2011 (registered as IR102584 with GID: 3539833 [F_1(*IR29 / Horkuch*)]). F_1 plants were confirmed by SSR marker RM493 at the University of Dhaka, Bangladesh and were advanced to F_2 in Plant Physiology Division of BRRI (Bangladesh Rice Research Institute). About 500 F_2 progenies from the cross were planted in the field at BRRI to be advanced to the generation F_3.

3.1.1.2 Isolation of F_1 DNA from plant tissue:

178 plants were selected randomly. The leaves of these F_1 plants were pooled, cut finely, crushed to powder in liquid nitrogen and DNA was isolated using CTAB method (Doyle 1987). Materials for DNA isolation and detailed procedure are included in appendix A. 1.

3.1.1.3 Quality assessment and quantification of DNA

The quality of DNA is very important to obtain good results and for long-term storage. It is also important to know the exact concentration of the DNA for correct PCR amplification used later.

3.1.1.3.1 Quantification of DNA by Nanodrop Spectrophotometer

This special spectrophotometer can measure the concentration of nucleic acid (DNA and RNA), protein samples and other with only 1µL of sample within few seconds, thus named so. It also ensures the quality of the samples by drawing the standard curve. The procedure to measure the concentration of DNA is given here.

- The spectrophotometer was selected to measure nucleic acid sample. The wavelength was fixed with 260 and 280 nm for nucleic acid analysis.
- The nozzle of the machine was first cleaned with soft tissue paper and was initialized with PCR graded water.
- The blank was set with appropriate buffer which was used to dissolve the DNA (TE buffer was used here).
- 1µL of sample DNA from each tube was loaded on the nozzle one by one. The lid was then closed and OD was measured. The machine showed the concentration of the sample in ng/µL, its standard curve with the absorbance ratio of 260 nm and 280 nm.

3.1.1.3.2 Comparison of sample DNA with λ DNA standard

- Stock DNA samples were diluted to 10× and 20×.
- 1µL of diluted samples were loaded in the wells of 0.8% agarose gel followed by 50 and 100 ng of λ DNA standard.
- Electrophoresis and staining with Ethidium Bromide was carried out. DNA concentration was estimated by visual comparison of the fluorescence of each sample with the standards under UV light.
- The quality of the samples was also checked by observing any smear of degraded DNA or lower size bands of RNA.

3.1.1.4 DNA amplification through Polymerase Chain Reaction (PCR)

3.1.1.4.1 Primer

Primer pair for RM493 was used to amplify DNA from the leaves. SSR markers are very useful markers. Each primer-pair typically identifies a single locus, which have many alleles because of the high mutability of SSR loci and thus show polymorphism. The chromosomal location, annealing temperatures and amplified product size ranges are summarized in appendix A. 2.

3.1.1.4.2 Preparation of the Master mixture

Master mixture was prepared for 180 reaction samples containing buffer, dNTPs, Mg^{2+}, specific primer pairs and Taq polymerase in a sterile 1.5mL eppendorf tube (table 3.1).

Table 3.1: Preparation of Master mixture for PCR

Components	1 reaction (µL)	180 reactions (µL)	Final Concentration
PCR buffer (10×)	1.5	270.0	1x
dNTP (10mM)	1.5	270.0	0.1 mM
$MgCl_2$ (25 mM)	1.0	180.0	1.66 mM
Forward primer (50ng/ µL)	0.5	90.0	0.33µM
Reverse primer (50ng/ µL)	0.5	90.0	0.33µM
Taq DNA polymerase	0.5	90.0	0.75U
dH_20	9.5	1710	
Total	**15**	**2700.0**	

3.1.1.4.3 PCR reaction preparation

- At first genomic DNA, 20% DMSO and ultra pure water were dispensed in the labeled PCR tubes prior to adding the master mixture.
- The mixture was then denatured at 95°C for 5 min and immediately transferred into ice.

- After mixing and spin, 5.5µL of the above master mix was added to each PCR tube.
- Taq DNA polymerase was added to the tubes just before the start of the reaction.
- Finally the tubes were subjected to spin and transferred to Thermocycler for the amplification reaction.

Table 3.2: Preparation of sample and control tubes with DNA, DMSO and ddH_2O.

Tube	DNA(50ng/ µL)	DMSO (20%)	ddH_2O	Total Volume
Sample tube	1.0 µL	2.0µL	6.5µL	9.5µL
Control tube	0.0µL	2.0µL	7.5µL	9.5µL

3.1.1.4.4 Thermal cycling profile used in PCR

The thermal cycling profiles programmed in PCR machine to amplify the gene by polymerase chain reaction (PCR) for 35 cycles are as follows in figure 3.1 and table 3.3.

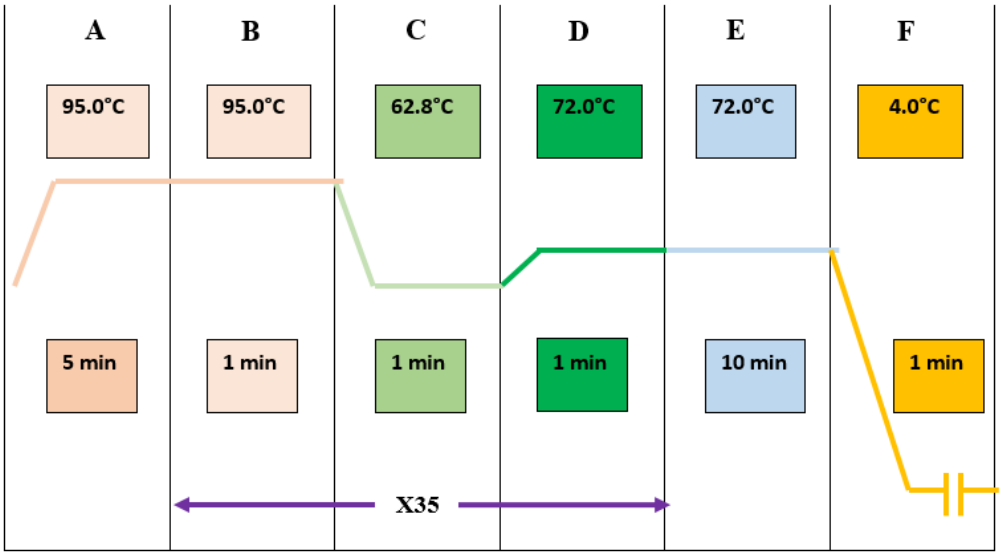


Figure 3.1: An illustration of PCR Cycle. A) initial denaturation, B) Denaturation, C) Annealing, D) Elongation, E) Elongation, F) Final extension. For different primers different anneal temperatures and elongation times were employed.

Table 3.3: Thermal cycling program for PCR amplification

Steps	Temperature (°C)	Time (minutes)	No. of cycles
Initial denaturation	95	5	1(First)
Denaturation	95	1	35
Annealing	55	1	
Elongation	72	1	
Final extension	72	7	1(Last)

3.1.1.5 Visualization of the amplified products

The amplified PCR products were visualized by agarose gel electrophoresis.

3.1.2 Isolation, quality assessment and quantification of F_2 DNA

Sprouted F_2 seeds were sown in the Styrofoam sheets floating in trays containing Yoshida's culture solution under net house condition.

For DArT assays, 50 - 100 ng/ µL suspended in TE were submitted.

3.1.3 Genotyping of F_2 DNA using DArT based SNP (DArTseq™) markers

In this study, a new approach based on traditional DArT and next-generation sequencing technique, called as DArTseq™ (Raman, Cowley et al. 2014), was used to analyze the genome of the rice population. DArTseq™ is a genotyping technology that detects all types of DNA variation (SNP, indel, CNV, methylation). The traditional method called Diversity Arrays Technology (DArT) is a microarray-based DNA marker technique for genome-wide discovery and genotyping of genetic variation. Simultaneous scoring of hundreds of restriction site based polymorphisms between genotypes is possible in DArT and it does not require DNA sequence information or site specific oligonucleotides. DArT uses an array of individualized clones from a genomic representation that is prepared

from amplified restriction fragments. The genomic representation may not be random but based on some prior representation such as knowledge of restriction enzymes more effective for gene-rich regions. Labeled genomic representations of individuals to be genotyped, such as the progeny of a segregating population, are then hybridized to the arrays. The polymorphisms scored are the presence versus absence of hybridization to individual array elements. DArT can detect DNA polymorphisms by scoring the presence or absence of specific DNA sequences in a defined genomic representation (i.e., a representative subset of genomic fragments) through hybridization to microarrays (Jaccoud, Peng et al. 2001; Wenzl, Carling et al. 2004).

DArTseq Overview

At the core of DArTseq technology is a genome complexity reduction concept. In order to reduce genome complexity, many methods have been developed. The DArTseq methods provide a significant advantage via an intelligent selection of genome fraction corresponding predominantly to active genes. This selection is achieved through the use of a combination of restriction enzymes which separate low copy sequences (most informative for marker discovery and typing) from the repetitive fraction of the genome.

The DArTseq method deploys sequencing of the representations on the Next Generation Sequencing (NGS) platforms. Though there is no microarray in the present method, it is still called DArT because the polymorphic sites on rice genome have already been previously identified by Array technology and this information is used to find the restriction enzymes that cut the maximum diverse sites. The RE cut sites are then amplified and sequenced. An illustration of DArT based SNP genotyping method is given in figure 3.2.

As modern breeding moves rapidly in this direction, especially in larger organisations, DArTseq is increasingly used in crop improvement applications.

Complexity reduction

DArTseq works by reducing the complexity of a DNA sample to obtain a 'representation' of that sample. The method of complexity reduction relies on a combination of restriction enzyme digestion and adapter ligation, followed by amplification (Wenzl, Carling et al. 2004). However, a wide range of alternative methods can be used to prepare genomic representations for DArT.

DArTseq Data Types

DArTseq generates two types of data:

1. Scores for "presence/absence" (dominant) markers, called SilicoDArTs as they are analogous to microarray DArTs, but extracted "in silico" from sequences obtained from genomic representations.

2. SNPs in fragments present in the representation.

It is also possible to extract Copy Number Variation (CNV) polymorphism information from some DArTseq representations.

The "0/1" scores are based on a range of DNA variation types: SNPs and small indels in restriction enzyme recognition sites, larger insertions/deletions in restriction fragments and at lower frequency, methylation variation at restriction sites when methylation sensitive enzymes are used in complexity reduction methods.

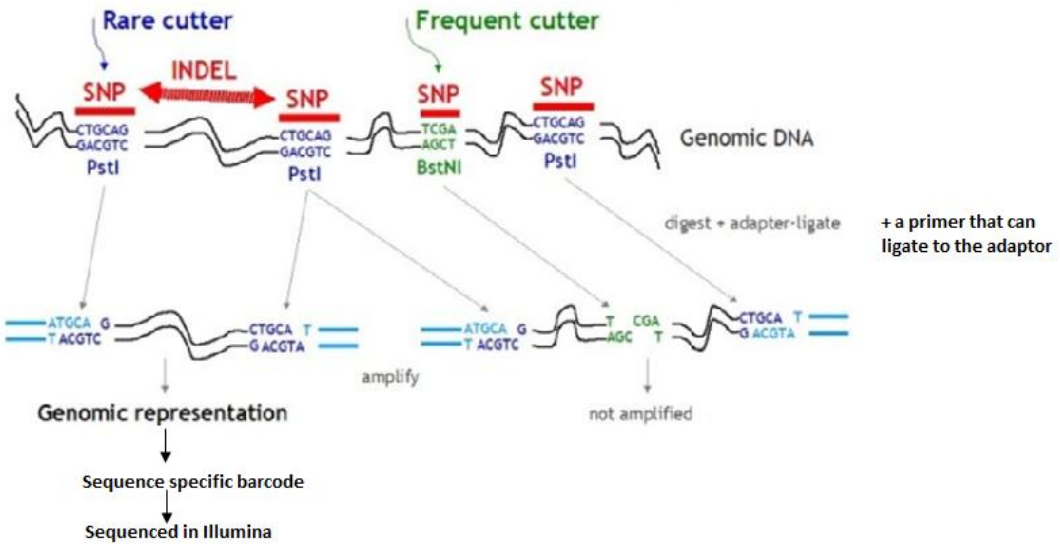


Figure 3.2: DArT based SNP genotyping (DArTseq) method

DNA samples of 174 F_2 plants were sent to Australia for genotyping. Contact Address is: Andrzej Kilian, Director, Diversity Arrays Technology,Bldg 3, Lv D, University of Canberra, Kirinari st., Bruce,ACT 2617, Australia.

To avoid leaks and cross contamination of samples, DNA was sent in two fully skirted, V-shape bottom 96 well PCR plates. The wells were sealed by strips of 8 clear flat caps. The plates were wrapped in glad wraps and packaged in a rigid box.

3.1.3.1 Marker filtration from DArTseq analysis

A number of 12760 marker data was available from DArTseq analysis. About one third of the markers were found to be non-polymorphic and were removed.

After removal of the non-polymorphic markers, 4087 polymorphic markers were kept.

The distorted markers, markers showing distortion from the Mendelian segregation ratio (1:2:1), were removed. It was performed in Chi-square method using R. The markers having a P value of greater than 0.05 (P>0.05) maintained the Mendelian segregation ratio (1:2:1) and were considered as non-distorted. Markers with a lower P value (P<0.05) were considered as distorted and were

removed. A sample datasheet of Chi-square analysis of marker distortion is given in **figure 3.3.**

AlleleID	HK	IR29	Heterozygote	Chisq Value	P Value
3443827|F|0--12:A>C	33	48	93	3.41379310344828	0.181427972145712
100021261|F|0--18:G>A	35	40	99	3.59770114942529	0.165488996180197
3052367|F|0--15:T>C	44	38	91	0.884393063583815	0.642623327130135
3452736|F|0--43:C>G	38	45	90	0.84971098265896	0.653864267358909
3763204|F|0--13:T>C	37	40	97	2.40229885057471	0.300848210558072
3442626|F|0--58:A>G	44	41	89	0.195402298850575	0.906919896791066
3052040|F|0--27:T>G	38	41	95	1.57471264367816	0.455046202223014
5406791|F|0--19:G>A	40	44	90	0.390804597701149	0.822503699195518
3048372|F|0--25:C>T	41	44	89	0.195402298850575	0.906919896791066
3449142|F|0--15:T>A	33	45	96	3.51724137931034	0.172282330816188
3048958|F|0--59:G>A	31	45	98	5.03448275862069	0.0806818707154285
3058528|F|0--19:C>T	34	41	99	3.8735632183908	0.144167190292904
100013054|F|0--17:G>T	32	45	97	4.24137931034483	0.119948876616417
3053446|F|0--34:G>A	34	47	93	2.77011494252874	0.250309413779816
3048740|F|0--19:C>T	37	41	96	2.04597701149425	0.35951890878583
3057829|F|0--22:C>T	30	46	98	5.72413793103448	0.0571503956636256
3058305|F|0--34:C>T	38	38	98	2.7816091954023	0.248874979815399
3062387|F|0--44:A>G	31	46	97	4.88505747126437	0.0869407231516099
5141144|F|0--66:A>G	31	47	96	4.80459770114942	0.090509645795994
3756798|F|0--39:A>G	40	38	96	1.90804597701149	0.38518829416266
3056500|F|0--11:G>C	30	49	95	5.62068965517241	0.0601842356291272
3049412|F|0--45:C>T	31	45	98	5.03448275862069	0.0806818707154285
3452358|F|0--62:G>A	36	46	92	1.72413793103448	0.422287479634612
3048944|F|0--48:G>A	31	50	93	4.97701149425287	0.0830339476374353
3055469|F|0--7:A>C	34	51	89	3.41379310344828	0.181427972145712
3762988|F|0--10:C>T	37	51	86	2.27586206896552	0.320481401328101
3763932|F|0--15:T>C	37	50	87	1.94252873563218	0.378604040588727

Figure 3.3: Sample datasheet of Chi-square analysis of marker distortion.

A number of 2700 non-distorted, polymorphic markers were kept after removal of the distorted markers.

3.1.3.2 Marker correlation analysis

From the filtered non-distorted, polymorphic markers, one marker every 1Mb was selected. Where there was a gap of more than 1Mb between two markers, the correlation of each marker with its flanking five markers was analyzed by the software Minitab version 17. The linked markers had a high correlation coefficient (R) value and low P value (<0.05) and were used in the analysis. The non-correlated markers were replaced by another marker located nearby that showed good correlation. Markers that are far apart do not show correlation. If there is no correlation between nearby markers, there are two possibilities. Either there was a double crossover or chiasma during meiosis or the quality of the data obtained for that marker was poor. The steps of correlation analysis are described below.

1. Minitab17 software was installed.

2. An excel file was created that included the marker names and allele information. The alleles were annotated as different numbers. 1, 0 and 2 corresponded to *IR29*, *Horkuch* and heterozygous allele respectively.
3. Minitab17 was opened.
4. Selected excel file was pasted.
5. "Basic statistics" was selected from "Stat" menu.
6. "Correlation" was selected from "Basic statistics" menu.
7. All the markers displayed were selected and "Ok" was clicked to complete data input.
8. Result sheet appeared showing the correlation coefficient and P value for each and every marker analyzed.

3.1.4 Linkage Mapping

A linkage map covering all 12 chromosomes is necessary for whole genome mapping in rice. Individual maps were constructed using MAPMAKER 3.0 (Lander *et al.* 1987).

There are two basic stages to construct a linkage map with MAPMAKER:

1. To get the data into the format that MAPMAKER needs.
2. To construct a genetic map for the marker data.

For linkage mapping analysis of marker data in MAPMAKER, a data file was prepared with *txt extension containing information on mapping population type, genotype data of number of markers, number of phenotypic data of quantitative traits, coding scheme of the data set (figure 3.4). The selected DArTseq™ markers were renamed to a simplified form starting with SF, then comes the chromosome number and the serial number of the marker. For example the fifth marker on chromosome 2 would be like: SF25. To get the data into MAPMAKER, the data must first be placed into a 'raw' file in an appropriate format.

The very first line of the raw data file was cross type:

data type xxxx

where xxxx is one of the allowed data types. In this study, it was F_2 intercross.

The second line of the raw file should contain a list of three numbers, separated by spaces, such as:

174 291 12

The first is the number of individuals scored for phenotype (200), second is number of markers scored (291) and third is the number of quantitative traits scored (12). This may be zero, if there is no quantitative trait data is present.

After the first two lines, the file contained the genetic locus data. For each locus, the name of the locus was listed, preceded by an asterisk ("*") followed by one or more spaces and the genotypic data for all individuals in order. The scores of all markers were converted into genotype codes according to the scores of the parents; 'A' for *IR29*, 'B' for *Horkuch*, 'H' for heterozygous genotype and '–' for the missing data. The file was saved as Tab delimited txt file. The file was saved in the same folder as MAPMAKER program.

Whenever the "prepare data" command is issued, MAPMAKER looks for a file with the same name as the raw data file and the extension ".prep" (on UNIX, truncated to ".pre" on DOS). If this file is present, it is assumed to contain MAPMAKER commands, which are automatically executed after the data are prepared. These "initialization files" serve as a useful way to setup MAPMAKER in the appropriate state for working with a particular data set.

Typical actions in an initialization file might be to:

- set various MAPMAKER options or parameters
- declare the names of chromosomes, classes, anchor loci, etc
- set the framework orders of chromosomes, particularly for MAPMAKER/QTL
- precompute two-point data and find linkage groups
- set various named sequences

To load a data set into MAPMAKER/QTL, "framework" maps are needed to be provided for any chromosome to be scanned. When a map order for some chromosomes is known, it is often convenient to place this in an initialization file in order to quickly have a data set ready for MAPMAKER/QTL.

data	type	f2	Intercross														
174	339	30															
*SF011	A	H	H	A	H	B	H	H	B	B	H	H	H	B	H	H	H
*SF012	A	H	H	A	H	B	A	H	B	B	H	H	H	H	H	H	H
*SF013	A	H	H	A	H	B	A	H	B	B	H	H	H	H	H	H	H
*SF014	A	H	H	A	H	B	A	H	B	B	A	H	H	H	H	H	H
*SF015	A	H	H	A	H	B	A	H	H	B	A	H	H	H	H	H	H
*SF016	A	B	H	A	H	B	A	H	H	B	A	H	H	H	H	H	H
*SF018	A	B	H	A	H	B	A	H	H	B	A	H	H	B	H	H	H
*SF019	A	B	A	A	H	B	A	H	H	B	A	H	H	B	H	B	H
*SF0111	A	B	H	A	H	H	A	H	H	B	A	H	H	H	H	B	H
*SF0112	A	B	H	A	H	A	A	H	B	H	A	H	H	H	H	B	H
*SF0113	A	B	H	A	H	A	A	A	B	H	A	H	H	H	H	B	A
*SF0114	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	B	A
*SF0115	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	B	A
*SF0116	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	B	A
*SF0117	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	B	A
*SF0118	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	B	A
*SF0119	A	B	H	A	H	A	A	A	B	A	A	H	H	H	B	B	A
*SF0120	A	B	H	H	H	A	A	A	B	A	A	H	H	H	B	B	A
*SF0121	A	B	H	H	A	A	A	A	B	A	A	A	A	A	H	B	A
*SF0123	A	B	A	H	A	A	A	A	B	A	A	A	A	A	H	B	A
*SF0124	H	B	A	H	A	A	H	A	B	A	H	H	A	A	H	B	A

Figure 3.4: Sample input data for MAPMAKER.

At first, the raw data file was loaded for analysis. Linkage between markers is usually calculated using odds ratios (the ratio of linkage vs no linkage). It is expressed as the log of the ratio and is called a LOD value or LOD score.

To determine whether any two markers are linked, MAPMAKER calculates the maximum likelihood distance and corresponding LOD score between the two markers: if the LOD score is greater than some threshold, and if the distance is less than some other threshold, then the markers will be considered linked. By default, the LOD threshold is 3.0, and the distance threshold is 80 Haldane cM in MAPMAKER 3.0. Linked markers are grouped together into 'linkage groups,' which represent chromosomal segments or entire chromosomes. For the purpose of finding linkage groups, MAPMAKER considers linkage *transitive*. That is, if marker A is linked to marker B, and if B is linked to C, then A, B, and C will be included in the same linkage group.

3.1.4.1 Map construction and integration

Individual maps for each population were constructed using MAPMAKER 3.0 (Lander et al. 1987). The steps of map construction are as follows:

1. Because MAPMAKER runs in DOS only, DOS emulator was opened and MAPMAKER was installed.
2. 12 groups were defined using the "MAKE CHROMOSOME" command.

3. The "ANCHOR" command was then used to locate marker loci.
4. When analysis is started with a new data set, MAPMAKER's "prepare data" command is used.
5. "photo tutorial.out" was used to save the output of current file.
6. "order" command was used to find a linear order of the markers on chromosome.
7. The "map" command produced the linkage map.

The distances (in centiMorgans) were calculated using the Haldane mapping function.

The map was drawn using Windows QTL Cartographer version 2.5.

3.1.5 Phenotypic evaluation at F_3 population

3.1.5.1 Seedling stage phenotyping

3.1.5.1.1 Growth condition

Two hundred F_3 progenies from *IR29/Horkuch* cross were randomly chosen for phenotypic characterization against salinity in controlled condition at seedling stage. Seeds were germinated on soaked filter paper in Petri dishes and were kept inside a seed germinator with 30˚C and 75% relative humidity condition. Well germinated seedlings of same size were planted on netted Styro-foam sheet floating in plastic tray containing Yoshida culture solution (Cock, Yoshida et al. 1976) in 3 replicates following Incomplete Block Design (balanced), where small homogenous blocks (plastic trays) were inserted in to replications. Each block accommodated 19 lines and two parents & one tolerant check (FL378). Only 4 progenies from each lines/parents/check were placed in rows per block and all 22 genotypes in each block were allocated with complete randomization. The Yoshida solution was changed once a week and pH of the solution was kept 5.0±0.5 throughout the experimental period. Salt stress was applied 12 days after seeding by exchanging the normal Yoshida solution by saline Yoshida solution, but the salt was gradually applied at 2dS increment per day, starting from 6 dS on 13 day old seedling till 12 dS on day 4. The progenies were screened from 18th may to 16th June, 2013. Temperature and humidity recorded at that period were more or less similar (averaging 27^{0}C at night and 34.9^{0}C at day).

Parameters like SES (Standard Evaluation System) score, chlorophyll content, stomatal conductance, shoot and root relative water content, shoot and root length, shoot and root dry weight, Na^+/K^+ ratio were measured on the salt stressed plants. The overall salinity tolerance at seedling stage was evaluated mainly based on the value of leaf damage score named as SES score (IRRI, 1976) where a scale of 1-9 corresponding from highly tolerant to extremely sensitive was used.

Figure 3.5: Measurement of stomatal conductance and SES score

Table 3.4: Standard evaluation system for rice (IRRI, 1976)

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

3.1.5.1.2 Stomatal conductance

Stomatal conductance is the measure of the rate of passage of carbon dioxide (CO_2) entering, or water vapor exiting through the stomata of a leaf. Stomata are small pores on the top and bottom of a leaf that are responsible for taking in and expelling CO_2 and moisture from and to the outside air. The Leaf Porometer measures the rate at which this happens.

How the Leaf Porometer Works: The Leaf Porometer measures the stomatal conductance of leaves by putting the conductance of the leaf in series with two known conductance elements. By measuring the humidity difference across one of the known conductance elements, the water vapor flux is known. The conductance of the leaf can be calculated from these variables. The humidity at three places are known: inside the leaf, and at both of the humidity sensors. The Leaf Porometer effectively calculates the resistance between the inside and outside of the leaf: the stomatal conductance. Resistance is measured between the leaf and the first humidity sensor, and the first and second sensors. The following diagram (figure 3.6) schematically illustrates this:

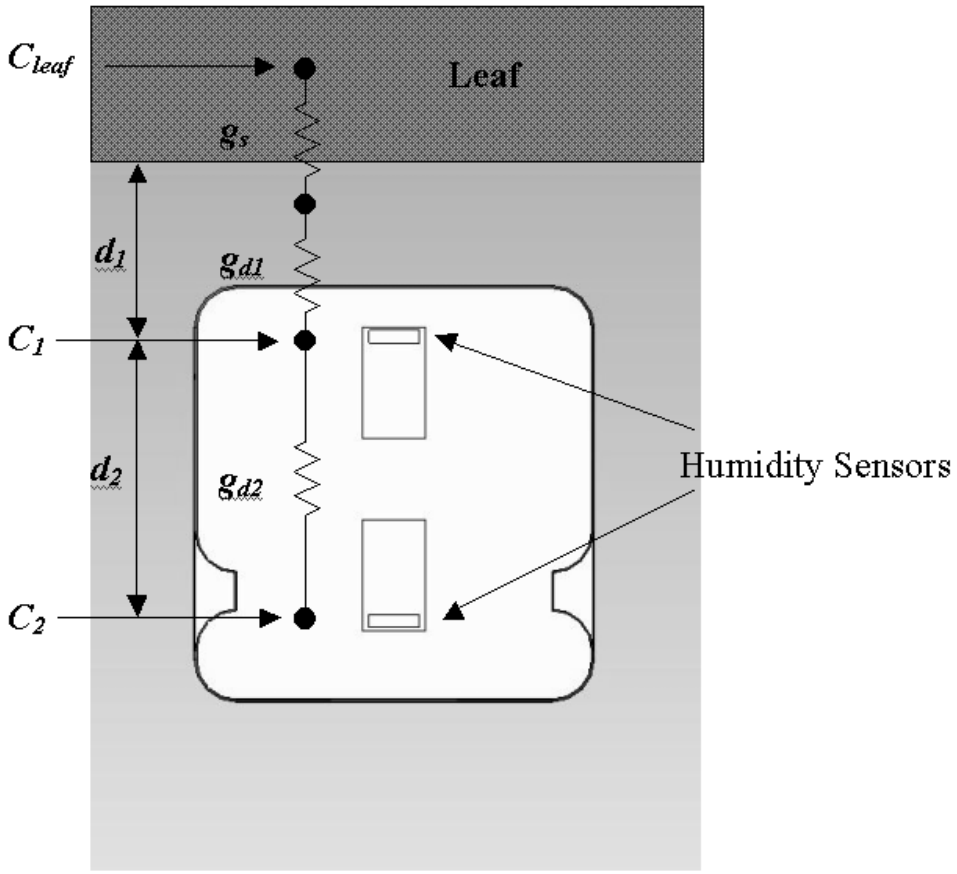


Figure 3.6: Measurement of stomatal conductance by a porometer

The parameters listed above represent the following:

Cleaf The mole fraction of vapor inside the leaf

C1 The mole fraction of vapor at node 1

C2 The mole fraction of vapor at node 2

gs Stomatal conductance of the leaf surface

gd1 Vapor conductance of the diffusion path between leaf surface and node 1

gd2 Vapor conductance of the diffusion path between node 1 and node 2

d1 Distance between the leaf surface and the first humidity sensor

d2 Distance between the two humidity sensors. In this experiment, stomatal conductances of fully opened young leaves were measured after 7 days of salt stress by a Decagon Leaf Porometer (sensor serial LPS1283) (Decagon inc., USA)

during a bright sunny day from 11 am to 2 pm. A control population of unstressed plants was also present.

3.1.5.1.3 Relative water content

Relative water content of shoot and root was measured from the percent ratio of the difference between fresh and dry weight and the difference between turgid and dry weight. The root and shoot samples were weighed (W) and hydrated immediately afterward to full turgidity for 24 hour under normal light and temperature. The hydration was carried out by floating on de-ionized water in closed Petri dishes. After hydration the samples were taken out of water and were well dried of any surface moisture quickly with tissue paper and immediately weighed to obtain fully turgid weight (TW). The samples were then oven dried at 800^0C for 24 hours and weighed (after being cooled down) to determine dry weight (DW).

Calculation:

RRWC = [(W-DW)/ (TW-DW)] x 100

Where,

W = sample fresh weight

TW = Sample turgid weight

DW = Sample dry weight

(Barrs and Weatherley 1962)

3.1.5.1.4 Chlorophyll content measurement

Fresh leaves were cut into pieces and 100 mg put into a bottle containing 12 ml of 80% acetone. After 48 hours, absorbance was taken at two different wavelengths; 645 nm and 663 nm for Chlorophyll a and b (Cock, Yoshida et al. 1976).

Calculation:

Chlorophyll a measurement: (0.00802 × A663)

Chlorophyll b measurement: (0.0202 × A645)

Total Chlorophyll: [(0.00802 × A663) + (0.0202 × A645)]

Total amount of chlorophyll content: [{(0.00802 × A663) + (0.0202 × A645)} × V/W}]

Where,
A = absorbance,
V = volume,

W = weight
(Chutia and Borah 2012)

3.1.5.1.5 Sodium and potassium concentration

Plants were washed in flowing tap water for 30 sec and oven dried for the measurement of sodium and potassium concentrations in seedling shoot and root. Dried leaves from each replicate were pooled, ground and analyzed by a flame photometer Sherwood 410 (Sherwood, UK) after 48 h of extraction with 1N HCl following the procedure described by (Cock, Yoshida et al. 1976). Concentrations of Na^+ and K^+ were expressed as percent of dry weight and mmole/g dry weight.

3.1.5.1.6 Length measurement of root and shoot

Individual plants were divided into two parts: root and shoot immediately after taking out of hydroponic solution. Then the lengths (cm) were measured using ruler.

3.1.5.1.7 Shoot and root dry weight

Shoot and root dry weights were measured after drying in a hot air circulating oven for 72h at 70°C (ALP, Japan).

3.1.5.2 Reproductive stage phenotyping

For reproductive stage characterization 100 similar F_3 lines was selected based on SES scores during seedling stage phenotyping, where extreme tails of 25% each were chosen from 200 lines. Phenotyping at reproductive stage was carried out in a Net house with controlled saline environment by the method described (Gregorio, Senadhira et al. 1997). Rice field soils were sun-dried first and then ground by mortar and pestle. Small perforated plastic buckets were filled by the grounded soils and were kept into large plastic bowl. Each bowl can accommodate 6 pots. Incomplete Block Design (partially balanced) with 2 replications of each of 100 selected F_3 plants, *Horkuch, IR29* parent were taken and were non-randomly distributed.

NaCl salt at 8dS/m was applied at 35 days after seeding of sprouted seeds to the soil according to (Gregorio, Senadhira et al. 1997). Salt was applied by replacing tap water with saline water (8 dS/m) in all bowls and the level/volume of water

in each bowl was marked and the evaporated volume replenished each day. The population was phenotyped in the *T. Aman* season (July to December) in 2013. All cultural managements i.e. fertilizer, weed and disease-insect managements were done according to the recommendation of (Gregorio, Senadhira et al. 1997) and BRRI, 2013. Important parameters like flag leaf Na^+/K^+ ratios, spikelet fertility, 1000-grain weight, grain yield, days to flowering and maturity, effective tillers, shoot and root weight, panicle branching, plant height, spikelet damage, panicle exsertion, seed length and breadth, leaf weights were measured.

Days to flowering: Flowering time is the period in which a plant produces flowers (bud), typically after the vegetative stage when it's been actively growing. Days to flowering was recorded as the duration in days from seeding to the appearance of floral buds.

Days to maturity- It was recorded as the duration in days from seeding to the time when more than 80% of the grains on the panicles were fully ripened.

Plant height- The length between base of the plant and the tip of the longest leaf blade was measured as plant height.

Panicle type- Panicles were classified according to their mode of branching, angle of primary branches, and spikelet density: (1) compact, (2) intermediate, and (3) open.

Panicle exsertion- The exsertion of the panicle above the flag leaf sheath after anthesis was classified as:

- well exserted- the panicle base appeared way above the collar of the flag leaf blade.
- moderately well exserted- the panicle base was above the collar of the flag leaf.
- just exserted- the panicle base coincided with the collar of the flag leaf.
- partly exserted- the panicle base was slightly beneath the collar of the flag leaf blade.
- enclosed- the panicle was partly or entirely enclosed within the leaf sheath of the flag leaf. Rating was based on the majority of plants in the plot.

Panicle exsertion was scored in percentages, from 0 to 100% exsertion.

Panicles per plant- It was recorded as the number of effective tillers per plant.

Panicle length- Length of panicles was measured in centimeters from the base to the tip of the panicle.

Total tiller and effective tiller- Tillers are branches that develop from the leaf axils at each unelongated node of the main shoot or from other tillers during vegetative growth. An effective tiller is one which bears a panicle on which the grains will ripen fully.

Flag leaf length- It was measured in centimeters from the collar to the tip of the flag leaf.

Seed length and breadth- Seed length and breadth was measured using a digital slide calipers.

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

Table 3.5 (A): Classification of seed length

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.

Table 3.5 (B): Classification of seed breadth

Size	Length in mm
Slender	>3.0
Bold	2.0-3.0
Round	<2.0

Spikelets per panicle- Total number of spikelets includes filled, partially filled, and unfertilized spikelets. Spikelets per panicle was estimated, by dividing the total number of spikelets by the number of panicles.

Spikelet damage- Spikelet damage is the visual damage of spikelets caused by salt stress creating sterile spikelets. Scores were given in percentages.

Percent fertility- The percent fertility was a ratio of the number of grains to the total number of spikelets.

$$\text{Percent fertility} = \frac{\text{Filled spikelets x 100}}{\text{Total spikelets}}$$

1000 grain weight - A random sample of 1000 well-developed, whole grains dried to 13% moisture content was weighed on a precision balance to give the 1000-grain weight.

$$\text{Thousand ground weight} = \frac{\text{(Total filled grain weight) x 1000}}{\text{Filled spikelets/panicle}}$$

Yield- Plant yield is a complex which is measured as such-

$$\text{Yield} = \frac{\text{(Spikelet/panicle) x (Panicle per plant) x (1000 grain weight) x (\%fertility)}}{\text{1000 x 100}}$$

3.1.6 QTL mapping and analysis

A significant *P* value obtained for differences between mean trait values indicate linkage between marker and QTL due to recombination. The closer a marker is from a QTL, the lower the chance of recombination occurring between marker and QTL. Therefore, the QTL and marker will be usually be inherited together in the progeny, and the mean of the group with the tightly-linked marker will be significantly different ($P < 0.05$) to the mean of the group without the marker. When a marker is loosely-linked or unlinked to a QTL, there is independent segregation of the marker and QTL. In this situation, there will be no significant difference between means of the genotype groups based on the presence or absence of the loosely linked marker. Unlinked markers located far apart or on different chromosomes to the QTL are randomly inherited with the QTL. So, no significant differences between means of the genotype groups will be detected.

A typical output from interval mapping is a graph with markers comprising linkage groups on the *x* axis and the test statistic on the *y* axis (figure 3.7). The peak or maximum must also exceed a specified significance level in order for the QTL to be declared as 'real' (i.e. statistically significant). The determination of significance thresholds is most commonly performed using permutation tests (Churchill and Doerge 1994). The phenotypic values of the population are 'shuffled' while the marker genotypic values are held constant (i.e. all marker-trait associations are broken) and QTL analysis is performed to assess the level of false positive marker-trait associations (Churchill and Doerge 1994; Haley and Andersson 1997; Hackett 2002). This process is then repeated (e.g. 500 or 1000 times) and significance levels can then be determined based on the level of false positive marker-trait associations.

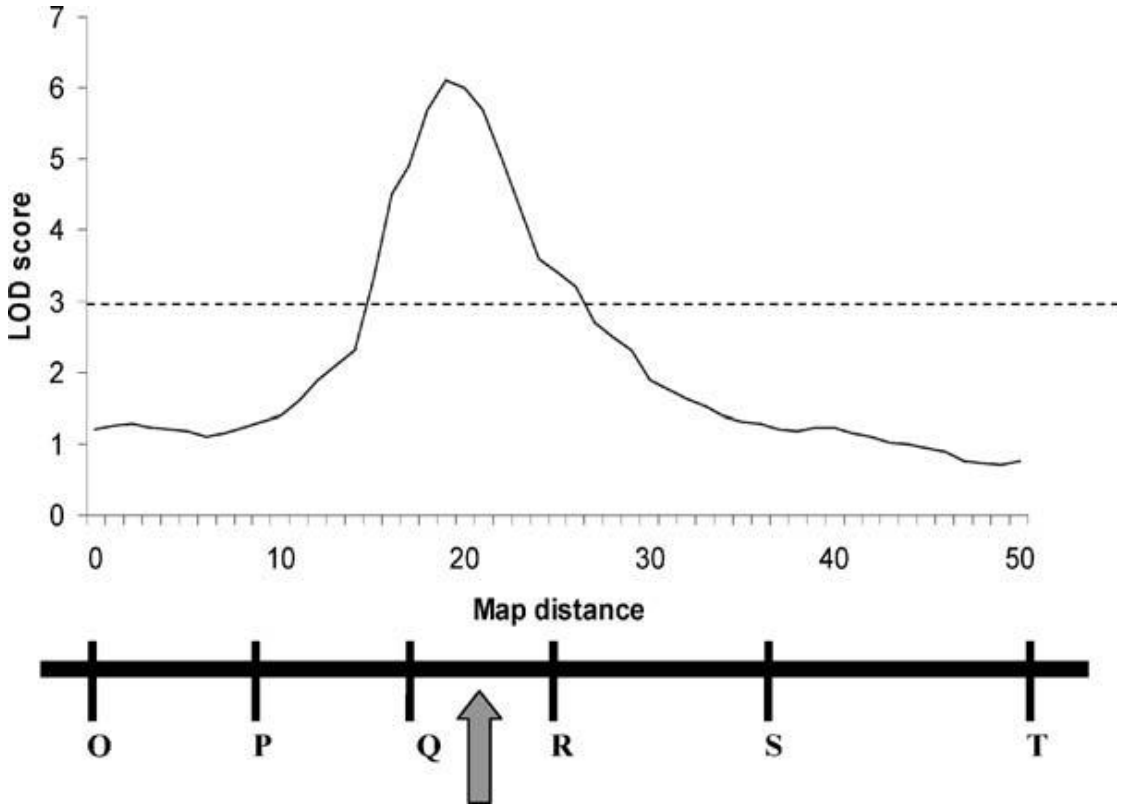


Figure 3.7: Hypothetical output showing a LOD profile for chromosome 4. The dotted line represents the significance threshold determined by permutation tests. The output indicates that the most likely position for the QTL is near marker Q (indicated by an arrow). The best flanking markers for this QTL would be Q and R (Collard et al., 2005).

Two separate QTL maps were made for seedling and reproductive stages. The *.raw data and *.map file generated by MAPMAKER were kept in the same folder and were used as input files. QTL analysis was achieved by composite interval mapping conducted with QTL Cartographer version 1.15 (by C.J. Basten, B.S. Weir, and Z.B. Zeng, Department of Statistics, North Carolina State University) with model 6 using the program Srmapqtl to identify significant background markers and having a window size of 10 cM. Permutation testing indicated that a LOD score of 3.5 is suitable as the genomewide 5% significance threshold for this set of data. QTLs with LOD scores between 2.5 and 3.5 were considered as putative QTLs. Two separate QTL maps were drawn for seedling and reproductive stages using the software MapChart.

*SF1216	B	H	H	H	A	B	H	B	B	H
*SF1217	B	H	H	H	A	B	H	B	B	H
*SF1218	B	H	H	H	A	H	B	B	H	H
*SF1219	B	H	H	H	A	H	B	B	H	H
*SF1220	B	H	H	H	A	H	B	B	H	H
*SF1221	B	H	H	H	H	H	B	B	A	H
*SF1222	B	H	A	H	H	H	B	B	A	H
*SF1223	B	H	A	B	H	H	B	B	A	A
*SF1225	H	H	A	H	H	A	H	H	A	A
*SF1226	H	H	H	H	H	A	H	H	A	A
*SF1227	H	H	H	H	H	A	H	H	A	A
	1	2	3	4	5	6	7	8	9	10
*TotalChl	.	.	5.653761	4.535014	4.882305	4.598737	.	.	5.480281	.
*A+BChl	.	.	5.308842	4.155527	4.674182	4.375536	.	.	5.267342	.
*Chla/b	.	.	2.276069	2.736657	2.601254	3.497136	.	.	3.559821	.
*SRWC	.	.	68.57838	72.95088	67.7206	63.40857	.	.	73.41093	.
*RRWC	.	.	87.99179	111.2725	82.74889	95.47972	.	.	90.63669	.
*SES	.	.	5.166667	4.111111	4.944444	6.277778	.	.	5.722222	.
*SC	.	.	666.4333	400.9333	388.5667	540.1	.	.	388.6	.
*SL	.	.	39.83333	40.16667	42.83333	33.75	.	.	34	.
*RL	.	.	10.93333	9.722222	8.677778	7.555556	.	.	8.888889	.
*SDW	.	.	0.6857	0.838433	0.8303	0.399633	.	.	0.6287	.
*RDW	.	.	0.357267	0.11	0.107467	0.0466	.	.	0.0695	.
*K/Na	.	.	0.188073	0.24306	0.215168	0.191261	.	.	0.217824	.

Figure 3.8: Sample input data of genotype and phenotype for analysis by QTL Cartographer.

3.2 QTL Validation

The generation was advanced up to F_5. In this study, both molecular and physiological analyses were done to confirm the presence of desired QTLs in specific F_5 progenies.

3.2.1 QTL Validation: Molecular Analysis

3.2.1.1 Significance study of the QTLs

The statistical significance of the level each QTL is affecting its corresponding phenotype or on any other phenotype was assessed with one-way analysis of variance (ANOVA) using the software R at $P < 0.05$ significance level.

3.2.1.2 QTL selection for molecular validation

Three seedling and one reproductive QTL were selected for validation at molecular level. The seedling QTLs were total chlorophyll, root length and stomatal conductance and the reproductive QTL to be confirmed was third leaf length. The donor allele for total chlorophyll, root length and third leaf length was *Horkuch*. The donor allele for stomatal conductance was *IR29*. The QTLs were selected based on their LOD score, R^2 value, that is, the phenotypic variation each QTL is causing and its donor allele.

3.2.1.3 Selection of positive and negative plants

The images of specific chromosomes carrying the QTLs were created using the software graphical genotype 2 (GGT2) and from those images, plants having a combination of good QTLs were primarily selected. The plants were finally selected by matching the initially selected plants with the allele type of genotype data obtained from DArTseq™ analysis. When the QTLs with desired *Horkuch* alleles were located in a big *Horkuch* chunk (3Mb) in the chromosome with no *IR29* or heterozygous DNA nearby were designated as positive plants. And the QTLs inside an *IR29* chunk (3Mb) with no *Horkuch* allele nearby were called negative plants. Similarly, plants with desired *IR29* QTLs inside an *IR29* derived chromosomal chunk were called positive plants. They were called negative when these QTLs were present in a *Horkuch* chunk. The positive and negative plants for each trait were selected from the GGT images. These specific QTL regions were

amplified using primers for nearby microsatellite markers and designed primers for SSR.

3.2.1.4 Primer design

SSR primers were designed using the SNP containing DNA sequence provided by DArT. 300 bp upstream and downstream sequence of the SNP provided by DArT was retrieved by BLAST from the website www.gramene.org. This sequence was used to design SSR primer from batchprimer 3.

Table 3.6: Details of selected QTLs and nearby markers.

Primer Name	Sequence	QTL	Chromosome	Position	Donor Allele
RM13642	ATATGGATACAGGCCAGCATTGG CTGAGCCATCAAGTGCCTTTCC	TLL_1_H	2	25.41	*Horkuch*
RM22073	AAGAAGTTCTGCCTCAGCCAGTTCG CCTCCGTCGTCTCCTCCACTATCG	Tch _H	7	27.53	*Horkuch*
drtSSR 3452265	AGCCACTCAGCAATAGGAC ATTTTTGCCATCCCTCTT	RL_H	2	28.48	*Horkuch*
drtSSR 3050109	CTCCCCTAGCTTAGGTCATAG AAGGACAATTTCAGAAACCAT	SC_2_I	5	15	*IR29*

3.2.1.5 DNA amplification using SSRs

Molecular studies were done using Simple Sequence Repeat (SSR) marker system to confirm the presence of predicted QTLs in the plants where the QTLs of interest were supposed to be located. Total genomic DNA was isolated according to CTAB method reported by (Doyle 1987; Doyle 1990). DNA was quantified and then used to employ specific SSR markers. DNA amplification reactions were carried out using a pair of SSR primers. All primers were synthesized by Integrated DNA

Technologies, Inc, USA. Polymerase chain reactions were performed in 15µl reaction mixture. The PCR reaction mix contains PCR buffer, 25mM MgCl, 1mM of each dNTPs, Taq polymerase, 2µl (05 picomole/µl) of each reverse and forward primers and 50ng/µl genomic DNA. The PCR amplification program was as follows: initial denaturation 95° C for 05 min, denaturation 35 cycles with 95° C for 01 min, annealing 55° C for 01 min, extension 72° C for 01 min and final extension was set 72° C for 07 min. The amplified product was observed using polyacrylamide gel electrophoresis. Amplified PCR products of microsatellite were scored for the presence of distinct allelic pattern of each marker allele-genotype combination.

3.2.2 QTL Validation: Physiological Analysis at seedling stage

3.2.2.1 Plant selection

The progenies which were advanced to F_5 by single seed descent, were selected based on the presence of major or minor seedling and reproductive QTLs. Plants with good combination of both seedling and reproductive QTLs under stress conditions were given highest priority. Based on that, the best 27 plants were chosen for seedling stage screening having a total of 7 to 11 QTLs appendix C. 2.

3.2.2.2 Experimental design at seedling stage

The phenotypic screening for the salinity tolerance at seedling stage was done by the method described by (Gregorio 1997). The experiment was conducted at the Net house, of the Plant Biotechnology Laboratory, University of Dhaka (during September-October, 2016). Seeds of the selected plants were incubated at 50° C for 5 days to break dormancy. Then seeds were rinsed several times with distilled water and placed in Petri dishes with moistened filter paper and incubated at 37° for 72 hours to germinate.

In this experiment, tolerant and susceptible checks were *Horkuch* (IRGC 31804) and *IR29* (IRGC 30412) respectively. The selected 27 F_5 plants, one *Horkuch* parent (tolerant check) and one *IR29* parent (sensitive check) with 16 replicates of each were used in this experiment. Among them, 8 replicates were used for 'Control'

(no salt) whereas rest of the 8 replicates as 'Stress' (12 dS m^{-1} NaCl); so a total of (27+1+1) × 16= 464 plants were used for seedling stage screening. In each floater, one replicate of all the 27 F_5 plants, one *Horkuch* and one *IR29* parent were sown in a completely randomized (CRD) design. At 14 days of seedling age (four leaf stage), 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 by 2 dSm^{-1} every day until it finally reached 12 dSm^{-1}. The pH of the culture solution was adjusted daily to 5 by adding either NaOH or HCl to avoid Fe deficiency (Yoshida et al., 1976) and the solutions were renewed twice a week. The sensitivity of each seedling was scored when the sensitive checks were almost dead. Different other parameters were measured for both conditions, as described in section 3.1.4.1.

Figure 3.9: A view of representative floaters in seedling stage screening experiment. Two control floaters and two floaters containing 12dS m^{-1} salt solution are seen at left and right sides, respectively. The picture was taken 14 days after applying 12dS m^{-1} salt.

Table 3.7: Experimental design of seedling screening at F_5.

Plant type	Number	Condition	
		Control	Stress
Horkuch	1	8 replicates	8 replicates
IR29	1	8 replicates	8 replicates
F_5 progeny	27	8 replicates	8 replicates

3.2.2.3 Physiological screening of selected F_5 plants: Reproductive stage

3.2.2.3.1 Plant selection

As described previously (section 3.7.2.1), F_5 progenies with good combination of seedling and reproductive QTLs were selected. Based on that, the best 10 plants were chosen for reproductive stage screening ranging from lowest 9 to highest 13 QTLs appendix C. 3.

3.2.2.3.2 Experimental design at reproductive stage

This experiment was carried out at Plant Physiology Division Net house of BRRI (September - December, 2016) by the soil based method described by (Gregorio 1997). Seed dormancy breaking and germination were same as described in section 3.1.1.1. Six replicates (3 for control and 3 for stress) of each 10 selected F_5 plants, *Horkuch* parent (tolerant check) and *IR29* parent (sensitive check) were taken. So, a total (10+1+1) × 6= 72 plants were used for reproductive stage screening; where half of them were used for control and half of them for stress condition. 12 big plastic bowls (6 for control and 6 for stress) with a capacity to accommodate 6 pots were taken. Tap water was added to the bowls containing perforated plastic pots filled with fertilized puddle soil. 3 of the control bowls and 3 of the stress bowls were randomly selected for the *Horkuch* parent (tolerant check) and *IR29* parent (sensitive check). After randomly assigning 2 pots for the

parents in each of the 3 randomly selected bowls of control and stress, the rest of the pots were randomly assorted for the selected F_5 plants. As one pot contains a single plant and so each pot could be considered as one experimental unit.

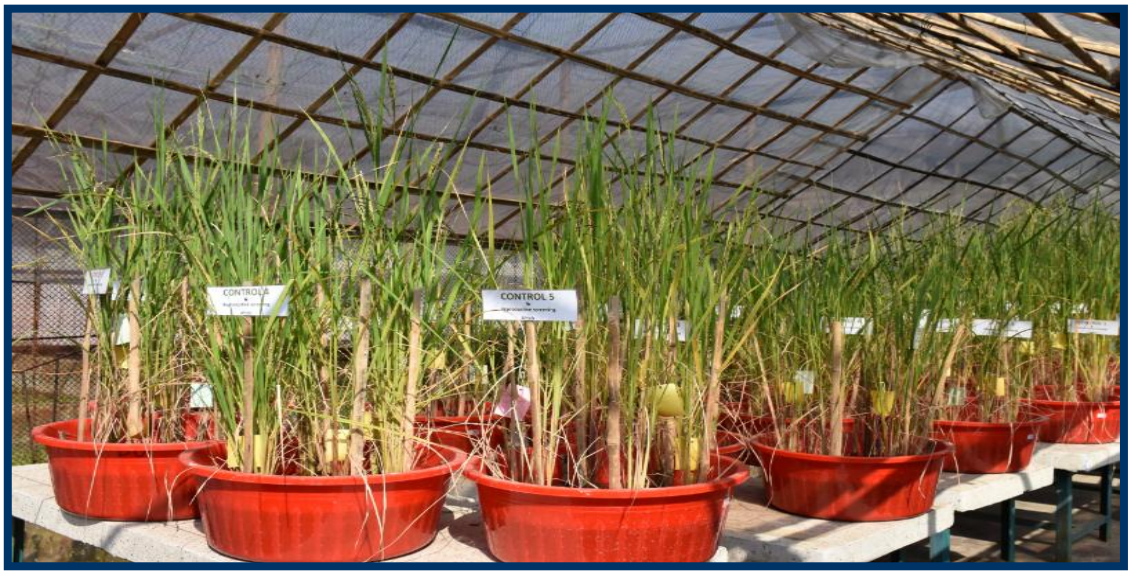


Figure 3.10: Physiological screening of F_5 progeny under 10dSm^{-1} salt stress

Table 3.8: Experimental design of reproductive screening of F_5 progeny.

Plant type	Number	Condition	
		Control	Stress
Horkuch	1	3 replicates	3 replicates
IR29	1	3 replicates	3 replicates
F_5 progeny	10	3 replicates	3 replicates

When the seedlings were 30 days old, salinity stress (NaCl) at EC of 10 dS m^{-1} were applied to the bowls by replacing tap water with saline water and maintained by keeping the volume same with water on a daily basis until maturity. All cultural managements i.e. fertilizer, weed and disease-insect managements were done

according to the recommendation of (Yoshida 1976) and (BRRI, 2013). At physiological maturity all plants were harvested and different phenotypic parameters including yield and its components were measured and recorded as described in section 3.1.4.2.

CHAPTER 4 *RESULTS*

Pages 72 - 130

4.1 Background

Bangladesh is situated near sea level which makes it particularly vulnerable to climate change-induced salinity. The productivity of cultivable lands is threatened by increased salinity. So, developing salt tolerant varieties with high yield is a crying need. Rice landraces, such as *Horkuch* from Satkhira, endemic to the coastal regions can be suitable donors for salt tolerance traits into the genetic background of high-yielding cultivars. For this purpose it is important to map the genetic loci of *Horkuch*, so that specific loci can be introgressed into high yielding rice using DNA markers linked to these genetic regions. Rice is extremely sensitive to salinity stress at certain phases of two stages of its life cycle, seedling and reproductive. Salt tolerance is a multi-genic trait and it is expected that multiple QTLs (quantitative trait Loci) for seedling and reproductive level tolerance will be found. The current work focused on generating QTL maps of seedling and reproductive stages of *Horkuch*, QTLs related to both salt tolerance and yield. Also, confirmation of the QTLs in advanced generation by physiological screening of both developmental stages under salt stress condition as well as validation at molecular level by SSR markers.

With a view to attaining this goal, an F_2 mapping population was generated by crossing *Horkuch*, a salt tolerant, low yielding landrace, with a salt sensitive high-yielding rice variety, *IR29*. *Horkuch* has been shown to be tolerant at both seedling and reproductive stages of salinity stress (Lisa, Elias et al. 2011). To generate a genetic linkage map, genotyping was done at F_2 generation by using DArTseq™ technology. Phenotypic screening was done at F_3 generation and $F_{2:3}$ strategy was used for quantitative trait locus (QTL) mapping which has been implemented in many studies of rice to explore the genetic basis of traits involved in salinity stress for different growth stages.

Furthermore the population was advanced up to F_5 generation to obtain homozygosity for fixation of the identified salt tolerance loci. The mapped QTLs were confirmed at F_5 at both molecular and physiological level. The location of DNA markers linked to the identified QTLs were spotted. Also, plants with multiple major QTLs were identified as potential donors to be used in breeding lines. An outline of the study is given in figure 4.1.

The result chapter is divided into two sub-chapters:

(A) QTL mapping and

(B) Confirmation of QTLs.

Chapter A (QTL mapping) includes-

(i) Genotyping of F_2 plants followed by linkage map construction and

(ii) Seedling and reproductive stage phenotypic screening of F_3 plants, to generate different QTL maps for both developmental stages.

Chapter B (Confirmation of QTLs) includes-

(i) Phenotypic characterization of F_5 progeny at both developmental stages and

(ii) Molecular validation of some identified QTLs by SSR markers.

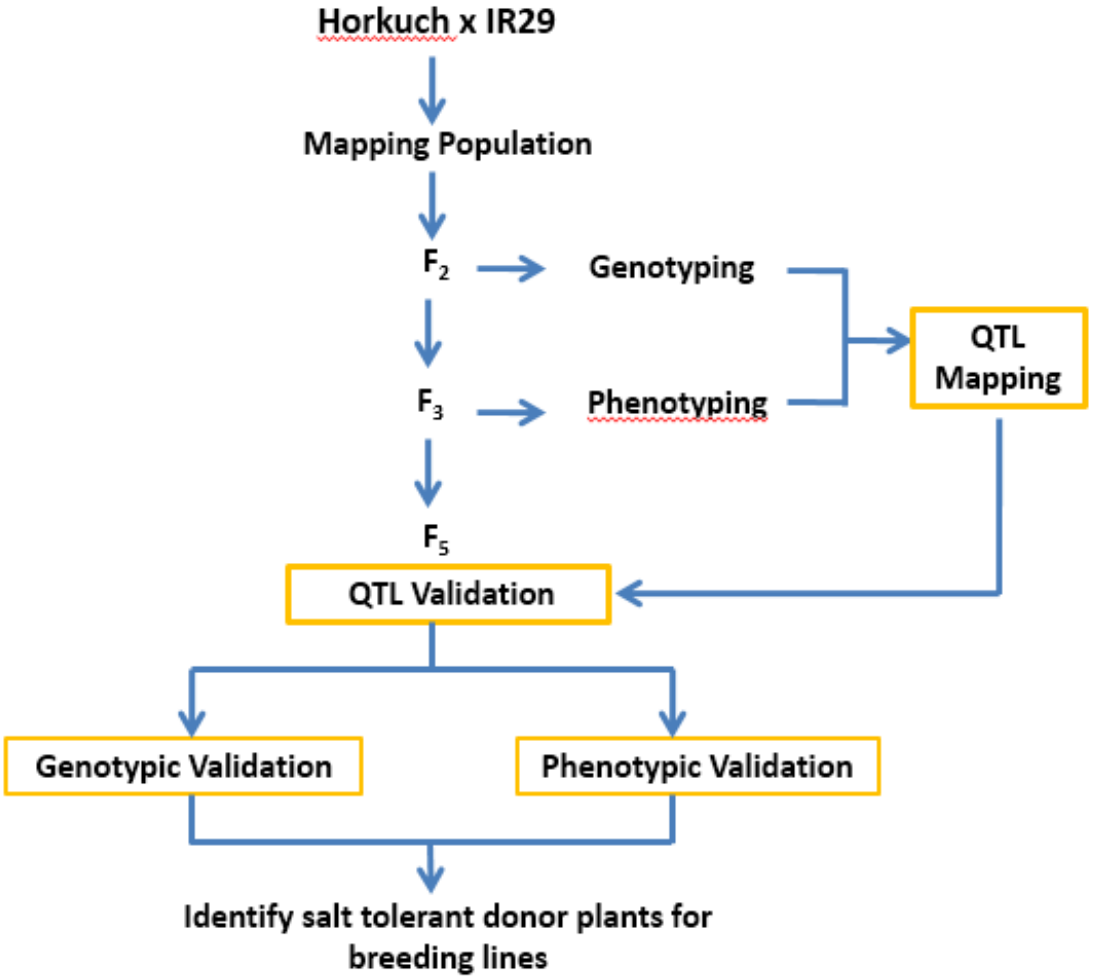
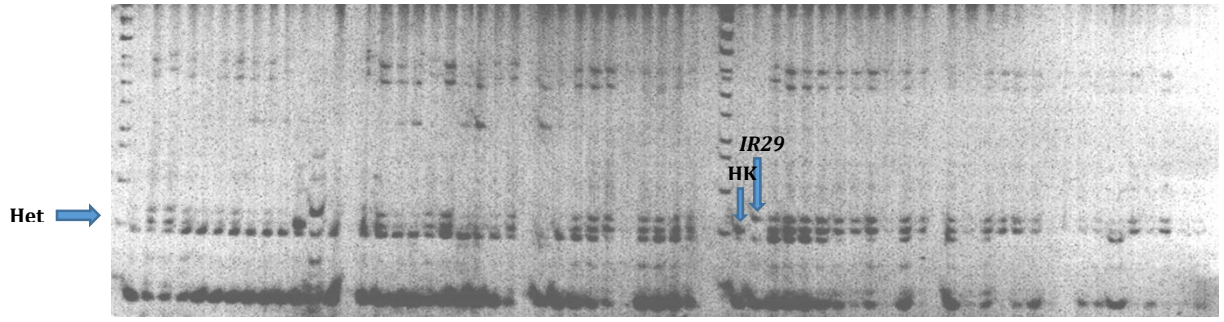


Figure 4.1: Outline of the study.

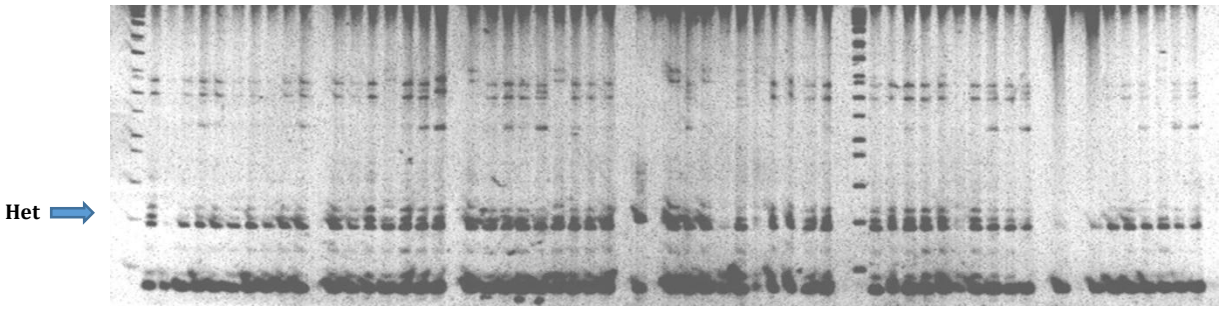
Chapter 4A:
QTL Mapping

4.2 Confirmation of heterozygosity of the F_1 progeny

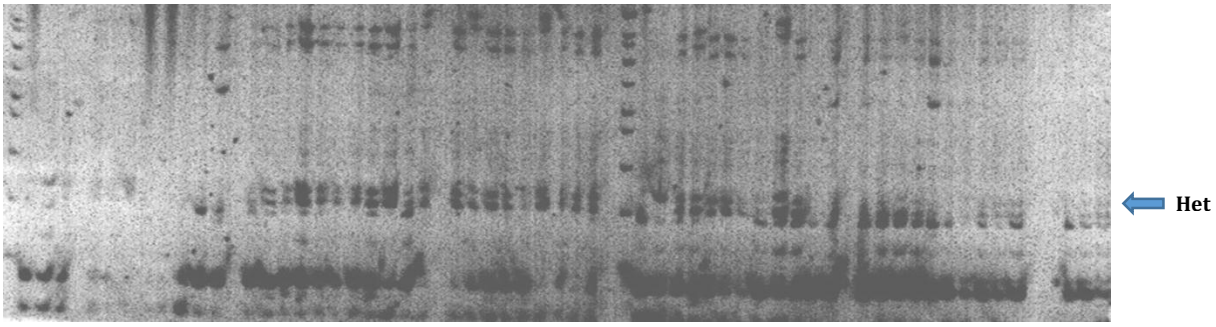
The F_1 plant DNA were amplified by a pair of primers of the polymorphic SSR marker RM493. As F_1 plants should be heterozygous at all loci, the amplified DNA showed bands for both parents at the particular locus (figure 4.2).



Gel-1



Gel-2



Gel-3

Figure 4.2: Parental and F_1 DNA run in PAGE. HK=*Horkuch*, Het=Heterozygotes

4.3 QTL mapping

A quantitative trait locus (QTL) is a section of DNA (the locus) which correlates with variation in a phenotype (the quantitative trait). Usually the QTL is linked to, or contains, the genes which control that phenotype. Most agronomically important traits are controlled by more than one gene called quantitative traits. QTL mapping is the process of locating genes with effects on quantitative traits using molecular markers. (McCough and Doerge 1995; Paterson 1996; Mohan, Nair et al. 1997). Its aims are to detect which regions (QTL) of the genome affect the trait and to describe the effect of the QTL on the trait.

QTL analysis detects an association between phenotype and the genotype of markers. QTL mapping can be divided into two parts:

(i) Linkage map construction by genotyping through markers

(ii) Phenotypic screening of the population

A significant difference between phenotypic means of the groups, depending on the marker system and type of population, indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait. In the current study, QTL mapping was performed in $F_{2:3}$ strategy where F_2 population was genotyped with DArTSeq™ markers (DArT based SNP markers) and F_3 population was phenotyped at both seedling and reproductive stages. Outputs of genotyping, linkage map construction and phenotyping are discussed in sections 4.3.1, 4.3.2 and 4.3.3, respectively.

4.3.1 Genotyping of F_2 DNA

As mentioned in chapter 3, section 3.1.1.1, 500 F_2 progenies from *IR29/Horkuch* cross were planted in the field at BRRI to be advanced to the generation F_3. The F_2 plants showed diverse phenotypes. They showed all types of phenotypic characteristics, that is, *Horkuch, IR29* and intermediate types (figure 4.3).

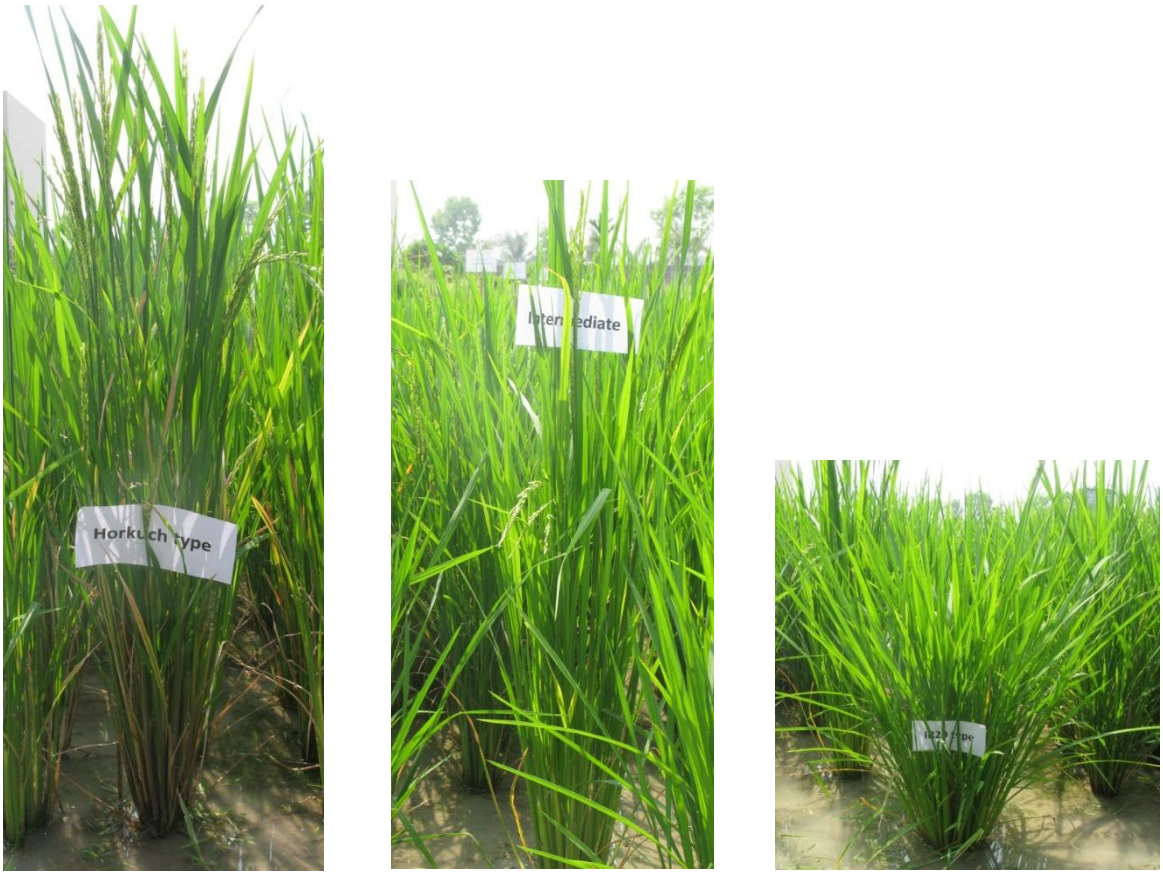


Figure 4.3: F_2 plants with parental and intermediate phenotype.

Randomly chosen 174 F_2 DNA was genotyped using DArTseq technique. The output of the assay has been provided in detail in an excel sheet (appendix A. 3). Snapshots of sample datasheet are given in figures 4.4 (A and B). The headers of the rows indicate:

[1]: Order number where sample belongs to - important for multi-orders reports
[2]: DArT plate barcode
[3]: client plate barcode
[4]: well row position
[5]: well column position
[6]: genotype name

CloneID	AlleleID	AlleleSequenceREF	leleSequenceSI	immedSequen	ice_omeridio	Rice_omeridi	ice_omeridio	Rice_omeridi
43|F|0-11:G>A-11	1243|F|0--11:G	TATCCTGGCAATT	ATCCTGGCAAT	ATCCTGGCAATTTATTGACCT		0	0	999
64|F|0-41:G>A-41	2264|F|0--41:G	CAGCAAATTTGCC	AGCAAATTTGC	AGCAAATTTGCCGTCCCCTCC		0	0	999
08|F|0-28:G>A-28	0008|F|0--28:G	GCACCCCCTTGTA	CACCCCCTTGT	ATTCCCTTGCACCCCCTTGTA		0	0	999
72|F|0-61:T>C-61	3272|F|0--61:T	GCAACACGGCCGA	CAACACGGCCG	CAACACGGCCGACAATCCAAC		0	0	999
05|F|0-51:C>T-51	18723|F|0--51:	AGTCTTATTAGTT	GTCTTATTAGT	GTCTTATTAGTTGGTCATAGT		0	0	999
05|F|0-61:C>T-61	2005|F|0--61:C	AGTCTTATTAGTT	GTCTTATTAGT	GTCTTATTAGTTGGTCATAGT		0	0	999
68|F|0-56:T>G-56	1568|F|0--56:T	TGGTGGTGTAGCT	GGTGGTGTAGC	GGTGGTGTAGCTCAATTCATT		0	0	999
95|F|0-44:T>C-44	0195|F|0--44:T	TGGGCAGATGCTA	GGGCAGATGCT	GGGCAGATGCTATATTAGAGC		0	0	999
69|F|0-22:G>C-22	1369|F|0--22:G	ATGCACTAGTTAT	TGCACTAGTTA	ACAGCATTTATGCACTAGTT		0	0	999
69|F|0-28:A>T-28	8769|F|0--28:A	ATTTTTAGGTAAA	TTTTTAGGTAA	ATTATTTTTAGGTAAAAATCT		0	0	999
34|F|0-31:T>C-31	2734|F|0--31:T	TAATGGGCGGAGT	AACGGGCGGAG	AATGGGCGGAGTTGCCATCCT		0	0	999
883|F|0-5:A>G-5:A	50883|F|0--5:A	GTCATACAGAATG	TCATACAGAAT	TCATACAGAATGTAAAATATT		0	0	999
92|F|0-16:A>G-16	9392|F|0--16:A	TCAGACAATTTGG	CAGACAATTTG	CAGACAATTTGGTACTTCAAA		0	0	999
768|F|0-9:T>C-9:T	50768|F|0--9:T	ACTGCCAATATTA	CTGCCAATATT	TCTCAAGGAT	24.1_chromo	9401	1	2.2046E-12
10|F|0-34:G>A-34	2410|F|0--34:G	AATTGTGTTCCTG	ATTGTATTCCT	ATTGTGTTCCT	24.1_chromo	17762	1	1.4213E-27
62|F|0-27:G>T-27	8862|F|0--27:G	TCAACTCAATAAG	CAACTCAATAA	CAACTCAATAA	24.1_chromo	26912	1	1.4213E-27
77|F|0-33:C>T-33	3577|F|0--33:C	CGAAGCAATTACA	GAAGTAATTAC	CGAGGAGGAA	24.1_chromo	110519	1	2.5517E-11
97|F|0-42:A>T-42	2697|F|0--42:A	AGTAAAAGTATGC	GTAAAAGTATG	GTAAAAGTATG	24.1_chromo	137259	1	1.4213E-27
97|F|0-52:T>C-52	18962|F|0--52:	AGTAAAAGTATGC	GTAAAAGTATG	GTAAAAGTATG	24.1_chromo	137259	1	6.6126E-26
537|F|0-8:T>C-8:T	58537|F|0--8:T	CTTTGCTAGTCTT	TTTGCTAGTCT	TTTGCTAGTCT	24.1_chromo	141232	1	3.0766E-24
27|F|0-43:T>C-43	9527|F|0--43:T	TGCAAACTCAGTT	GCAAACTCAGT	GCAAACTCAGT	24.1_chromo	157306	1	1.1065E-23
91|F|0-10:C>G-10	8191|F|0--10:C	CGCCGCAACTGAT	GCCGCAACTGA	GCCGCAACTGA	24.1_chromo	158899	1	8.554E-25

Figure 4.4 (A): Sample datasheet

And the header of each column defines:

CloneID: Unique identifier for the sequence in which the SNP marker occurs.

AlleleSequence: In 1 row format: the sequence of the Reference allele. In 2 rows format: the sequence of the Reference allele is in the Ref row, the sequence of the SNP allele in the SNP row.

SNP: In 2 rows format: this column is blank in the Reference row, and contains the base position and base variant details in the SNP row. In 1 row format: contains the base position and base variant details.

SnpPosition: The position (zero indexed) in the sequence tag at which the defined SNP variant base occurs.

CallRate: The proportion of samples for which the genotype call is either "1" or "1", rather than "-".

OneRatioRef: The proportion of samples for which the genotype score is "1", in the Reference allele row.

OneRatioSnp: The proportion of samples for which the genotype score is "1", in the SNP allele row.

FreqHomRef: The proportion of samples which score as homozygous for the Reference allele.

FreqHomSnp: The proprtion of samples which score as homozygous for the SNP allele.

FreqHets: The proportion of samples which score as heterozygous.

PICRef: The polymorphism information content (PIC) for the Reference allele row.

PICSnp: The polymorphism information content (PIC) for the SNP allele row.

AvgPIC: The average of the polymorphism information content (PIC) of the Reference and SNP allele rows.

AvgCountRef: The sum of the tag read counts for all samples, divided by the number of samples with non-zero tag read counts, for the Reference allele row.

AvgCountSnp: The sum of the tag read counts for all samples, divided by the number of samples with non-zero tag read counts, for the SNP allele row.

RepAvg: The proportion of technical replicate assay pairs for which the marker score is consistent.

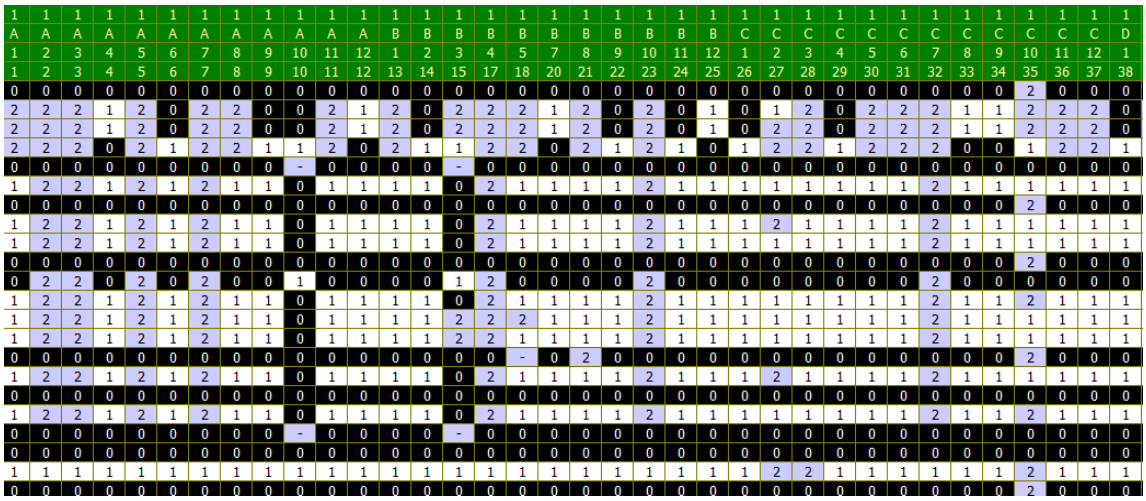


Figure 4.4 (B): Sample genotype data

As mentioned in chapter 3, section 3.1.3.1, a number of 12760 marker data was available from DArTseq analysis. The positions of the SNP markers were given in basepair. About one third of the markers were found to be non-polymorphic and were removed, leaving 4087 polymorphic markers. After removal of the polymorphic but distorted markers, 2174 markers were left (appendix A. 4). There were 267 markers on average on each chromosome, ranging from a lowest

number of 176 (Chr 4) to a highest number of 428 (Chr 3). They were evenly distributed on most of the chromosomes. In some cases, distortions were observed, especially on chromosome 4, 8 and 12 (figure 4.5 A-E).

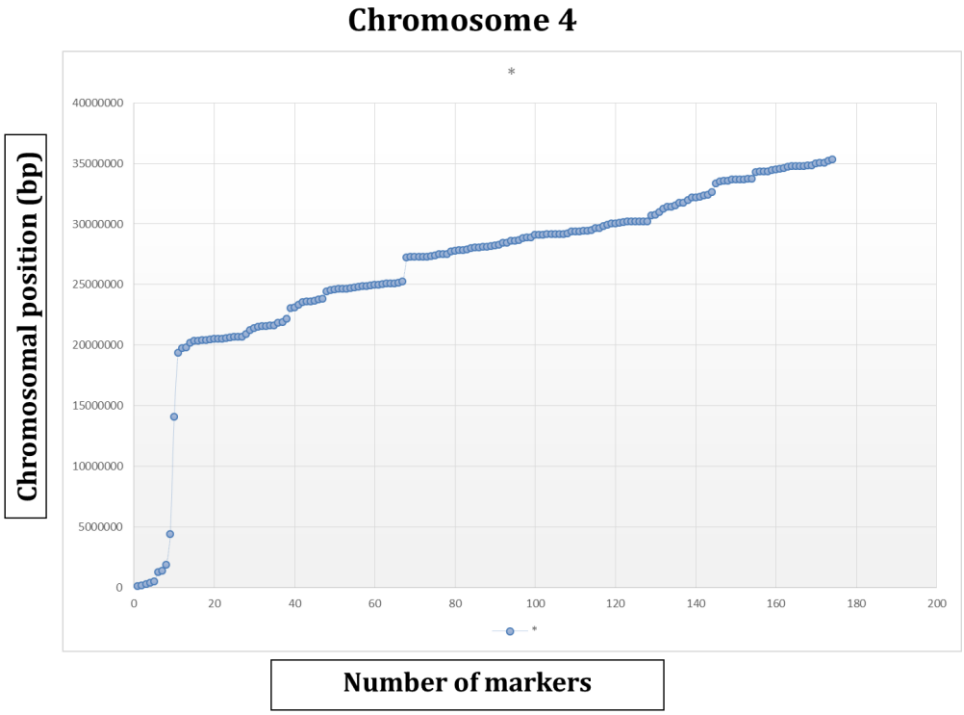


Figure 4.5 (A): Marker distribution on chromosome 4.

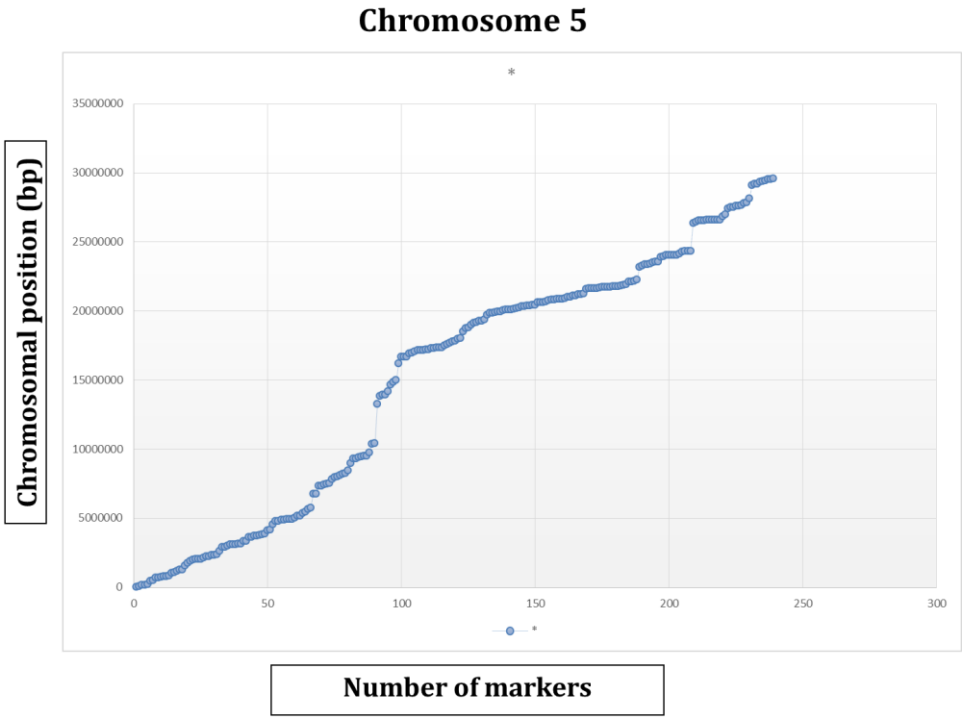


Figure 4.5 (B): Marker distribution on chromosome 5.

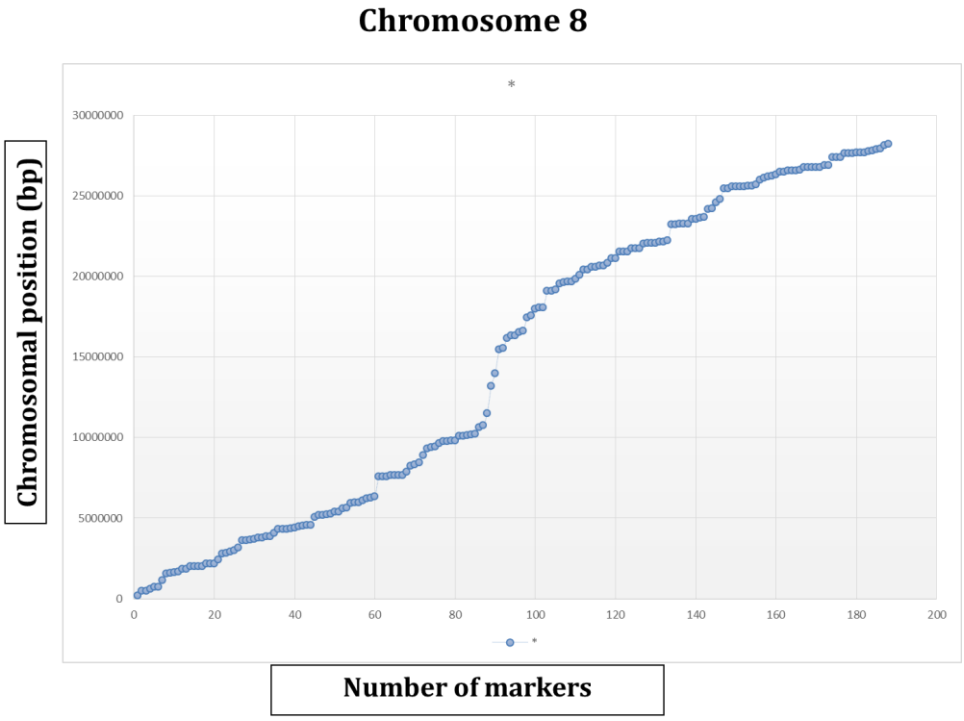


Figure 4.5 (C): Marker distribution on chromosome 8.

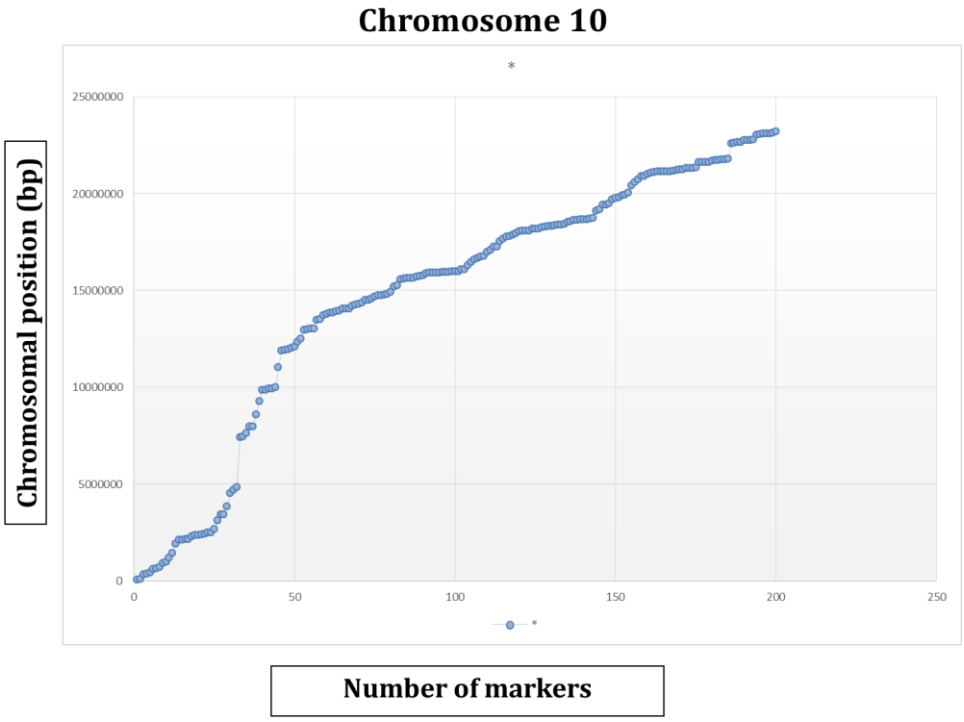


Figure 4.5 (D): Marker distribution on chromosome 10.

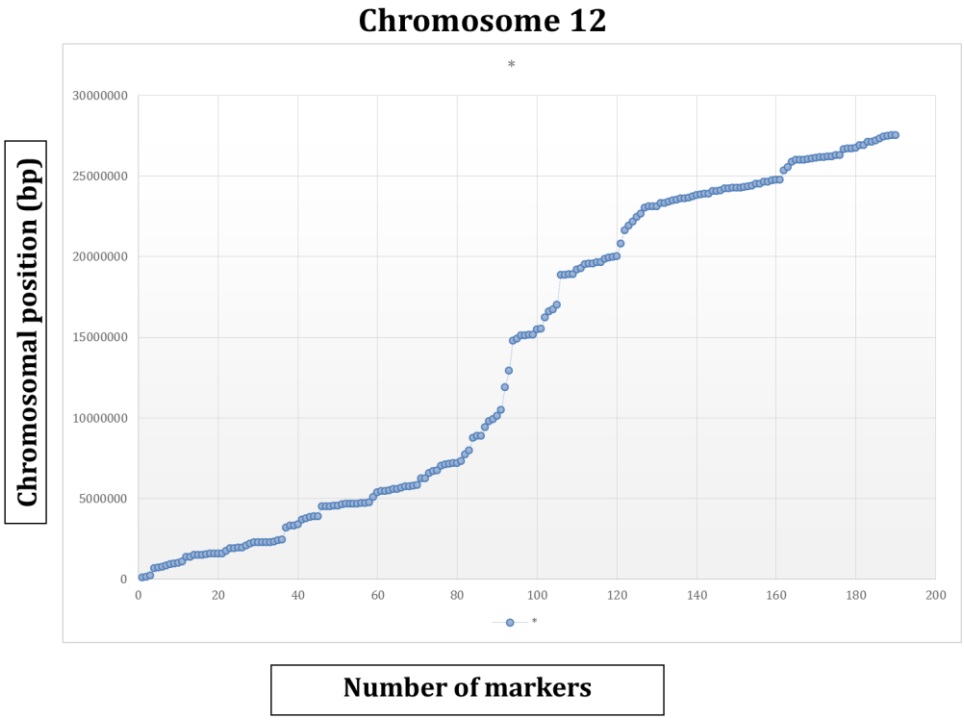


Figure 4.5 (E): Marker distribution on chromosome 12.

4.3.1.1 Marker Segregation

The expected genotypic ratio in the F_2 population should be 1:2:1 for homozygous *Horkuch*, heterozygous *Horkuch*/*IR29* and homozygous *IR29*, respectively. From chi-square analysis, it was found that 33% of the polymorphic markers showed deviation from the expected Mendelian segregation ratio and showed segregation distortion. Among the distorted markers, 42.62% were skewed towards *Horkuch*, 49.25% towards *IR29* and 8% were skewed towards the heterozygous genotype (appendix A. 4, figure 4.6).

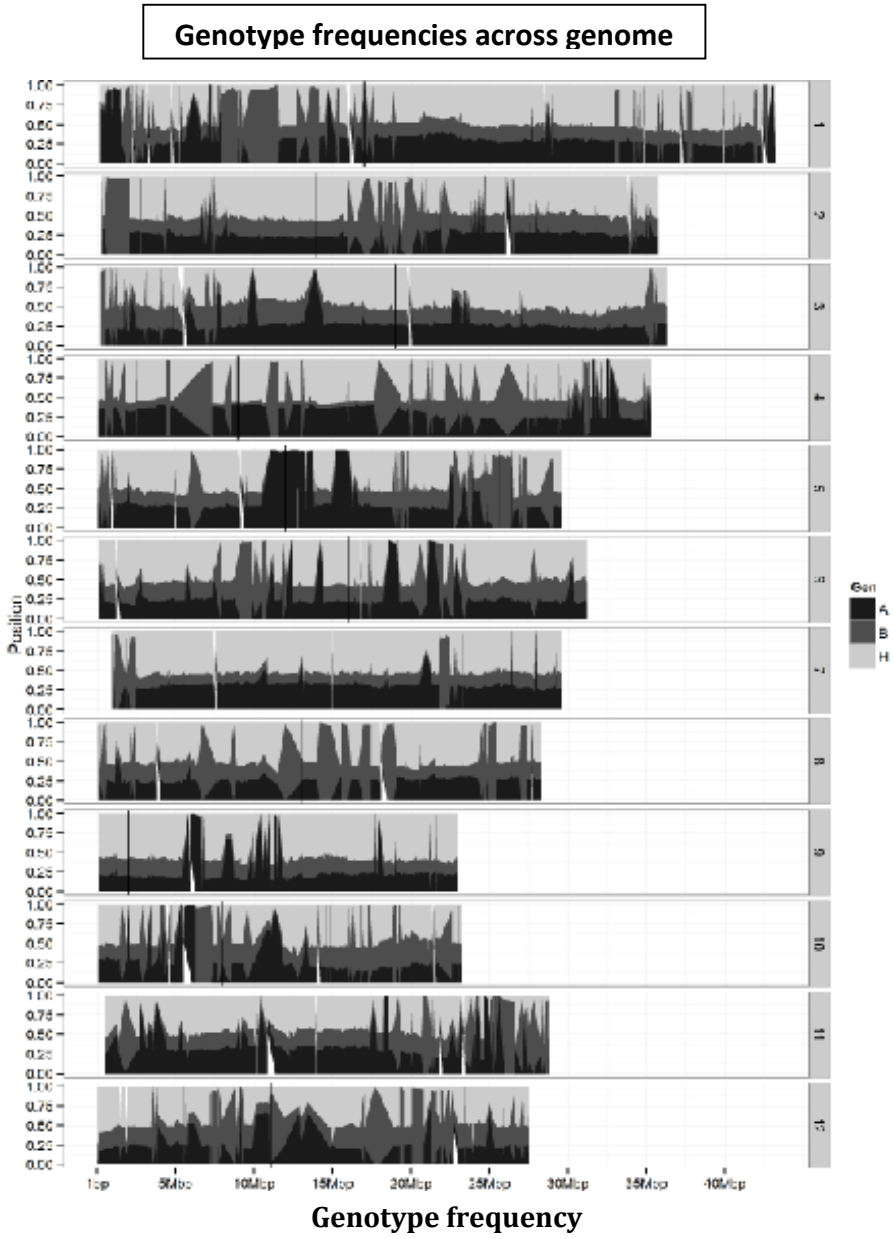


Fig 4.6: Genotype frequencies across genome. A=*IR29*, B=*Horkuch*, H=Heterozygous.

As mentioned in chapter 3, section 3.1.3.2, one marker every 1Mb was selected from the filtered non-distorted, polymorphic markers. Where there was a gap of more than 1Mb between two markers, the correlation of each marker with its flanking five markers was analyzed by the software Minitab version 17. The linked markers had a high correlation coefficient (R) value and low P value (<0.05) and

were used in the analysis. After correlation analysis, 291 markers were selected to make a linkage map. A sample of the output of correlation analysis between nearby markers is shown in figure 4.7 where the upper value denotes correlation coefficient (R) and the lower value denotes P value. In this case, all markers are well correlated with each other and have a good significance.

Correlation: 3445706|F|08, 3988591|F|05, 3051961|F|03, 3048599|F|02, 3997368|F|05, ...

	3445706|F|08:A>T	3988591|F|056:G>	3051961|F|032:A>	3048599|F|026:G>
3988591|F|056:G>	0.927 0.000			
3051961|F|032:A>	0.784 0.000	0.820 0.000		
3048599|F|026:G>	0.674 0.000	0.711 0.000	0.811 0.000	
3997368|F|052:T>	0.572 0.000	0.579 0.000	0.638 0.000	0.797 0.000
5409445|F|09:G>T	0.473 0.000	0.457 0.000	0.553 0.000	0.676 0.000
3053993|F|010:T>	0.571 0.000	0.552 0.000	0.638 0.000	0.743 0.000

Figure 4.7: Sample of correlation analysis between markers.

4.3.2 Linkage mapping

A linkage map covering all 12 chromosomes is necessary for whole genome mapping in rice. In this study, individual maps were generated using MAPMAKER 3.0**.** The positions of the markers were given in cM. The total map length for the 12 chromosomes was 2257 cM, with each chromosome varying from 128.8 cM to 241 cM (table 4.1). Average distance between adjacent markers on each chromosome was 8.11 cM. A genetic linkage map associated with salt tolerance was drawn using Windows QTL Cartographer version 2.5 and is shown in figures 4.8 A and B. The marker order and distances including physical and genetic distance on each individual chromosome are given in appendix A. 5.

Chromosome Number	Chromosome Size (cM)	Average distance between markers (cM)
1	236.6273	7.170524
2	230.6761	8.238432
3	238.6649	8.523746
4	241.1017	8.313852
5	181.0997	8.231805
6	167.3068	8.36534
7	170.7382	7.760827
8	178.5488	8.92744
9	128.8368	7.1576
10	138.7271	8.160418
11	170.6979	7.758995
12	174.4687	8.723435

Table 4.1: Size of chromosomes and average distance between markers (Mapped by MAPMAKER 3.0).

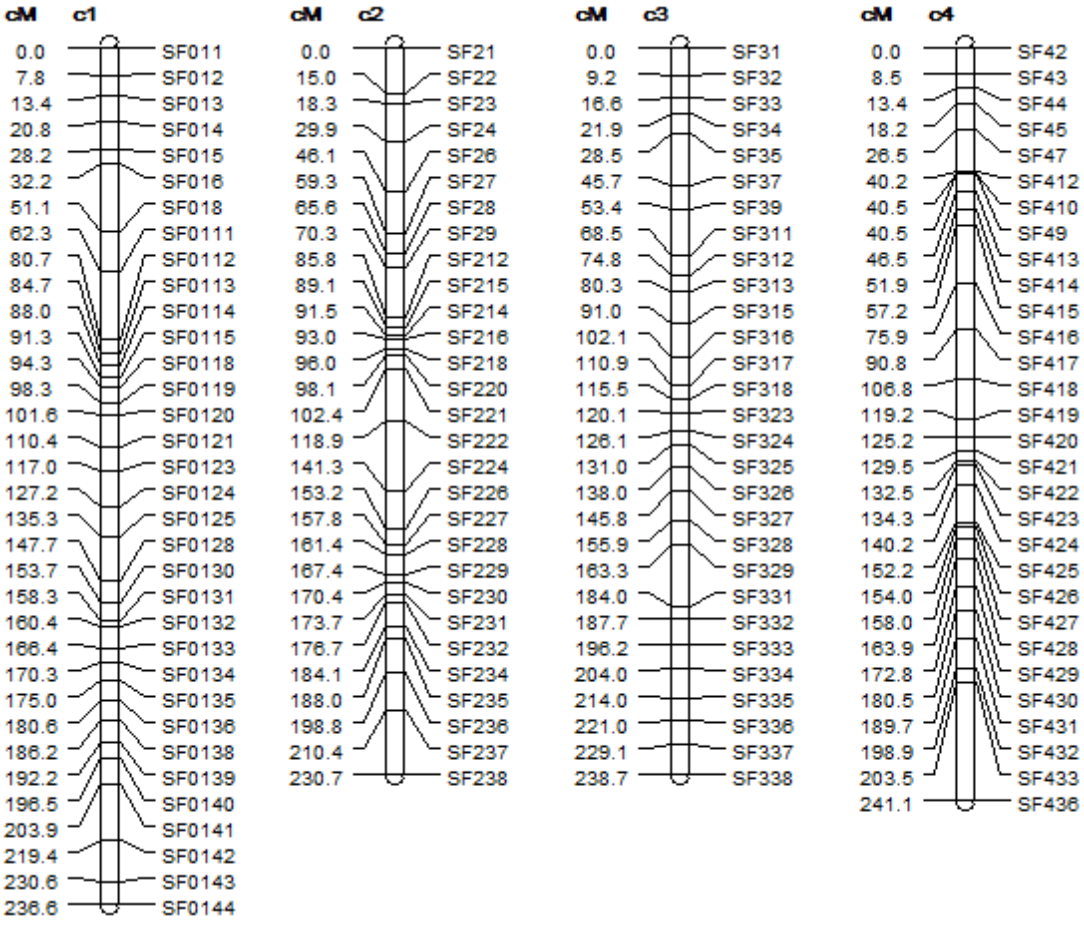


Figure 4.8 (A): Linkage map of chromosome 1-4.

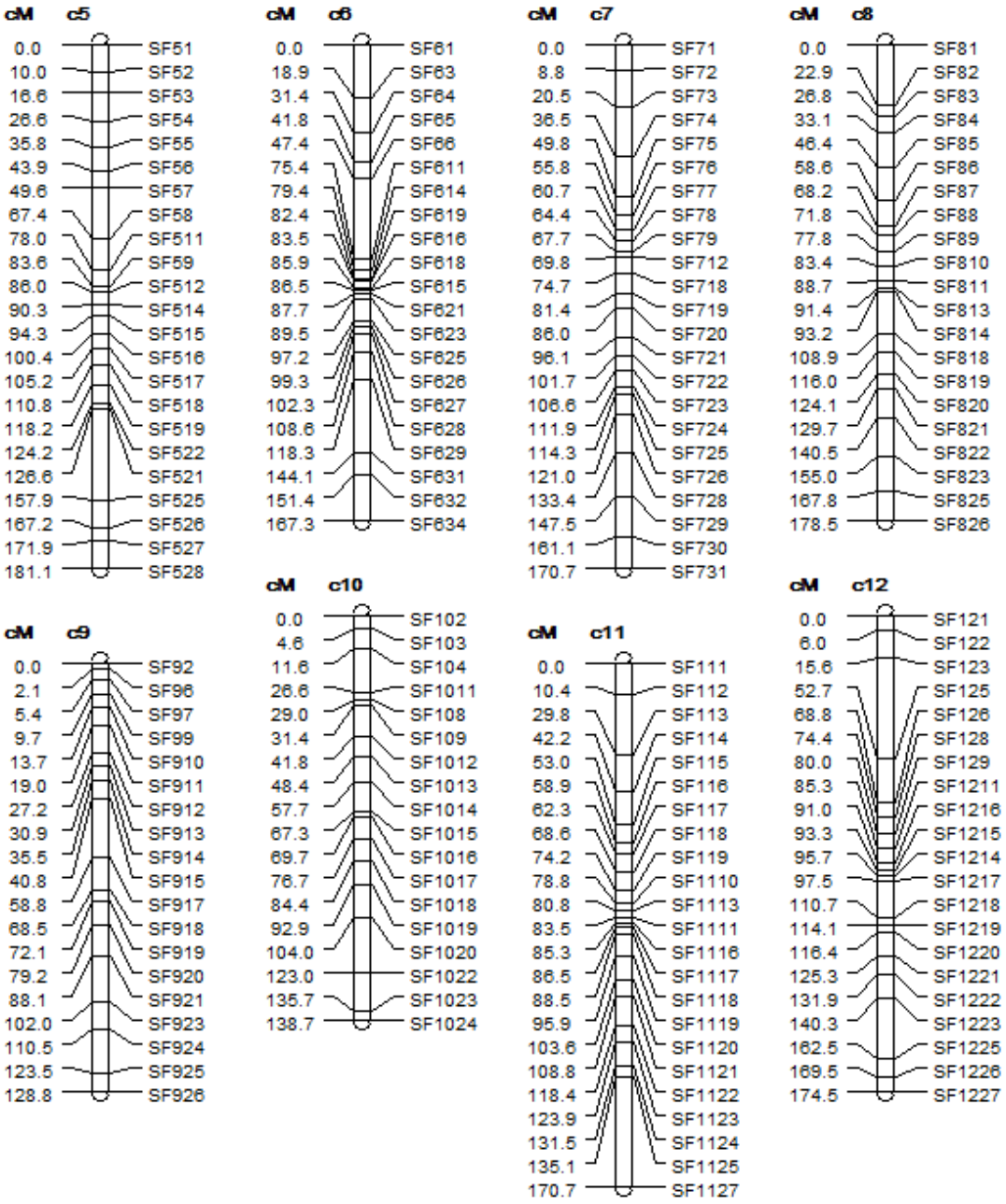


Figure 4.8 (B): Linkage map of chromosome 5-12.

4.3.3 Phenotyping of F_3 Progeny

In this study, $F_{2:3}$ strategy was used for quantitative trait locus (QTL) mapping which has been implemented in many studies of rice to explore the genetic basis of traits involved in salinity stress for different growth stages (Austin and Lee 1996; Ding, Ma et al. 2011). In this strategy, genotyping is done in F_2 generation, one generation earlier than the generation that was evaluated for phenotypic traits (F_3). Two separate QTL maps were generated for seedling and reproductive stages. Seedling and reproductive stage phenotypic screening will be discussed in detail in section 4.4.1 and 4.4.2, respectively.

4.3.3.1 Seedling stage phenotyping

Effects of salt stress on F_3 seedlings were observed. Physiological responses of plants and correlations among different traits under salt stress were studied (appendix B.1).

Table 4.2: Ranges and mean values of seedling traits measured in F_3 population and values of both parents. SL: Shoot length, RL: Root length, SRWC: Shoot relative water content, RRWC: Root relative water content, SDW: Shoot dry weight, RDW: Root dry weight, Total Chl: Total chlorophyll

Trait Name	Range in F_3 population	Mean value of the population	*Horkuch*	*IR29*
SES*	3.33-8.94	6.0	7.08	8.81
SC	140.13-896	425.39	366.26	307.00
SDW** (gm)	0.21-1.0	0.68	0.72	0.22
RDW (gm)	0.04-0.72	0.15	0.07	0.03
SRWC (%)	39.16-87	70.31	61.24	70.63
RRWC (%)	42.51-100	86.47	79.2	91.13
Average** K^+/Na^+	0.06-0.29	0.14	0.15	0.04
Total Chl (mg/gm)	1.41-13.88	4.6	6.4	3.0
SL* (cm)	26.1-99.28	39	41.7	21.74
RL (cm)	6.80-26.50	11	11.44	7.68

*The asterisks denotes significant correlation with the trait to cross direction (*** for P < 0.001, ** for P <0.01 and * for P <0.05)*

Some plants were severely affected by salinity and clearly exhibited symptoms of salt injury, such as leaf burning, chlorosis and stunted growth under salt stress with a SES score of 7.0 or more. Among the population, 15% of plants showed very good performance under salt stress having SES score of below 5. Fifty percent of the genotypes had intermediate SES scores from 5.1-7.0. 17% of the progeny plants showed sensitivity towards salt and had a SES score of 7-9. So, according to the scoring system followed (IRRI, 1996), half of the progenies were moderately tolerant. SES score is given to salt stressed plants where a scale of 1-9 corresponding from highly tolerant to extremely sensitive was used.

From plots obtained by R, the frequency distribution of the traits among the population was observed (Figure 4.9). The frequency of SES score was evenly distributed among the population and the mean value (6.0) was much lower than both parents (*Horkuch*: 7.08 and *IR29*: 8.81), indicating that the progeny plants performed better than the parents under salt stress. It may be an example of transgressive segregation. Transgressive segregation is the formation of extreme phenotypes, or transgressive phenotypes, observed in segregated hybrid populations compared to phenotypes observed in the parental lines (Nolte and Sheets 2005). The appearance of these trangressive (extreme) phenotypes can be either positive or negative in terms of fitness. Transgressive segregation may be the result of combination of favorable alleles at different loci were from both parents (Ali, Xu et al. 2017). Pyramiding the favorable alleles of different loci might be responsible for the transgressive segregation in the progeny.

Root relative water content was lower in tolerant *Horkuch* parent (79.2%) than in sensitive *IR29* (91.13%) under salt stress. The mean value of the population (86.47%) was very close to that of the tolerant parent *Horkuch*.

Chlorophyll content which is a measure of high photosynthetic efficiency (Liu, Duan et al. 2009), was found to be higher in *Horkuch* (6.40 per 100mg) than in *IR29* (3.0 per 100mg) under salt stress condition. The mean value (4.60 per 100 mg) of the population was in between the chlorophyll content of two parents.

Stomatal conductance was found to be higher in *Horkuch* (366.26) than in *IR29* (307) under salt stress condition. The frequency was evenly distributed and the mean value (425.4) of the population was much higher than both parents.

Root and shoot lengths were found to be longer in *Horkuch* (11.44 cm and 41.7 cm, respectively) than in *IR29* (7.68 cm and 21. 74 cm, respectively) under salt stress condition. In both cases, the mean values of the population (11 cm and 38.96 cm, respectively) were close to that of the tolerant parent *Horkuch.*

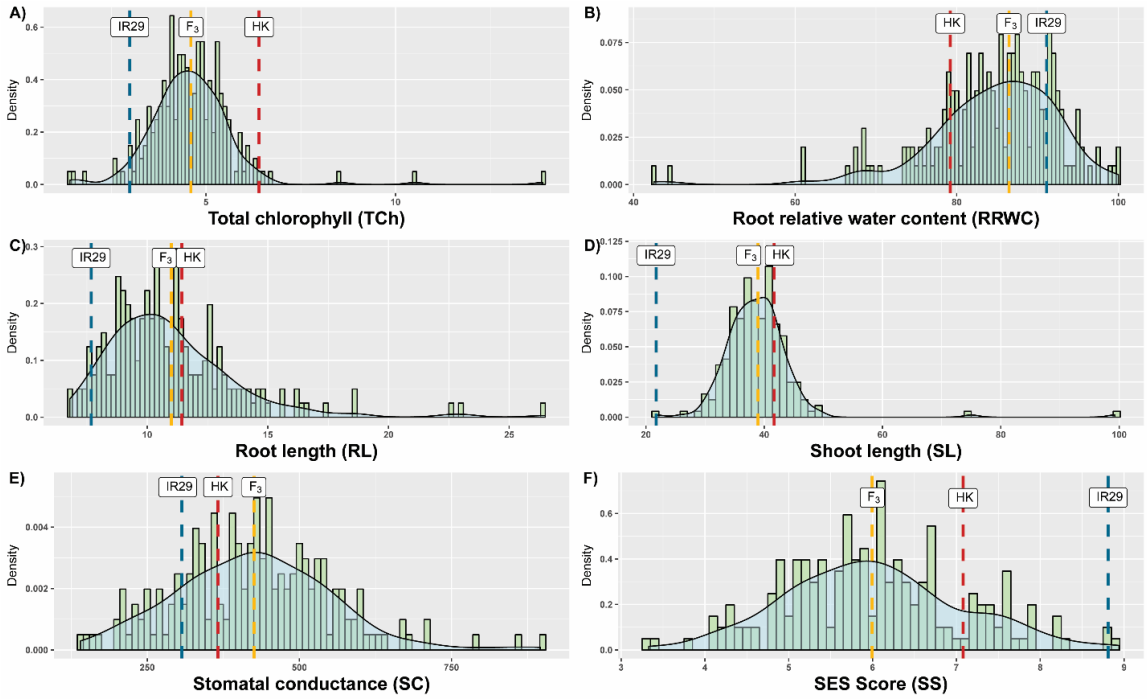


Figure 4.9: Frequency distribution of the seedling stage traits of F_3 generation.

4.3.3.2 Reproductive stage phenotyping

Effects of salt stress on F_3 progeny plants at reproductive stage were observed. Yield parameters like filled grain number, filled grain weight, total grain weight, number of total and effective tillers, spikelet fertility, primary and secondary branching, panicle damage under stress etc. were studied. Physiological responses

of plants in reproductive stage and correlations among different traits under salt stress were studied (appendix B.2).

Table 4.3: Ranges and mean values of reproductive traits measured in F_3 population and values of both parents. DF: Days to flowering, PH: Plant height, ET: Effective tiller, PB: Primary branching, SB: Secondary branching, SeL: Seed length, SeB: Seed breadth, PL: Panicle length, PF: Percent fertility, ThGW: Thousand grain weight.

Trait Name	Range in F_3 population	Mean value of the population	*Horkuch*	*IR29*
DF (days)	53-102.5	68.5	92.25	72.625
PH** (cm)	39.75-114.5	82.53	117.2	56.31
ET	2.5-14.75	9.0	9.125	7.66
PB	4.7-9.43	7.1	6.16	6.04
SB	2.92-12.9	7.30	5.25	4.85
SeL (mm)	5.91-9.18	7.87	7.25	8.35
SeB (mm)	1.76-2.45	2.14	2.356	1.94
PL* (cm)	15.25-28.42	21.2	24.15	16.4
PF (%)	2.9-75.87	42.8	49.45	48.4
ThGW (gm)	15-35.23	21.24	23.27	15.41

*The asterisks denotes significant correlation with the trait to cross direction (*** for P < 0.001, ** for P <0.01 and * for P <0.05)*

As expected, the traits were segregated in the F_3 population. For every parameter studied, some plants resembled *Horkuch*, while the others resembled *IR29* (figure 4.10 A-D). Some plants were tall, compact tillering pattern with more culm strength, had late flowering, low yielding with short and bold grains and more salt tolerant like *Horkuch* while some were short, spread type tillering with less culm strength, had early flowering, were high yielding with long and slender grains and salt sensitive like *IR29*. However, most plants had a combination of phenotypic traits from both parents.

A)

Horkuch IR29 F3 (I-83)

I_083: Resembling sensitive parent IR29, compact type

B)

Horkuch IR29 F3 (I-43)

I_043: Taller than both parents, spread type

A)

Horkuch IR29 F3 (I-50)

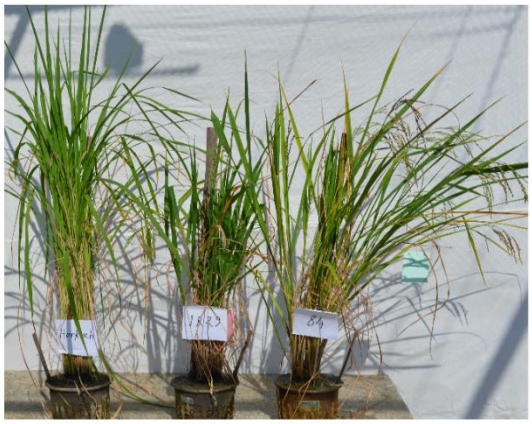
I_050: Tolerant, compact type, early flowering

B)

Horkuch IR29 F3 (I-80)

I_080: More sensitive than both parents

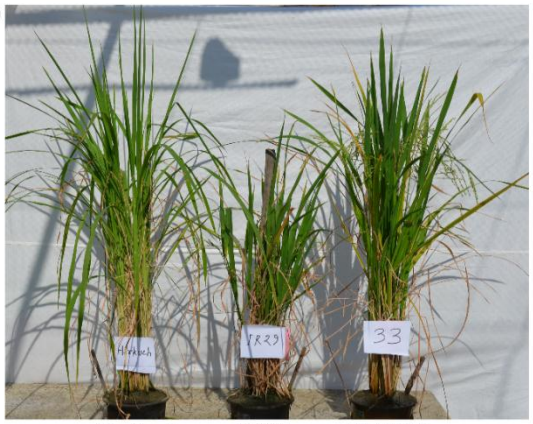
A)



Horkuch IR29 F3 (I-84)

I_084: Tolerant, early flowering, high yielding, spread type

B)



Horkuch IR29 F3 (I-33)

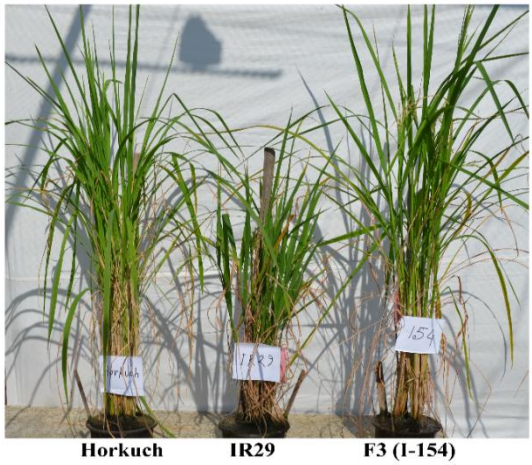
I_033: Tolerant, compact type, early flowering

A)

Horkuch IR29 F3 (I-167)

I_167: Early flowering, high yielding, taller than both parents, spread type

B)



Horkuch IR29 F3 (I-154)

I_154: Tolerant, late flowering, compact type

Figure 4.10 (A): Variations observed in F_3 progeny plants in traits such as days to flowering (early and late), level of salt tolerance, plant height, tillering pattern (compact and spread type), yield.

Figure 4.10 (B): Spikelet damage

Figure 4.10 (C): Variation in grain types and colour

Figure 4.10 (D): Variation in panicle branching and length

From plots obtained by R, the frequency distribution of the traits among the population was observed (figure 4.11). Panicle length was longer in the tolerant *Horkuch* parent (24.15 cm) than in sensitive *IR29* (16.40 cm) under salt stress. The frequency of panicle length was evenly distributed among the population and the mean value of the population (21.18 cm) was in between the panicle lengths of the parents.

Thousand grain weight was higher in tolerant *Horkuch* parent (23.27 gm) than in sensitive *IR29* (15.4 gm) under salt stress. The mean value of the population (21.24 gm) was close to that of the tolerant parent *Horkuch*. Also, the weight of filled grain was higher in tolerant *Horkuch* parent (4.65 gm) than in sensitive *IR29* (3.31 cm) under salt stress. The mean value of the population (5.80) was much higher than both parents, indicating that the progeny plants performed better than the parents.

Percent fertility was higher in salt tolerant, low yielding parent *Horkuch* (49.45%) than in salt sensitive, high yielding parent *IR29* (48.39%) under salt stress. The mean value of the population (42.8%) for percent fertility was in between the value of two parents.

Days to flowering was higher in tolerant *Horkuch* parent (92.25) than in sensitive *IR29* (72.62) under salt stress. The mean value of the population (68.5) was lower than both parents, which is a desired trait, indicating that the progeny plants performed better than the parents.

Plant height was higher in tolerant *Horkuch* parent (117.2 cm) than in sensitive *IR29* (56.31 cm) under salt stress. The mean value of the population (82.53 cm) was close to that of the shorter parent *IR29*.

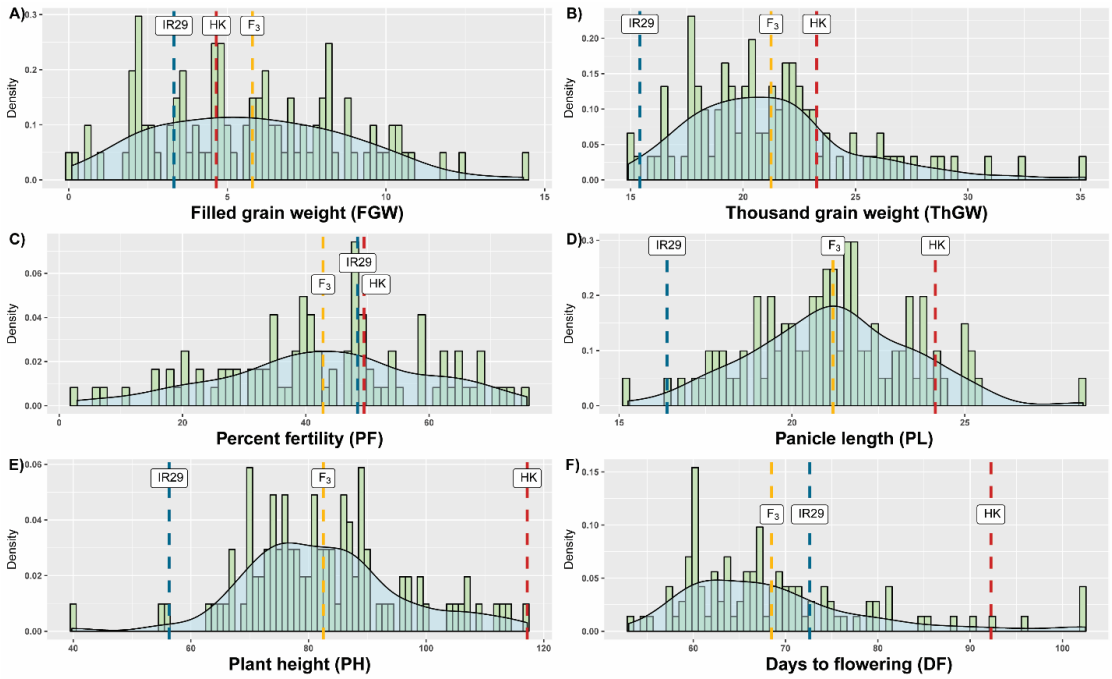


Figure 4.11: Frequency distribution of filled grain weight, thousand grain weight, percent fertility, panicle length, plant height and days to flowering in F_3 population.

4.3.4 Correlation between physiological traits

QTLs for traits correlated were often mapped in the same chromosomal regions (Abler, Edwards et al. 1991; Paterson, Damon et al. 1991; Veldboom, Lee et al. 1994). This trend was observed in this study and will be discussed in detail in chapter 5. The correlation between traits was evaluated by regressing phenotypic values of one trait on those of another trait. The correlation among traits are presented in figure 4.12 (A-C). For most of the traits, the direction (+/-) and degree

of correlation was consistent with previous observations (Xiao and Yuan 1988). These results supported the fact that the trait correlation may be attributed to the effect of pleiotropy or to the very close linkage of genes, as many of the QTLs located in close proximity are well correlated (discussed in section 5.2). Combination of best correlated traits in seedling and reproductive stage may yield superior plants.

4.3.4.1 Seedling vs seedling

The correlations between character pairs were computed at P <0.05 in R using trait averages. SES score showed significant negative correlation with shoot dry weight, root length, shoot length, total chlorophyll and stomatal conductance indicating that plants having higher SES score or sensitive plants have less shoot weight, shoot and root length and reduced stomatal conductance. No significant association was found between SES score and shoot and root relative water content.

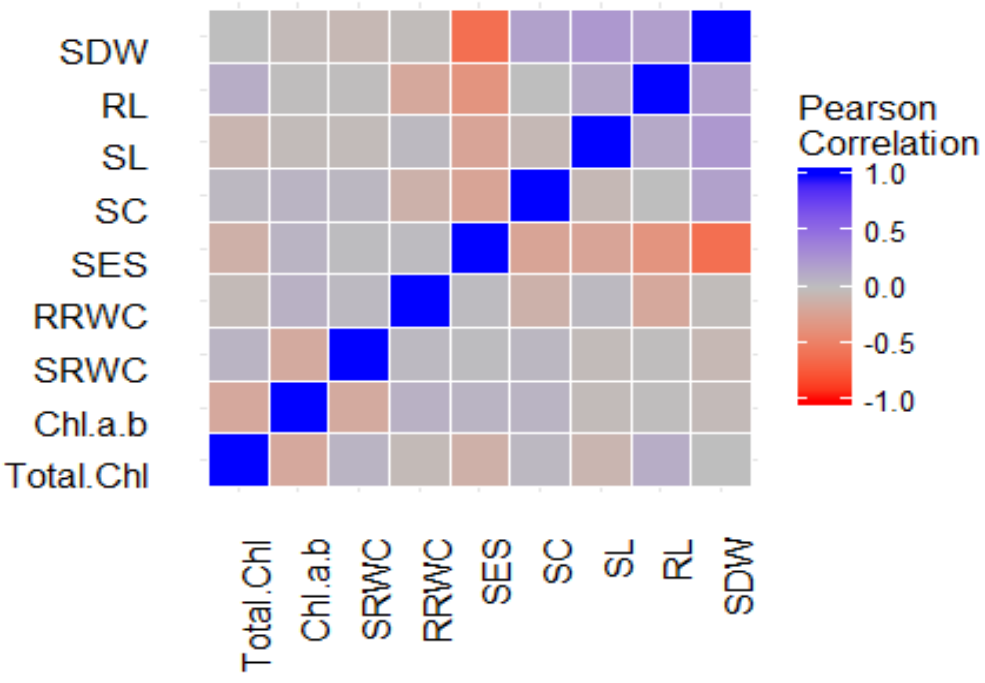


Figure 4.12 (A): Correlation between traits at seedling stage.

4.3.4.2 Reproductive vs reproductive

The correlations between character pairs were computed at P <0.05 in R using trait averages. Significant negative correlation was observed between days to flowering and yield, weight of filled grain, number of total and effective tillers, primary and secondary branching. Days to flowering showed significant positive correlation with plant height, total tiller and thousand grain weight. Plant height showed significant positive correlation with thousand grain weight and yield. Significant positive correlation was observed between flag leaf weight and yield, thousand grain weight, weight of filled grain, primary and secondary branching.

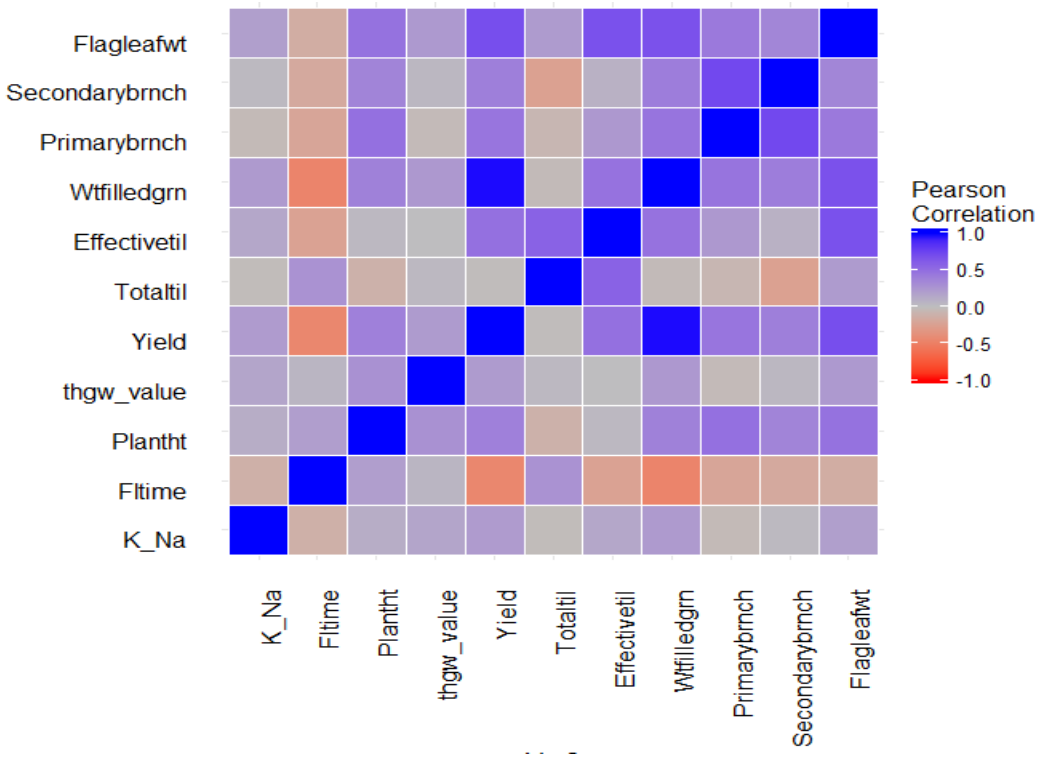


Figure 4.12 (B): Correlation between traits at reproductive stage.

4.3.4.3 Seedling vs reproductive

The correlations between character pairs were computed at P <0.05 in R using trait averages. Significant positive and negative correlations were observed between seedling and reproductive traits. Seedling shoot K^+/Na^+ ratio showed significant positive correlation with thousand grain weight, yield, weight of filled

grain, total grain weight and effective tiller, primary and secondary branching . Also, total chlorophyll and root length showed positive correlation with weight of filled grain, primary and secondary branching.

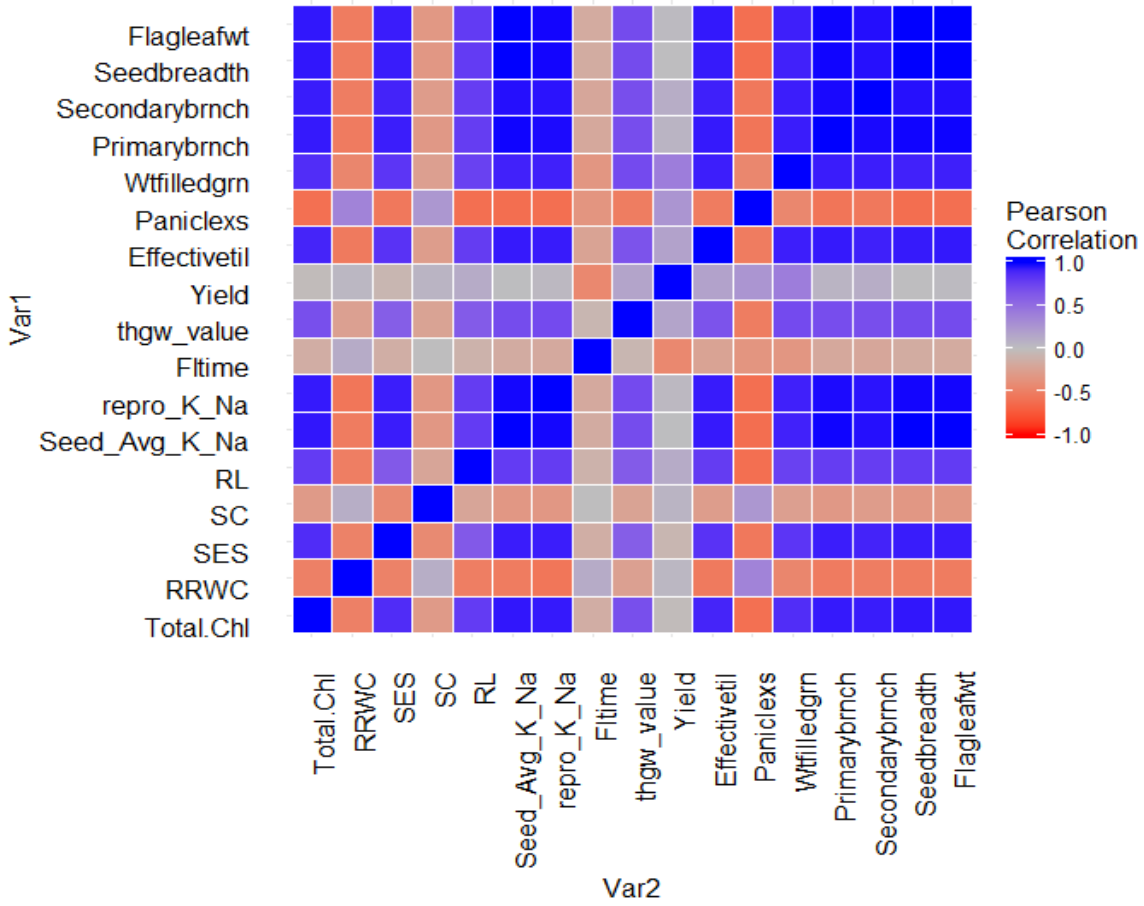


Figure 4.12 (C): Correlation between traits at seedling and reproductive stage.

4.3.5 QTL mapping of salt tolerance-related traits at the rice seedling stage

Fifty nine QTLs controlling fifteen traits were mapped on the QTL map**.** Eleven QTLs that contributed less than 1% to the total phenotypic variance were excluded. The output of QTLs by the software QTL Cartographer is given below (figure 4.13).

Flowering Time

Chromosc	.Marker	Position	LOD Score	.Additive.	.Dominan	R2	Donor of positive allele
1	21	157.73	2.4428	0.5616	-5.5297	0.0711	A
1	24	166.38	2.3395	0.9662	-5.1986	0.0723	A
1	30	196.54	7.1348	-1.7709	-9.0605	0.0553	B
2	27	200.83	3.832	-5.3628	-3.1737	0.0592	B
2	28	218.42	4.3768	-6.5918	-6.7676	0.0383	B
3	11	96.99	2.0577	5.0555	4.1424	0.0072	A
3	25	210.01	4.2888	-6.1813	-3.1385	0.0755	B
3	27	227.03	3.1933	-4.7533	-1.9787	0.0759	B
4	29	233.53	2.0274	2.5159	-4.6097	0.1032	A
6	5	73.43	5.6337	5.8954	-6.517	0.3076	A
9	15	98.11	2.633	0.3272	-7.4326	0.1012	A
9	16	105.98	2.432	-0.135	-6.8271	0.0893	B
9	17	120.51	3.8105	2.2344	-6.9485	0.1407	A
9	18	127.52	3.897	3.2663	-5.9032	0.1552	A
11	2	26.42	2.0955	2.7405	-5.2005	0.1145	A
11	10	78.77	2.8704	-4.8449	-1.8874	0.055	B
11	14	86.48	2.8924	-4.5612	-1.5306	0.0635	B

Figure 4.13: Sample output by QTL cartographer for flowering time QTLs. Here, A=*IR29* allele, B=*Horkuch* allele.

Here, the higher the value of the additive effect, the more dominant the effect of the gene (or genes) underlying the QTL. The position of the QTLs were given in cM. The physical location of the QTLs (in basepair) were calculated from the positions of the adjacent SNP markers. The direction of additive effect determines the donor of the allele (*Horkuch* or *IR29*). Positive additive effect indicates A allele and negative additive effect indicates B allele. Here, A=*IR29* allele, B=*Horkuch* allele.

A robust QTL associated with total chlorophyll was detected at position 157.47 cM on chromosome 7, with a LOD score of 5.44. It explained 14.25% of the total phenotypic variance. The donor of the allele was *Horkuch*, the salt tolerant parent. Six QTLs were mapped for K^+/Na^+ ratio on chromosomes 1, 3, 6, 7, 8 and 9, three of which were real and three were putative QTLs. QTLs with LOD scores between 2.5 and 3.5 were considered as putative QTLs and QTLs with LOD score > 3.5 were considered as real QTLs (section 3.1.6). Only the *Horkuch* alleles (two of them), that explained 5.23-9.32% of the phenotypic variation were taken into consideration. Six QTLs were also detected for root length, of which three were major and three were putative QTLs.

Three QTLs, for which the donor alleles were *Horkuch*, were considered for further analysis. One of which showed large effect on root length at 173.75 cM on chromosome 2, with a LOD score of 8.35 and explained 16.5% of the total phenotypic variance. Two QTLs were identified for stomatal conductance on chromosome 4 and 5, showing a large effect on SC explaining 13.1% and 20.5% of the total phenotypic variance, respectively. The donor of both the alleles were *IR29*. Three QTLs were mapped for SES (reverse) on chromosomes 1 and 7. Among these, the QTLs located on chromosome 7 had higher R^2 than the other QTLs and explained 9.56% of the total phenotypic variation. A summary of the statistics of all significant QTLs (LOD >2.5) is presented in appendix B. 6. Map of identified true QTLs (LOD>3.5) of seedling stage in all chromosomes have been depicted in figure 4.14 A and B.

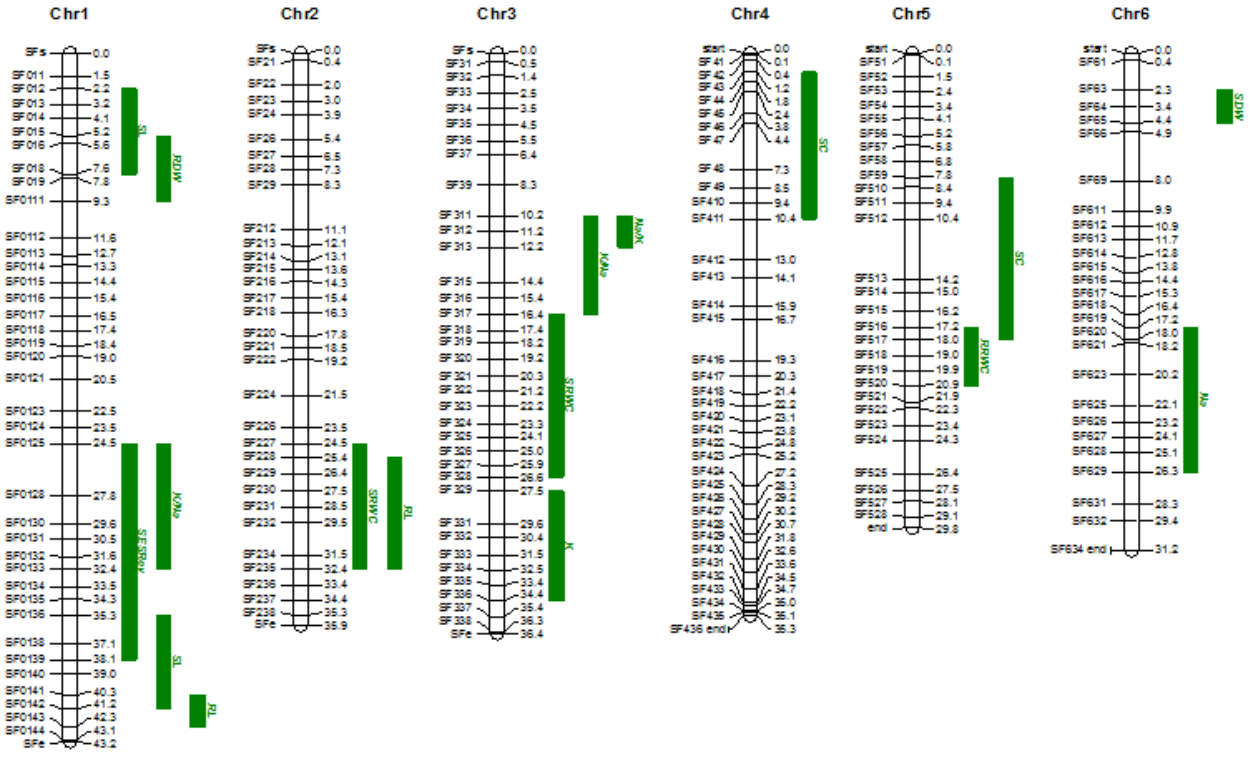


Fig 4.14 (A) QTL map of identified QTLs of seedling stage in chromosomes 1-6 (position given in megabase pair).

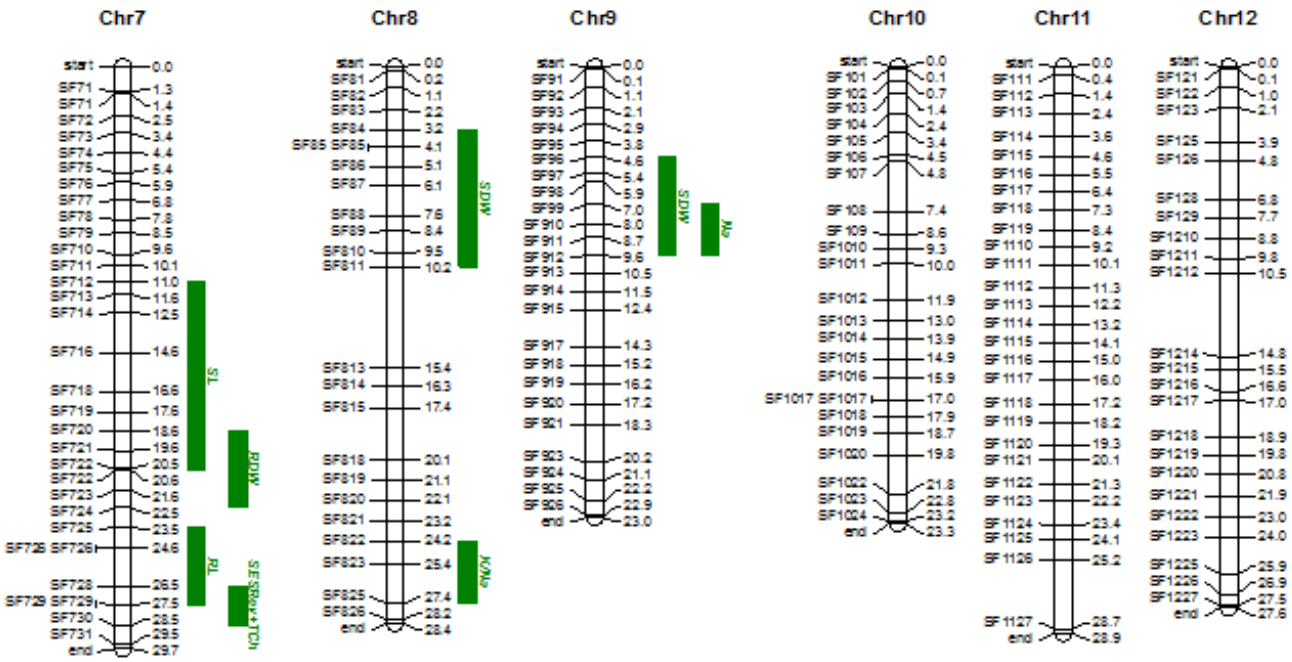


Fig 4.14 (B) QTL map of identified QTLs of seedling stage in chromosomes 7-12 (position given in megabase pair).

QTL likelihood curves of the LOD score for seedling traits are shown in figures 4.15 (A-E).

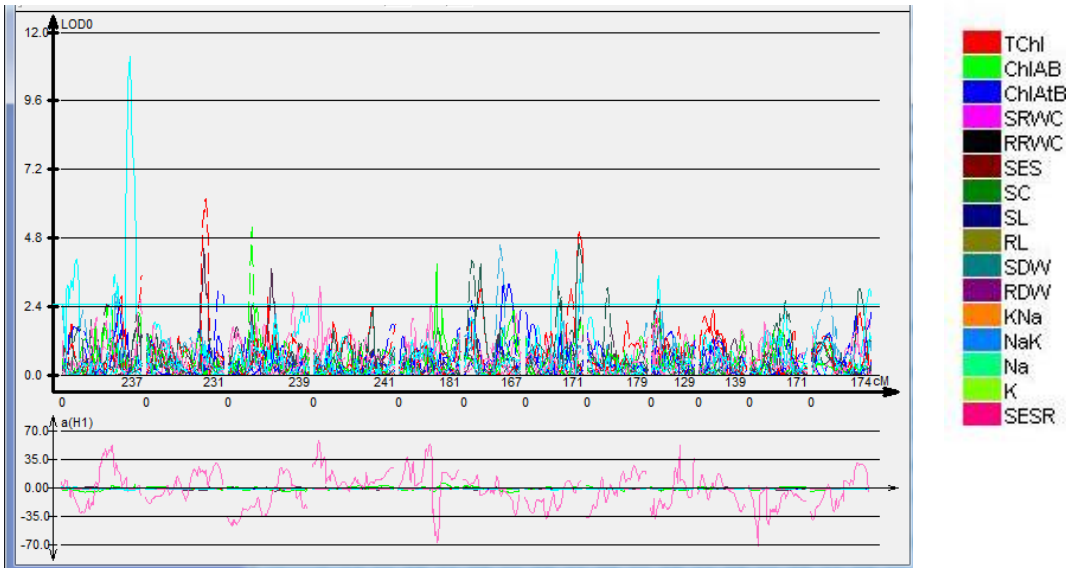


Figure 4.15 (A): Seedling QTLs drawn by WinQTL cartographer.

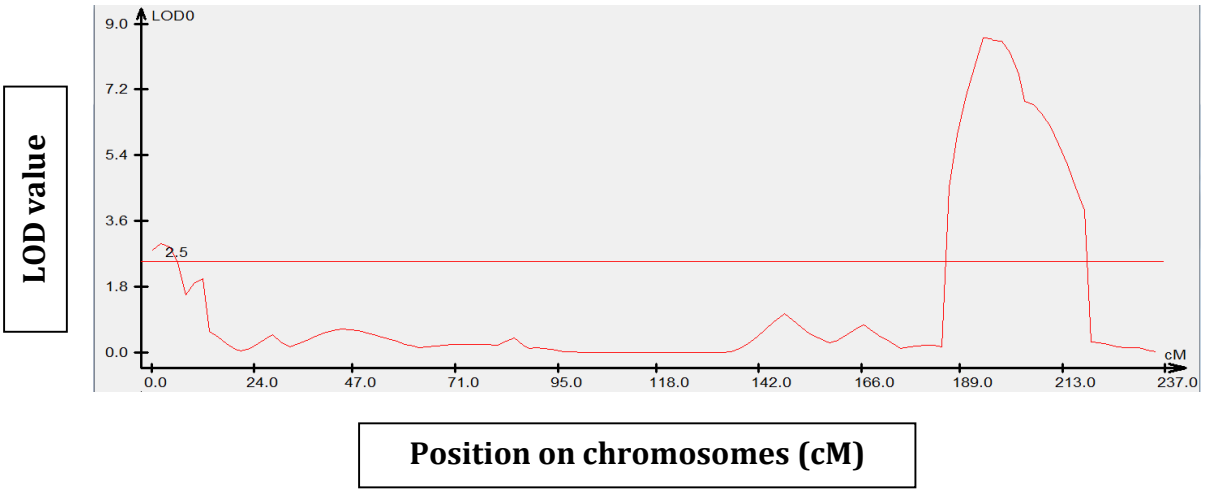


Figure 4.15 (B): Shoot length QTL (Chromosome 1)

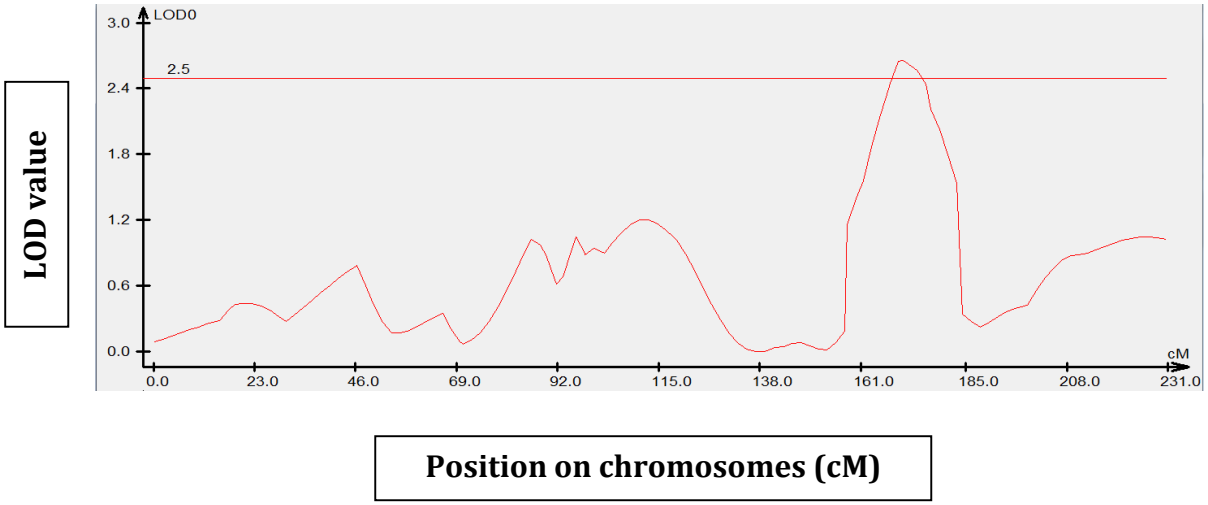


Figure 4.15 (C): Root length QTL (Chromosome 2)

4.3.6 QTL mapping of salt tolerance and yield under salt stress at the rice reproductive stage

A hundred and ninety nine QTLs controlling thirty nine traits were mapped on the QTL map**.** Thirty two QTLs explaining less than 1% to the total phenotypic variance were excluded. A couple of putative QTLs affecting thousand grain weight were detected on chromosomes 3 and 7. The QTL on chromosome 3, a *Horkuch* allele, explained 13.88% of the total phenotypic variation and was considered for further study. A QTL associated with K^+/Na^+ ratio was mapped on chromosome 4, for which the donor was *Horkuch*. A robust QTL associated with primary branching was detected at position 114.36 cM on chromosome 1, with a LOD score of 7.08. It explained 37.2% of the total phenotypic variance. Branch number in a panicle is directly associated with rice productivity (Miura, Ikeda et al. 2010). Three QTLs associated with panicle length were identified on chromosomes 1 and 3, explaining 12.96-27.68% of the total phenotypic variance. Two QTLs for yield were mapped on chromosomes 1 and 10. The one on chromosome 1 had a LOD score of 3.78 and explained 11.83% of the total phenotypic variance. Map of identified true QTLs (LOD>3.5) of seedling stage in all chromosomes have been depicted in figures 4.16 A, B and C.

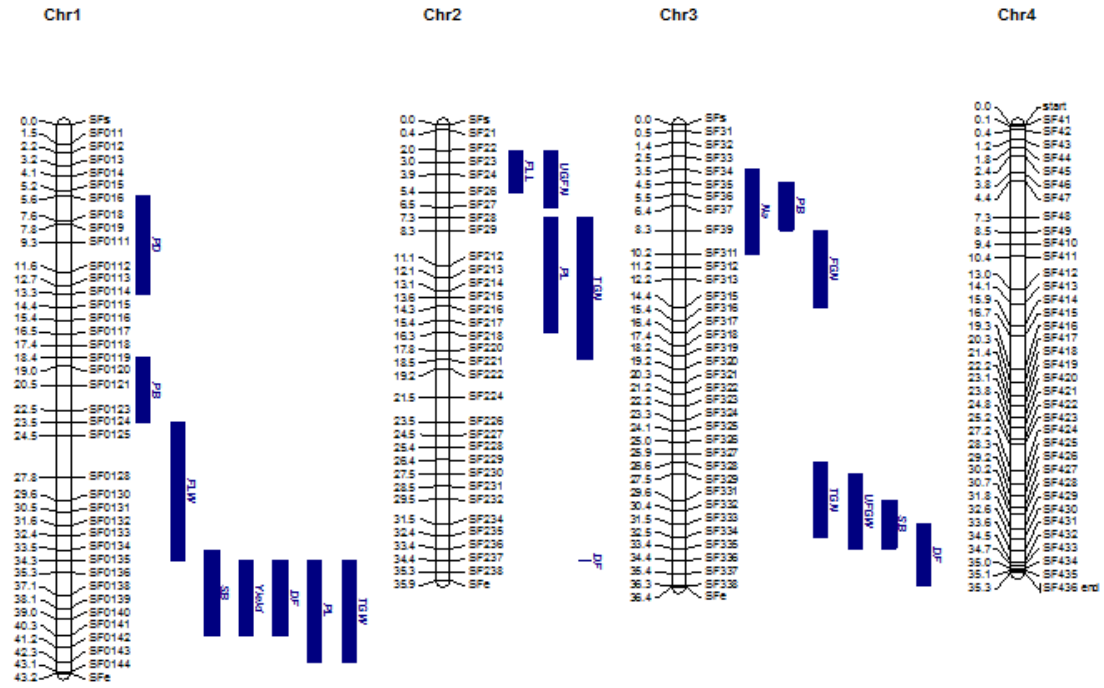


Fig 4.16 (A) QTL map of identified QTLs of reproductive stage in chromosomes 1-4 (position given in megabase pair).

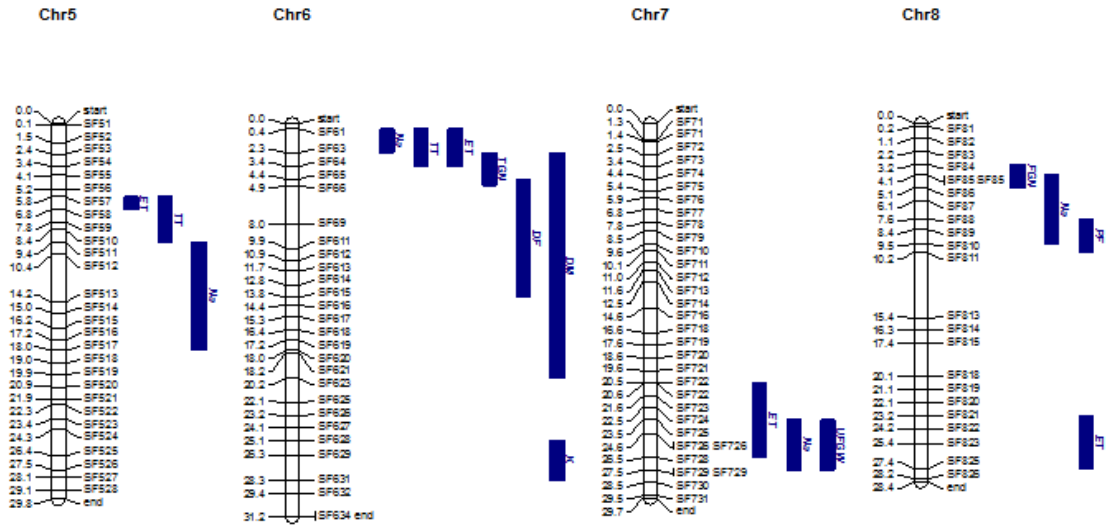


Fig 4.16 (B) QTL map of identified QTLs of reproductive stage in chromosomes 5-8 (position given in megabase pair).

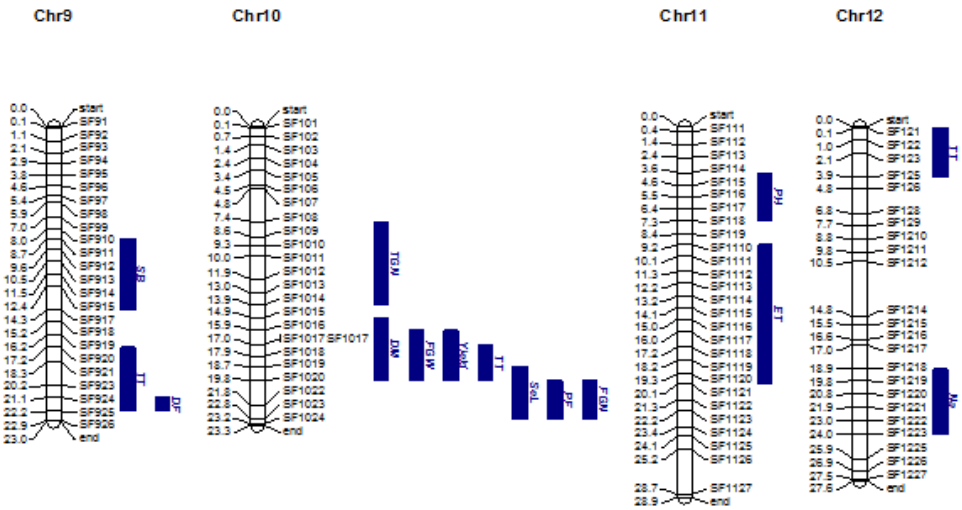


Fig 4.16 (C) QTL map of identified QTLs of reproductive stage in chromosomes 9-12 (position given in megabase pair).

A summary of the statistics of all significant QTLs (LOD >2.5) is presented in **appendix B.7**.

QTL likelihood curves of the LOD score for reproductive traits are shown in figures 4.17 (A-E).

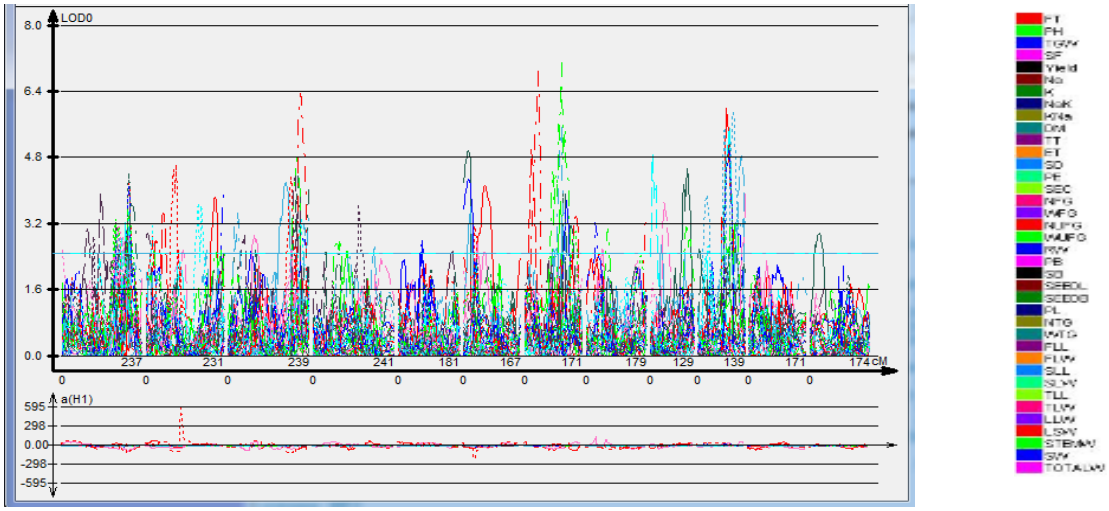


Figure 4.17 (A): Reproductive QTLs drawn by WinQTL cartographer.

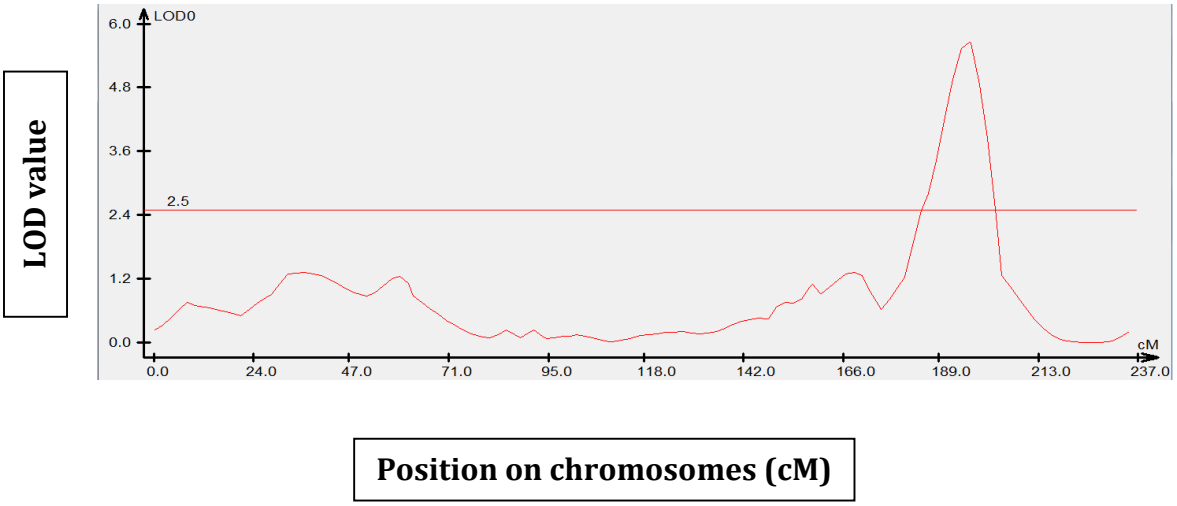


Figure 4.17 (B): Plant height QTL (Chromosome 1)

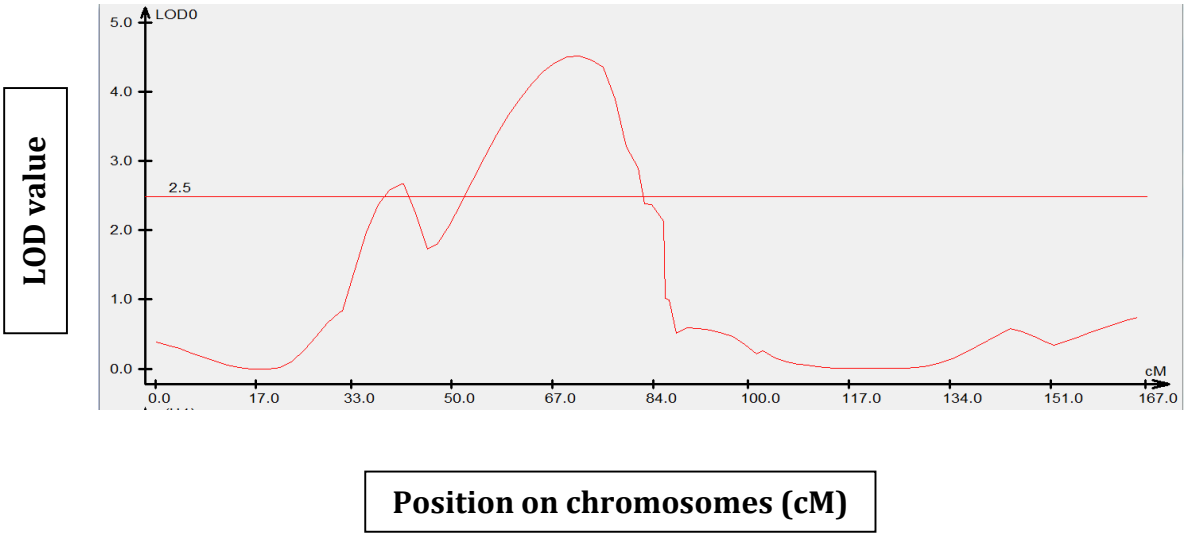


Figure 4.17 (C): Days to flowering QTL (Chromosome 6)

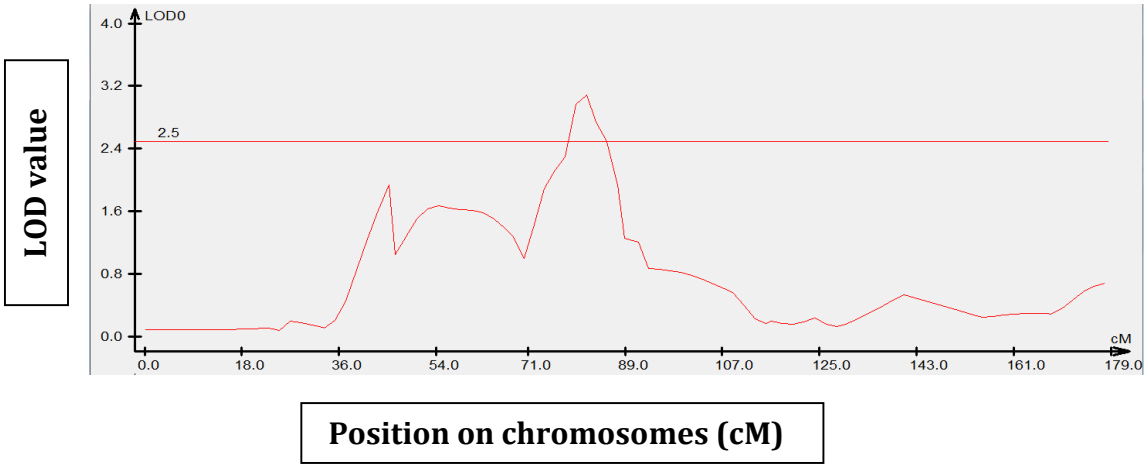


Figure 4.17 (D): Percent fertility QTL (Chromosome 8)

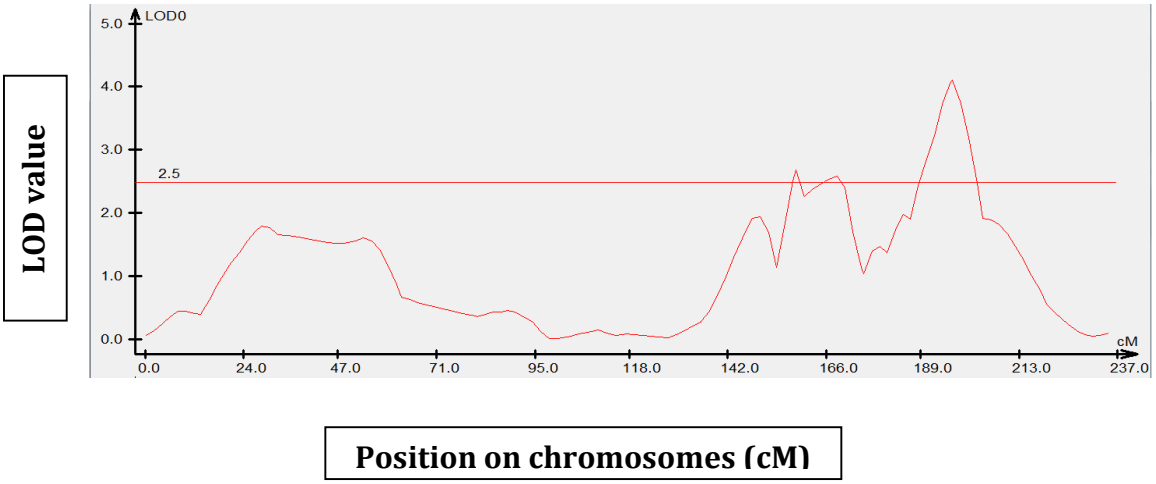


Figure 4.17 (E): Yield QTL (Chromosome 1)

Chapter 4B:
QTL Validation

4.4 QTL Validation

4.4.1 Finding QTLs with significant effects

One way ANOVA of the major QTLs were performed to identify the QTLs that have significant effect on the corresponding phenotypes. The QTLs with a P value of less than 0.05 had significant effect on the corresponding phenotype. A number of seedling and reproductive QTLs had been found to affect their phenotypes significantly. The output have been given in appendix C.1.

Besides these, The total chlorophyll QTL on chromosome 7 affected phenotypes such as SES and reproductive phenotypes such as thousand grain weight, seed breadth, total and effective tiller. It also affected weights like flag leaf, second leaf, third leaf, lower leaf, straw and total weights (figure 4.18 B).

SES value had an influence on a number of reproductive QTLs such as filled grain number, spikelet fertility, effective tiller, plant height and culm strength. Total chlorophyll significantly affected plant height and days to flowering QTL and root length was affected by third leaf length and days to flowering QTLs (appendix C.1).

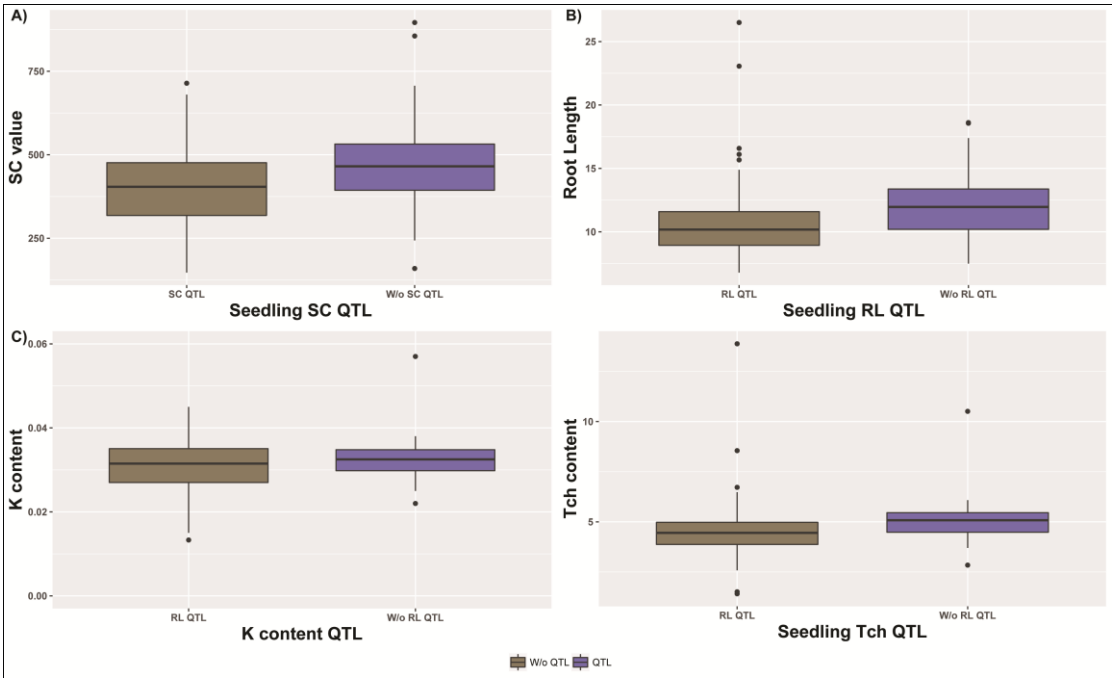


Figure 4.18 (A): Boxplots showing QTLs having significant effects on corresponding phenotypes. Abbreviations: SC: Stomatal Conductance, RL: Root Length, Tch: Total Chlorophyll. For each trait, the brown box indicates phenotype of plants having corresponding QTL and the blue box indicates phenotype of plants without corresponding QTL.

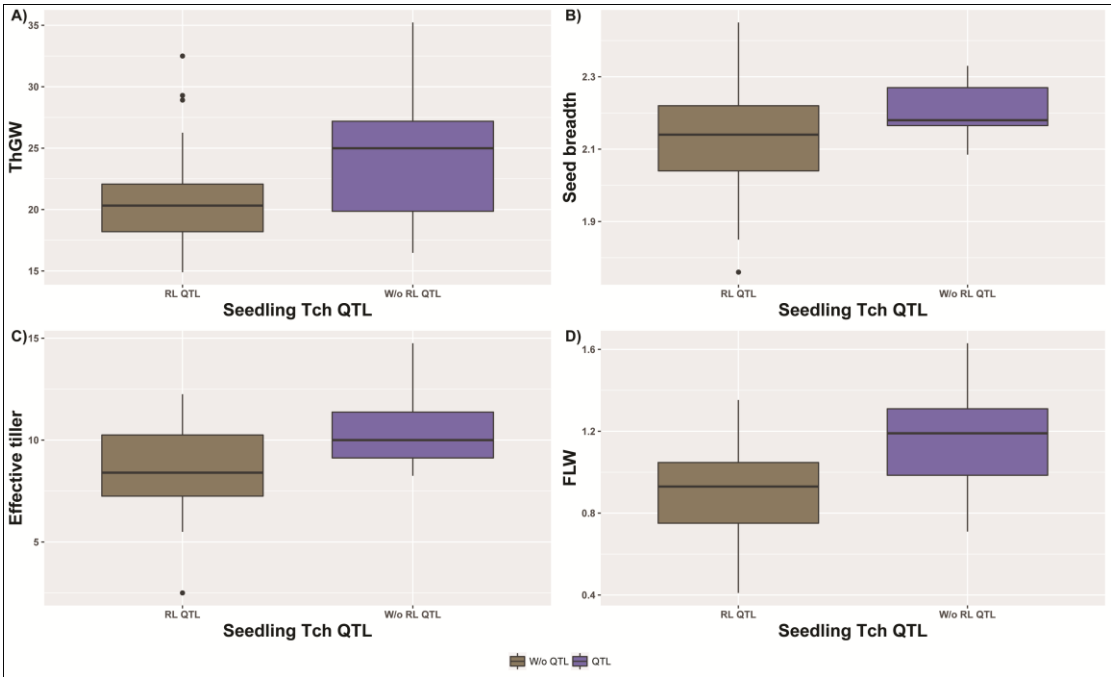


Figure 4.18 (B): Boxplots showing total chlorophyll QTL having significant effects on reproductive phenotypes. Abbreviations: Tch: Total Chlorophyll, FLW: Flag leaf weight, FLW. For each trait, the brown box indicates phenotype of plants having corresponding QTL and the blue box indicates phenotype of plants without corresponding QTL.

4.4.2 Physiological analysis for the confirmation of the presence of desired QTLs in F_5 progenies

The outcome of the study aimed at validation of QTLs by both molecular and physiological analysis. As the effect of salinity on rice growth varies across physiological stages (Lutts, Kinet et al. 1995), physiological analyses were done both at seedling and reproductive stages when they are most sensitive to salt stress.

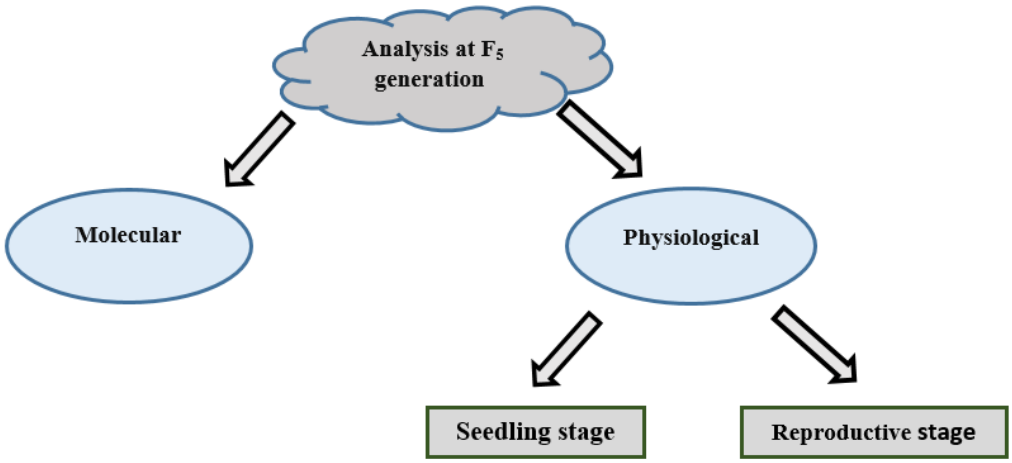


Figure 4.19: An overview of the analysis for the validation of desired QTLs

Results of phenotypic evaluation at seedling and reproductive stage will be stated in section 4.4.2.1 and 4.4.2.2. Section 4.4.3 will deal about molecular analysis thus confirmation of QTLs by SSR markers. Finally, plants with confirmed presence of multiple desired QTLs will be chosen that can be used as novel donor for breeding salt tolerant cultivars.

4.4.2.1 QTL validation at seedling stage

4.4.2.1.1 Selection of plants for seedling stage phenotypic analysis at F_5 generation

As described previously (section 3.2.1.3), plants were selected based on combination of QTLs from both seedling and reproductive stages that could confer salt tolerance and yield simultaneously. The images of individual chromosomes carrying the QTLs were created using the software graphical genotype 2 (GGT2) and from those images, plants having a combination of good QTLs were primarily selected. The plants were finally selected by matching the initially selected plants with the allele type of genotype data obtained from DArTseq™ analysis. Twenty seven plants were selected for analysis at seedling stage and the best 10 plants out of these 27 were screened at reproductive stage under salt stress condition

(appendix C.2 and C.3). Individual plants with QTLs and their adjacent marker information are given in appendix B. 5.

In figure 4.20 (A), F_3 progeny I_80 containing multiple seedling is shown. Four chromosomes (chr 2, 3, 7 & 8) of plant I_80 carry 7 seedling QTLs. The QTLs are root length (RL) on chromosome 2; K^+ content and K^+/Na^+ ratio on chromosome 3; SES score, root dry weight (RDW) and total chlorophyll on chromosome 7 and shoot dry weight (SDW) on chromosome 8. Similarly, in figure 4.20 (B), F_3 progeny I_170 containing multiple reproductive QTLs (image by GGT2) is shown. Four chromosomes (chr 1, 2, 4 & 8) of plant I_170 carry 7 reproductive QTLs. The QTLs are panicle length, filled grain weight and seed breadth on chromosome 1; days to flowering on chromosome 2; panicle exsertion and seed length on chromosome 4 and percent fertility on chromosome 8.

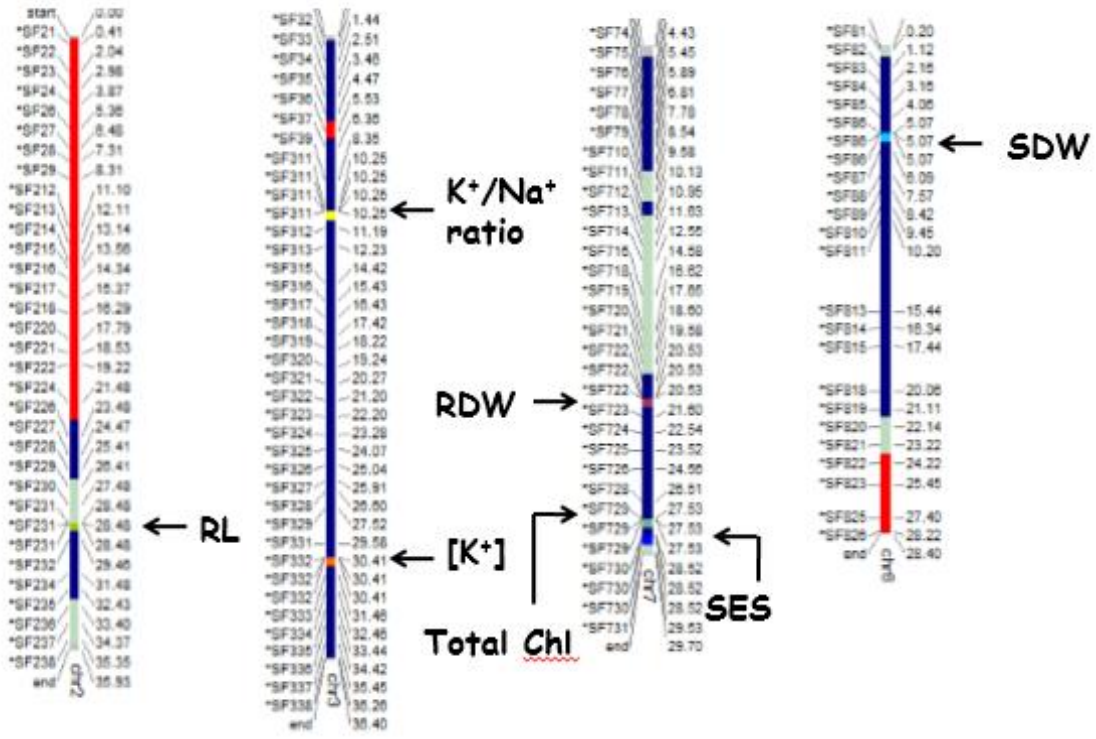


Figure 4.20 (A): F_3 progeny I_80 containing multiple seedling QTLs (image by GGT2).

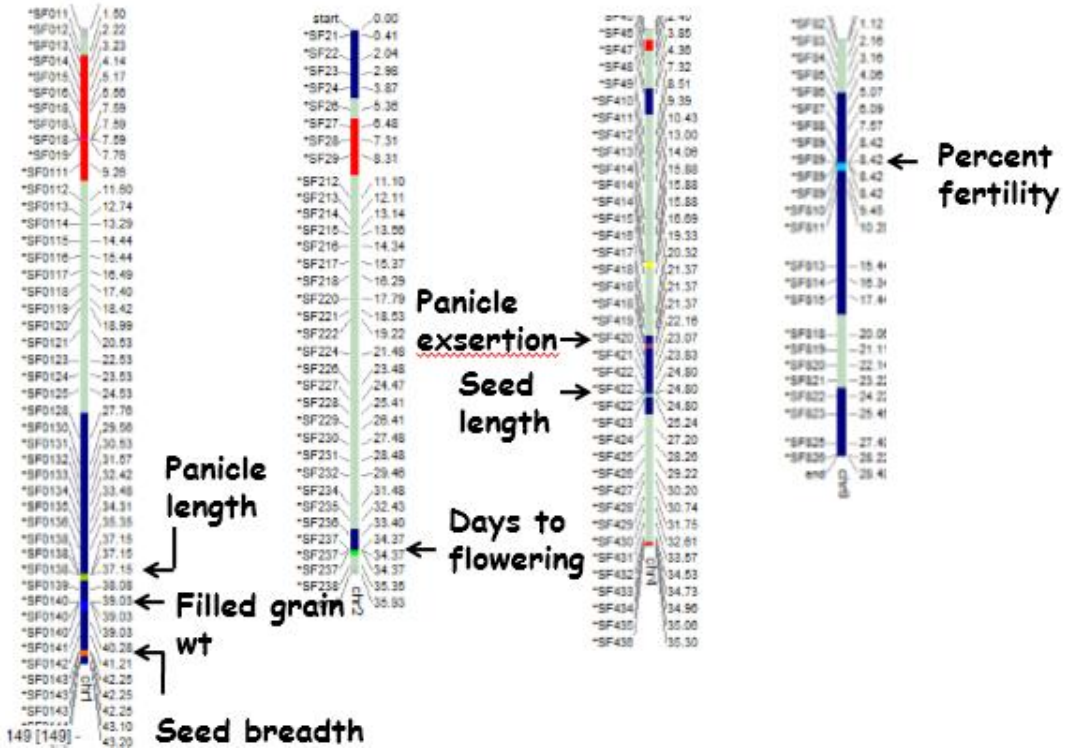


Figure 4.20 (B): F_3 progeny I_170 containing multiple reproductive QTLs (image by GGT2).

4.4.2.1.2 Phenotypic evaluation of F_5 population at seedling stage

Selected plants with QTLs for salt tolerance in seedling stage like total chlorophyll, SES, K^+/Na^+ ratio, stomatal conductance, shoot dry weight, root dry weight were studied and their effects on phenotype were observed (figure 4.21).

In seedling stage, plants exposed to 12 dSm^{-1} salt stress were scored on the extent of damage caused by salt (SES score) when the sensitive check *IR29* in each floater was almost dead. The tolerant parent (*Horkuch*) and the sensitive parent (*IR29*) differed significantly in their visual score having an average score of 5.66 and 8.0, respectively.

Some Plants were severely affected by salinity and clearly exhibited symptoms of salt injury, such as leaf burning, chlorosis and stunted growth under salt stress with a SES score of 7.0 or more. 31% of the screened seedling were tolerant (score <5), ~46% were moderately tolerant (score 5-5.9) and ~23% were sensitive (score >6). So, according to the scoring system followed most of the progenies were moderately tolerant (Seedling stage data provided in appendix B.3).

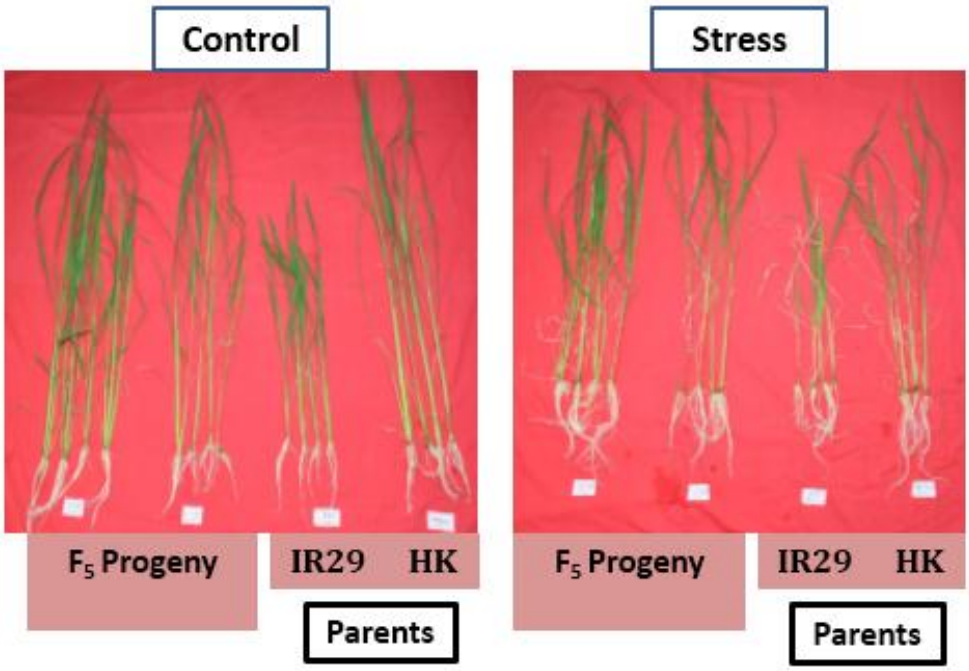


Figure 4.21: *Horkuch* (*HK*), *IR29* parents and F_5 progenies both at control and stress conditions.

From plots obtained by R, the frequency distribution of the traits among the population was observed (figure 4.22). The frequency of SES score was evenly distributed among the population and the mean value (5.24) was lower than both parents (*Horkuch*: 5.66 and *IR29*: 8), indicating that the progeny plants performed better than the parents under salt stress.

Chlorophyll content was found to be higher in *Horkuch* (3.74 mg/gm) than in *IR29* (2.44 mg/gm) under salt stress condition. There was a fall of 56% and 31% in chlorophyll content in *IR29* and *Horkuch* respectively, under salt stress. The mean value of the population was higher than both parents (4 mg/gm).

Root and shoot lengths were found to be longer in *Horkuch* (14.83 cm & 37.33 cm, respectively) than in *IR29* (12.735 cm and 29 cm, respectively) under salt stress condition. In both cases, the mean values of the population were higher than both parents, 15.3 cm for root length and 46.4 cm for shoot length. The root length of *IR29* was reduced by 19.5% under stressed condition, while that of *Horkuch* remained unchanged. The shoot length reduced by 47% and 31% in *IR29* and *Horkuch*, respectively.

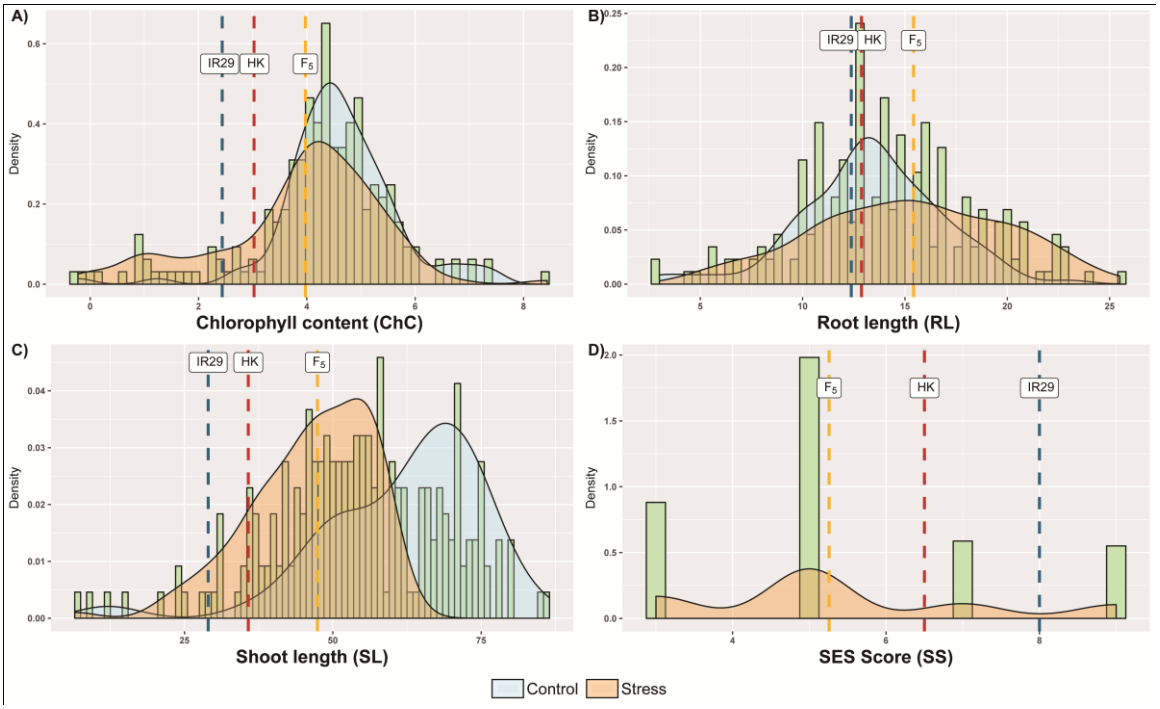


Figure 4.22: Distribution curve of different parameters measured in seedling stage: Mean value distributions of SES score, root length (RL), shoot length (SL) and chlorophyll content are shown here.

4.4.2.2 Phenotypic evaluation of F_5 population at reproductive stage

Plants with QTLs that are related to yield parameters like filled grain number, filled grain weight, total grain weight under stress etc. were studied and their effects on phenotypes were observed. Physiological responses of plants in reproductive stage under salt stress were studied. (Reproductive stage data provided in appendix B. 4).

10 best plants with good combination of seedling and reproductive QTLs were screened. In figure 4.23 (A & B), comparative pictures of whole plant and panicle damage of four F_5 plants with their parents, three with good yield related traits are shown. *Horkuch* and *IR29* had panicle damage of 10% and 30%, respectively, whereas the good plants I_074, I_170 and I_033.

Plant I_033 and I_143 which had 10 QTLs, including some yield related QTLs like filled grain number, spikelet fertility, seed length etc. showed increased percent fertility, 3.42% and 37.66% in I_033 and I_143, respectively, under salt stress condition (figure 4.24). I_170 showed a small decrease in percent fertility (2.2%). The salt tolerant, low yielding parent *Horkuch* also showed 7.8% increase in percent fertility under salt stress. As observed from the figure 4.24, I_143 and I_170 showed less reduction in yield and filled grain weight than the remaining 8 plants in reproductive stage. Plant I_033 and I_143 also showed less reduction in panicle length than other F_5 plants and both parents.

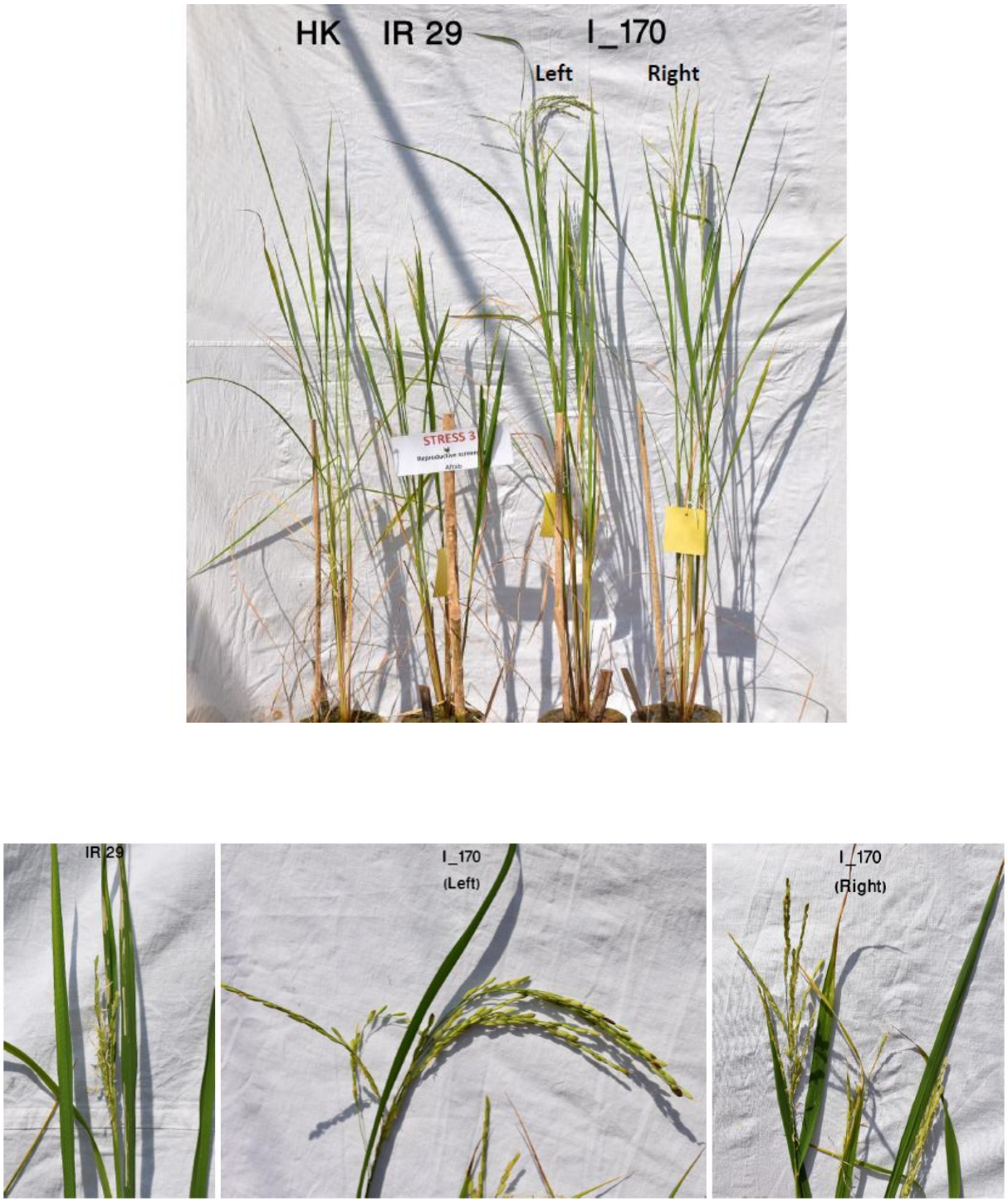


Figure 4.23 (A): Panicle damage

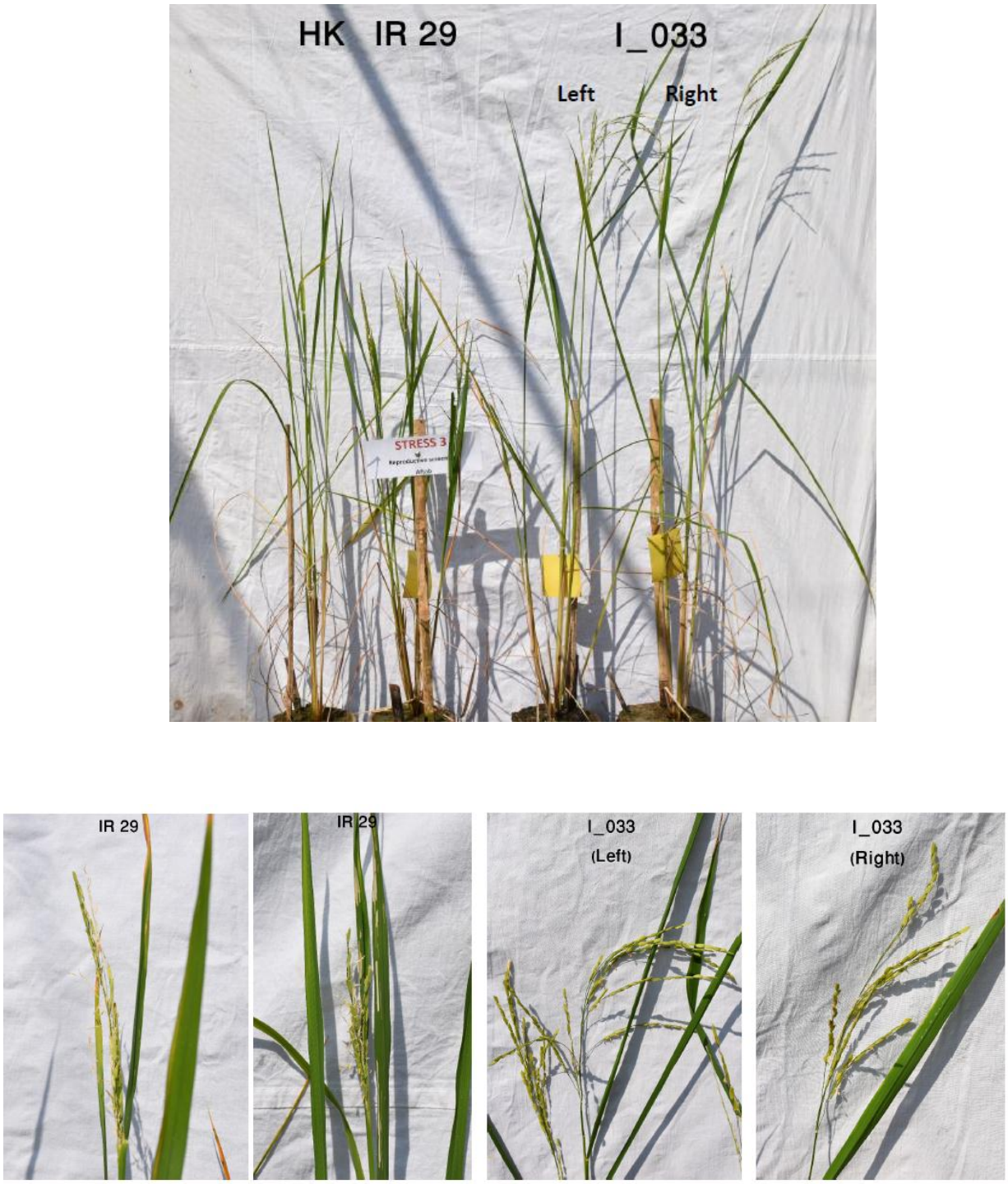


Figure 4.23 (B): Panicle damage

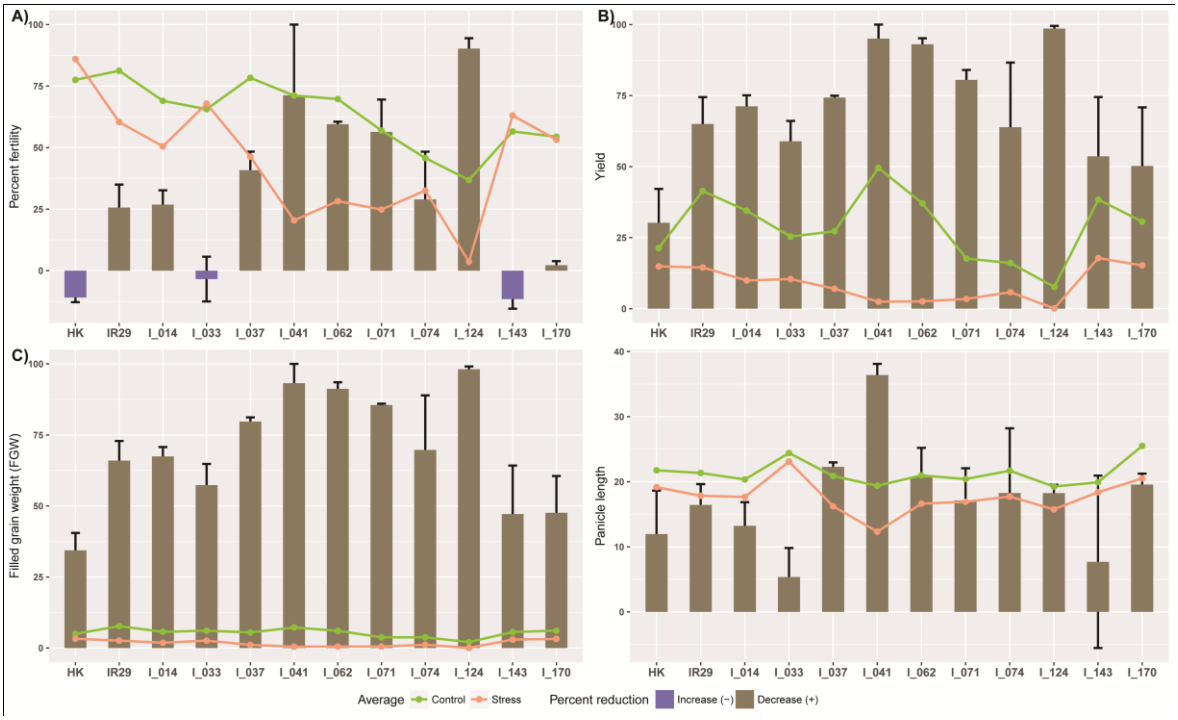
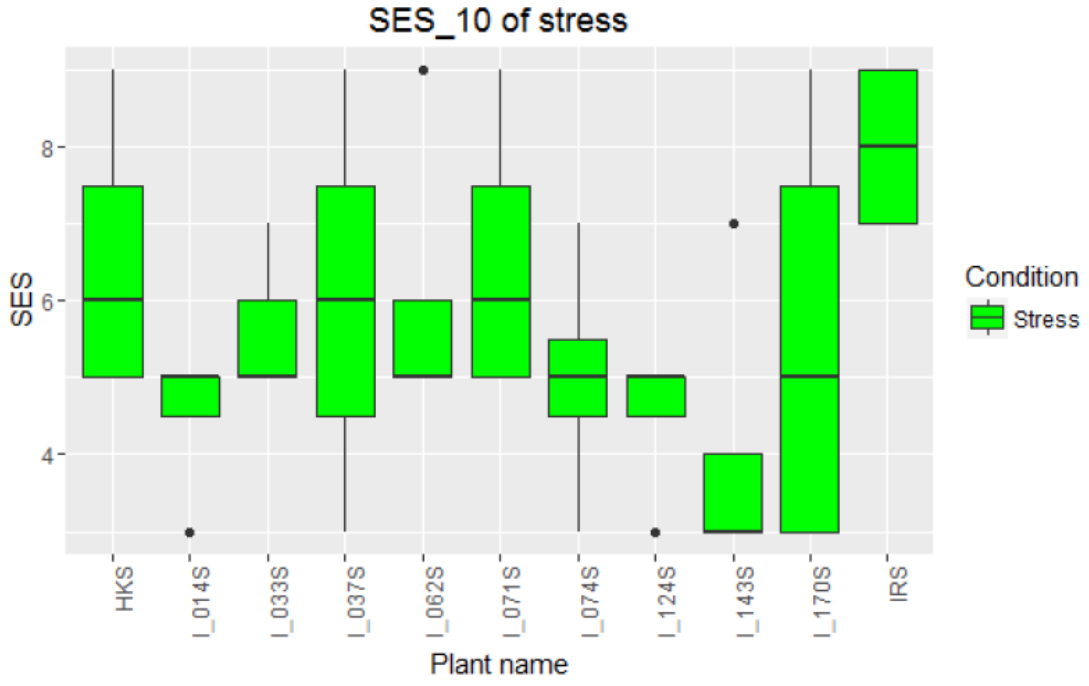


Figure 4.24: Percent reduction graph for (A) percent fertility, (B) yield (C) filled grain weight and (D) panicle length, under salt stress

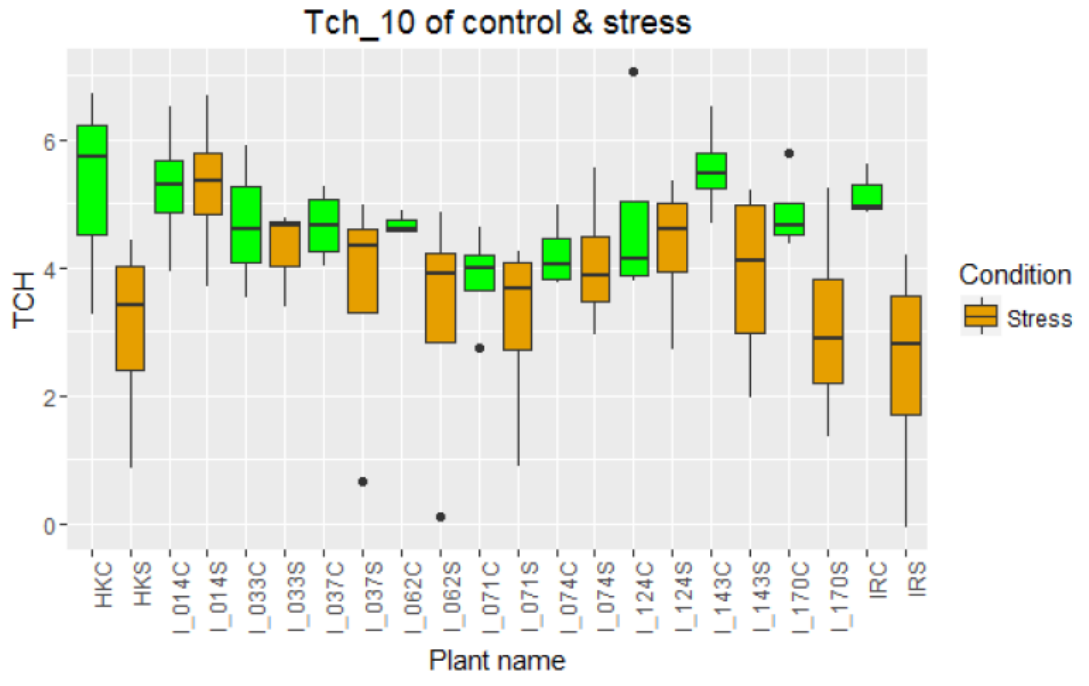
4.4.2.2.1 Plants that performed good both in seedling and reproductive stage

A few plants were identified that performed good in both developmental stages (figure 4.25). Plant I_033 and I_074 was moderately tolerant to salinity in seedling stage, with a SES score of 5.66 and 5, respectively and a good total chlorophyll (0.035 and 0.04 mg/gm). I_033 also performed good in reproductive stage with better percent fertility (67.85%) and longer panicle length (23.1 cm). I_074 had increased effective tiller (4.66 cm) in reproductive stage. I_143 was tolerant to salinity at seedling stage (SES score 4). Later in reproductive stage, it was found to have better percent fertility (63.1%). So, plant I_033, I_074 and I_143 showed

tolerance in seedling stage and performed well in reproductive stage. These plants can be used as donors for introgression into farmer popular rice variety.



(A)



(B)

Figure 4.25: Seedling stage performance of the plants selected for reproductive screening. (A) SES score and (B) total chlorophyll.

4.4.3 Molecular analysis for the confirmation of the presence of desired QTLs in F_5 progenies: Validation of QTLs by SSR markers

SSR markers near to the QTL regions were selected for validation. A total of 4 SSR markers linked to QTLs were found to be polymorphic between two parents (*Horkuch* and *IR29*) (table 3.6). They were selected for confirmation of presence of desired QTLs.

As previously described, one way ANOVA of the major QTLs were performed to identify the QTLs that have significant effect on the corresponding phenotypes (figure 4.26). Among the QTLs with significant effects, three seedling and one reproductive QTL were selected for molecular validation. The seedling stage QTLs were- stomatal conductance, root length, total chlorophyll and the reproductive QTL was third leaf length.

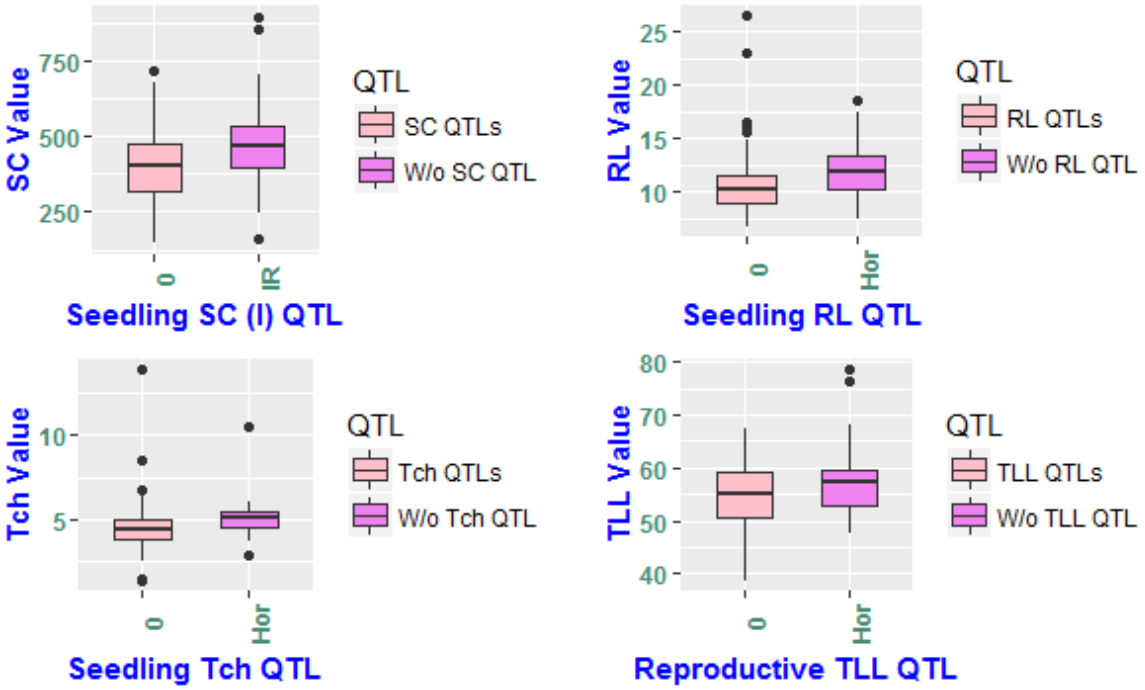


Figure 4.26: Boxplots showing QTLs having significant effects on corresponding phenotypes. Abbreviations: SC: Stomatal Conductance, RL: Root Length, Tch: Total Chlorophyll, TLL: Third leaf length.

4.4.3.1 Confirmation of a single QTL

4.4.3.1.1 Total chlorophyll

Total chlorophyll QTL was mapped at chromosome 7 (figure 4.27 A) and an SSR marker RM22073 was found to be associated with this QTL. So, this marker was selected to confirm the presence of this QTL. The positive allele for this QTL was *Horkuch* and negative allele was *IR29*. Through Graphical genotyping (GGT 2.0) analysis, positive and negative plants for this QTL were selected (figure 4.27 B). Among the selected plants, 8 out of 10 positive plants gave distinctive *Horkuch* banding pattern and 7 out of 9 plants gave distinctive *IR29* banding pattern (Figure 4.27 C).

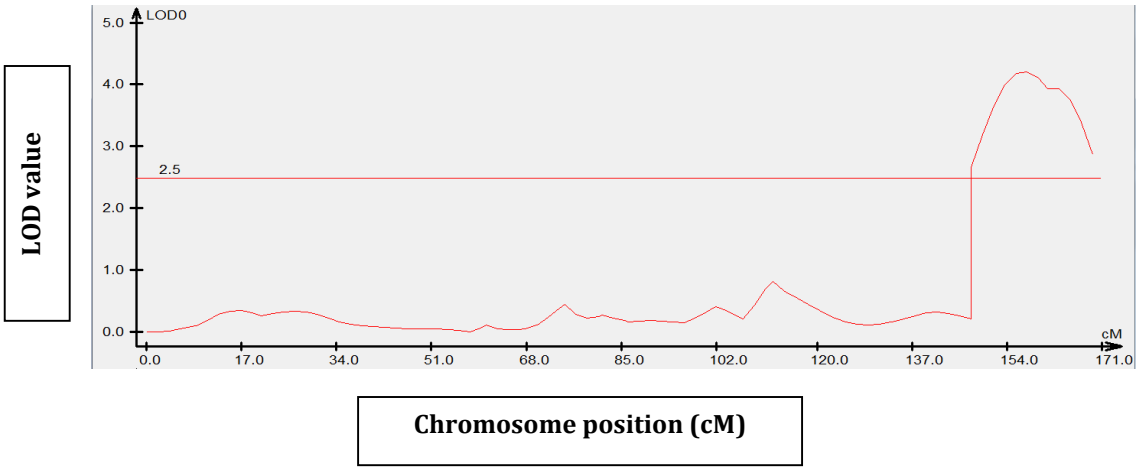


Figure 4.27 (A): Total chlorophyll QTL was mapped at chromosome 7

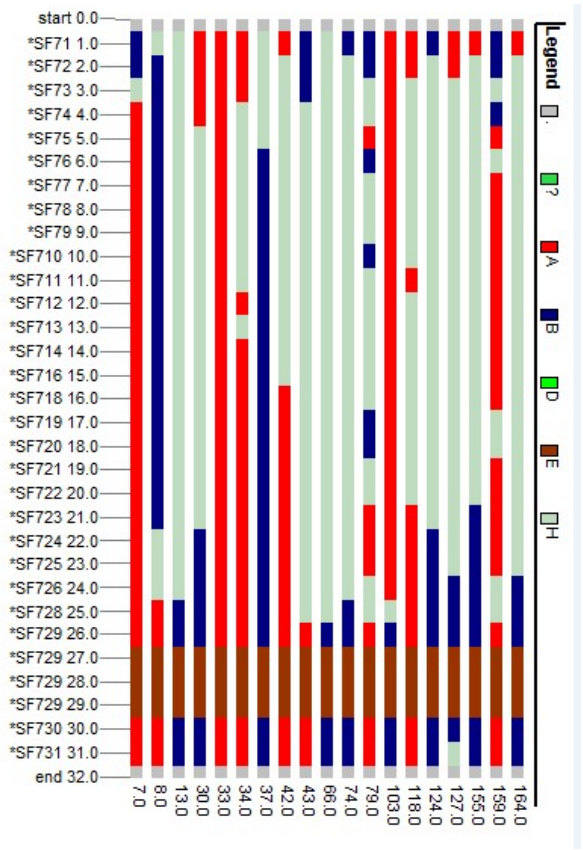


Figure 4.27 (B): GGT analysis for total chlorophyll QTL at chromosome 7. Chromosomal region of all plants used for analysis are shown here. Here legends A, B, D, E, H represents *IR29* allele, *Horkuch* allele, missing regions, location of total chlorophyll QTL and heterozygous allele respectively.

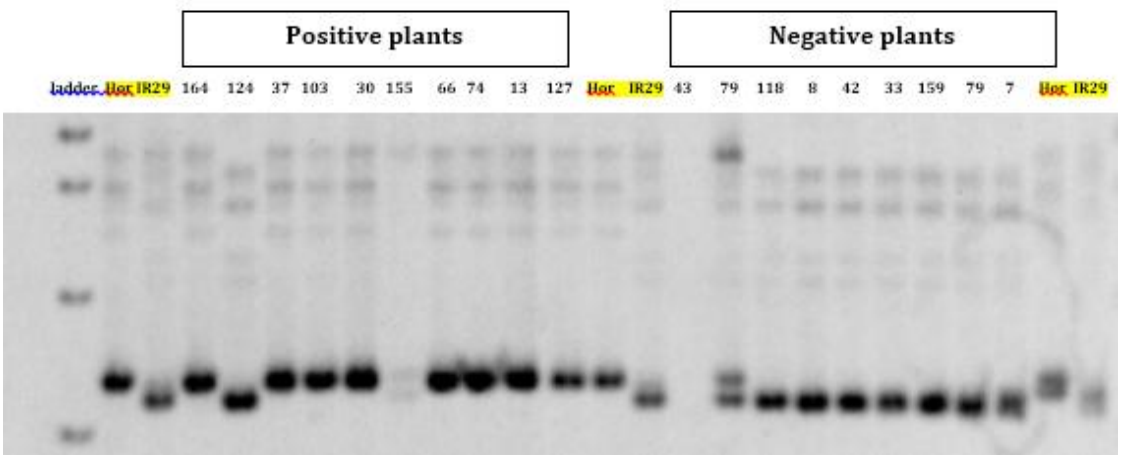


Figure 4.27 (C): Use of RM22073 marker for validation of total chlorophyll QTL at chromosome 7.

4.4.3.1.2 Root length

Root length QTL was mapped at chromosome 2 (figure 4.28 A) and an identified SSR marker drtSSR 3452265 was found to be associated with this QTL. So, this marker was selected to confirm the presence of this QTL. The positive and negative alleles for this QTL was *Horkuch* and *IR29*, respectively. Through Graphical genotyping (GGT 2.0) analysis, positive and negative plants for this QTL were selected (figure 4.28 B). Among the selected plants, 9 out of 14 positive plants gave distinctive *Horkuch* banding pattern and 11 out of 15 plants gave distinctive *IR29* banding pattern (figure 4.28 C).

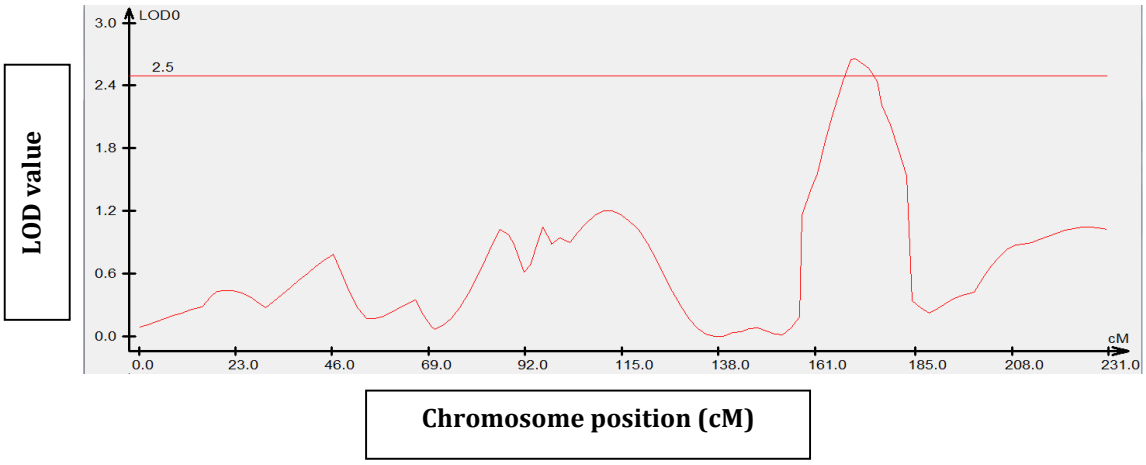


Figure 4.28 (A): Root length QTL was mapped at chromosome 2

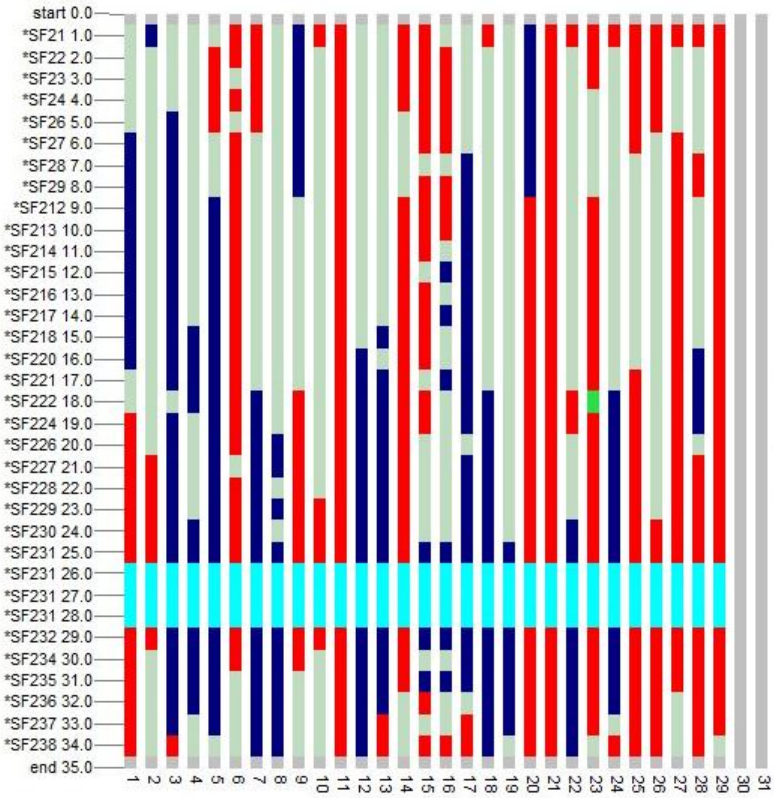


Figure 4.28 (B): GGT analysis for root length QTL at chromosome 2. Chromosomal region of all plants used for analysis are shown here. Here legends A, B, D, E, H represents *IR29* allele, *Horkuch* allele, missing regions, location of root length QTL and heterozygous allele respectively.

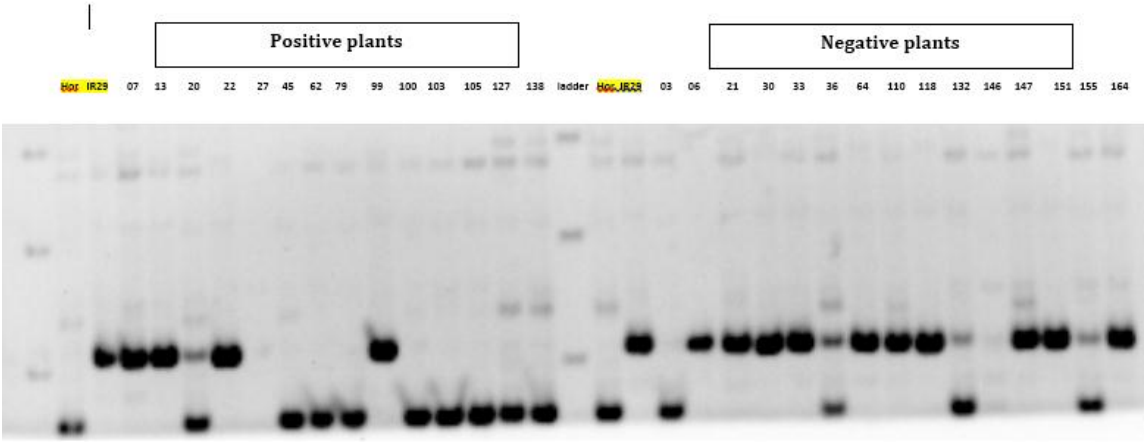


Figure 4.28 (C): Use of drtSSR 3452265 marker for validation of root length QTL at chromosome 2.

4.4.3.1.3 Stomatal conductance

Stomatal conductance QTL was mapped at chromosome 5 (figure 4.29 A) and an identified SSR marker drtSSR 3050109 was found to be associated with this QTL. So, this marker was selected to confirm the presence of this QTL. The positive and negative alleles for this QTL was *IR29* and *Horkuch*, respectively. Through Graphical genotyping (GGT 2.0) analysis, positive and negative plants for this QTL were selected (figure 4.29 B). Among the selected plants, 15 out of 19 positive plants gave distinctive *IR29* banding pattern and 8 out of 14 plants gave distinctive *Horkuch* banding pattern (figure 4.29 C).

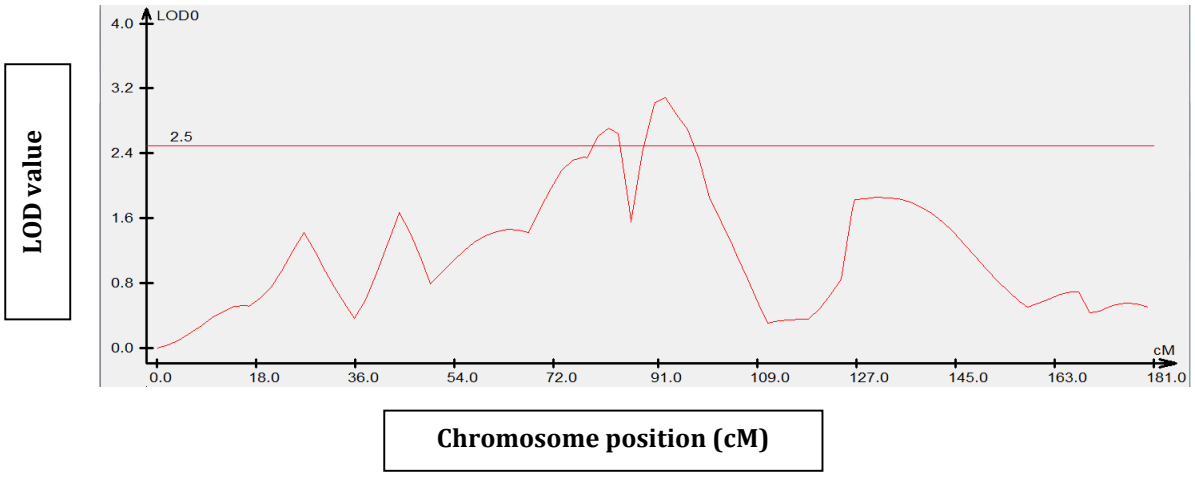


Figure 4.29 (A): Stomatal conductance QTL was mapped at chromosome 5

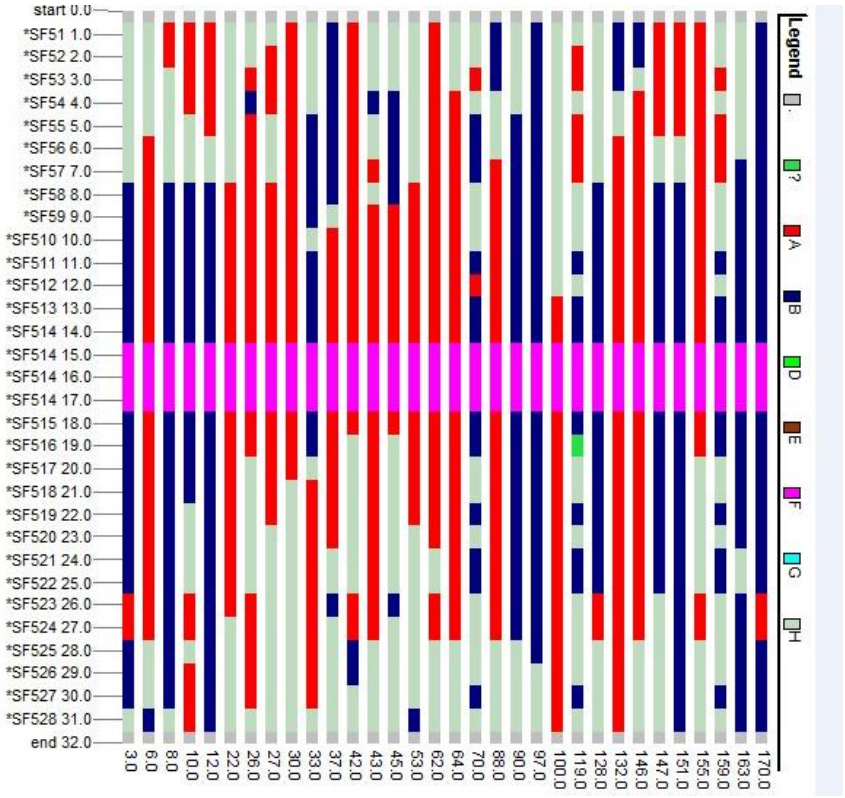


Figure 4.29 (B): GGT analysis for stomatal conductance QTL at chromosome 5. Chromosomal region of all plants used for analysis are shown here. Here legends A, B, D, F, H represents *IR29* allele, *Horkuch* allele, missing regions, location of SC QTL and heterozygous allele respectively.

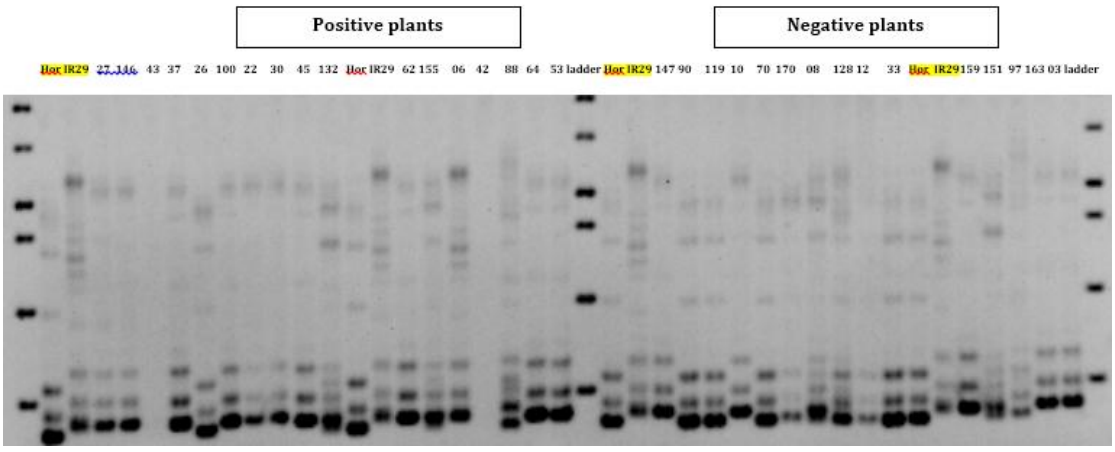


Figure 4.29 (C): Use of drtSSR 3452265 marker for validation of stomatal conductance QTL at chromosome 5. Here legends A, B, D, F, H represents *IR29* allele, *Horkuch* allele, missing regions, location of SC QTL and heterozygous allele respectively.

4.4.3.1.4 Third leaf length

Third leaf length QTL was mapped at chromosome 2 and SSR marker RM13642 was found to be associated with this QTL. So, this marker was selected to confirm the presence of this QTL. The positive and negative alleles for this QTL was *Horkuch* and *IR29*, respectively. Through Graphical genotyping (GGT 2.0) analysis, positive and negative plants for this QTL were selected (figure 4.30 A). Among the selected plants, 6 out of 8 positive plants gave distinctive *Horkuch* banding pattern and 4 out of 8 plants gave distinctive *IR29* banding pattern (figure 4.30 B).

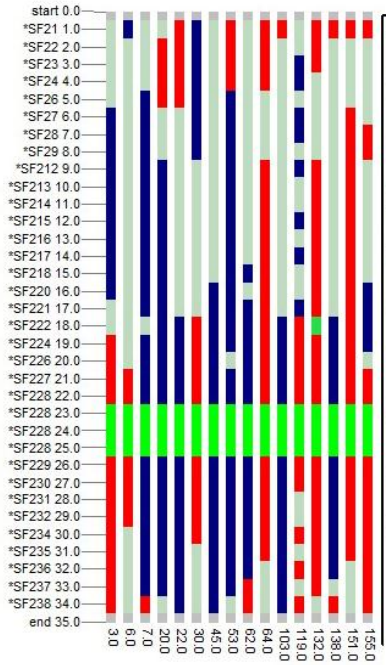


Figure 4.30 (A): Position of the QTL on chromosome 2 of positive and negative plants for third leaf length QTL (image produced by GGT 2). Here, red, blue, grey and green regions represent *IR29* allele, *Horkuch* allele, heterozygous allele and third leaf length QTL, respectively.

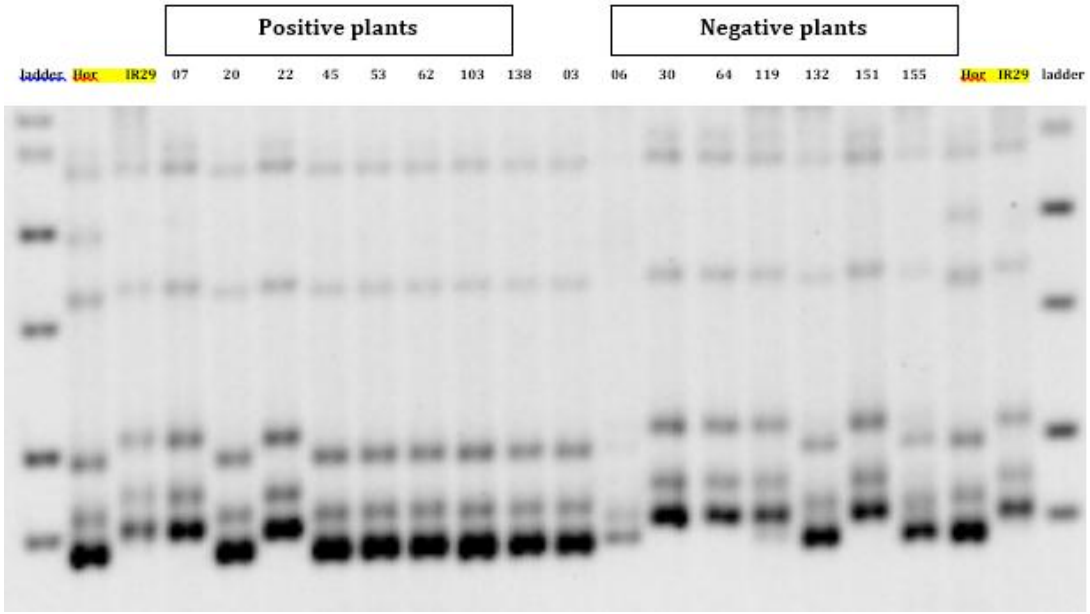


Figure 4.30 (B): Use of RM13642 marker for validation of third leaf length QTL at chromosome 2.

A list of positive and negative plants containing the desired QTLs have been identified (table 4.4) from the molecular validation. These plants with desired QTLs can be used as donors in marker assisted breeding.

Table 4.4: List of confirmed positive (presence of desired QTL) and negative (absence of desired QTL) plants.

QTL Name	Positive plant	Negative plant
Total Chlorophyll QTL	I_164, I_037, I_103, I_030, I_066, I_074, I_013, I_127	I_118, I_008, I_042, I_033, I_159, I_079, I_007
Root length	I_006, I_021, I_030, I_033, I_064, I_110, I_118, I_147, I_151, I_164	I_045, I_062, I_079, I_100, I_103, I_105, I_127, I_138
Stomatal conductance	I_027, I_146, I_037, I_100, I_022, I_030, I_045, I_132, I_062, I_155, I_006, I_064, I_053	I_090, I_119, I_070, I_170, I_128, I_012, I_033, I_159, I_151, I_063, I_003
Third leaf length	I_020, I_045, I_053, I_062, I_103, I_138	I_030, I_064, I_119, I_151

CHAPTER 5 *DISCUSSION*

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Increasing salinity is a major environmental obstacle that is contributing to global climate change and posing serious threat to rice production especially in low lying countries like Bangladesh because of its limited arable lands (Haque, Jahiruddin et al. 2015). Rice being a salt sensitive cereal, is most sensitive to salinity at the early seedling stage and during panicle formation, whereas it is relatively tolerant during early germination, active tillering and maturity (Singh and Flowers 2010). Salinity tolerance is a polygenic trait which comprises genes from both of the sensitive developmental stages of rice growth (Moradi and Ismail 2007) but these stages are poorly correlated. This emphasizes the importance of discovering the contributing traits of these two developmental growth phases of rice. Recent advances in understanding molecular mechanism of plant stress responses aided in enabling the dissection of QTLs (Quantitative Trait Loci) responsible for salt tolerance (Negrão, Courtois et al. 2011). However, identifying major QTLs contributing to salt tolerance and their introgression in high yielding rice varieties will significantly enhance and stabilize rice productivity in problem soils (Ismail, Heuer et al. 2007). But a major constraint is high yielding varieties are susceptible to salt stress and their production in Bangladesh will decrease by 15.6% in coastal districts where soil salinity is predicted to exceed 4 dSm-1 (Dasgupta, Hossain et al. 2014). This current study focused on mapping QTLs of a tolerant, low yielding Bangladeshi rice landrace *Horkuch*. The QTLs were validated by molecular and physiological analysis. This chapter analyses different parts of this study in detail and compares the results obtained with previous studies.

5.1 Previously reported QTLs

A good number of QTLs found in our study had previously been reported.

Root Length:

Six QTLs were found on chromosome 1, 2, 7, 8, 9 and 10 in our study. Three of them were reported in previous studies (table 5.1). The one on chromosome 2 were also validated in our study using SSR marker.

Table 5.1: Previously reported QTLs for root length

Chromosome	Position of QTLs in our study (Mb)	Position found in references (Mb)	References
2	28.48	28.3-30.2, 29.54	(Nguyen, Nguyen et al. 2002; Price, Steele et al. 2002)
7	24.55	24.08, 25.1, 25.47-26.52, 24.08-25.1	(Yadav, Courtois et al. 1997; Nguyen, Nguyen et al. 2002; Zheng, Yang et al. 2003; Xu, Li et al. 2004)
10	8.59	8.65-8.67	(Price, Steele et al. 2002; MacMillan, Emrich et al. 2006)

Source: http://archive.gramene.org/qtl/

Root Dry Weight

Seven RDW QTLs were found in our study. Three of them were previously reported in Lian, Xing et al. 2005 (table 5.2).

Table 5.2: Previously reported RDW QTLs

Chromosome	Position of QTLs in our study (Mb)	Position found in references (Mb)	References
2	34.37	35.66	(Lian, Xing et al. 2005)
6	2.33	2.65-2.77	(Lian, Xing et al. 2005)
7	20.52	21.87	(Lian, Xing et al. 2005)

Source: http://archive.gramene.org/qtl/

Total Chlorophyll

In our study, two QTLs for total chlorophyll t were found. The QTL on chromosome 4 was reported in a couple of studies (Ping and Ancheng 1996; Bing, Wei-Ya et al. 2006). However, the QTL on chromosome 7, at 28.22 Mb had a higher LOD score of 5.44 and caused a phenotypic variation of 14.25%. This QTL was validated at F_5 generation by SSR markers. Furthermore, this QTL was found to have influenced several other seedling as well as reproductive QTLs such as SES score, thousand grain weight, total and effective tillers, leaf weights, etc. (Section 4.7.1). As described by previous study (Lisa, Elias et al., 2011), an increase in expression of photosynthesis-related genes as well ROS scavengers suggested that this could be the reason for the better yield performance of *Horkuch*. Our finding about the total chlorophyll QTL and its capability of controlling reproductive traits *indica*te that this might be a factor responsible of the unique characteristics of *Horkuch*.

Days to flowering QTL

In this study, six QTLs for days to heading were found. All the QTLs except the one on chromosome 11 were reported previously and are mentioned in table 5.3.

Table 5.3: Previously reported QTLs for days to flowering

Chromosome	Position of QTLs in our study (Mb)	Position found in references (Mb)	References
1	39	39.68-41.96, 40.56	(Li, Yu et al. 2003; Mei, Li et al. 2005)
2	34.37	35.36, 33.84-35.66, 35.36, 35.13, 33.61-33.09	(Yamamoto, Lin et al. 2000; Moncada, Martinez et al. 2001; Li, Yu et al. 2003; Septiningsih, Prasetiyono et al. 2003; Mei, Li et al. 2005)
3	32.45	32.94, 31.04-31.19, 33.38, 31.32-31.39, 32.35	(Sarma, Gill et al. 1998; Yamamoto, Lin et al. 2000; Takahashi, Shomura et al. 2001; Li, Yu et al. 2003; Thomson, Tai et al. 2003)
6	4.93	3.92, 5.89-8.06	(Takeuchi, Hayasaka et al. 2001; Mei, Li et al. 2005)
9	22.17	22.19	(Xiao, Li et al. 1996; Suh, Ahn et al. 2005)

Source: http://archive.gramene.org/qtl/

In our study, a QTL controlling shoot Na^+/K^+ ratio at the seedling stage was found at 24.53 Mb, supported by another previous study (Koyama, Levesley et al. 2001). A major QTL controlling shoot Na^+/K^+ ratio called Saltol is located at 10.6–11.5 Mb on chromosome 1 (Thomson, de Ocampo et al. 2010). But no QTL controlling either Na^+/K^+ ratio or Na^+ or K^+ content was found in this region. Several QTLs controlling Na^+/K^+ ratio were found on chromosomes 3, 6, 7, 8 and 9. Some of them

are not previously reported. These QTLs can be fine mapped to find any novel strategy controlling the salt tolerance in *Horkuch*.

Yield QTL

In this study, three yield QTLs were found. One of the QTLs on chromosome 10 (at 11.94 Mb) was reported previously (Xiao, Li et al. 1996), where the QTL was reported to be located at 11.71-12.9 Mb. It explained 5.5% of the phenotypic variance of the F_3 population.

None of the QTLs for seed length and breadth found in our study matched with any of the previously reported QTLs.

5.2 Overlapping QTLs

Shoot length & plant height

Five reproductive QTLs for plant height were found in this study. Two of them, the ones on chromosomes 1 (39 Mb) and 3 (35.55 Mb) coincided with the shoot length QTLs found in seedling stage. The location of the QTL on chromosome 1 matched with those found in a study (Fukuda and Terao 2015). According to Fukuda and Terao, 2015, the most effective QTL in increasing the shoot length qSL1, was detected in the same region as qCL1 which increased culm length, suggesting that the qSL1 region increased the plant growth both at seedling and harvesting stages. The region corresponding to qSL1 contained genes that affect the plant height: D61 (Yamamuro, Ihara et al. 2000), D10 (Arite, Iwata et al. 2007), THIS1 (Liu, Zhang et al. 2013), Psd1 (Li, Xia et al. 2014), CIGR (Kovi, Zhang et al. 2011), SD1 (Sasaki, Ashikari et al. 2002; Ashikari, Sakakibara et al. 2005; Yano, Takashi et al. 2012) and SPS (Ishimaru, Ono et al. 2004). Among them, SD1, which encodes for gibberellin 20 oxidase-2 (OsGA20ox2), is well known as a semi-dwarf gene (Sasaki, Ashikari et al. 2002). OsGA20ox2 is the enzyme involved in the biosynthesis of gibberellin (Sasaki, Ashikari et al. 2002). The most effective QTL

explaining the highest phenotypic variation (R^2) of shoot length, qSL1, was located in the same chromosomal region as qCL1, which affects culm length.

As demonstrated by other studies (Abler, Edwards et al. 1991; Paterson, Damon et al. 1991; Veldboom, Lee et al. 1994)correlated traits often have QTLs mapping to the same chromosomal locations. The same trend was observed in the current study. A QTL cluster was observed at 39 Mb of chromosome 1 where QTLs of plant height, days to flowering, number and weight of filled grain, total grain number and straw weight were found. Among these, plant height and filled grain weight showed very good correlation in F_3 reproductive phenotyping (section 4.3.4). Plant height and days to flowering also showed positive correlation. QTLs for flag leaf weight and total grain weight were located on chromosome 1 (38 Mb). F_3 phenotypic values of flag leaf weight and filled grain weight were also well correlated as mentioned in section (4.3.4). Effective tiller and flag leaf weight QTLs on chromosome 8 (25.44 Mb) showed good correlation in F_3 reproductive phenotyping. Trait correlations may result from either pleiotropic effects of single genes, or from tight linkage of several genes controlling the traits.

Chromosome 10 harbors a number of QTLs like total grain weight, filled grain number, filled grain weight, unfilled grain weight, total tiller, yield, seed length, and percent fertility. All these QTL are close together in one region at around 35 cM. It seems likely that these chromosomal areas appear to be involved in yield and yield related traits. The question then arises whether there is a single QTL affecting multiple traits or whether there are several QTLs affecting separate traits, but located adjacent to each other. Problems arise in finding useful QTL for a particular trait when there are numerous QTL associated with it, as the smaller their individual contribution, the more difficult they are to detect. This highlights one of the problems of QTL analysis. It is not yet possible to discern whether significant effects at several linked markers are due to a common QTL or due to several linked QTLs (Koyama, Levesley et al. 2001).

It can be concluded from the above that in this study, the QTL analysis was entirely consistent and hence validated with the known QTLs in rice. Some additional QTLs were reported which therefore may be unique to *Horkuch*.

5.3 Marker Segregation and Distortion

Segregation distortion is a common phenomenon in segregated populations derived from crosses between divergent parents in rice (Matsushita, Iseki et al. 2003). It is caused by the deviation of the observed genotypic frequency from expected Mendelian segregation ratios within a segregating population. Segregation distortion is being increasingly recognized as a potent evolutionary force that is influenced by many factors, for example, mapping population, marker types, genetic transmission, gametic and zygotic selections, non-homologous recombination, gene transfer, transposable elements, environmental agents and so on (Kinoshita 1995; Knox and Ellis 2002; Yamagishi, Takeuchi et al. 2010).

Higher segregation distortion ratio in interspecific population than that in intraspecific population has been reported in many plants (Xu, Zhu et al. 1997; Lin 2005; Li, Lin et al. 2007). *IR29* is a salt sensitive *indica* rice variety whereas *Horkuch* is a salt tolerant rice variety of coastal region of Bangladesh that was reported to cluster with salt tolerant and aromatic rice varieties (Yesmin, Elias et al. 2014).

There are different reports about the amount of segregation distortion in rice, between *indica* and *japonica* subspecies. One report observed 43.7% and 40.2% segregation distortion in F_2 reciprocal crosses between *indica* and *japonica* varieties, respectively (Kim, Jang et al. 2014). Others have reported segregation distortion ranges from 12-35% in *indica* and *japonica* F_2 crosses and reciprocal populations (Wu, Ko et al. 2010). In our study, 33% of the polymorphic DArTseq™ markers showed deviation from the expected Mendelian segregation ratio and

showed segregation distortion (Section 4.3.1). So, it shows similarity with previous studies.

Cytoplasmic and genetic factors are two of the major influencing factors causing segregation distortion. Genetic factors include pollen tube competition, lethal pollen, preferential fertilization, sterility and chromosome translocation. Several genetic factors have been identified in plant species such as maize (Zhang, Wan et al. 2006), rice (Bing, Qi-Ming et al. 2006; Gutiérrez, Carabalí et al. 2010) soybean (Zhang, Chen et al. 1997; Liu, Wu et al. 2000).

Segregation distortion was directly or indirectly associated with the effect of cytoplasm. The effect of cytoplasmic factors on the transmission of nuclear marker genes in barley (Goloenko, Davydenko et al. 2002) and effects of cytoplasmic factors on SD in rice (Kim, Jang et al. 2014) have been reported. In a study (Razzaque, Khan et al. 2016), the IH F_2 population (*IR29* mother) used in our study and its HI reciprocal population (*Horkuch* mother) was genetically analyzed with polymorphic SSR markers. A genetic linkage map of the F_2 populations was first constructed using SSR markers. Then the frequency of distorted marker alleles was assessed. Out of the 25 common markers, 16 markers were deviated from the expected Mendelian segregation ratio in both reciprocal F_2 populations *i*ndicating effects of nuclear genetic factors on SD.

Similar distortion in both reciprocal F_2 populations indicates that nuclear genetic factors are responsible for SD whereas if the markers are distorted in only one of the reciprocal F_2 populations, a cytoplasmic effect can be inferred. Based on the results of the study, it was inferred that SD was influenced by both nuclear and cytoplasmic factors in reciprocal F_2 populations.

Effect of gametophytic and zygotic factors on SD has been reported in rice. Zygotic selection causing SD is explained by those markers which are distorted in both

female-and male segregating populations. The gametophytic (female and male gamete function) and zygotic selection as mechanisms underlying SD can be inferred from the distorted marker patterns (Kim, Jang et al. 2014). In Razzaque, Khan *et al* (2016), SD of 22 and 15 markers were found to be influenced by gametic and zygotic selections simultaneously in the HI and IH population whereas 13 markers by either zygotic or gametic selection. So it can be concluded that gametic and zygotic selections had big effects on SD in the F_2 reciprocal populations.

Moreover, in Razzaque, Khan *et al* (2016), all the markers on chromosome 4 and 11 showed significant deviation in both populations. All but one marker on chromosome 5 deviated significantly. In our study, chromosome 4 was the most distorted one (section 4.3.1). Chromosome 5 too showed distortion. Information of the loci, genetic elements and other factors responsible for SD in rice are important for the selection of breeding cultivars, and could also aid the development of molecular breeding programs.

If the salt tolerance determinants of *Horkuch* are to be put into *indica* backgrounds for breeding salt tolerant rice, a bridging rice cultivar, intermediate in genetic distance between *indica* and *aromatic* varieties may be required. However, the exact comparative genetic distance among the *indica* rice *IR29*, *aromatic Horkuch* and *japonica* rice needs to be determined.

5.4 Linkage Map

Construction of linkage maps are often complicated in the presence of SD as it is known to affect recombination frequency and thus the construction of genetic linkage map. The program MapMaker could only use 291 out of 339 markers for linkage analysis. Some programs, for example, MapManager and Mapdisto can handle segregation distortion by providing options for calculating linkage distances of distorted markers, and several algorithms were developed to adjust recombination frequency in such cases (Lorieux, Ndjiondjop et al. 2000; Zhu,

Wang et al. 2007). LOD ≥ 3.0 was used in linkage map construction using DArT data. LOD ≥ 3.0 is considered as the presence of a linkage between the two loci. However, in our study, QTLs with LOD score between 2.5 and 3.5 was considered as putative QTLs as some of the QTLs with LOD < 3 matched with previously identified, well known QTLs. For example, in our study, the plant height QTL found on chromosome 1 (at 39 Mb) matches the position of the previously identified Sd1 (semi-dwarf) gene responsible for the short stature of plant (Sasaki, Ashikari et al. 2002). But in this study, the LOD score was 2.92 for this QTL. QTLs with LOD score between 3.5 and 4.5 were considered as real QTLs.

5.5 DArT based SNP genotyping

In another unpublished work with restriction site associated DNA based marker (ddRAD), construction of genetic map was very difficult due to segregation distortion of the markers as well as lack of coverage in the whole genome. DArT markers are intelligently designed, where the locations are supervised from previous diversity array based information. In DArTseq™ genotyping, restriction enzymes cut only the gene rich regions of DNA. These regions control the phenotypes and this might be one of the reasons that QTL mapping through DArT based SNP genotyping was more successful than it was with ddRAD. These RADseq based genotyping (both DArTseq and ddRAD) are very useful in reducing the complexity of the genome, by sequencing the desired regions, ultimately generating a large number of robust SNP markers.

5.6 Association between stages

Weak association between seedling and reproductive stage salt tolerance have been reported before (Moradi, Ismail et al. 2003; Moradi and Ismail 2007). In this study, the effects of QTLs on phenotypes were assessed by one way ANOVA (Section 4.7.1). Many of the seedling QTLs seemed to have significant effects on reproductive traits. Especially, total chlorophyll QTL had significant effect on yield

related traits such as thousand grain weight, total and effective tiller, leaf weights etc. This QTL for total chlorophyll on chromosome 7 (at 27.53 Mb) was not reported before. In the study by Lisa, Elias et al. 2011, it was proposed that an increase in expression of photosynthesis-related genes as well as ROS scavengers could have been the reason for the better yield performance of *Horkuch*. Salt tolerant genotypes were found to have maintained relatively higher photosynthetic function during both vegetative and reproductive stages in another study (Moradi and Ismail 2007). Therefore, the total chlorophyll QTL affecting a number of reproductive traits are relevant with previous findings. The presence of the total chlorophyll QTL (on chromosome 7) found in our study were also confirmed using SSR marker. This QTL can be fine mapped and candidate gene analysis can be performed to be used for MAS to develop high yielding varieties.

5.7 Selective Phenotyping

In our study, selective phenotyping method was applied in the reproductive stage characterization of F_3 lines. As mentioned in chapter 2 (section 3.1.4.2), 100 similar F_3 lines was selected based on SES scores during seedling stage phenotyping, where extreme tails of 25% each were chosen from 200 lines.

Quantitative trait locus (QTL) experiments provide valuable clues for identifying genetic elements responsible for quantitative trait variation (Lander and Botstein 1989; Lynch and Walsh 1998; Rapp 2000). The power of a genetic mapping study depends on the heritability of the trait, the number of individuals included in the analysis, and the genetic dissimilarity among them, that is, the number of recombination events occurring in the segregating population. For best results, QTL experiments require that large numbers of individuals be genotyped and phenotyped for the quantitative trait of interest. Since this can be a costly endeavor, cost-saving strategies such as selective phenotyping are applied, in which a selected portion of the genotyped individuals is phenotyped (Jin, Lan et al. 2004)

Selective phenotyping efficiency decreases as the number of unlinked loci used for selection increases, and approaches random selection in the limit. However, when phenotyping is expensive, and a small fraction can be phenotyped, the efficiency of selective phenotyping is high compared to random sampling, even when >10 loci are used for selection (Sen, Johannes et al. 2009).

5.8 Fixation of alleles

The QTLs identified in this study were validated at both physiological and molecular level. The progeny was advanced up to F_5 generation. At molecular level, four QTLs were validated with nearby SSR markers. In recombinant inbred lines (RIL), the alleles are fixed at homozygous state. Recombinant inbred lines (RILs) can serve as powerful tools for genetic mapping. An RIL is formed by crossing two inbred strains followed by repeated selfing to create a new inbred line whose genome is a mosaic of the parental genomes.

However, the percentage of fixation ranged from 62.5% to 79% in our study. Third leaf length QTL was 62.5% fixed, where the desired alleles were present in homozygous state in 10 out of 16 plants. Root length QTL showed 69% fixation in F_5, where the desired alleles were present in homozygous state in 20 out of 29 plants. Stomatal conductance QTL showed 70% fixation in F_5, where desired alleles were present in homozygous state in 23 out of 33 plants. And total chlorophyll QTL showed 79% fixation in F_5, where desired alleles were present in homozygous state in 15 out of 19 plants. The F_5 generation should be further advanced to get 100% fixation of desired alleles.

5.9 Complementary action of genes from the two parents is the major genetic basis of transgressive segregants

Transgressive segregation is the term used to describe the phenomenon in which individuals in segregating populations out-perform the parents. It has been

observed in the progeny of inter- and sub-specific crosses of rice, but the underlying genetic basis of this phenomenon has not been experimentally determined. Transgressive segregants were observed for all traits examined in the current study. Similar phenomena were observed in a study (Xiao, Li et al. 1996), where two elite homozygous lines, 9024 (*indica*) and LH422 (*japonica*) were crossed. Transgressive segregants were found in all traits observed. For those traits for which two or more significant QTLs were detected, both parents were found to possess QTL alleles which increased phenotypic values. The occurrence of such transgression could be directly associated with the inheritance of complementary QTL alleles from the two parents. For example, six significant QTLs were detected for plant height. *I* alleles were associated with an increase in plant height at two of the six QTLs, and *J* alleles at the other four. When graphical genotypes of the transgressive segregants were constructed based on their marker genotypes, transgressive segregants taller than the *J* parent were found to possess "tall" alleles at four or more of the plant height QTLs. In contrast, transgressive segregants shorter than the *I* parent possessed "short" alleles at four or more of the six QTLs. In another study (Pang, Chen et al. 2017), a designed QTL pyramiding experiment was carried out to develop high yielding “Green Super Rice” varieties with significantly improved tolerance to salt stress and grain yield. The QTL mapping results *indica*ted that favorable alleles at different salt tolerance loci were derived from both parents and grandparents. Thus, pyramiding the favorable alleles of different salt tolerance to loci was responsible for the transgressive segregations for salt tolerance in the progeny.

5.10 Salt effect on physiological traits: Identification of donor plants for marker assisted breeding

In this study, *Horkuch* is the salt tolerant, low yielding parent, whereas *IR29* is the sensitive, high yielding parent. The salt tolerance and yield related traits were distributed in the segregating population after the cross was advanced. After seedling and reproductive phenotyping both in the F_3 and F_5 generations, the

frequency distribution of the traits were studied and it was found that for every trait, some progeny plants performed better than the parents, which may be explained as transgressive segregation. These better performing plants can be used as donor plants for marker assisted breeding develop high yielding, salt tolerance rice cultivars.

High photosynthetic efficiency is the main target of rice breeding in the 21st century (Wang 1995; Peng 2000). Identifying the locations of genes or groups of genes controlling chlorophyll content and photosynthetic rate in salt stress conditions is useful to further understand the molecular genetic mechanisms of photosynthesis. This information can be practically applied in breeding programs that use MAS or mapping. Photosynthetic rate (PR) directly affects the biomass and yield of crops. In rice, higher leaf photosynthetic rate was reported to be correlated with greater biomass (Ohno 1976; Shen 1980; Chen, Xu et al. 1995) and grain yield (Xu and Shen 1994). Two studies (Ohno 1976; Cao, Zhai et al. 2001) also reported significant differences among PR in different rice varieties and suggested that high yielding varieties could be developed by selecting varieties with high PR.

In reproductive screening, the mean of the population was better than parents in case of thousand grain weight and days to flowering. A shorter days to flowering and a higher thousand grain weight under salt stress are desired traits for the development of an improved variety. Salinity delays heading in rice, which negatively affects a number of yield components (Grattan, Zeng et al. 2002). Salinity interferes with rice growth and development, plant adaptation and stress responses. Salinity causes sterility in rice if imposed during pollination and fertilization (Pearson and Bernstein 1959). Another study (Akbar, Yabuno et al. 1972) found that salinity causes panicle sterility in only some rice varieties, suggesting some genetic control. Salinity stress affects seed germination, seedling growth, leaf size, shoot growth, shoot and root length, shoot dry weight, shoot fresh weight, number of tillers per plant, flowering stage, spikelet number, percent

of sterile florets and productivity (Zeng and Shannon 2000; Läuchli and Grattan 2007; Moradi and Ismail 2007; Munns and Tester 2008).

For validation of QTLs by physiological screening, ten F_5 plants with good combination of seedling and reproductive QTLs were screened at reproductive level. As mentioned in section 4.7.2.2, plant I_033, I_143 and I_170 performed well in the reproductive screening.

Few plants were identified whose performances was superior in both developmental stages (section 4.7.2.2.1). Plant I_033, I_074 and I_143 showed tolerance in seedling stage and performed well in reproductive stage. So, F_5 plants with a combination of multiple QTLs performed well in seedling as well as reproductive stages of development under salt stress. Best performing plants under salt stress can be used as donor plant for introgression into farmer popular rice varieties. This will be possible since markers can now be developed for the QTL traits and used for ensuring introgression into different high yielding backgrounds. These selected individuals are expected to show high salinity tolerance along with higher yield potential and thus can be directly used for breeding purposes.

5.11 Conclusion

The main objective of the study was the characterization of salt tolerant Bangladeshi rice landrace *Horkuch*. QTL mapping of both seedling and reproductive stages was done in $F_{2:3}$ strategy using DArTseq™ markers. Major QTLs like total chlorophyll, stomatal conductance, root length were discovered and validated at F_5 progeny by both physiological screening and molecular validation through SSR markers. The target of this process was to develop donor plants for breeding lines to develop salt tolerant, high yielding rice cultivars. Considering all the experiments, the outcomes of the study can be summarized as:

1. In this study, the QTL mapping of Horkuch, a naturally salt tolerant, low yielding rice landrace endemic to Bangladesh, has been done for the very first time.
2. QTL mapping of salt tolerance and other yield related traits in both seedling and reproductive stages of *Horkuch* has been performed. Some major QTLs identified in seedling stage were K^+/Na^+ ratio, total chlorophyll, stomatal conductance, root length. Among the major QTLs identified in reproductive stage were thousand grain weight, yield, percent fertility, primary and secondary branching, days to flowering.
3. Correlation study among different 'tolerance' and 'yield' related traits has been performed and pleiotropic effects have been identified.
4. Distorted chromosomal regions have been identified that is supported by previous studies on sub-specific crosses like *indica* and *japonica*.
5. Some overlapping QTLs or QTL clusters have been identified. Successful introgression of these clusters would ensure an easy transfer of multiple QTLs and QTL pyramiding as well.
6. Many of the QTLs found in this study had been reported in previous studies, which ensures the authenticity and acceptability of this study.
7. As DArT markers are intelligently designed, DArT based SNP genotyping was shown to be more effective than other RADseq marker based genotyping such as ddRAD.
8. Association between some seedling and reproductive stage QTLs have been found.
9. A QTL controlling total chlorophyll was found that influences a number of yield related traits. It supports the previous finding (Lisa, Elias et al., 2011) that an increased photosynthetic rate is responsible for the better performance of *Horkuch*. This QTL can be fine mapped for candidate gene analysis.
10. Presence of QTLs have been confirmed by molecular and phenotypic validation at both developmental stages at the F_5 generation.
11. Pyramided QTLs of desirable seedling and reproductive traits in progeny plants have been identified.

12. Transgressive segregants have been found for most traits in both F_3 and F_5 population, which is indicative of pyramiding of favorable alleles.
13. Plants with a combination of several QTLs have been identified at F_5 that also showed tolerance to salinity at seedling stage and had better yield properties in reproductive stage. The plants can act as donors in breeding lines to develop high yielding, salt tolerant rice varieties. It is worth mentioning that seedling and reproductive QTLs have never been combined and tested before to produce more tolerance. The ability to combine seedling and reproductive QTLs in a single genotype and achieve greater tolerance was one of the major findings of the study. However, the alleles were not 100% fixed at homozygous state in F_5. More advancement is needed to develop these as fixed lines. However markers linked to the important QTLs have now been identified and can be used to introgress multiple QTLs into high yielding genotypes.

Understanding the mechanism of high salinity stress and subsequently developing salinity tolerant crops can be a solution for increasing food production. There is a need for developing new varieties with higher yield potential and stability across environments, climates and geographic locations. A number of novel QTLs related to salt tolerance and yield had been identified in this study. Fine mapping of these QTLs and identification of candidate genes can lead to a better understanding of salinity tolerance mechanism and also, to the development of transgenic rice through gene cloning. Besides, useful QTL clusters have been identified that might be easily introgressed into other varieties, as there are identified markers linked to the QTLs. Plants with pyramided seedling and reproductive QTLs have been developed for the first time. Horkuch is a local landrace and breeding lines developed from this donor plants would hopefully more stable with respect to salt tolerance and yield. This study is encouraging for our country of diverse germplasms of rice where there are still many more landraces with unique qualities which can be characterized and high quality rice can be produced to ensure food security.

APPENDIX

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Appendix A

A. 1. *Procedure of plant DNA isolation in CTAB method*

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 to a 15 ml screw cap tube containing 5 ml of preheated (65^{o} C) CTAB buffer.
3. Incubation at 65^{o} 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^{o} 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 was 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 was washed with 3-5 ml ice cold 70% ethanol. The washing and precipitation steps were repeated thrice.

19. Finally, the pellet was completely dried and dissolved in minimum TE solution and the DNA stock was stored at –20° C.

20. Quality and quantity of DNA for all extracted samples was checked by gel electrophoresis and NanoDrop spectrophotometer.

A. 2. The chromosomal location, annealing temperatures and amplified product size ranges of RM493.

Marker Name	Chromosome number	Chromosomal location (Mb)	Annealing Temp	Amplified product size
RM493	1	12.28	55°C	211 bp

A. 3. DArTseq output data

A. 4. Segregation distortion

A. 5. Marker details

Since the supplementary files are large in size they are deposited in a website. The supplementary files from A. 3 to A. 5 are temporarily available in the following URL: http://www.pbtlab.du.ac.bd/SFKTHESIS/thesis_sfk.zip.

Appendix B

B. 1. F_3_Seedling data

B. 2. F_3_Reproductive data

B. 3. F_5_Seedling data

B. 4. F_5_Reproductive data

B. 5. Plant selection with QTLs

Since the supplementary files are large in size they are deposited in a website. The supplementary files from B. 1 to B. 5 are temporarily available in the following URL: http://www.pbtlab.du.ac.bd/SFKTHESIS/thesis_sfk.zip.

B. 6. List of all seedling QTLs identified (Putative QTL: P value 2.5-3.5. True QTL > 3.5). A=*IR29* allele, B=*Horkuch* allele.

QTL Name	Chr.	Marker	Position	LOD Score	Additive	Dominance	R2	Donor of positive allele
(SESRev)	1	1	0.01	2.5231	0.3163	-0.0342	0.0509	A
Shoot Dry Weight	1	6	50.19	3.3023	0.0599	-0.0052	0.0701	A
Na^+	1	8	62.31	2.5404	-0.286	0.0438	0.0561	B
K^+	1	15	101.58	2.5853	0.0037	0.0335	0.0183	A
Na^+/K^+ ratio	1	19	143.34	2.7824	-1.2809	-0.6985	0.033	B
Root Dry Weight	1	24	166.38	2.5124	0.0068	0.0102	0.001	A
K^+/Na^+ ratio	1	32	227.46	3.0163	-0.0148	0.0108	0.0932	B
K^+	1	33	230.64	2.6738	-0.0245	0.0072	0.0672	B
Root Dry Weight	2	28	224.42	2.6801	0.0093	-0.0069	0.0956	A
Shoot Length	3	28	229.1	2.4519	-0.9551	0.2252	0.0446	B
A+B Chlorophyll	4	11	57.17	2.5417	0.2204	0.4519	0.0191	A
Total Chlorophyll	4	25	172.77	2.9129	-0.1677	-0.3195	0.0002	B

Root Dry Weight	6	2	18.96	2.8713	0.0069	0.0087	0.0041	A
K^+/Na^+ ratio	6	13	91.47	3.2191	-0.003	0.0238	0.0523	B
Shoot Dry Weight	6	14	97.24	2.6802	0.0542	0.0138	0.035	A
Chl a/b	6	17	112.66	3.4775	-0.4882	0.2633	0.1363	B
Root Dry Weight	6	18	140.26	3.3577	0.0096	-0.0093	0.1202	A
K^+/Na^+ ratio	7	1	0.01	3.3208	0.0138	0.0177	0.0016	A
Shoot Dry Weight	7	10	69.79	2.6728	-0.0526	-0.0025	0.0442	B
A+B Chlorophyll	7	17	111.95	2.5026	0.2597	0.3114	0.0009	A
Na^+/K^+ ratio	7	22	165.15	2.5267	-0.3969	-1.7309	0.042	B
Root Length	8	10	87.44	2.707	-0.6518	0.4696	0.0723	B
Root Length	9	4	11.73	2.9747	0.739	-0.5321	0.0867	A
K^+/Na^+ ratio	9	5	15.71	2.5354	0.0159	0.0039	0.0346	A
K^+	9	5	13.71	2.7332	0.0231	0.0108	0.0233	A
Shoot Length	9	6	19.02	2.5297	1.1392	0.1666	0.0313	A
Root Length	10	6	39.39	2.5428	0.5456	-0.5439	0.0653	A
Shoot Length	11	17	103.57	2.4609	0.3906	1.2451	0.0014	A
Na^+	12	3	39.61	3.1536	-0.0346	0.6083	0.0424	B
Na^+/K^+ ratio	12	5	68.81	2.8672	1.6564	1.0854	0.0003	A
A+B Chlorophyll	12	17	139.91	3.0391	0.243	-0.2361	0.0959	A
Shoot Length	1	6	42.19	5.0213	1.5427	-0.8978	0.1437	A
Root Dry Weight	1	7	59.09	3.616	0.0119	-0.0036	0.1073	A
K^+/Na^+ ratio	1	20	147.76	4.4921	0.02	0.0093	0.0462	A
(SESRev)	1	21	153.73	4.4182	0.4278	0.1571	0.0528	A
Shoot Length	1	30	198.54	12.7785	-2.6545	-0.6158	0.1747	B
Root Length	1	33	230.64	3.5306	0.6807	-0.4398	0.0825	A
Shoot Relative Water Content	2	21	169.42	4.134	-3.2393	-2.3466	0.0289	B

Root Length	2	23	173.75	8.3572	-1.1418	0.1773	0.165	B
K^+/Na^+ ratio	3	8	68.52	6.385	0.0064	0.0285	0.0165	A
Na^+/K^+ ratio	3	8	68.52	5.7909	-0.2523	-2.151	0.0195	B
Shoot Relative Water Content	3	16	126.07	4.8077	2.8562	3.834	0.0023	A
K^+	3	23	187.67	3.5834	-0.0309	-0.004	0.0613	B
Stomatal Conductance	4	4	18.22	5.1552	27.6473	-70.9728	0.1317	A
Stomatal Conductance	5	12	92.3	4.8805	73.4585	-44.9533	0.2056	A
Root relative water content	5	15	109.2	3.9097	-7.7536	-4.5504	0.0077	B
Shoot Dry Weight	6	2	22.96	5.5358	0.0474	0.0736	0.0068	A
A+B Chlorophyll	6	4	45.81	3.9027	0.3378	-0.2523	0.1574	A
Na^+	6	16	104.32	5.9273	-0.4526	-0.2985	0.0412	B
Shoot Length	7	13	90.05	4.1415	-0.9983	1.1853	0.0987	B
Root Dry Weight	7	15	101.69	3.9528	-0.0181	-0.0012	0.0776	B
Root Length	7	19	131.01	3.6643	-0.8149	-0.051	0.0615	B
Total Chlorophyll	7	21	157.47	5.4418	-0.4917	0.0114	0.1425	B
(SESRev)	7	22	161.15	3.672	-0.2936	0.3043	0.0956	B
Shoot Dry Weight	8	6	58.66	3.8591	-0.0477	0.0479	0.1076	B
K^+/Na^+ ratio	8	19	157.05	3.5667	0.0007	0.0249	0.0305	A
Shoot Dry Weight	9	5	17.71	4.268	0.0801	0.0604	0.001	A
Na^+	9	5	17.71	3.5586	-0.5844	-0.0411	0.0654	B
Shoot Length	12	19	168.49	3.4915	-1.1873	0.1465	0.0616	B

B. 7. List of all reproductive QTLs identified (Putative QTL: P value 2.5-3.5. True QTL: P > 3.5). A=*IR29* allele, B=*Horkuch* allele.

QTL Name	Chr.	Marker	Position	LOD Score	Additive	Dominance	R2	Donor of positive allele
Flowering time	11	14	86.48	2.8924	-4.5612	-1.5306	0.0635	B
Plant Height	1	30	200.54	2.9268	-8.2403	-0.5323	0.1027	B
Plant Height	3	28	237.1	3.0146	-4.8382	0.8387	0.1278	B
Plant Height	7	20	147.38	2.982	2.999	-5.0521	0.1311	A
Plant Height	10	7	41.77	3.2365	-8.6463	2.1427	0.175	B
1000 grain weight	3	18	138.05	3.0462	-1.2993	1.5417	0.1388	B
1000 grain weight	9	10	40.84	2.4755	1.6832	0.3741	0.0593	A
Percent Fertility	2	3	24.31	2.9912	-8.4584	4.7297	0.1773	B
Percent Fertility	6	5	69.43	2.7038	0.5316	12.6577	0.0803	A
Percent Fertility	7	11	76.75	3.0644	-0.5681	12.5456	0.1409	B
Percent Fertility	7	16	108.64	2.8398	3.5337	13.3703	0.0348	A
Yield	10	7	41.77	3.4276	-17.2041	-8.3762	0.0557	B
K^+ content	1	25	172.35	2.9176	0.0159	0.0021	0.0896	A
K^+ content	4	11	59.17	2.9747	-0.0163	-0.024	0.0506	B
Na^+/K^+ ratio	12	6	78.43	3.3466	-0.337	0.2085	0.4202	B
K^+/Na^+ ratio	4	29	239.53	2.5495	-0.4148	-0.4287	0.0126	B
K^+/Na^+ ratio	6	19	144.09	2.4692	0.2319	-0.4175	0.1001	A
Days to maturity	2	3	18.31	2.5457	6.2261	3.8677	0.0138	A
Days to maturity	7	20	133.38	2.6004	-5.2756	1.7872	0.08	B
Total tiller	5	13	96.27	3.4038	1.7669	-0.1869	0.1169	A
Total tiller	10	1	4.01	2.5399	-2.2412	-0.4746	0.0401	B
Effective tiller	5	2	11.98	2.941	-1.035	-0.6042	0.0239	B
Spikelet damage	2	28	230.42	2.5726	-2.1605	-0.15	0.0935	B
Spikelet damage	10	12	78.7	3.0788	-1.0159	-2.3256	0.0012	B
Panicle exsertion	4	14	110.77	2.685	-1.1293	2.9087	0.3876	B

Number of filled grain	1	20	149.76	2.5301	-20.2263	84.2916	0.0951	B
Weight of filled grain	1	24	166.38	2.8231	0.1115	1.9082	0.0532	A
Weight of filled grain	1	30	196.54	3.3741	-0.0557	2.1203	0.0721	B
Number of unfilled grain	3	25	206.01	2.8784	-68.1902	-31.7285	0.0561	B
Rachis weight	1	3	13.42	2.7465	0.1696	-0.028	0.1076	A
Rachis weight	9	17	112.51	2.5237	0.1153	-0.1251	0.1176	A
Rachis weight	12	7	84.06	2.9122	-0.2657	-0.0878	0.0101	B
Primary branching	1	8	74.31	2.8558	0.5441	0.0091	0.1188	A
Primary branching	1	29	192.24	2.6384	-0.2227	0.3794	0.1131	B
Primary branching	4	23	157.97	2.4504	0.2974	-0.2894	0.1175	A
Secondary branching	1	16	116.36	2.9308	-0.3812	1.024	0.1385	B
Secondary branching	1	20	147.76	2.6298	-0.5548	1.002	0.1819	B
Seed length	2	27	202.83	2.6685	-0.2039	0.0312	0.0993	B
Seed length	4	18	132.51	2.9243	-0.1701	0.1613	0.1299	B
Seed length	4	27	197.69	2.4517	-0.1192	0.1993	0.0955	B
Seed breadth	1	33	234.64	3.4193	-0.07	-0.032	0.0627	B
Seed breadth	4	28	198.91	2.7939	-0.0511	0.0355	0.1287	B
Seed breadth	5	6	45.95	2.5379	-0.0634	-0.0444	0.0268	B
total grain no	1	24	166.38	2.5474	47.1477	101.2697	0.0109	A
total grain no	3	10	88.36	2.8327	82.1416	-11.9344	0.1123	A
Weight of total grain	6	3	33.42	3.1588	-0.9323	2.0353	0.2188	B
Weight of total grain	11	2	22.42	2.8964	0.1339	2.4543	0.0593	A
Yield	10	7	41.77	3.4276	-17.2041	-8.3762	0.0557	B
flag leaf length	1	12	93.32	2.857	-0.5575	-2.3407	0.0214	B
flag leaf length	1	22	158.35	3.3534	0.6809	2.3556	0.0322	A
flag leaf length	1	29	192.24	3.3922	-1.3266	1.6102	0.1835	B
flag leaf length	2	28	230.42	2.6207	1.5748	-0.3179	0.1204	A

flag leaf length	7	2	10.86	2.594	-1.2761	1.403	0.1524	B
Weight of flag leaf	3	22	184.02	2.8853	-0.1256	-0.0462	0.0424	B
Weight of flag leaf	5	20	157.93	2.5001	-0.1107	0.0399	0.0998	B
Weight of flag leaf	7	3	34.47	2.4641	-0.1124	-0.0669	0.0232	B
Weight of flag leaf	8	19	161.05	2.8619	-0.1097	0.0299	0.1221	B
Weight of second leaf	1	24	166.38	2.8772	0.0971	0.2114	0.0103	A
Third leaf length	3	26	214.02	3.2294	-3.0585	1.8016	0.1361	B
Third leaf length	8	18	142.52	3.3889	-5.9234	0.103	0.1232	B
Weight of third length	5	8	67.41	2.7766	0.1139	-0.0816	0.0926	A
Weight of third length	9	15	96.11	3.1097	0.1041	-0.2184	0.1173	A
Lower leaf weight	2	27	198.83	3.2571	-0.116	0.0832	0.1145	B
Leaf sheath weight	3	26	214.02	2.9384	-0.3185	-0.0212	0.0752	B
Leaf sheath weight	10	16	123	2.7376	-0.2815	0.0262	0.0811	B
Straw weight	2	27	198.83	3.1549	-2.0628	-1.2434	0.0358	B
Straw weight	3	5	28.54	2.8112	-1.7215	-1.6009	0.0111	B
Total weight	1	31	211.94	3.1143	-3.3956	0.5491	0.124	B
Total weight	8	9	79.81	3.4053	0.5059	4.4599	0.0946	A
Total weight	9	17	122.51	2.546	2.8032	-0.9665	0.09	A
Total weight	10	11	71.68	3.2337	-3.0618	0.4349	0.0997	B
Total weight	10	16	125	2.9096	-2.6599	0.729	0.099	B
Total weight	11	22	159.1	3.421	1.2868	5.5935	0.0151	A
Flowering time	1	30	196.54	7.1348	-1.7709	-9.0605	0.0553	B
Flowering time	2	28	218.42	4.3768	-6.5918	-6.7676	0.0383	B
Flowering time	3	25	210.01	4.2888	-6.1813	-3.1385	0.0755	B
Flowering time	6	5	73.43	5.6337	5.8954	-6.517	0.3076	A
Flowering time	9	18	127.52	3.897	3.2663	-5.9032	0.1552	A

Plant Height	11	7	62.26	3.5606	-3.7527	5.9567	0.1704	B
Percent Fertility	8	9	81.81	3.5356	-6.8614	-11.7423	0.0168	B
Percent Fertility	10	17	137.73	4.8746	8.2761	9.0261	0.0436	A
Yield	1	29	196.24	3.7839	-1.4921	22.6748	0.1183	B
Yield	10	14	92.91	6.2208	26.3737	-4.3708	0.3223	A
Na^+ content	3	6	47.69	6.209	0.0104	0.0011	0.1841	A
Na^+ content	5	13	94.27	4.5317	0.0074	-0.0025	0.1658	A
Na^+ content	6	1	6.01	3.7867	0.0086	-0.001	0.1487	A
Na^+ content	7	19	123.01	3.5335	0.0076	0.0053	0.0341	A
Na^+ content	8	9	79.81	3.5359	0.0065	-0.0057	0.1673	A
Na^+ content	12	16	127.27	3.9088	0.0066	-0.0054	0.1664	A
K^+ content	6	19	144.09	3.7955	0.0066	-0.0124	0.1464	A
Na^+/K^+ ratio	6	4	41.81	5.6366	-0.1528	-0.3525	0.0744	B
Days to maturity	6	5	57.43	5.3167	1.827	-13.0228	0.1187	A
Days to maturity	10	13	84.45	7.0217	-8.1047	-5.9272	0.0585	B
Total tiller	5	7	61.59	4.4569	2.3728	0.1067	0.1414	A
Total tiller	6	1	12.01	8.6293	1.9576	2.224	0.036	A
Total tiller	9	16	105.98	4.7523	0.588	-2.2882	0.1592	A
Total tiller	10	14	94.91	4.8141	-1.6888	-1.178	0.0483	B
Total tiller	12	3	29.61	3.6047	0.7311	-2.7039	0.1537	A
Effective tiller	5	8	67.41	4.4828	1.0706	-0.5819	0.1675	A
Effective tiller	6	1	16.01	5.6983	1.0425	0.9926	0.0369	A
Effective tiller	7	19	121.01	6.2359	-1.4558	-0.2191	0.1533	B
Effective tiller	8	19	163.05	3.5275	-0.9566	0.3632	0.1326	B
Effective tiller	11	2	26.42	3.7768	-0.8698	0.894	0.1714	B
Effective tiller	11	16	99.87	4.4864	1.093	-0.6329	0.1911	A
Spikelet damage	1	7	59.09	3.5458	-0.4295	3.4072	0.1077	B
Number of filled grain	1	30	196.54	3.6572	-1.0415	97.923	0.0871	B
Number of filled grain	3	10	84.36	3.8207	77.0456	-2.7066	0.1611	A

Number of filled grain	8	5	46.36	6.4065	-158.744	-82.8383	0.0108	B
Number of filled grain	10	17	137.73	4.0647	60.2477	63.3632	0.0322	A
Weight of filled grain	10	13	90.45	5.5849	2.4211	-0.2428	0.2628	A
Number of unfilled grain	2	5	48.07	4.0432	-41.1944	-96.2951	0.0128	B
Number of unfilled grain	7	21	149.47	3.6356	-71.5948	9.8537	0.1745	B
Weight of unfilled grain	1	30	196.54	3.542	-0.066	0.2127	0.1376	B
Weight of unfilled grain	3	16	130.07	3.4088	0.2559	-0.0015	0.132	A
Weight of unfilled grain	3	25	208.01	4.8415	-0.2219	-0.1489	0.0658	B
Weight of unfilled grain	7	20	137.38	3.912	-0.1827	-0.1572	0.0434	B
Primary branching	1	16	114.36	7.0888	-0.5974	0.4452	0.372	B
Primary branching	3	6	47.69	4.81	-0.1401	-0.6845	0.0157	B
Primary branching	3	25	208.01	4.6321	-0.4809	0.3365	0.2471	B
Secondary branching	1	28	186.25	5.509	-1.2253	1.0518	0.3206	B
Secondary branching	3	25	204.01	5.5279	-1.1876	0.8258	0.298	B
Seed length	10	16	127	4.9064	0.2641	-0.1064	0.2172	A
Seed breadth	9	10	40.84	4.0228	0.0734	0.0362	0.0571	A
Panicle length	1	16	110.36	4.4509	-1.3662	0.2093	0.1587	B
Panicle length	1	28	186.25	5.9995	-2.433	0.5055	0.2768	B
Panicle length	2	12	93.02	4.148	-0.5017	1.4495	0.1296	B
total grain no	1	30	196.54	5.0061	-40.2676	145.8781	0.1765	B
total grain no	2	10	89.16	4.0609	-106.56	-46.2207	0.0696	B
total grain no	3	22	184.02	4.2208	-122.559	-19.4087	0.1171	B
total grain no	6	3	31.42	4.6939	-85.1152	141.1726	0.283	B
Weight of total grain	1	29	196.24	3.8035	-0.2098	2.2249	0.09	B

Weight of total grain	10	7	41.77	3.9188	-1.8343	-0.6641	0.0716	B
Weight of total grain	10	13	90.45	5.666	2.5434	-0.6084	0.288	A
Yield	1	29	196.24	3.7839	-1.4921	22.6748	0.1183	B
Yield	10	14	92.91	6.2208	26.3737	-4.3708	0.3223	A
flag leaf length	2	4	31.96	3.5814	-0.5677	2.5235	0.2945	B
Weight of flag leaf	1	21	157.73	5.2619	0.1481	0.1822	0.0406	A
Weight of flag leaf	1	29	196.24	4.7135	-0.0415	0.1715	0.1583	B
Weight of flag leaf	7	1	2.01	3.5084	-0.1341	-0.0226	0.0747	B
Length of second leaf	10	4	26.62	3.6381	2.1319	1.8797	0.0133	A
Length of second leaf	10	15	103.99	5.0166	3.7515	-0.7772	0.2349	B
weight of second leaf	8	4	35.15	3.6376	-0.2109	-0.2128	0.0105	B
Third leaf length	2	3	18.31	5.0238	4.2377	0.6598	0.1247	A
Third leaf length	2	20	161.38	4.7369	-3.5437	-0.0513	0.1361	B
Weight of third length	3	25	210.01	7.9655	-0.2668	0.0235	0.2722	B
Weight of third length	7	4	38.48	9.5532	-0.261	-0.2772	0.0172	B
Weight of third length	8	20	175.82	4.0909	-0.1402	-0.1128	0.03	B
Lower leaf weight	1	31	203.94	4.5145	-0.1807	-0.0157	0.1085	B
Lower leaf weight	3	4	21.89	3.5248	-0.2086	-0.0656	0.0241	B
Lower leaf weight	3	28	237.1	5.4814	-0.1522	0.0889	0.199	B
Lower leaf weight	8	6	66.66	3.7923	-0.0974	0.1385	0.1286	B
Leaf sheath weight	2	28	230.42	5.1721	-0.1056	-0.5703	0.0342	B
Leaf sheath weight	12	7	80.06	4.0199	-0.8883	-0.0751	0.0637	B
Stem weight	2	1	2.01	4.036	0.5268	-0.1691	0.1466	A

Stem weight	2	28	230.42	3.5915	0.0223	-0.6632	0.0603	A
Stem weight	3	25	212.01	5.4004	-0.6375	-0.347	0.0784	B
Straw weight	7	12	81.43	3.8078	-0.1611	-3.3323	0.0808	B
Straw weight	8	6	62.66	5.671	-1.6319	3.491	0.2222	B
Straw weight	9	16	105.98	5.274	2.0582	-3.3142	0.2399	A
Straw weight	10	15	109.99	3.6636	-1.659	-2.7091	0.0194	B
Total weight	2	24	178.75	3.8752	-3.1856	-0.4356	0.1019	B
Total weight	3	5	28.54	4.4168	-3.278	-2.5323	0.0281	B
Total weight	3	28	237.1	4.3921	-2.8436	2.5217	0.1835	B

Appendix C

C. 1. Effect of QTLs on phenotypes based on one way ANOVA.

Stage	QTL name	Donor Allele	Phenotype	Chr No.	Marker	P value
Seedling	Stomatal Conductance (SC_1_I)	IR29	Stomatal Conductance	4	SF45	0.000371 ***
Seedling	Stomatal Conductance (SC_2_I)	IR29	Stomatal Conductance	5	SF514	0.01672 *
Seedling	Root length (RL_H)	Horkuch	Root length	2	SF231	0.001228 **
Seedling	K content (K_H)	Horkuch	K content	3	SF332	0.0004237 ***
Seedling	Total chlorophyll (total chl_H)	Horkuch	Total chlorophyll	7	SF729	0.02458 *
Reproductive	Repro_FGN_2_I + SF1	IR29	FGN	10	SF1023	0.03732 *
Reproductive	TLL_1_H	Horkuch	TLL	2	SF228	0.03876 *
Reproductive	Flowering time	IR29	Flowering time	6	SF66	0.03359 *
Reproductive	Seed length	IR29	Seed length	10	SF1022	0.00261 **
Reproductive	FLW_2	Horkuch	FLW_2	7	SF71	0.0008932 ***
Reproductive	Effective tiller	Horkuch	Effective tiller	7	SF726	0.002948 **
Reproductive	Repro_PB	IR29	Yield	1	SF0111	0.0449 *
Reproductive	FLW_1	Horkuch	Yield	1	SF0139	0.02699 *
Reproductive	Culm strength	Horkuch	Culm strength	3	SF335	0.004892 **
Seedling	Total chlorophyll (total chl_H)	Horkuch	SES	7	SF729	0.004927 **
Seedling	Total chlorophyll (total chl_H)	Horkuch	Thousand grain weight (reproductive)	7	SF729	0.00289 **
Seedling	Total chlorophyll (total chl_H)	Horkuch	Seed breadth (reproductive)	7	SF729	0.02607 *
Seedling	Total chlorophyll (total chl_H)	Horkuch	Total tiller (reproductive)	7	SF729	0.04625 *
Seedling	Total chlorophyll (total chl_H)	Horkuch	Effective tiller (reproductive)	7	SF729	0005481 ***
Seedling	Total chlorophyll (total chl_H)	Horkuch	Flag leaf weight (reproductive)	7	SF729	0.0001399 ***
Seedling	Total chlorophyll (total chl_H)	Horkuch	Second leaf weight (reproductive)	7	SF729	0.005971 **

Seedling	Total chlorophyll (total chl_H)	Horkuch	Third leaf weight (reproductive)	7	SF729	0.0004423 ***
Seedling	Total chlorophyll (total chl_H)	Horkuch	Lower leaf weight (reproductive)	7	SF729	0.001736 **
Seedling	Total chlorophyll (total chl_H)	Horkuch	Straw weight (reproductive)	7	SF729	0.01006 *
Seedling	Total chlorophyll (total chl_H)	Horkuch	Total weight (reproductive)	7	SF729	0.002535 **

*The asterisks denotes significant correlation with the trait to cross direction (*** for P < 0.001, ** for P <0.01 and * for P <0.05)*

C. 2. Selected plant list with QTL name* and numbers. All these plants were used in seedling stage screening.

QTL Names	Plant ID	Total QTLs	Seedling QTLs	Seedling QTLs	Reproductive QTLs	Reproductive QTLs Names
SES	I_023	8	5	K^+/Na^+, SES, total chlorophyll, SC, SDW	3	TLL, SeB, FLW
	I_154	8	3	K^+/Na^+, SES, total chlorophyll	5	FGN, PB, SeB, TLL, CS
	I_126	7	3	K^+/Na^+, SES, total chlorophyll	4	FGN, SeB, FLW, ET
	I_066	7	3	K^+/Na^+, SES, total chlorophyll	4	FGN, FGN+SF, SF, SeL
	I_175	7	3	SES, total chlorophyll, RDW	4	PB, TLL, ET, CS
K^+/Na^+	I_080	7	5	K^+/Na^+, (2), SES, SC, RRWC	2	FGN, PB
	I_045	9	5	K^+/Na^+, (2), SC, RL	4	TLL (2), SeB, CS
	I_061	9	4	K^+/Na^+, (2), SC, RRWC	5	SF, FLW, TLL, CS, PD

	I_146	9	3	K^+/Na^+, SES, SC	6	FGN, SeB, TLL, CS, DF, PD
Total Chl	I_184	9	4	K^+/Na^+, SES, total chlorophyll, SC	5	FGN+SF, PB, SeL, SeB, TLL
	I_040	10	6	K^+/Na^+, SES, total chlorophyll, SC (2)	4	TLL, FLW, ET, CS
	I_090	11	7	K^+/Na^+, K^+, total chlorophyll, SC (2), RDW, RRWC	4	FGN, ET, FLW, PD
SC	I_155	10	5	K^+, SES, total chlorophyll, SC (2)	5	FGN, SF, FLW, DF, PH
	I_043	9	4	K^+/Na^+, SC (2), SDW	4	ThGW, FGN+SF, SeL, FLW
	I_054	9	3	K^+/Na^+, SC (2)	6	ThGW, PB, SeB, TLL, FLW, CS
	I_022	10	5	K^+/Na^+, SC (2), RL	5	ThGW, PB, SeL, FKW, PH
	I_195	10	4	SC (2), SDW, RL	6	FGN+SF, SeL, FLW, TLL, DF, PD
SDW/RDW	I_196	9	4	SES, total chlorophyll, SC, SDW	5	FGN, SF, FLW, DF, PH
	I_151	9	5	SES, SC, SDW, RRWC	4	ThGW, FGN+SF, SeL, FLW
	I_050	9	3	K^+/Na^+, K^+, SDW	6	ThGW, PB, SeB, TLL, FKW, CS

	I_176	7	2	SC, SDW	5	ThGW, PB, SeL, FKW, PH
	I_026	9	3	SC (2), SDW	6	FGN+SF, SeL, FKW, TLL, DF, PD
	I_119	9	4	K^+, SC (2), SDW	5	FGN+SF, SF, FKW, ET, PD
THGW	I_032	8	3	K^+/Na^+, K^+, SC	5	ThGW, FGN+SF, FGN, TLL, CS
	I_142	7	3	K^+/Na^+, RL, RRWC	4	ThGW, FGN+SF, TLL, PH
	I_182	7	3	SC (2), SDW	4	ThGW, FGN, PB, FLW
	I_131	9	4	K^+/Na^+, SC, RL, RDW	5	ThGW, SeB, ET, TLL, FLW

C.3. Selected F_5 plant list for reproductive screening with QTL names and numbers. These plants were also used in seedling stage screening.

Plant ID	Total QTLs	Seed_QTLs	Names	Repro_QTLs	Names
I_014	13	6	K^+/Na^+, K^+, SC (2), RDW, RL	7	FGN+SF, SeB, TLL (2), PH, CS, PD
I_062	12	6	K^+/Na^+, K^+, SC (2), RDW, RL	6	ThGW, FGN+SF, SeL, ET, TLL, FLW
I_124	12	4	SES, Total chlorophyll, RL, SC	8	ThGW, SF, SeL, ET, TLL (2), FKW, CS
I_170	12	4	K^+/Na^+, SDW, SC, RRWC	8	FGN+SF, FGN, SF, PB, SeL, SeB, FLW, DF
I_074	11	5	SES, K^+/Na^+, K^+, Total chlorophyll, SDW	6	FGN+SF, SeL, SeB, FLW, FKW, PD
I_033	10	4	K^+/Na^+, K^+, SC (2)	6	FGN+SF, SeL, TLL, PH, FLW, CS
I_037	10	5	SES, K^+/Na^+, Total chlorophyll, SC, RDW	5	FGN, SF, SeL, SeB, ET
I_071	10	3	K^+/Na^+, K^+, SC	7	FGN+SF, FGN, SeL, SeB, TLL, FLW, CS
I_143	10	2	K^+/Na^+, K^+	8	FGN+SF, FGN, SeL, SeB, PB, TLL, CS, DF
I_041	9	4	K^+/Na^+, K^+, RRWC, RL	5	ThGW, SeB, PB, TLL, DF

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