

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 F₃, 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 F₁ 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 [F₁(*IR29* / *Horkuch*)]). F₁ plants were confirmed by SSR marker RM493 at the University of Dhaka, Bangladesh and were advanced to F₂ in Plant Physiology Division of BRRI (Bangladesh Rice Research Institute). About 500 F₂ progenies from the cross were planted in the field at BRRI to be advanced to the generation F₃.

3.1.1.2 Isolation of F₁ DNA from plant tissue:

178 plants were selected randomly. The leaves of these F₁ 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 1 μ L 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).
- 1 μ L 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/ μ L, 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 \times and 20 \times .
- 1 μ L 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 (μ L)	180 reactions (μ L)	Final Concentration
PCR buffer (10 \times)	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/ μ L)	0.5	90.0	0.33 μ M
Reverse primer (50ng/ μ L)	0.5	90.0	0.33 μ M
Taq DNA polymerase	0.5	90.0	0.75U
dH ₂ O	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, 5.5 μ L 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 ddH₂O.

Tube	DNA(50ng/ μ L)	DMSO (20%)	ddH ₂ O	Total Volume
Sample tube	1.0 μ L	2.0 μ L	6.5 μ L	9.5 μ L
Control tube	0.0 μ L	2.0 μ L	7.5 μ L	9.5 μ L

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.

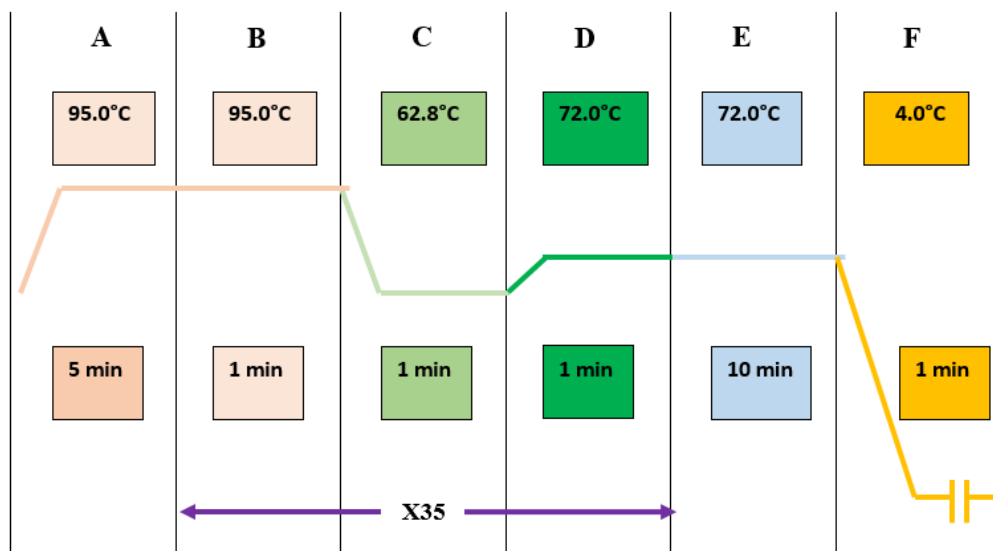


Figure 3.1: An illustration of PCR Cycle. A) Initial Denaturation, B) Denaturation, C) Annealing, D) Elongation, E) Final Extension. For different primers different anneal temperatures and elongation times were employed.

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 F₂ DNA

Sprouted F₂ 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/ μ L suspended in TE were submitted.

3.1.3 Genotyping of F₂ 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.

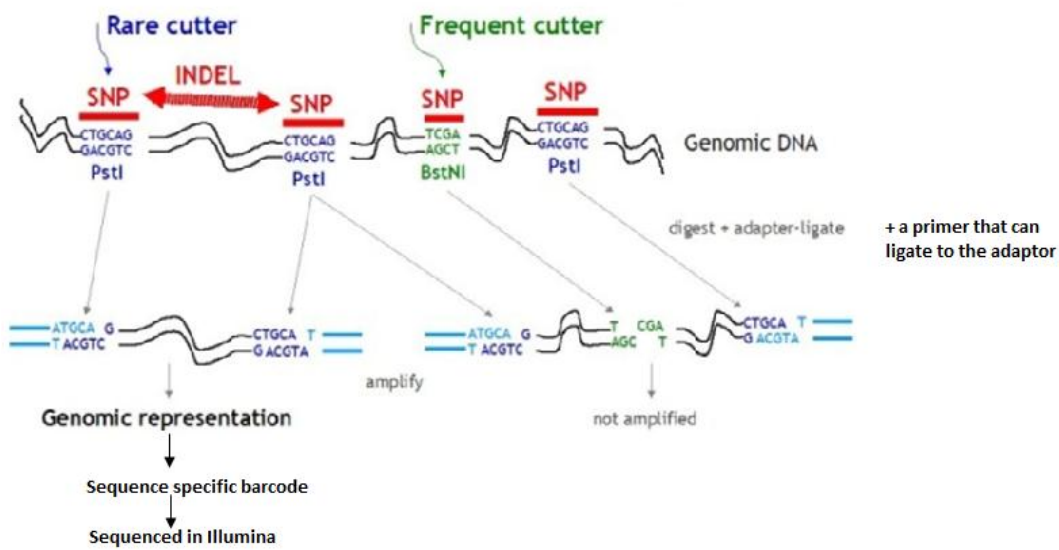


Figure 3.2: DArT based SNP genotyping (DArTseq) method

DNA samples of 174 F₂ 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
3763032 F 0--15:T>C	37	50	87	1.04252873563218	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 F₂ 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 (174), 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																	
*SF01	A	H	H	A	H	B	H	H	B	B	H	H	H	B	H	H	H	H	H
*SF02	A	H	H	A	H	B	A	H	B	B	H	H	H	H	H	H	H	H	H
*SF03	A	H	H	A	H	B	A	H	B	B	H	H	H	H	H	H	H	H	H
*SF04	A	H	H	A	H	B	A	H	B	B	A	H	H	H	H	H	H	H	H
*SF05	A	H	H	A	H	B	A	H	H	B	A	H	H	H	H	H	H	H	H
*SF06	A	B	H	A	H	B	A	H	H	B	A	H	H	H	H	H	H	H	H
*SF08	A	B	H	A	H	B	A	H	H	B	A	H	H	B	H	B	H	H	H
*SF09	A	B	A	A	H	B	A	H	H	B	A	H	H	B	A	H	B	H	H
*SF011	A	B	H	A	H	H	A	H	H	B	A	H	H	H	H	H	H	B	H
*SF012	A	B	H	A	H	A	A	H	B	H	A	H	H	H	H	H	B	H	H
*SF013	A	B	H	A	H	A	A	A	B	H	A	H	H	H	H	H	B	A	A
*SF014	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	H	B	A	A
*SF015	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	H	B	A	A
*SF016	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	H	B	A	A
*SF017	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	H	B	A	A
*SF018	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	H	B	A	A
*SF019	A	B	H	A	H	A	A	A	B	A	A	H	H	H	H	B	B	A	A
*SF020	A	B	H	H	H	A	A	A	B	A	A	H	H	H	H	B	B	A	A
*SF021	A	B	H	H	A	A	A	A	B	A	A	A	A	A	A	H	B	A	A
*SF023	A	B	A	H	A	A	A	A	B	A	A	A	A	A	A	H	B	A	A
*SF024	H	B	A	H	A	A	H	A	B	A	H	H	A	A	H	B	A	A	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°C at night and 34.9°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 (CO₂) 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 CO₂ 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:

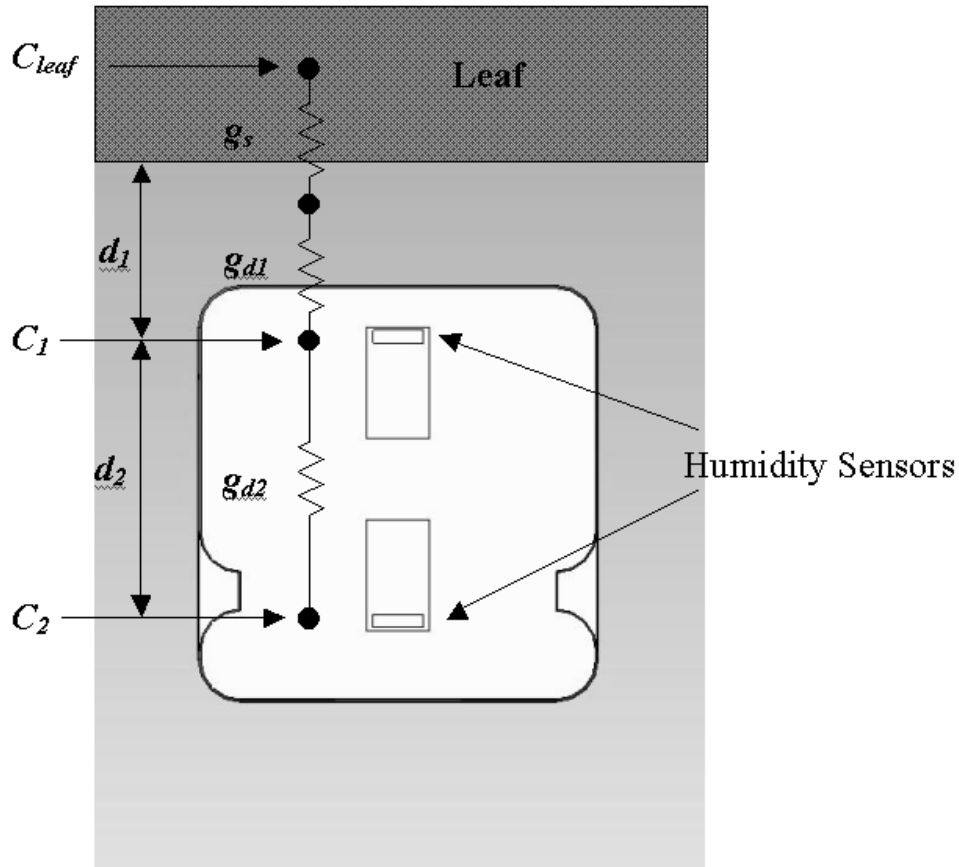


Figure 3.6: Measurement of stomatal conductance by a porometer

The parameters listed above represent the following:

C_{leaf} The mole fraction of vapor inside the leaf

C_1 The mole fraction of vapor at node 1

C_2 The mole fraction of vapor at node 2

g_s Stomatal conductance of the leaf surface

g_{d1} Vapor conductance of the diffusion path between leaf surface and node 1

g_{d2} Vapor conductance of the diffusion path between node 1 and node 2

d_1 Distance between the leaf surface and the first humidity sensor

d_2 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°C for 24 hours and weighed (after being cooled down) to determine dry weight (DW).

Calculation:

$$RRWC = [(W-DW) / (TW-DW)] \times 100$$

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: $(0.00802 \times A_{663})$

Chlorophyll b measurement: $(0.0202 \times A_{645})$

Total Chlorophyll: $[(0.00802 \times A_{663}) + (0.0202 \times A_{645})]$

Total amount of chlorophyll content: $[(0.00802 \times A_{663}) + (0.0202 \times A_{645})] \times V/W$

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 F₃ 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 F₃ 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 exertion, 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 exertion- The exertion of the panicle above the flag leaf sheath after anthesis was classified as:

- well exerted- the panicle base appeared way above the collar of the flag leaf blade.
- moderately well exerted- the panicle base was above the collar of the flag leaf.
- just exerted- the panicle base coincided with the collar of the flag leaf.
- partly exerted- 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 exertion was scored in percentages, from 0 to 100% exertion.

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.

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

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.

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

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

$$(\text{Spikelet/panicle}) \times (\text{Panicle per plant}) \times (\text{1000 grain weight}) \times (\% \text{fertility})$$

$$\text{Yield} = \frac{\text{—————}}{1000 \times 100}$$

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.

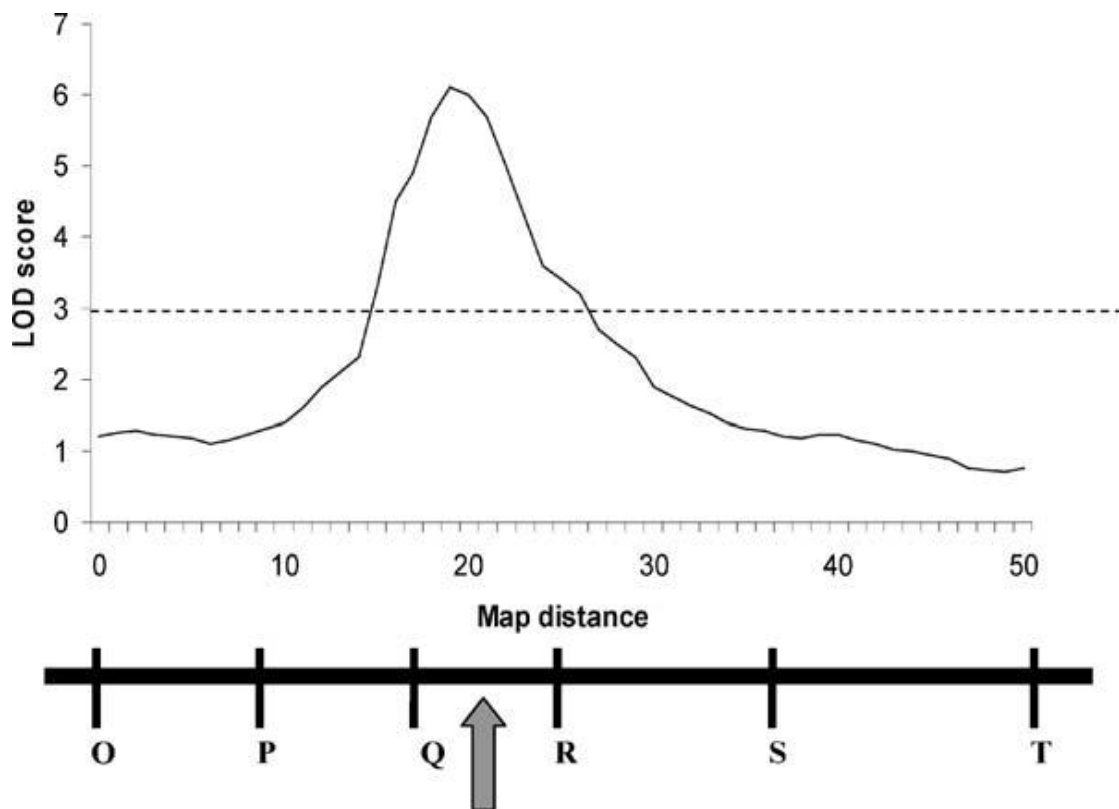


Figure 3.7: Hypothetical output showing a LOD profile for chromosome 4. The dotted line represents the significance threshold determined by permutation tests. The output indicates that the most likely position for the QTL is near marker Q (indicated by an arrow). The best flanking markers for this QTL 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 Smapqtl 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 F₅. In this study, both molecular and physiological analyses were done to confirm the presence of desired QTLs in specific F₅ 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, R² 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	ATATGGATACAGGCCAGCA TTGG CTGAGCCATCAAGTGCCTTT CC	TLL_1_H	2	25.41	<i>Horkuch</i>
RM22073	AAGAAGTTCTGCCTCAGCCA GTTTCG CCTCCGTCGTCTCCTCCACT ATCG	Tch_H	7	27.53	<i>Horkuch</i>
drtSSR 3452265	AGCCACTCAGCAATAGGAC ATTTTTGCCATCCCTCTT	RL_H	2	28.48	<i>Horkuch</i>
drtSSR 3050109	CTCCCCTAGCTTAGGTCATA G AAGGACAATTTTCAGAAACC AT	SC_2_I	5	15	<i>IR29</i>

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 15 μ l reaction mixture. The PCR reaction mix contains PCR buffer, 25mM MgCl, 1mM of each dNTPs, Taq polymerase, 2 μ l (05 picomole/ μ l) of each reverse and forward primers and 50ng/ μ l 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 F₅ 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 F₅ 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' ($12 \text{ dS m}^{-1} \text{ NaCl}$); so a total of $(27+1+1) \times 16 = 464$ 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 12 dS m^{-1} salt solution are seen at left and right sides, respectively. The picture was taken 14 days after applying 12 dS m^{-1} salt.

Table 3.7: Experimental design of seedling screening at F₅.

Plant type	Number	Condition	
		Control	Stress
<i>Horkuch</i>	1	8 replicates	8 replicates
<i>IR29</i>	1	8 replicates	8 replicates
F ₅ progeny	27	8 replicates	8 replicates

3.2.2.3 Physiological screening of selected F₅ plants: Reproductive stage

3.2.2.3.1 Plant selection

As described previously (section 3.7.2.1), F₅ 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 BRRRI (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 F₅ plants, *Horkuch* parent (tolerant check) and *IR29* parent (sensitive check) were taken. So, a total $(10+1+1) \times 6 = 72$ 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 F₅ plants. As one pot contains a single plant and so each pot could be considered as one experimental unit.

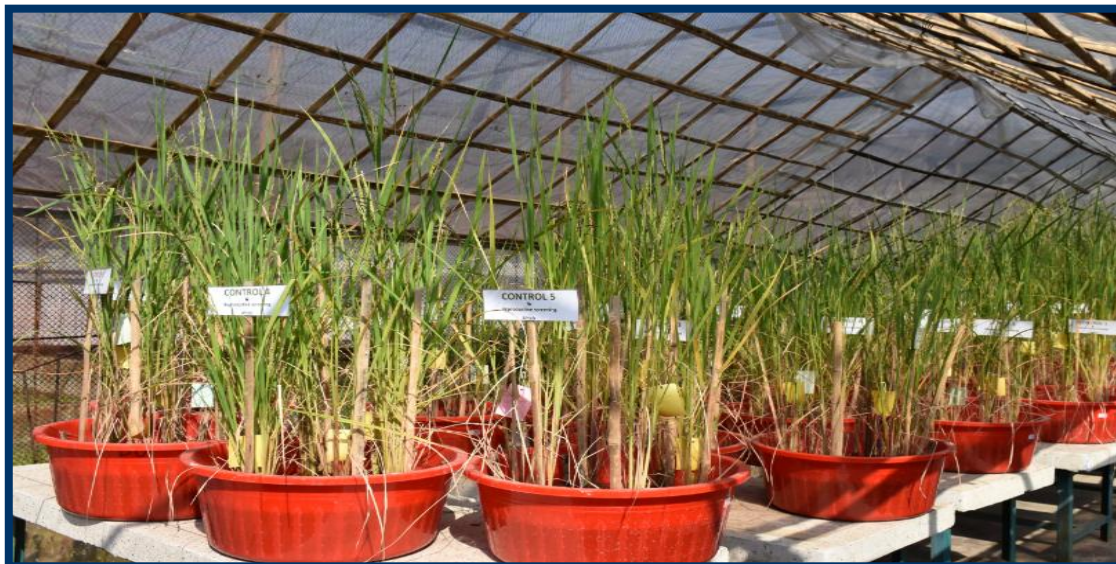


Figure 3.10: Physiological screening of F₅ progeny under 10dSm⁻¹ salt stress

Table 3.8: Experimental design of reproductive screening of F₅ progeny.

Plant type	Number	Condition	
		Control	Stress
<i>Horkuch</i>	1	3 replicates	3 replicates
<i>IR29</i>	1	3 replicates	3 replicates
F₅ 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.

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