CHARACTERIZATION OF THE COASTAL RICE LANDRACES OF BANGLADESH WITH MICROSATELLITE DNA MARKERS AND SALT TOLERANCE DETERMINANTS

M. Phil. Thesis

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BY

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CERTIFICATE

This is to certify that Md. Rafiqul Islam has conducted thesis work entitled "Characterization of the Coastal Rice Landraces of Bangladesh with Microsatellite DNA Markers and Salt Tolerance Determinants" under my supervision for partial fulfillment of the degree of Master of Philosophy in Biochemistry and Molecular Biology from the University of Dhaka. The work or any part there of has not been submitted any where for any other degree.

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Dedication

This is dedicated to my beloved wife Most. Saida Akter, who continues to be my greatest inspiration, infinite patience and continued support to my academic carrier.

Md. Rafiqul Islam

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CONTENTS

		Page
Acknowledgements		i-ii
Contents		iii-vii
List of figures		viii-x
List of tables		xi
List of abbreviations		xii-xv
Abstract		xvi
Chapter 1: Introduction		**01-08**
1.1	Rice: A grain food for mitigating world hunger	01
1.2	Food security remains a challenge before Bangladesh	01
1.3	Soil salinity: A serious global threat to rice production	02
1.4	Saline zones of Bangladesh and rice cultivation in those areas	03
1.5	Demand for the improvement of salt tolerant crop	05
1.6	Incorporation of salt tolerant trait into modern varieties	06
1.7	Rice germplasm and its assessment by markers	06
1.8	Salt stress inducible genes: implication for tolerance in rice	07
1.9	Objectives of the present research work	08
Chapter 2: Review Of Literature		**09-39**
2.1	Soil salinity	09
2.2	Adverse effects and consequences of NaCl on plants	09
	2.2.1 Shortage of water with consequences	09
	2.2.2 Toxicity of ion and protection mechanism	11
	2.2.3 Altered nutritional status	12
2.3	Mechanisms of salt tolerance	12
	2.3.1 Control at the whole plant level	13
	2.3.2 Control at the organelle level	13
	2.3.3 Control at the molecular level: ion homeostasis	14
2.4	Proposed six-phase breeding strategy for enhancing salt tolerance in crop plants	15
2.5	Progress in gene discovery for salt tolerance	17
	2.5.1 Gene isolation	18

2.5.2 Analysis of candidate genes 19
2.5.3 Plant biotechnology for enhancing salt tolerance 20
2.6 Current advancement in breeding for salt tolerance 21
2.7 Limitation of traditional breeding for salt tolerance 22
2.8 Molecular markers 22
2.8.1 Some commonly used molecular or DNA markers 23
2.8.1.1 Restriction fragment length polymorphism (RFLP) 23
2.8.1.2 Amplified restriction fragment length polymorphism (AFLP) 25
2.8.1.3 Random amplified polymorphic DNA (RAPD) 26
2.8.1.4 Microsatellites/simple sequence repeat polymorphisms (SSRPs) 27
2.8.1.5 Single nucleotide polymorphisms (SNPs) 29
2.8.1.6 Expressed sequence tags (ESTs) 30
2.8.2 Primary applications of DNA markers in plant breeding 30
2.8.3 Marker assisted selection (MAS) 30
2.8.3.1 Toward the development of MAS for salinity tolerance 31
2.8.3.2 Identification of markers linked to salt tolerance locus in Pokkali. 32
2.9 Genome coverage and distribution of SSRs/ Microsatellite primers along rice chromosomes. 32
2.10 Techniques for amplification of the target nucleotide sequences 33
2.10.1 Polymerase chain reaction (PCR) 33
2.10.2 Reverse transcriptase-PCR(RT-PCR) 35
2.11 Genes found to be linked with abiotic stress tolerance 36
2.11.1 Late embryogenesis abundant (lea) or lea related genes 36
2.11.2 Calmod (Z12828) 38
2.11.3 SalT 38
2.11.4 SOD 39

Chapter 3: DNA fingerprinting of traditional landraces using microsatellite primers **40-53**

3.1 Materials and Methods **40-48**

3.1.1 Plant genetic resources 40

3.1.1.1 Collection of seeds 40

3.1.1.2 Varieties selected for DNA fingerprinting with microsatellite primers 42

3.1.2 Isolation and quantification of plant genomic DNA 42

3.1.3 Rapid isolation and purification of high-activity Taq DNA polymerase 43

3.1.3.1 Checking enzymatic activity and optimization of stock dilution 43

3.1.3.1.1 Agarose gel run to detect any contaminated DNA 43

3.1.3.1.2 PCR with purified Taq DNA polymerase 43

3.1.4 Polymerase chain reaction (PCR) using microsatellite/ SSR primers 44

3.1.4.1 Preparation of dNTPs mixture 44

3.1.4.2 Dilution of the template DNA 44

3.1.4.3 Microsatellite/SSR primers and their dilution 44

3.1.4.4 Optimization of the annealing temperature of some primers 45

3.1.4.5 Hot start PCR 45

3.1.5 Visualization of the amplified PCR product 46

3.1.5.1 Agarose gel electrophoresis 46

3.1.5.2. Denaturing sequencing gel electrophoresis 46

3.1.5.3 Non-denaturing polyacrylamide gel electrophoresis 46

3.1.6 Gel documentation for polymorphic band patterns 46

3.1.6.1 Gel processing after electrophoresis 46

3.1.6.2 Image capturing 47

3.1.7 Scoring for polymorphic bands 47

3.1.8 Cluster analysis 48

3.2 Results **48-52**

3.2.1 Enzymatic activity checking of DNaseI treated Taq DNA polymerase 48

3.2.2 Annealing temperature optimization of some SSR primers 49

3.2.3 DNA fingerprinting of LRs using microsatellite primers 50

3.3 Discussion **53**

Chapter 4: DNA fingerprinting of traditional landraces using primers homologous to stress-related genes **54-64**

4.1 Materials and Methods **54-56**

4.1.1 Rice varieties selected for DNA fingerprinting with gene specific primers 54

4.1.2 PCR using primers homologous to stress-related genes 54

4.1.2.1 Gene specific primers 55

4.1.2.2 Restriction digestion of PCR amplified products 55

4.1.2.2.1 Restriction enzymes used for digestion 55

4.1.2.2.2 Model digestion reaction 56

4.2 Results **57-63**

4.2.1 DNA fingerprinting using primers linked to stress-related genes 57

4.2.2 Restriction digestion of the amplified product using primers homologous to the genes lea3, Calmod and salT 60

4.3 Discussions **64**

Chapter 5 : Expression study of some stress-linked genes under 100mM salt stress **65-81**

5.1 Materials and Methods **65-71**

5.1.1 Study of specific gene expression 65

5.1.1.1 Rice varieties selected for gene expression study 65

5.1.1.2 Application of salt stress on 18 days old rice seedlings 65

5.1.2 Isolation of total RNA after 100mM salt stress using TRIZOL reagent 68

5.1.3 One step RT-PCR with gene specific primers (GSP) 69

5.1.3.1 Gene specific cDNA primers 69

5.1.3.2 Designing of primers 69

5.1.3.3 RT-PCR (Reverse transcriptase-Polymerase chain reaction) 70

5.1.3.3.1 Important parameters for consideration during RT-PCR 70

5.2 Results **71-80**

5.2.1 Recovery responses of different traditional landraces at seedling stage along with Pokkali and IR29 71

5.2.2 RNA isolation and quantitation 72

5.2.3 Expression of oslea3 gene in seedling roots and leaves of a salt tolerant, Pokkali and a salt sensitive variety, IR29 exposed to salt stress 73

5.2.4 Expression of oslea3 gene in some traditional landraces 75

5.2.4.1 Expression in root 76

5.2.4.2 Expression in leaf/shoot 78

5.2.4.3 Constitutive expression of G3PDH gene 78

5.2.5 Expression of Calmod, salT and SOD genes in roots and leaves of selected traditional landraces at seedling stage 79

5.3 Discussions **81**

Chapter 6: Conclusion 82

Chapter 7: References 83-102

Appendix A **a-w**

Appendix B **x**

Appendix C **y-ww**

Appendix D **ww**

Appendix E **xx-yy**

LIST OF FIGURES

FIG. NO.	NAME OF THE FIGURE	PAGE NO.
Fig. 1.1	Trends in Bangladesh population, rice area, production and yield, 1973-2002	01
Fig: 1.2	Map showing variations in soil salinity levels in coastal areas of Bangladesh	04
Fig. 2.1	Schematic representation of radial movement of water through the root from the soil to the xylem	10
Fig. 2.2	Signaling pathways that regulate the expression and activities of ion transporters to maintain a low cytoplasmic concentration of Na^+ under salt stress	15
Fig. 2.3	Proposed six-phase breeding program for enhancing salt tolerance in crop plants	16
Fig. 2.4	Schematic diagram showing restriction fragment length polymorphism procedure	24
Fig. 2.5	Schematic diagram illustrating AFLP analysis	25
Fig. 2.6	The flow diagram illustrations CA/TG dinucleotide repeat polymorphisms	28
Fig. 2.7	The polymerase chain reaction	33
Fig. 2.8	Schematic representation of various methods for the amplification of RNA by RT-PCR	36
Fig. 3.1	Enzymatic activity of isolated Taq DNA polymerase for different dilutions	48
Fig. 3.2	Annealing temperature optimization of the SSR primer RM 221	50
Fig. 3.3a	Amplified DNA of traditional landraces, along with two MV, using microsatellite primer RM332 after resolution in 4% agarose gel	51
Fig. 3.3b	Amplified DNA of traditional rice cultivars from the southwest region using microsatellite primer RM172 after resolution in 6% polyacrylamide gel	51

Fig. 3.4	Dendogram of 16 traditional landraces along with Pokkali, IR65195, IR29 and IR36 using 60 microsatellite primers	52
Fig. 4.1a	Restriction enzyme (*Bgl*II) position within amplified product using the primer Calmod 5′3′	55
Fig. 4.1b	Restriction enzyme (*Xho*I) position within amplified product using the primer Calmod 5′3′	55
Fig. 4.1c	Restriction enzyme (*Hae*III) position within amplified product using the primer LEA3 5′3′	56
Fig. 4.1d	Restriction enzyme (*Taq*I) position within amplified product using the primer LEA3 5′3′	56
Fig. 4.2	DNA fingerprinting of 36 cultivars using the primer (SODAB026724) homologous to stress related gene Sod (superoxide dismutase)	59
Fig. 4.3	DNA fingerprinting of 36 cultivars using the primer (SalT5′3′) homologous to stress related gene salT	60
Fig. 4.4a	Restriction digestion *(TaqI)* of the amplified product of traditional landraces using primer LEA3 5′3′	62
Fig. 4.4b	Restriction digestion *(Hae*III*)* of the amplified product of traditional landraces using primer LEA3 5′3′	62
Fig. 5.1	Salt stress (100 mM) responses of some selected traditional landraces	71
Fig. 5.2	Shoot RNA samples after electrophoresis on 1.5% agarose gel	72
Fig. 5.3	RT-PCR of Pokkali root RNA using LEA3 NC primer.	73
Fig. 5.4	RT-PCR of Pokkali leaf RNA using G3PDH primer and LEA 3 NC primer	74
Fig. 5.5	RT-PCR of IR29 shoot RNA using G3PDH primer and LEA3 NC primer	75
Fig. 5.6	RT-PCR of root RNA with the primer LEA3 NC. 4hrs control samples (L2-11) and 4 hrs stressed samples (L13-22)	77

Fig.5.7	RT-PCR with stressed root RNA samples using LEA3 NC primer. 24hrs stressed samples (L2-11) and 6 days stressed samples (L13-22)	77
Fig. 5.8	RT-PCR with stressed leaf RNA samples using LEA3 NC primer. 24hrs stressed samples (L2-11) and 6 days stressed samples (L13-22)	78
Fig. 5.9	RT-PCR with the primer G3PDH. Leaf RNA of Boilam (L 2-10) and Root RNA of Boilam (L12-20)	79
Fig. 5.10	RT-PCR with 4hr control and stressed root RNA samples using the primer SalT NC. Control (L1-10) and stressed (L12-21)	80

LIST OF TABLES

TAB. NO.	NAME OF THE TABLE	PAGE NO.
Tab. 2.1	Resources for gene discovery in relation to molecular breeding	18
Tab. 3.1	Properties of experimental rice varieties	40
Tab. 4.1	Amplified products using gene specific primers and suitable restriction enzymes for digestion	56
Tab. 4.2	Primers, homologous to stress-related genes, used to check polymorphism in rice landraces	58
Tab. 4.3	Restriction digestion of the amplified product to find polymorphism	61
Tab. 4.4	Digestion of LEA3 5′3′ amplified product with *Taq*I and *Hae*III	63
Tab. 5.1	Constituents of stock solution	66
Tab. 5.2	Composition of nutrient culture solution	67
Tab. 5.3	Expression profile of oslea3 gene in leaf and root of some landraces at different time point	76
Tab. 5.4	Expressional study with some gene specific primers	80

LIST OF ABBREVIATIONS

ABA	➢ Abscisic acid
AEZ	➢ Agro-ecological zones
APS	➢ Ammonium per sulphate
BOC	➢ Bangladesh Oxygen Company
bp	➢ Base pair
BRRI	➢ Bangladesh Rice Research Institute
BSA	➢ Bovine serum albumin
C	➢ Celsius
cDNA	➢ Complementary DNA
cm	➢ Centimeter
cM	➢ Centimorgan
Conc.	➢ Concentration
CTAB	➢ Hexadecyl Trimethyl Ammonium Bromide
d	➢ Day(s)
2-D	➢ Two dimensional
ddH_2O	➢ Distilled deionized water
DEPC	➢ Diethyl pyrocarbonate
DMSO	➢ Dimethyl sulfoxide
DNA	➢ Deoxyribonucleic acid
DNase	➢ Deoxyribonuclease
dNTPs	➢ Deoxynucleotide triphosphate
dS/m	➢ Decisiemens per meter
DTT	➢ Dithiothreitol
EC	➢ Electrical conductivity
EDTA	➢ Ethylene diamine tetra acetic acid
e.g.	➢ For example

et al.	➢ and others
EtBr	➢ Ethidium bromide
etc	➢ Et cetera
Fig.	➢ Figure
g	➢ Gram
GEI	➢ Genotype environment interaction
Gha	➢ Giga hectare
ha	➢ Hectare
h/hr	➢ Hour
IAA	➢ Isoamyl alcohol
i.e.	➢ That is
IPTG	➢ Isopropyl thio-β-D-galactose
IRRI	➢ International Rice Research Institute
Kb/kb	➢ Kilobase
Kda	➢ Kilodalton
Km	➢ Kilometer
L	➢ Liter
LB	➢ Luria Broth
LRs	➢ Landraces
M	➢ Molar
MAS	➢ Marker assisted selection
mg	➢ Milligram
µg	➢ Microgram
Mha	➢ Mega hectare
min.	➢ Minute
ml	➢ Milliliter
µl	➢ Microliter
mM	➢ Millimolar

µM	➢ Micromolar
µm	➢ Micrometer
MOPS	➢ 3-[N-morpholino] propane sulfonic acid
MV	➢ Modern variety
MW/mol. wt.	➢ Molecular weight
N	➢ Normal
NaCl	➢ Sodium chloride
NaOH	➢ Sodium hydroxide
NCBI	➢ National Center for Biotechnology Information
ng	➢ Nanogram
nm	➢ Nanometer
No./no.	➢ Number
O.D.	➢ Optical density
PAGE	➢ Polyacrylamide gel electrophoresis
PCR	➢ Polymerase Chain Reaction
PMSF	➢ Phenyl methyl sulfoxyl fluoride
P^H	➢ Negative logarithm of hydrogen ion concentration
QTL	➢ Quantitative trait loci
RGP	➢ Rice genome project
RNA	➢ Ribonucleic acid
RNase	➢ Ribonuclease
rpm	➢ Rotation per minute
RT	➢ Room temperature
SDS	➢ Sodium dodecyl sulphate
Sec.	➢ Second
SOD	➢ Superoxide dismutase
Sol^n	➢ Solution
SSR	➢ Simple sequence repeat

TAE	➢ Tris acetate EDTA
TE	➢ Tris EDTA
TEMED	➢ N,N,N,N,-tetrametlyl ethylene diamine
Temp.	➢ Temperature
t/ha	➢ Ton per hectare
UV	➢ Ultraviolet
V/V	➢ Volume per volume
Viz.	➢ Videlicet
Vol.	➢ Volume

Abstract

Traditional rice landraces are grown by farmers in the saline coastal belt of Bangladesh which forms one ninth of the total cultivable land. These landraces are expected to be salt tolerant. This study was aimed at producing DNA-based fingerprinting as well as characterization with respect to genes linked to salinity tolerance.

Sixteen *Oryza sativa* L. landraces (LRs) from the saline-prone coastal zones of Bangladesh; as well as IRRI, BRRI rice varieties and salt tolerant control rice Pokkali were analyzed with 60 evenly distributed rice microsatellite DNA markers. A total of 196 reproducible polymorphic bands or alleles were identified after amplification of the genomic DNA of these rice cultivars. The microsatellite fingerprinting analysis revealed that the salt tolerant landraces of the coastal region have unique polymorphic loci, quite distinct from the popular salt tolerance donor Pokkali. The relatedness of these cultivars based on their shared alleles was computed using Jaccard's coefficient followed by clustering into a dendogram. Three major groups were apparent, at the coefficient value of 0.284. One group consisted of IR65195, IR36, IR29, Pokkali and Hida. Other two groups contain all the photoperiod sensitive landraces from the southwest saline prone coastal regions of Satkhira and Khulna.

DNA fingerprinting of 31 LRs along with Pokkali, Nonabokra, BR40, BR29, IR29 were also done using primers homologous to stress related genes. Primers homologous to the untranslated but transcribed regions (UTR) as well as part of the coding regions of lea3 (late embryogenesis abundant), salT and SOD (superoxide dismutase) genes amplified polymorphic DNA among Pokkali, sensitive IR29 and BR29 as well as other landraces. Primers homologous to UTR of lea3 grouped landraces into clusters previously observed using microsatellite markers corresponding to all the 12 rice chromosomes.

A gene expression study of stress linked genes was also conducted under 100mM salt stress by a one step RT-PCR (reverse transcriptase-polymerase chain reaction) technique using specific cDNA primers such as LEA3NC, SalTNC, CalmodNC, SOD(Cc2)A and SOD(Cc2)B. Differential expression for Lea3 gene was observed under stress only for particular/specific traditional landraces which was not observed in salt sensitive modern variety IR29. The increased expression in LRs corresponded to the situation found in Pokkali (salt tolerant control from Srilanka). Therefore LEA gene expression may help to identify rice landraces tolerant to salinity.

Chapter 1: Introduction

1.1 Rice: A grain food for mitigating world hunger

Rice (*Oryza sativa* L.) is the most important cereal and probably the world's most versatile crop. It is a staple food for almost half of the population of the world, most of whom live in Asia and Africa. More than 90% of all rice is produced and consumed in Asia (FAO, 1990).

In Bangladesh, rice, provides 60% of the total food energy of the country, is the main source of carbohydrate and also provides 70% of dietary protein for the majority of the population (Nutritional Survey of Bangladesh, 1989). Of our total daily intake of approximately 2100 calories, 75% is derived from rice. Genetic improvement has played a major role in increasing rice output over the past three decades. However, the rate of growth in rice fields in the highest yielding areas of Asia has slowed. This suggests that, a yield plateau has been reached. The major scientific challenges, facing rice researchers are to raise the maximum yield potential; close the yield gaps between potential yields and those achieved in practice and also to sustain current high yields and evolve stress tolerant and disease resistant varieties.

1.2 Food security remains a challenge before Bangladesh

In Bangladesh a commendable progress in rice production has been made even though the total does not meet the country's requirement. The yield growth during the period of 1973-2002 was almost at par with the growth of population and the increase in production came with little increase in cropped area (Fig 1.1).

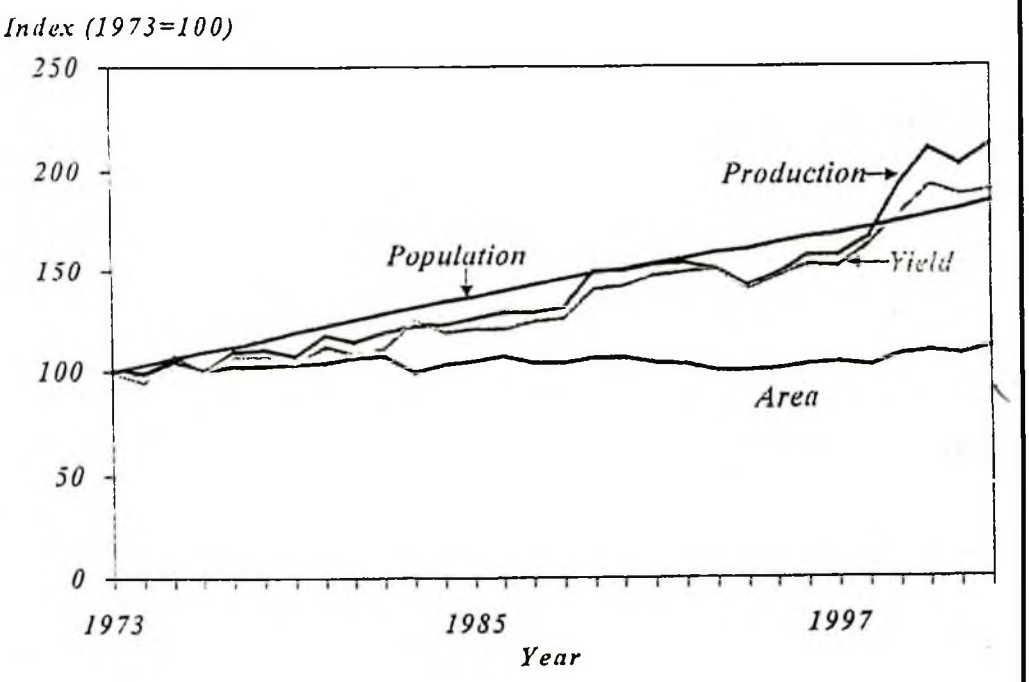

Fig. 1.1 Trends in Bangladesh population, rice area, production and yield, 1973-2002 (Hossain et al., 2003).

Food security will remain a major concern so long as population continues to increase. The country is still adding two million people every year, putting a pressure on the increase in food grain supplies at 0.56 million tons every year, to maintain the same level of per capita consumption (Hossain et al., 2003). While the increasing population puts a pressure on food, the unpredictable annual weather causes fluctuation in rice production threatening the food security of the country (Majumder and Das Gupta, 1996). In addition, per capita cultivable land has been reduced to 0.07 hectare from 0.15 hectare in 1965 due to the growth of urban population (The Daily Star, May 6, 1996). We are also lagging in adaptation of modern varieties of rice. An estimated 37 million tons of rice will be required to feed the growing population of Bangladesh by the year 2020. In such a situation, there is an urgent need to increase the present yields and to introduce those traits in rice, which will provide protection against various biotic (e.g. insects, diseases, weeds etc) and abiotic (e.g. salinity, drought, submergence, cold etc) constraints.

1.3 Soil salinity: A serious global threat to rice production

Soil salinity is a major abiotic stress in plant agriculture worldwide and an ever-present threat to crop yields, especially in countries where irrigation is an essential aid to agriculture. Although the tolerance of saline conditions by plants is variable, crop species are generally intolerant of one-third of the concentration of salts found in sea water (Flowers, 2004).

The demand for salt tolerant crops is likely to increase as population pressure and water shortages require greater productivity from arable land affected by primary or secondary salinity. Primary salinity of soil and groundwater is due to the weathering of naturally saline rocks and affects about 1.3 Gha out of the global land area of 13.4 Gha (Szabolcs, 1989). Secondary salinity is due to human activities and affects about 76 Mha out of 1.5 Gha of cropped land, including 56 Mha out of 280 Mha of irrigated lands (Yeo, 1999). That 20% of highly productive irrigated land should be affected compared with 2% of rainfed land reflects the major causes of secondary salinity, viz., the use of low quality irrigation water without adequate rainfall to leach out the deposited salt and the raising of saline water tables through the combination of excessive irrigation and poor drainage (Bennett and Khush, 2003).

The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flowers and

Yeo, 1995). World-wide, about 10×10^6 ha of irrigated land (Szabolcs, 1987) or the equivalent of all the irrigated land in Africa, are thought to be abandoned each year because of the adverse effects of secondary salinization and alkalisation.

1.4 Saline zones of Bangladesh and rice cultivation in those areas

Soil salinity is a major problem at coastal zone in Bangladesh. Bangladesh, bounded on the south by the Bay of Bengal, is subject to continual tidal saline water inundation that penetrates up to 167 Kms inside the country affecting 13 districts. It poses a great threat to the farming community of this vast area, accentuated by the Farakka Barrage in India that withdraws Ganges water allowing encroachment of saline water further inside the country (Panaullah, 1993).

Over 30% of the cultivable areas lie in the coastal zone. Out of 2.85 million hectares of coastal and offshore land, about 1.5 million hectares are affected by varying degrees of salinity. The coastal saline soils are distributed unevenly in 64 thanas of 13 districts, covering portions of 8 agro-ecological zones (AEZ) of the country (Fig. 1.2). In recent years, these have extended into the districts of Narail, Jessore and Magura, actually making a total of 16 districts (SRDI, 1998).

The Sundarban forest area spans one-third of the southern portion of 3 districts in Bangladesh and is known to be highly saline. From the west to east, it covers the districts of Satkhira, Khulna and Bagerhat. Salinity levels gradually decline from west to east; 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 Patuakhali, Barguna, Barisal and Pirojpur. These are slightly saline (2-4 dS/m), with some pockets being non-saline. The coastline extends north-east words, where there are the districts of Lakshmipur, Noakhali and Feni. The salinity in Noakhali is moderate (4-8 dS/m), and extends to larger areas compared to Lakshmipur and Feni. The coastal line moves southwards into Chittagong and Cox's Bazar, where there are pockets of high salinity as well as mild levels (Karim and Iqbal, 2001). In general, coastal salinity levels start gradually increasing from November at the beginning of the dry season and peaks in March until start of the annual rains, from April till October.

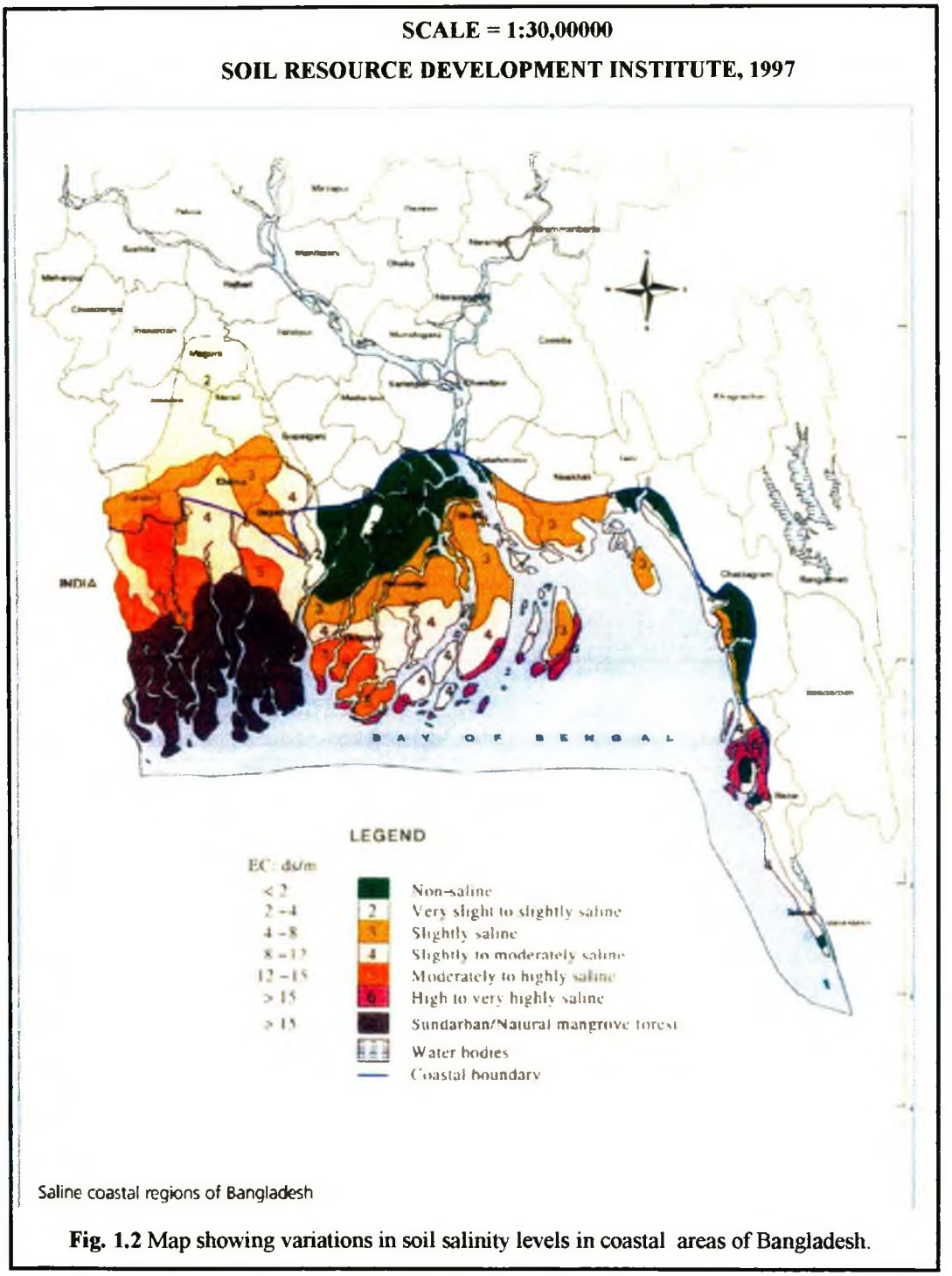

Fig. 1.2 Map showing variations in soil salinity levels in coastal areas of Bangladesh.

Soil salinity, at any time is maximum in the surface layers (0-15 cm), the salinity gradient being vertically much lower than topsoil salinity (Panaullah, 1993). Variations in the salinity levels from the west to the east coast are due to rivers flowing into the basin from the north and through into the Bay of Bengal (Karim and Iqbal, 2001). In the moderate to highly saline southwest coastal areas, farmers can only grow a single rice *(0. sativa* L.*)* crop during the monsoon season when the

salinity levels are relatively low. In the painted low lands of Satkhira and Khulna in the southwest, landraces still account for 80% of the top 20 rice varieties, despite introduction of modern varieties (Bose et al., 2001). Soil salinity in this region can be moderate even during the monsoon season, if there is lower than average or early rainfall. The popular landraces of this region are therefore well adapted to the prevailing soil stresses, including soil salinity. Farmer's preference for these landraces, despite introduction of modern varieties (Bose and Hossain, 2003) suggests greater adaptability of these landraces in the variable coastal environment, which includes unpredictable rainfall, variable water stagnation in low lands, depending upon the season, sea-water intrusion due to upstream withdrawal of water, again depending upon the amount of rainfall in the upper riparian areas in our neighboring country. The landraces are also adapted to the ion toxicities as well as deficiencies of the coastal soils (Lisa et al., 2004).

In the slight to moderate saline north eastern coast, farmers grow Aus rice, which is planted in the dry season in late March, when soil and canal water salinity levels are known to be moderate (Panaullah, 1993). Landraces in the eastern coast of Noakhali and Feni form 60% of the top 20 varieties grown there in the dry season (Bose et al., 2001). These varieties should therefore possess some resistance to salt stress, particularly at the seedling stage.

1.5 Demand for the improvement of salt tolerant crop

Earth is a salty planet, with most of its water containing about 30 g of sodium chloride per litre. Although the amount of salt affected land (about 900×10^6 ha) is imprecisely known, but its extent is sufficient to pose a threat to agriculture (Flowers and Yeo, 1995; Munns et al., 2002) since most plants and certainly most crop plants will not grow in high concentrations of salt; only halophytes (by definition) grow in > 400 mM NaCl concentrations. Consequently, salinity is a threat to food supply. Growth of the human population by 50%, from 6.1 billion in mid 2001 to 9.3 billion by 2050 (http://www.unfpa.org/swp/2001), means that crop production must increase if food security is to be ensured, especially for those who live on about $ 1 per day.

Given the amount by which food production will have to be increased, it seems reasonable to predict that changing the salt tolerance of crops will be an important aspect of plant breeding in the future, if global food production is to be maintained.

1.6 Incorporation of salt tolerant trait into modern varieties

Salinity is the most widespread soil problem in rice growing countries (Senadhira, 1987). There are approximately 400 million hectares of salt affected land in the world (Flowers et al., 1977) of which 5 million hectares are found in south and southeast Asia. Breeding for salinity tolerance in rice is difficult. This may be due to the involvement of several genes controlling this character as well as the insufficient knowledge of the mechanisms controlling rice salt tolerance (Akita and Cabuslay 1988; Yeo and Flowers, 1984).

Before planning a breeding strategy, it is necessary to recognize the following points-

- Saline soils vary widely in their physical, chemical and hydrological properties.
- Genetic variability for salinity exists among rice varieties.
- Tolerance varies with the age of the rice plant and its stage of development.
- The early seedling and flowering stages are more sensitive than the germination and vegetative stages.
- Grain yield is more affected than straw yield.
- Accumulation of K^+ in the plant decreases with increasing salinity.
- The discriminating level of salinity is around EC 8 dS/m.
- No single parameter confers to salt tolerance.

1.7 Rice germplasm and its assessment by markers

A large number of *Oryza sativa* L. landraces are found in the saline coastal belt of Bangladesh which have been shown to be excellent sources of genes for novel alleles (Evenson and Gollin,1997; Guevarra et al.,2001; Hoisington et al.,1999; Jackson,1999; Tanksley and McCouch,1997). More than 4000 traditional Bangladesh rice accessions or landraces have been collected and registered at a rice gene bank in the Bangladesh Rice Research Institute (BRRI) for medium-term storage and an identical set is held in trust at IRRI for longer storage (Bashar and Sarker,1997; Jackson,1999). Core selections of rice germplasm from many countries, including Bangladesh are being analyzed at IRRI for identification of new alleles and molecular markers for future use in enhancing the productivity of rice cultivars (IRRI; Medium Term Plan 2002-4).

For the assessment of any quantitative trait loci (i.e.salinity tolerance) genotypic information required in the form of markers (Flowers et al.,2000) as a tool for crop improvement. DNA markers can recover the gene after hybridization and thus facilitate the introgression of quantitative trait loci necessary to increase stress tolerance. Molecular marker techniques were used successfully to transfer alleles of interest from wild relatives into commercial cultivars (Tanksley and McCouch, 1997).

Use of simple sequence repeats or SSRs as genetic markers for rice germplasm assessment has gained popularity because of the technical efficiency of its use, its codominance and multi-allelic, highly polymorphic nature as well as its uniform distribution throughout the genome (McCouch et al.,1997;Temnykh et al.,2000). SSRs have been found to be of particular value in analysis of closely related rice genotypes (Nagaraju et al., 2002; Thanh et al.,1999).

1.8 Salt stress inducible genes: implication for tolerance in rice

Rice is a salt-sensitive crop species and soil salinity in irrigated land is a primary factor depressing yields in rice production. Plant geneticists have been able to compensate partially for this loss by breeding for increased salt tolerance in commercial varieties of rice, but the mechanism by which tolerance is conferred remains unclear. Until now, many rice cultivars have been investigated for salt-sensitivity. For example the indica varieties Pakkali, NonaBokra and BuraRata are low-yielding but tolerant to salinity stress where as the IR29, M-1-48 and Taichung are sensitive, high-yielding cultivars. Genetic studies revealed that salt tolerance of these varieties is principally due to additive gene effects, however, the underlying molecular mechanism for their salt tolerance has scarcely been investigated. In recent years, some of the salt-induced proteins, which were produced by the salt tolerant species, were identified by two-dimensional electrophoresis (Claes et al.,1990; Moons et al.,1995, 1997; Ramani et al.,1997; Singla et al.,1998). More than 35 polypeptides have been detected in this category. Prominent rice proteins responsive to salinity stress are the SalT, Lea (Group II, III), HSP100 family and OSR 40 proteins, active oxygen scavengers such as SOD and ascorbate peroxidase, betaine aldehyde dehydrogenase and several other novel and functionally unknown proteins . Technical advances in 2-D protein analysis makes it easy to identify accumulated proteins at specific stages of salinity stress, but mechanisms of metabolic changes and genetic regulation under salinity conditions remain unclear.

1.9 Objectives of the present research work

Soil salinity adapted traditional rice landraces are naturally found in the coastal belt of Bangladesh. These landraces are diverse with respect to their genomic sequences, morphology and saline stress response as well as yield components (Seraj et al., 2004). Genetic and physiological characterization of these landraces are important to indicate suitable target genes for transfer to modern high yielding rice varieties.

Detailed objectives:

1. Genetic composition of the traditional landraces of coastal saline areas will be characterized with the evenly distributed rice microsatellite DNA markers. The expected similarity and distinction, which may be found through microsatellite finger printing analysis, between the cultivars chosen for the study can be used as a valuable tool for further study of specific landrace and will suggest a proper choice of parents for breeding purposes.
2. Genomic DNA isolated from the traditional landraces will be amplified with the primers homologous to the coding region and part of the upstream as well as downstream region of some genes important in abiotic stress tolerance (i.e. Calmod, Sod, lea3, salT etc.). This will give information on genetic variability with respect to gene reported to be important for salt tolerance.
3. Salt induced expression pattern of some selected landraces will be studied by RT-PCR using cDNA primer (i.e. Calmod NC, Lea3 NC, SalT NC, SOD etc.) linked to the stress responsive genes. This will lead to information on any general correlation between gene expression and differences in salt tolerance of rice varieties.

Chapter 2: Review Of Literature

2.1 Soil salinity

A saline soil contains sufficient water soluble salts in the root zone to impair growth of crop plants. Salt injury depends on species, variety, growth stage, environmental factors and characteristics of the soil. Soil characteristics include salt source, nature and content of salts, distribution of salts (lateral, vertical and seasonal), p^H, organic matter content, nutrient status, water regime and other soil related toxicities. Hence, it is difficult to define saline soil precisely. The Soil Science Society of America (1979) ascribed salinity to soil that has an electrical conductivity (EC) greater than 2 dSm^{-1}, irrespective of sodium absorption ratio (SAR) and p^H values. However, the most widely accepted definition states that a saline soil gives an electrical conductivity in the saturation extract (EC_E) exceeding 4 dsm^{-1} at 25^0C, an exchangeable sodium percentage (ESP) less than 15 and p^H less than 8.5 (U.S. Salinity Laboratory Staff, 1954).

The salt content may range from 1 dSm^{-1} to 35 dSm^{-1}, pH from 2.5 to 8.5, organic carbon content from 1% to 50%, nutrient status from low to moderately high, texture form sandy to deep flooding (Akber and Seshu, 1992). These differences have important implications for breeding salt tolerant rice varieties (Akber, 1986).

2.2 Adverse effects and consequences of NaCl on plants

The impact of NaCl varies with its concentration, its rate of accumulation, the presence of other ions such as K^+, Mg^{2+} and Ca^{2+}, the duration of the stress, its time of onset relative to the major stages of plant development, the genotype of the plant and environmental factors such as temperature, humidity and light intensity (Bennett and Khush, 2003). Excessive amounts of Na^+ and Cl^- ions causes growth retardation, diminishing the yield and sometime death by creating three related problems, appearing one after the other, outlined below:

2.2.1 Shortage of water with consequences

Water moves from the soil to the vascular system of the root by a composite pathway that has transcellular, symplastic and apoplastic components (Steudle and Peterson, 1998). (Fig. 2.1)

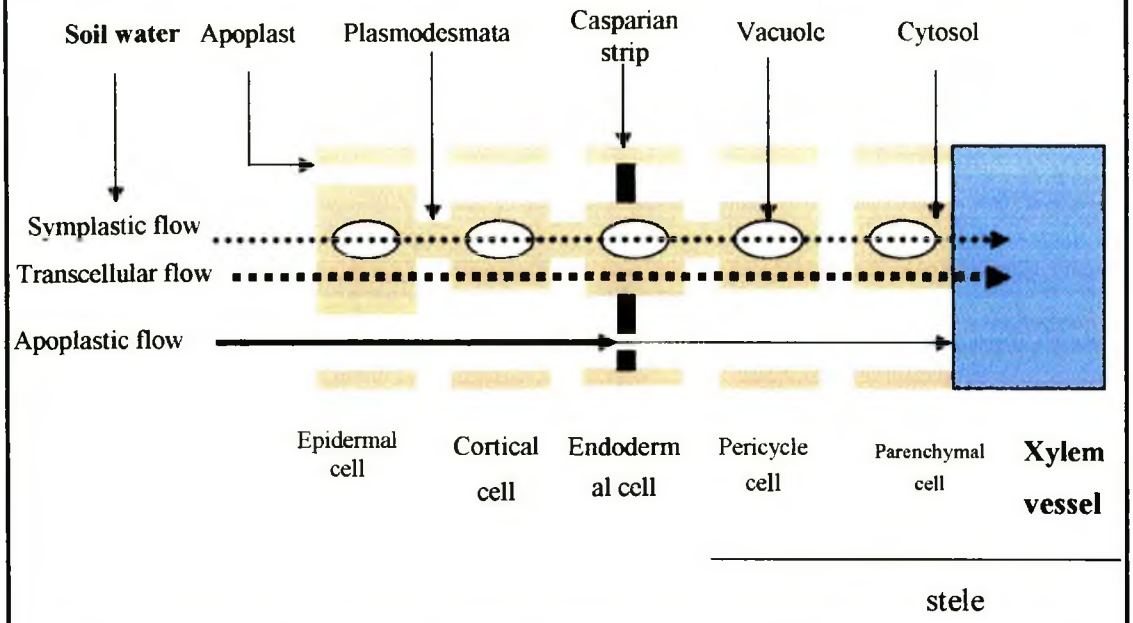

Fig. 2.1 Schematic representation of radial movement of water through the root from the soil to the xylem (Bennett and Khush, 2003).

Active water uptake into the xylem of plants is through the transcellular and symplastic pathways, driven by the difference in osmotic potential across the plasma membranes of root cells. Once in the xylem, the water flow is controlled by the leaf stomata aperture and the difference in vapor pressure between the intercellular spaces and air (Bennett and Khush, 2003). A third passive water uptake system is the apoplastic pathway, where water molecules move the intercellular spaces until hydrophobic barriers such as Casparian strip block their progress. If the osmotic potential of saline water balances the osmotic potential of the cytosol, net water uptake by the transcellular and synplastic pathway cease (Bennett and Khush, 2003). Salt-treated roots produce ABA, which is transported to the leaves via the Xylem and leads to stomatal closure. This response is crucial to the survival of the plant, because it can minimize the water deficit, caused by reduction in net water uptake. Plants may wilt at this time, which is countered by accumulation of osmotically active solutes such as glycine betaine and proline (Jagendort and Takabe, 2001; Strizhov et al., 1997).

The ABA-induced closure of stomata prevents CO_2 uptake for photosynthesis. Since the leaf continues to absorb light, photos carry on driving photosynthetic electron transport.

The lack of an ultimate electron acceptor causes the generation of active oxygen species when the products of the light reactions (electrons) and oxygen meet. The major product is superoxide O_2^- (Badger et al.,2000). Superoxide dismutase converts superoxide to H_2O_2, which can be converted non-enzymatically into the hydroxyl radical (OH^-), which can cause extensive damage to all cellular components (Ishida et al., 1999). H_2O_2 is detoxified by catalases and peroxidases and the reducing power of the electrons generated due to photosynthesis are used up for this purpose via several intermediates (Bennett and Khush, 2003). Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) acts as an oxygenase when there is lack of CO_2 due to stomatal closure. In this case the photosynthetic reducing power is used to scavenge ammonium ions. Increased production of glutamine synthetage, therefore, takes care of the ammonium ions and enhances stress tolerance (Hoshida et al., 2000).

In order to survive in the water deficit, water uptake by the symplastic and transcellular pathways has to resume in salt tolerant plants, even if partially. One of the main changes is the accumulation of Na^+ and Cl^- ions in the vacuoles of root cells, while K^+ ions accumulate in the cytosol. This differential compartmentation is done by ion selective transporters and channels, which remains incompletely understood and allows Na^+ and Cl^- ions to provide a high osmotic potential that restores water influx, while a high K^+/Na^+ ratio is maintained in the cytosol. The safe exploitation of NaCl for restoration of the difference in osmotic potential between the cytosol and soil water is found among halophytes and non-halophytes alike and serves as a major mechanism of salt tolerance.

2.2.2 Toxicity of ion and protection mechanism

The causes of the cytotoxicity of Na^+ and Cl^+ ions are unclear. It is unlikely that Na^+ owes its toxicity to its own chemical reactivity, although that remains a possibility for Cl^-, which is known to undergo certain enzymatic reactions of which methylation is probably the most conspicuous (Ni and Hager, 1999). The most obvious explanation for the toxicity of these ions is that they disrupt certain cellular processes by competing for crucial ionic interactions. Protein synthesis is a possible candidate as the most NaCl sensitive cellular process (Flowers and Dalmond, 1992).

Once Na^+ and Cl^- ions are transported to the shoot, they can interfere with the operation of metabolic pathways and alter the ion homeostasis of the cells and organelles. In some plants Cl^- is toxic, but in most plants it is Na^+ that is toxic and the Na^+/K^+ ratio that is the crucial factor (Bennett and Khush, 2003). The sensitivity of plants to salt is usually greatest during germination, seedlings growth and reproduction, but salt stress at any time during the growing season is liable to reduce yield.

Halophytes have a superior capacity to compartmentalize K^+ ions in the cytosol and Na^+ ions in the vacuole of older leaves (Yeo, 1998). When leaves senesce, most ions and other cell components are mobilized to younger tissues, Na^+ ions however, are poorly mobilized, presumably because of the lack of a channel or transporter to allow them to re-enter the cytosol. Some halophytes (i.e. *Porteresia coarctata)* may also dispose the ions through salt glands. Salt tolerant glycophytes such as rice have also been shown to efficiently partition Na^+ away from photosynthesizing leaves as well as the flag leaf (Moradi et al., 2003). Vacuolar Na^+/H^+ antiporters are responsible for compartmentalization of Na^+ into the vacuoles of older leaves and are thought to play an important role in salt tolerance (Blumwald et al., 2000).

2.2.3 Altered nutritional status

During the water deficit stage, the slow rate of water flow limits nutrient uptake. When water uptake resumes, Na^+ and Cl^- ions may directly inhibit uptake of other ions. The Na^+/K^+ ratio may climb to unacceptable levels in shoot tissues not only through the entry of Na^+ ions but also as a result of exclusion of K^+ ions. Salinity may also cause deficiencies of other ions, including Ca^{2+} and nitrate. On the other hand, high Ca^{2+} levels (>10mM) can often help to prevent these nutritional disorders (Huang and Redmann, 1995; Epstein, 1998).

2.3 Mechanisms of salt tolerance

Mechanisms of salt tolerance take place at three levels of organization: whole plant, cellular and molecular (Munns et al., 2001)

2.3.1 Control at the whole plant level

Salt tolerance depends on the ability of the plant to control the transport of salt at five sites as summarized below:

i. *Selectivity of uptake by root cells*: it is still unclear which cell types control the selectivity of ions from the soil solution.

ii. *Loading of the xylem*: there is evidence for a preferential loading of K^+ rather than Na^+ by the cells of the stele.

iii. *Removal of salt from the xylem* in the upper part of the roots, the stem, petiole or leaf sheaths.

iv. *Loading of the phloem*: there is little retranslocation of Na^+ or Cl^+ in the phloem, particularly in the more tolerant species. This ensures that salt is not exported to growing tissues of the shoot.

v. *Excretion through salt glands or bladders*: only halophytes have these specialized cell types.

2.3.2 Control at the organelle level

2.3.2.1 Ion compartmentation

Mechanism for salt tolerance at the cellular level involve keeping the salt out of the cytoplasm and sequestering it in the vacuole of the cell. Na^+ compartmentation in the vacuole requires energy dependent transport and it is an economical means of preventing Na^+ toxicity in the cytosol because the Na^+ can be used as an osmolyte in the vacuole to help to achieve osmotic homeostasis.

The importance of the vacuolar transporters (Na^+-H^+ antiporter) for plant salt tolerance is understood by the finding that over expression of AtNHX1 appeared to improve salt tolerance substantially (Apse et al., 1999).

2.3.2.2 Osmolyte biosynthesis

Plant accumulate various compatible osmolytes in the cytosol, thus lowering the osmotic potential to sustain water absorption from saline soil solutions (Zhu et at., 1997).

The organic solutes that accumulate most commonly under salinity are proline and glycine betaine, although other molecules (i.e. sucrose, trehalose, fructans, fructose, glycerol etc.) can accumulate to lesser degrees (Hasegawa et al., 2000).

Glycine betaine preserves thylakoid and plasma membrane integrity after exposure to saline solutions (Rhodes and Hanson, 1993). An adaptive biochemical function of osmoprotectants is the scavenging of reactive oxygen species that are by products of hyperosmotic and ionic stresses and cause membrane dysfunction and cell death (Bohnert and Jensen, 1996). Compatible solutes at high concentrations can reduce inhibitory effects of ions on enzyme activity to increase thermal stability of enzymes.

2.3.3 Control at the molecular level: ion homeostasis

Various ion transporters are the terminal determinants of ionic homeostasis. Na^+ competes with K^+ for uptake through common transport systems (K^+-selective channels, non-selective cation channels etc.). H^+-pumps, HKT (high affinity K^+ transporter), LCT (low affinity cation transporter) are important ion transport system that control Na^+ influx and efflux across the plasma membrane to maintain ion homeostasis. Recently, the SOS stress-signaling pathway was identified to be a pivotal regulator of plant ion homeostasis and salt tolerance (Hasegawa et al.,2000b; Sanders, 2000). A diagram of the relevant transporters and Ca^{2+} dependent stress signaling pathway involved in Na^+ homeostasis is shown in Fig. 2.2 (Zhu, 2003).

High salinity results in increased cytosolic ca^{2+}. In the presence of Ca^{2+} SOS3 activates SOS2 kinase activity which acts as a downstream component in the SOS3 signal transduction pathway leading to K^+ and Na^+ homeostasis and plant salt tolerance. Presumably, both hyperosmolarity and ion-specific signals of salt stress are sensed by plant cells. Although ion-specific signals are probably more important then hyperosmolarity in the regulation of Na^+ transport, osmotic stress also plays a role. (Fig. 2.2).

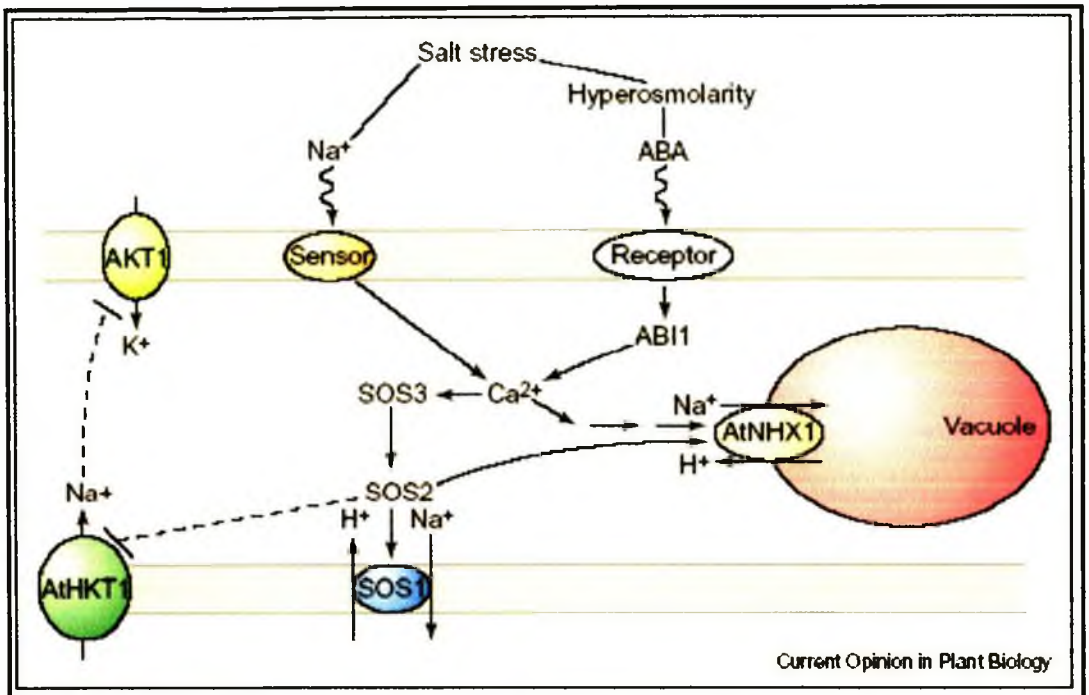

Fig. 2.2 Signaling pathways that regulate the expression and activities of ion transporters to maintain a low cytoplasmic concentration of Na^+ under salt stress. Excessive Na^+ and hyperosmolarity are each perceived by unknown sensors. The $Ca2^+$-responsive SOS3-SOS2 protein kinase pathway mediates Na^+ regulation of the expression and activities of Na^+ transporters. Hyperosmolarity is proposed to induce the synthesis of ABA, which in turn upregulates the transcripion of AtNHX1 and other ion-transporter genes. The potential negative regulation of AtHkT1 by SOS3-SOS2 and of AKT1 by intracellular Na^+ is also indicated by broken lines ending in t-bars.

2.4 Proposed six-phase breeding strategy for enhancing salt tolerance in crop plants

The new breeding strategy, which is focused on increasing productivity under salt stress rather than increasing salt tolerance as such, consists of six stages (Fig. 2.3).

According to the proposed breeding program, the output of the Pre-Breeding phase is a donor of salt tolerance that is backcrossed to either existing lines (an elite high-yielding variety or a high-quality traditional variety) or to the output of the Core Breeding Program. In conjunction with phases 2-4, research on natural resource management identifies management options that are consistent with the target environment, the breeding objectives and the resources of the farming community.

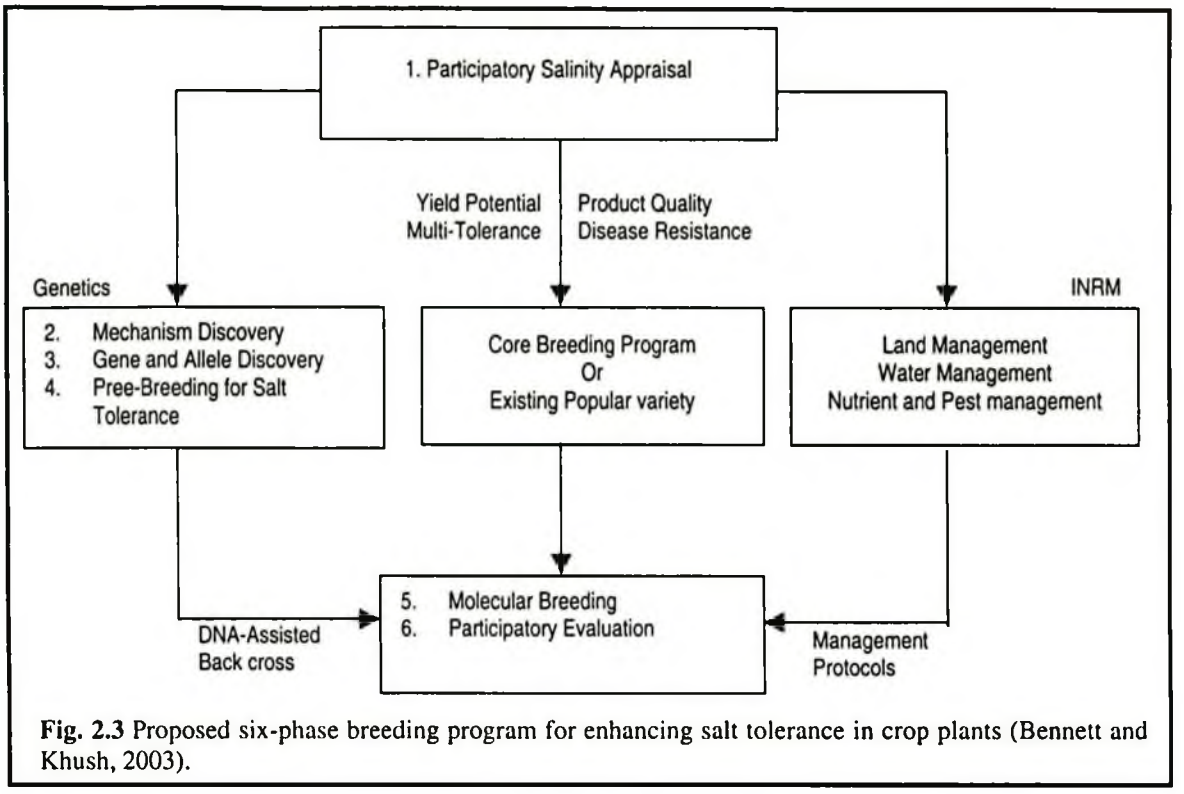

Fig. 2.3 Proposed six-phase breeding program for enhancing salt tolerance in crop plants (Bennett and Khush, 2003).

Salt stress during germination and seedling establishment might be addressed by pre-treatment (hardening) of seeds or seedlings to elicit hidden salt tolerance (Mandal and Handoo, 1998). In the case of some rice varieties, 1mM silicate enhances salt tolerance because of its ability to reduce the by-pass or apoplastic water flow that is responsible for much Na^+ uptake in this species (Yeo et al., 1999).

The output of the gene and allele discovery phase would be a set of useful alleles of different genes, each gene controlling a different mechanism contributing to salinity tolerance of the type identified by the salinity appraisal. Alleles of major QTLs would be selected using closely flanking DNA markers such as SNPs or SSRs (McCouch et al., 1997).

During pre-breeding for salt tolerance phase, DNA-based selection is used to pyramid a comparatively small set of known genes, transgenes and finely mapped major QTLs for relevant aspects of salt tolerance. DNA-based selection tools are again employed in the molecular breeding phase to transfer the entire set of salt-tolerance genes from the donor into various elite recipient lines by back crossing. The recipient lines may be high yielding modern cultivars or low-yielding traditional cultivars prized for their high product quality (Bennett and Khush, 2003).

The above mentioned strategy differs from the traditional breeding strategy in several key areas as follows:

a. it is based on a detailed physiological, biochemical and molecular understanding of the impact of salt on plant growth and reproduction.

b. the GEI of each mechanism should be much simpler and more easily controlled than the GEI for salt tolerance as a whole.

c. instead of using just one or two donors of salt tolerance as parents in the breeding program, multiple donors are exploited.

d. donors are selected not on the basis of their own performance under salt stress but for their capacity to contribute useful alleles conferring specific mechanisms of salt tolerance.

e. the alleles may correspond to known major genes, finely mapped major QTLs or transgenes altered by recombinant DNA technology.

f. the salt tolerance trait is initially assembled by gene pyramiding using robust molecular tools during pre-breeding and only subsequently transferred to elite varieties by DNA-assisted backcrossing during molecular-breeding and

g. the strategy begins and ends with farmers.

2.5 Progress in gene discovery for salt tolerance

Plant scientists have developed a complex set of tools and resources (Table 2.1) for the isolation and functional analysis of genes and their use in molecular breeding (Bennett and Khush, 2003).

Table 2.1 Resources for gene discovery in relation to molecular breeding

Type	Resources
Genetic	Germplasm collections
	Breeding lines
	Segregating populations
	Insertional and deletional mutants
	Specific transformants
Phenotyping	Field-based screens
	Screens in managed environments
	Physiological measurements
	Biochemical assays
Molecular	Large and small-insert genomic libraries
	cDNA libraries
	Cloned DNA markers
	Antibodies
	Metabolomics
Bioinformatics	Databases of DNA and protein sequences
	Genetic and physical maps
	Proteomic databases
	Databases on mutant libraries
	Databases on phenotypic and molecular characterization
	Software

2.5.1 Gene isolation

There are at least five pathways of gene isolation. Two pathway feature the isolation of cDNA clones, while the other pathways involve the isolation of genetic clones. The most through going pathway of gene isolation is the sequencing of the entire genome of a reference variety. Nipponbare in the case of rice (Sasaki and Burr, 2000). A more targeted method of isolating genes concerned with salt tolerance would be to use mutagenesis combined with classical or forward genetics (Bennett and Khush, 2003). Insertion mutagenesis is preferred over point or deletion mutagenesis because the process of gene interruption inserts a tag of known sequence that facilitates isolation of the disrupted gene (Parinov and Sundaresan, 2000). Genetic analysis including complementation is used to confirm that the mutated gene is related to the altered trait.

2.5.2 Analysis of candidate genes

An isolated gene may become a positional candidate gene for salt tolerance if it maps close to a major gene, QTL or mutation affecting this trait, well characterized mapping populations segregating for salt tolerance may consist of recombinant inbred lines (RILs), doubled haploid lines (DHLs), near isogenic introgression lines (NIILs) and advanced backcross lines (ABLs). A DNA-based genetic map provides a common framework for location of trait-related genetic loci and isolated genes encoding known or unknown proteins (Bennett and Khush, 2003). A typical mapping population of ~ 200 lines may allow precise mapping of major genes (<1cM) but major QTLs will be mapped to only about 10 cM (Kearsey and Farquhar, 1998). For rice, 1cM corresponds on average to about 300 Kb or about 30 genes, so that additional techniques are needed to identify which of these 30 positional candidates genes is the actual gene of interest. All 30 genes could be examined by macroarray or microarray analysis to determine which genes are expressed in the same tissues and at the same time and in response to the same signals as the trait of interest (Bennett and Khush, 2003).

Considerable progress has already been made in gene discovery for salt tolerance in plants arising from mutagenesis, cDNA library analysis, microarray analysis, proteomics, QTL mapping and transformation.

2.5.2.1 Mutation in SOS genes

A xylem-loading function of SOS1 would be consistent with sos1 mutant plants accumulating less Na^+. Preferential expression of SOS1 at the symplast/xylem boundary would also help explain the K^+ transport defect of sos1 mutant plants. Shi et al. (2002) proposed that SOS1 functions in retrieving Na^+ from the xylem stream under severe salt stress, where as under wild salt stress it may function in loading Na^+ into the xylem.

Gong et al. (2001) studied 89 genes regulated by exposure of Arabidopsis to 160mM NaCl for 4h. Six genes were expressed differentially in wild type and sos3 seedlings.

Five of these genes were expressed similarly is sos1 and sos3 seedlings but one was expressed similarly in sos1 and wild type seedlings. This gene encodes vegetative storage protein2 (VSP2). VSP2 is regulated presumably by SOS2/3 independently of SOS1, whereas the expression of the others is SOS1-dependent. VSP2 is postulated to be an effector of salt tolerance.

2.5.2.2 Microarray analysis of transcriptome under salt stress

Plants have both cellular and intercellular mechanisms of salt tolerance. Well known genes of salt-responsiveness were highly expressed at 90min, along with genes for detoxification of active oxygen species.

A microarray of ~ 1,300 cDNAs identified 63 drought or cold inducible genes; of which 40 were novel in terms of stress-inducibility. Microarray analysis to transcripts from transgenic plants over-expressing the drought, cold and salt-responsive transcription factor DREBIA (Kasuga et al., 1999) identified 12 genes that are targets for this factor, including 6 novel targets.

2.5.2.3 Upregulation of specific genes

Application of proteomics to the analysis of salt responsiveness in plants is in its infancy. Salekdeh et al. (2002) used 2D-E to compare salt-induced changes in root proteins of Pokkali and IR29. Five root proteins were responsive to salt (RS1-5), of which two (RS4 and RS2) were identified to be homologues of ascorbate peroxidase and caffeoyl-CoA O-methyl transferase (CCOMT) respectively. Ascorbate peroxidase catalyzes the conversion of H_2O_2 to H_2O and O_2 and helps to protect cells from active oxygen species. Both ascorbate peroxidase and CCOMT contribute to lignifications. In the case of salt-stress, increased lignifications may help to reduce the by-pass water flow that allows Na^+ ions to enter roots via an apoplastic route (Yeo et al., 1999).

2.5.3 Plant biotechnology for enhancing salt tolerance

Many genes have been reported to enhance salt tolerance when over-expressed in transformed plants (see Appendix A, section A1.). One of the most detailed analyses is that of Zhang et al. (2001) who engineered salt tolerance in *Brassica* plants using the Na^+/H^+-antiporter of Arabidopsis that promotes vacuolar sodium accumulation. Transgenic plant showed no loss in yield.

When DREB1 was expressed from the stress-inducible rd29A promoter, plants looked normal and showed enhance tolerance of salinity, drought and cold (Kasuga et al., 1999). Winicov (2000) showed that root-specific transcription factor Alfin1 is an essential gene for root growth and it's over expression in transgenic plants confers a many-fold increase in root growth under normal and saline condition and significantly increases plant growth and salt tolerance. A new arabidopsis cDNA was identified that encodes a functional homologue of the yeast Dbf2 Kinase (Lee et al., 1999). This cDNA enhanced salt, drought, cold and heat tolerance upon over expression in yeast or tobacco cells.

2.6 Current advancement in breeding for salt tolerance

Over the last 30 years, salt-tolerant varieties of many crops and forest species were developed by breeding institutes around the world. By intensive breeding programs, new varieties of major crops were generated by traditional methods combining salt tolerance with other desirable traits. In these programs, a donor of salt tolerance was crossed with an elite recipient line and segregating generations were advanced with subsequent replicated selection for multiple traits (Bennett and Khush, 2003).

Central Soil Salinity Research Institute in Karnal, India, has successfully released several salt-tolerant varieties of rice, wheat, barley, mustard and sugarcane (Mishra, 1996). Wheat variety Job666 has released for highly saline and alkaline areas of Rajasthan, India (Garg, 1998). Anther culture-derived line IR51500-AC11-1, suitable for coastal salinity (Zapata et al., 1991), has developed by IRRI, Los Banos, Philippines.

Re-combinant inbred lines (RILs) derived from crosses between salt sensitive high-yielding and salt-tolerant traditional varieties are now being used as donors in breeding programs for coastal areas of Asia (Gregorio et al., 2002). These RILs are superior donors of salt tolerance in part because of their superior agronomic traits, including semi-dwarf stature and high harvest index. RILs are also being developed from crosses between varieties different in salt tolerance mechanisms.

2.7 Limitation of traditional breeding for salt tolerance

Increase in salt tolerance can certainly be achieved by breeding. The difficulty lies in combining this complex trait (see section 2.3) with high-yield; tolerance of other abiotic stresses including submergence, drought; resistance to pests and diseases (Bennett and Khush, 2003). Gregorio et al. (2002) have emphasized the importance of developing salt-tolerant varieties that also posses tolerance to iron toxicity, aluminium toxicity, phosphorus deficiency and zinc deficiency.

The traditional breeding approach consists of:

i. screening germplasm collections for donors of salt tolerance
ii. crossing a donor with an elite line and advancing the F_1 hybrid to about F_7 or F_8 generations selecting for traits and concurrent selection for traits that confer high yield and salt tolerance.

The low efficiency of this breeding approach is due principally to the :

i. difficulty of recovering elite genotypes with salt tolerance traits
ii. genetic complexicity of salt tolerance
iii. large genotype X environment interactions (GEI) and the problem of controlling relevant environmental variables during field-based selection

The advent of genetic engineering raises the possibility of introducing a series of salt-tolerance genes directly into an elite variety by transformation to confer tolerance without breaking up the complex desirable traits of the recipient (see table in Appendix A1.).

2.8 Molecular markers

Molecular or DNA markers can be defined as a sequence of bases at a unique physical location in the genome, which varies sufficiently between individuals that it's pattern of inheritance can be tracked through families or populations and/or it can be used to distinguish among cell types. A marker may or may not be used as part of a gene (Seraj, 2001).

DNA markers are based on naturally occurring variations in DNA sequence that result from point mutations, random deletions or insertions or the presence of varying numbers of repeated copies of a DNA fragment (Patwary, 2004). A molecular marker should have some desirable properties such as (Chawla, 2002):

i. It must be polymorphic as it is the polymorphism that is measured from genetic diversity studies
ii. Co-dominant inheritance
iii. A marker should be evenly and frequently distributed throughout the genome
iv. It should be easy, fast and cheap to dectect
v. It should be reproducible

Unfortunately, no single molecular marker meets all these requirements. A wide range of molecular markers is available that detect polymorphism at the DNA level.

2.8.1 Some commonly used molecular or DNA markers

2.8.1.1 Restriction fragment length polymorphism (RFLP)

This technique detect, length difference in homologous fragments between different DNA molecules which are originated by point mutation, insertions or deletions (Fig. 2.4).

Uses of RFLP :

i. it permits direct identification of a genotype or cultivar in any tissue at any developmental stage in an enovironment independent manner.
ii. RFLPs are codominant markers, enabling heterozygotes to be distinguished from homozygotes.
iii. it has a discriminating power that can be at the species/ population (single locus probes) or individual level (multi locus probes).
iv. the method is simple as no sequence specific information is requird.
v. RFLP markers have been used extensively to create genetic maps on chromosomes of a large number of plant species.
vi. RFLP markers have been used also in studies of genetic variations, DNA fingerprinting, phylogenetic studies, hybridization studies, genetic segregation, and as markers linked to genes of interest.

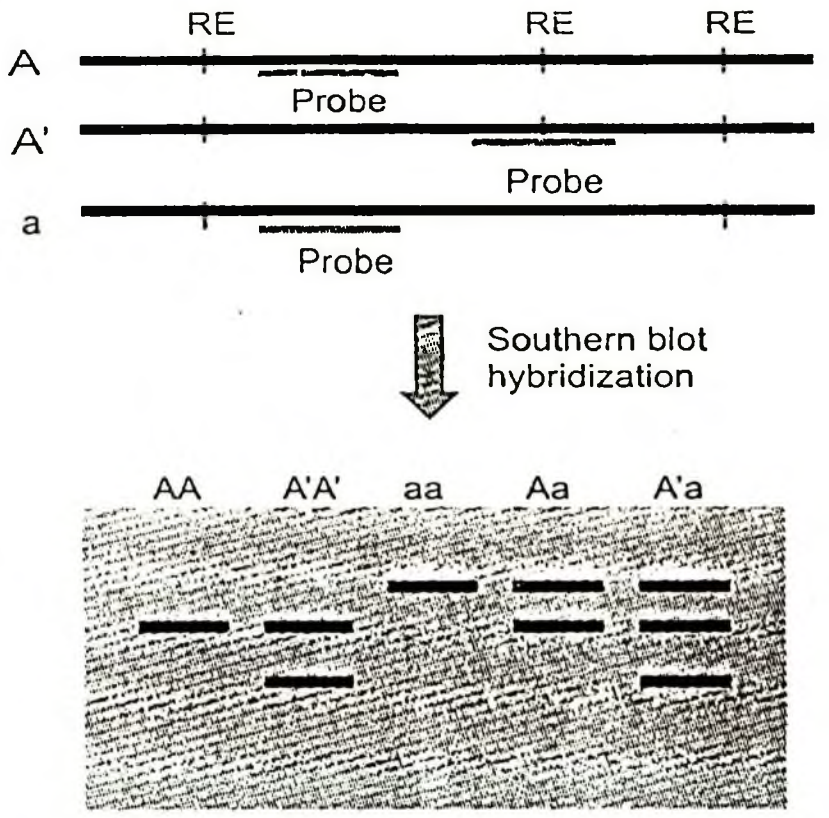

Fig. 2.4 Schematic diagram showing restriction fragment length polymorphism procedure (Patwary, 2004).

RFLP has some limitations, such as:

i. conventional RFLP analysis requires relatively large amount of highly pure DNA.

ii. a constant good supply of probes that can reliably detect varation are needed.

iii. it is laborious and expensive to identify suitable marker/restriction enzyme combinations from genomic of cDNA libraries where no suitable single-locus probes are known to exist.

iv. RFLPs are time consuming as they are not amenable to automation.

v. RFLP work is carried out using radioactively labeled probes and therefore requires expertise in autoradiography.

However, once RFLPs are developed they are very powerful markers (Chawla et al., 2002: Patwary, 2004).

2.8.1.2 Amplified restriction fragment length polymorphism (AFLP)

AFLPs are co-dominant PCR-based markers (Fig. 2.5)

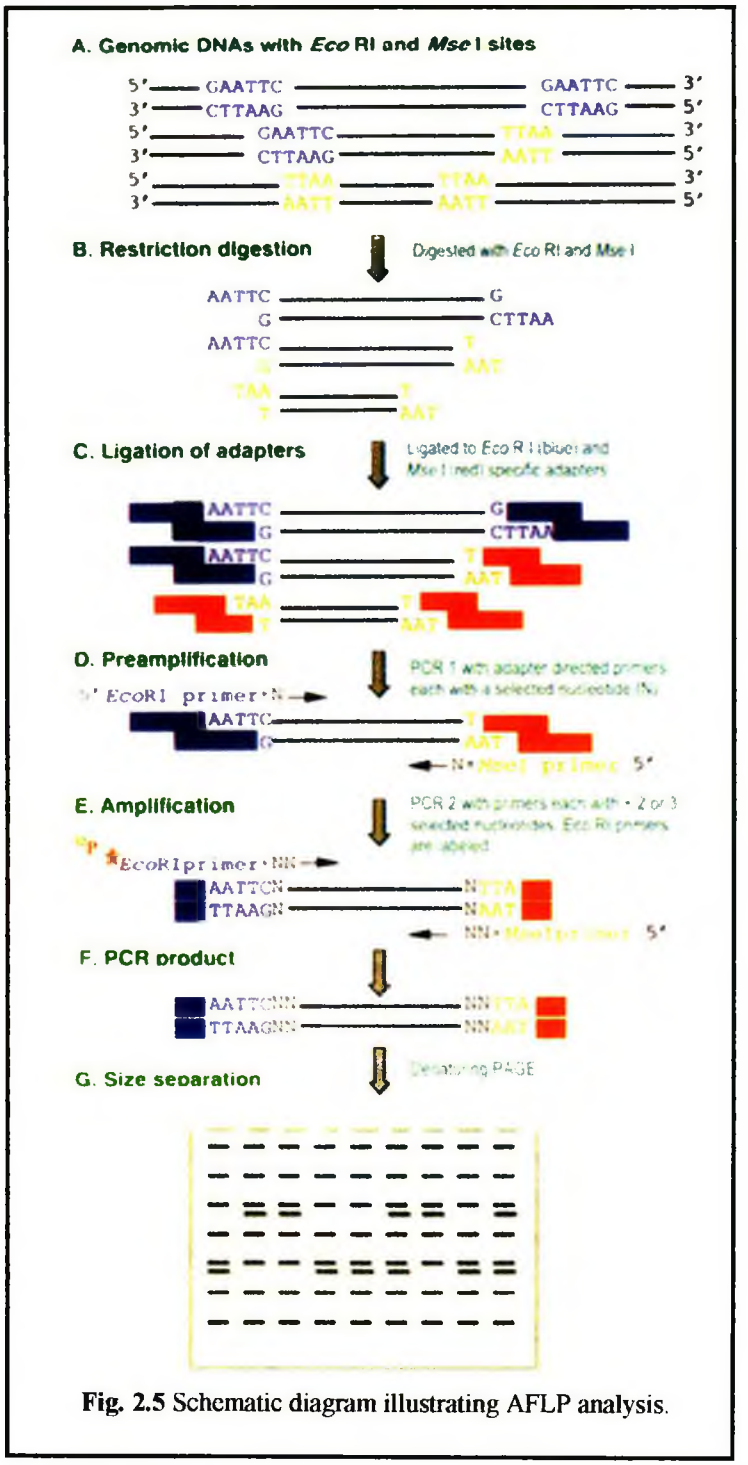

Fig. 2.5 Schematic diagram illustrating AFLP analysis.

Advantages of AFLP :

i. this technique is extremely sensitive.

ii. it has high reproduciblity, rendering it superior to RAPD.

iii. it has widescale applicability, proving extremely proficient in revealing diversity.

iv. it discriminates heterozygotes from homozygotes when a gel scanner is used.

v. it permits detection of restriction fragments in any background or complexity, including pooled DNA samples and cloned DNA segments.

vi. it is not only a simple fingerprinting technique, but can also be used for mapping.

Disadvantages of AFLP :

i. it is higly expensive and requires more DNA than is needed in RAPD (1 mg per reaction).

ii. it is technically more demanding than RAPDs, as it requires experience of sequencing gels.

iii. AFLPs are expensive to generate as silver staining, fluorescent dye, or radioactivity detect the bands (Chawla et al., 2002).

2.8.1.3 Random amplified polymorphic DNA (RAPD)

In this technique, a single non-specific 10 bases oligonucleotide primer is used to amplify genomic DNA. DNA amplified products are generated for each genomic region that happens to be flanked by a pair of 10 bases priming sites (inverted repeats), which are within 5000 base pairs of each other. The DNA sequence between inverted primer sites is amplified, and because the distance between primer sites can vary among individuals, different length fragments are generated. Because the primers are small and not specific to any given gene, many different fragments are produced after PCR amplification. Polymorphisms of amplified products are caused by loss or gain of priming sites due to base substitution or deletions in the priming sites, by one or more insertions that cause two inverted priming sites too distant to support amplification or by deletions and/or insertions that change the size of amplified products (Williams et al., 1990).

RAPD technique has several advantages as well as limitations. The advantages include non-requirement of template DNA sequence information to design primers, the need for only nanogranm quantities of DNA, temple DNA that can be of low

quality and / or purity, ability to generate markers in the regions containing repetitive sequences, an enormous number of polymorphisms can be identifited as available primers of arbitrary sequence are virtually unlimited, the procedure does not involve the use of radioactivity, is rapid and technically an easy process that is cost effective.

The techonology has a number of limitations :

i. RAPD polymorphisms are inherited as dominant-recessvie characters. This causes a loss of information relative to markers which show codominance.

ii. RAPD primers are relatively short, a mismatch of even a single nucleotide can often prevent the primer from annealing; hence, there is loss of band.

iii. the production of nonparental bands in the offspring of known pedigrees warrants its use with caution and extreme care.

iv. it is highly sensitive to reaction conditions that need to be strictly standardized. (Chawla et al., 2002: Patwary, 2004).

2.8.1.4 Microsatellites/Simple sequence repeat polymorphisms (SSRPs)/Simple sequence repeat length polymorphisms (SSRLPs)

Microsatellite DNA families are arrays of variable tandem repeat units of very short nucleotide sequence motifs, mostly from one to four base pairs long, flanked by unique sequences and are interspersed throughout the genome. The motifs are hypervariable (many allelic forms) among members of same species. The overall length of repeats are less than 1Kb, but it mostly varies from 70 to 200 base pairs.

A recent survey (Toth et al., 2000) on the distribution and abundance of SSRs in different eukaryotic genomes showed taxon-specific variation in the frequency of motif distribution, the presence of tri and tetra-nucleotide repeats in protein coding exons of all taxa and a significant difference in SSR distribution between coding and non-coding and between intergenic regions and introns. Development of SSR markers involves digestion of genomic DNA with one or more restriction enzymes, size selection of restricted fragments (usually 300-600 bp),

construction of a partial genomic library using these fragments, screening the library with SSR oligonucleotide probes of interest to isolate desirable clones, sequencing clones to identify flanked unique sequence to designed primers for PCR amplification, separation of PCR products by PAGE and exposing to X-ray films or using silver stain to observe polymorphisms (Fig.2.6).

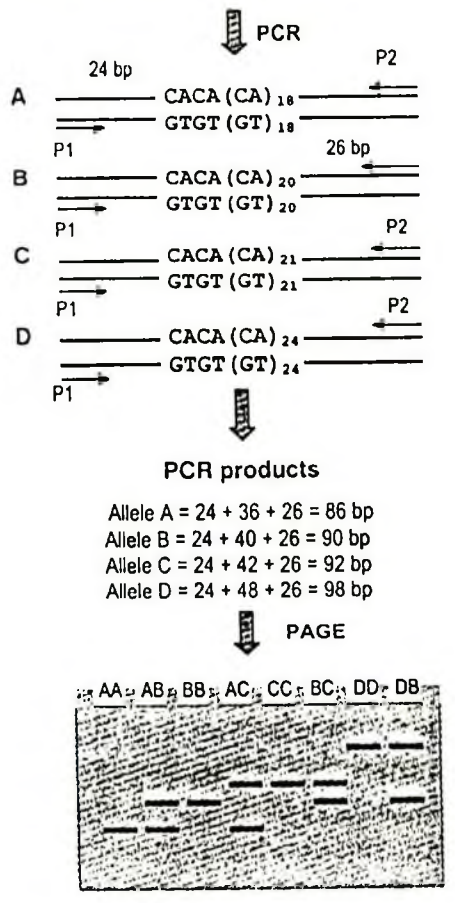

Fig. 2.6 The flow diagram illustrations CA/TG dinucleotide repeat polymorphisms. This locus shows four alleles as a result of variation in the number of (CA) repeats which is reflected on the polyacrylamide gel as polymorphisms (Patwary, 2004).

Simple sequence repeat polymorphisms (SSRPs) are valuable genetic markers as they are co-dominant, can detect a high level of allelic diversity and thus very useful in inter and intra-population studies;

easy to assay by PCR once the amplification primers are developed and because of their ability of detecting non-overlapping alleles of multiple SSRs from a multiplex PCR reaction. Another advantage of the technique is that, the microsatellite mutation rate of 10^{-4} (Dallas, 1992) generates new alleles fast enough to allow distinction of closely related accessions, but not so fast as to obscure relationships by homology (Meghen et al.,2001). The two primary limitations of SSR markers are their lengthy and expensive development and that, they are less suitable for studies involving less-related taxa. SSRPs are widely used in genotyping both plants and animal species. In plants, their applications include among others, linkage mapping (McCough et al.,1997; Roder et al.,1998; Joobeur et al.,2000; Weng et al.,2001; Yamanaka et al.,2001) genetic variation studies (Olufowote et al.,1997; Ariette et al.,2001; Guadagnuolo et al.,2001; Mariette et al.,2001) and studies of phylogenetic relationships (Bautista et al.,2001; Xu and Sun, 2001).

2.8.1.5 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms are bi-allelic markers. They are the most abundant type of DNA sequence variants in most organisms. It has been estimated that on an average one SNP occurs per 104 bp in the randomly sampled areas of the maize genome (Tenaillon et al., 2001) and one per 250-1000bp in the human genome (Collins et al.,1998). SNPs comprise RFLPs and other polymorphisms that do not create or abolish restriction sites. Several related techniques to create SNPs have been reported, but all of them use mismatches in PCR primers to create a polymorphism based on the target mutation (Newton et al.,1989; Ye et al.,1992; Neff et al.,1998). A recently reported techniques is tetra-primer ARMS-PCR (Ye et al.,2001) involving the use of four primers, a primer pair of "outer primers" that amplifies a small area of the genome encompassing the mutant allele and two "allele–specific primers" that each in combination with a outer primer amplify allele-specific regions (Patwary, 2004). The amplification is followed by electrophoretic separation of the products. If the targeted alleles are homozygous, two bands will appear on the gel, but if the alleles are hoterozygous three bands will be found.

2.8.1.6 Expressed sequence tags (ESTs)

ESTs are short (200 bp–500 bp) DNA sequences generated from the 3′ and 5′ ends of randomly selected cDNA clones. The purpose of EST sequencing is to scan rapidly for expressed genes and to provide a tag for each gene. The EST databases contain a potential wealth of valuable information about expressed genes. For example, ESTs can be used to find genes and DNA polymorphisms. Therefore EST sequences can be used as primers to amplify genes, which are expressed in a PCR-based approach. The latter can be very useful if one is looking for relatedness in a common function, such as biotic or abiotic stress tolerance (Seraj et al., 2001).

2.8.2 Primary applications of DNA markers in plant breeding

There are many applications for the use of DNA markers in breeding programs (Gregorio, 2001), some examples are listed below:

i. Improved access and utilization of germplasm resources
ii. Genetic analysis of breeding populations
iii. Parental selection and predicting progeny performance
iv. Marker assisted selection
v. Marker-accelerated backcross breeding
vi. Pyramiding genes from diverse sources
vii. Fingerprinting for impact assessment and protection of plant breeder's rights
viii. Comparative mapping
ix. Gene isolation, function and manipulation
x. Fingerprinting pests and pathogens

2.8.3 Marker-assisted selection (MAS)

In this technique, linkages are sought between DNA markers and agronomically important traits such as resistance to pathogens, insects and nematodes, tolerance to abiotic stresses, quality parameters and quantitative traits. Instead of selecting for a trait, the breeder can select for a marker that can be detected very easily in the selection scheme. The essential requirements for marker-assisted selection in a plant breeding program are as follows (Chawla, 2002):

i. Marker(s) should co-segregate or be closely linked (1 cM or less is probably sufficient for MAS) with the desired trait.

ii. An efficient means of screening large populations for the molecular marker(s) should be available. At present, this means relatively easy analysis based on PCR technology.

iii. The screening technique should have high reproducibility across laboratories.

iv. It should be economical to use and be user friendly.

Since the environment does not influence molecular markers, MAS should also prove useful to exercise selection for traits with low heritability and for distinguishing heterozygotes from homozygotes (in case of co-dominant markers).

2.8.3.1 Toward the development of MAS for salinity tolerance

In breeding rice for tolerance of salinity, a multiple stress tolerance traits have been emphasized. However, selection efficiency for these stresses using conventional selection is low. Thus, achieving multiple tolerance traits requires gene pyramiding through molecular marker-assisted breeding.

The genes governing salt tolerance in Pokkali were mapped through the use of F8 RILs derived from the cross between Pokkali and salinity-sensitive variety IR29. Phenotyping was done (in highly controlled environmental conditions) at the seedling stage using salinized nutrient solution (EC=12 dSm^{-1}) . Eighty RILs were selected from the extremes of the population in response to salinity and were used to construct an AFLP map consisting of 206 markers. A major gene for salinity tolerance was mapped on chromosome 1 (IRRI, 1997). The location of this gene may be the same as that of the salT gene in the Cornell map (Causses et al., 1994) which contains an open reading frame coding for a protein of 140 amino acid residues. This SalT mRNA accumulates very rapidly in leaf-sheaths and roots of mature plants and seedlings when subjected to salt (Claes, 1990).

A quantitative measure of the Na^+ and K^+ concentrations and Na^+:K^+ ratio absorption in the shoot at seedling stage in salinized condition, was taken from each line of the population.

QTLs governing high K^+ absorption low Na^+ absorption and low Na^+:K^+ ratio where mapped to five chromosomes. For high K^+ absorption, QTLs in addition to major gene in chromosome 1 were detected on chromosome 4 and 12 with 83.5 and 21.2% of the total phenotypic variation explained. For Na absorption there were QTLs on chromosome 1,10 and two in chromosome 3 and respectively explained 64.6, 35.6, 17.1 and 16.0% of the total variation. For Na^+:K^+ ratio, the three QTLs where mapped to chromosomes 1, 10 and 12 which respectively explained phenotypic variations of 64.3, 86.1 and 18.5%. A common major QTL was detected on chromosome 1 for the three traits putatively associated with salinity tolerance (IRRI, 1998). Recently Elahi et at.,(2004) linked the major QTLs for Na^+ and K^+ concentration as well as Na^+/K^+ to a 5 cM region of chromosome 1.

2.8.3.2 Identification of markers linked to salt tolerance locus in Pokkali

The work of Bonilla and his group (2002) had identified that a) RM23 and RM 5 were flanking markers to SALTOL region of chromosome 1, b) the RFLP marker C1733S is close to SALTOL. Microsatellite marker from choromosome 1, RM140 has been found to be linked to the major QTL for salt tolerance and low Na^+/K^+ ratio in Pokkali (Elahi et al., 2004)

The major QTLs responsible for salt tolerance of Pokkali are not shared by any of the traditional landraces of coastal Bangladesh (Seraj et al.,2004).

2.9 Genome coverage and distribution of SSR/Microsatellite primers along rice chromosomes

In general microsatellite or simple sequence repeat polymorphisms (SSRPs) markers are relatively evenly distributed throughout the linkage maps of the 12 rice chromosomes without obvious clustering in centromeric or telemeric regions (see Appendix A, section A2.).

2.10 Techniques for amplification of the target nucleotide sequence

2.10.1 Polymerase chain reaction (PCR)

The purpose of a PCR is to make a huge number of copies of a specific region of DNA. It exploits certain feature of DNA replication.

PCR is an iterative process, consisting of three elements: denaturation of the template by heat, annealing of the oligonucleotide primers to the single stranded target sequence(s) and extension of the annealed primers by a thermostable DNA polymerase (Sambrook et at., 2001) (Fig. 2.7).

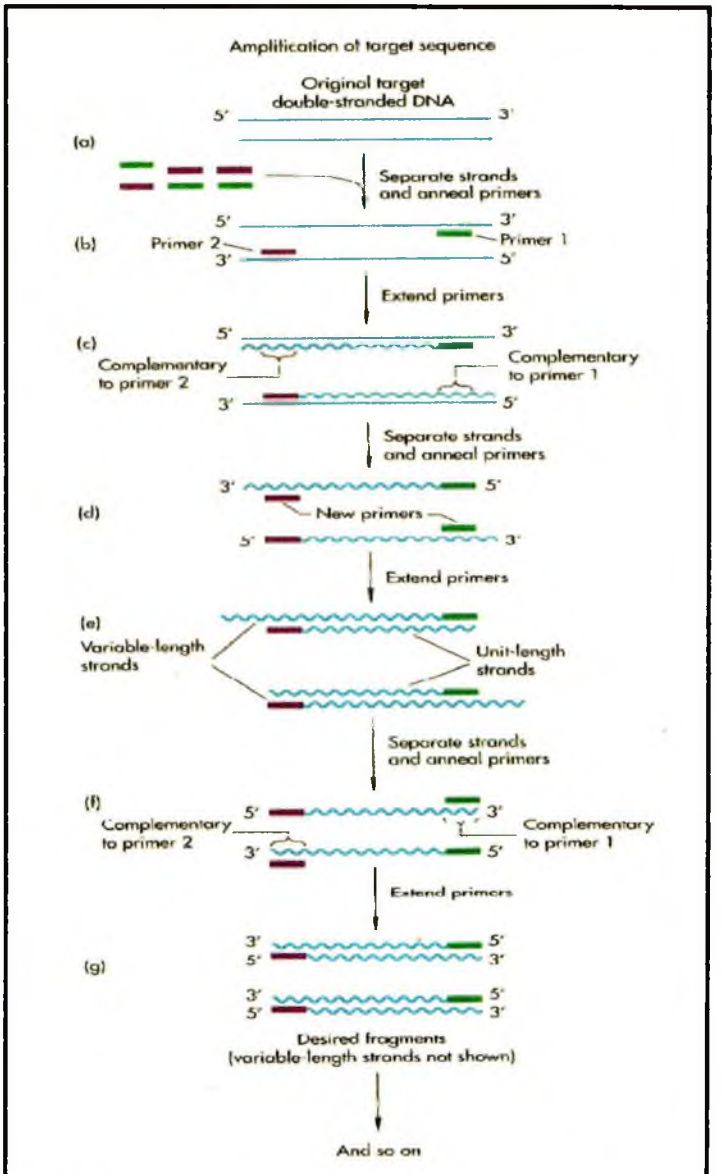

Fig. 2.7 The polymerase chain reaction (Watson et al., 1992).

2.10.1.1 Denaturation

Double stranded DNA templates denature at a temperature that is determined in part by their G+C content. The higher the proportion of G+C, the higher the temperature required to separate the strands of template DNA. The longer the DNA molecules, the greater the time required at the chosen denaturation to separate the two strands completely.

In PCR catalyzed by Taq DNA polymerase, denaturation is carried out at 94-95^{0}C, which is the highest temperature that the enzyme can endure for 30 or more cycles without sustaining excessive damage.

2.10.1.2 Annealing of primers to template DNA

The temperature used for the annealing step (Ta) is critical. If the annealing temperature is too high, the oligonucleotide primers anneal poorly, if at all, to the template and the yields of amplified DNA is very low. If the annealing temperature is too low, nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA. Annealing is usually carried out 3-5^{0}C lower than the calculated melting temperature of which the oligonucleotide primers dissociate from their templates.

However, it is better to optimize the annealing temperature by doing a gradient PCR.

2.10.1.3 Extension of oligonucleotide primers

Primer extension is carried out at or near the optimal temperature for DNA synthesis catalyzed by the thermostable polymerase, which in the case of Taq DNA polymerase is 72-78^{0}C. The length of time of the primer extension steps can be increased if the region of DNA to be amplified is long, however, for the majority of PCR experiments an extension time for 2 minutes is sufficient to get complete extension. For the last cycle of PCR, normally a longer extension time (3X longer than previous cycle) is preferred to allow completion of all amplified products.

2.10.1.4 Number of cycles

The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction and the efficiency of primer extension and amplification. The number of cycles is usually between 25 and 35. More cycle mean a greater yield of product. However, with increasing number of cycles the greater the probability of generating various artifacts (e.g. mispriming products). Theoretically the amplified products of a PCR is that by the end of 'n' cycle can be calculated by the following formula-

$N = 2P + (C-1) \times 2$ [This formula is applicable only after 3rd cycle]

Where, N = no. of amplified products.

P = no. of amplified products in preceding cycle

C= no. of cycle

2.10.2 Reverse transcriptase-PCR(RT-PCR)

Reverse transcriptase-PCR (RT-PCR) is a method used to amplify cDNA copies of RNA. Sensitive and versatile , RT-PCR is used to retrieve and clone the 5′ and 3′ termini of mRNAs and to generate large cDNA libraries from very small amounts of mRNA. In addition, RT-PCR can be easily adapted to identify mutations and polymorphisms in transcribed sequences and to measure the strength of gene expression when the amounts of available mRNA are limited and/or when the RNA of interest is expressed at very low levels.

During RT-PCR, the first step is the enzymatic conversion of RNA to a single-stranded cDNA template. An oligodeoxy-nucleotide primer is hybridized to the mRNA and is then extended by an RNA-dependent DNA polymerase to create a cDNA copy that can be amplified by PCR (Fig. 2.8).

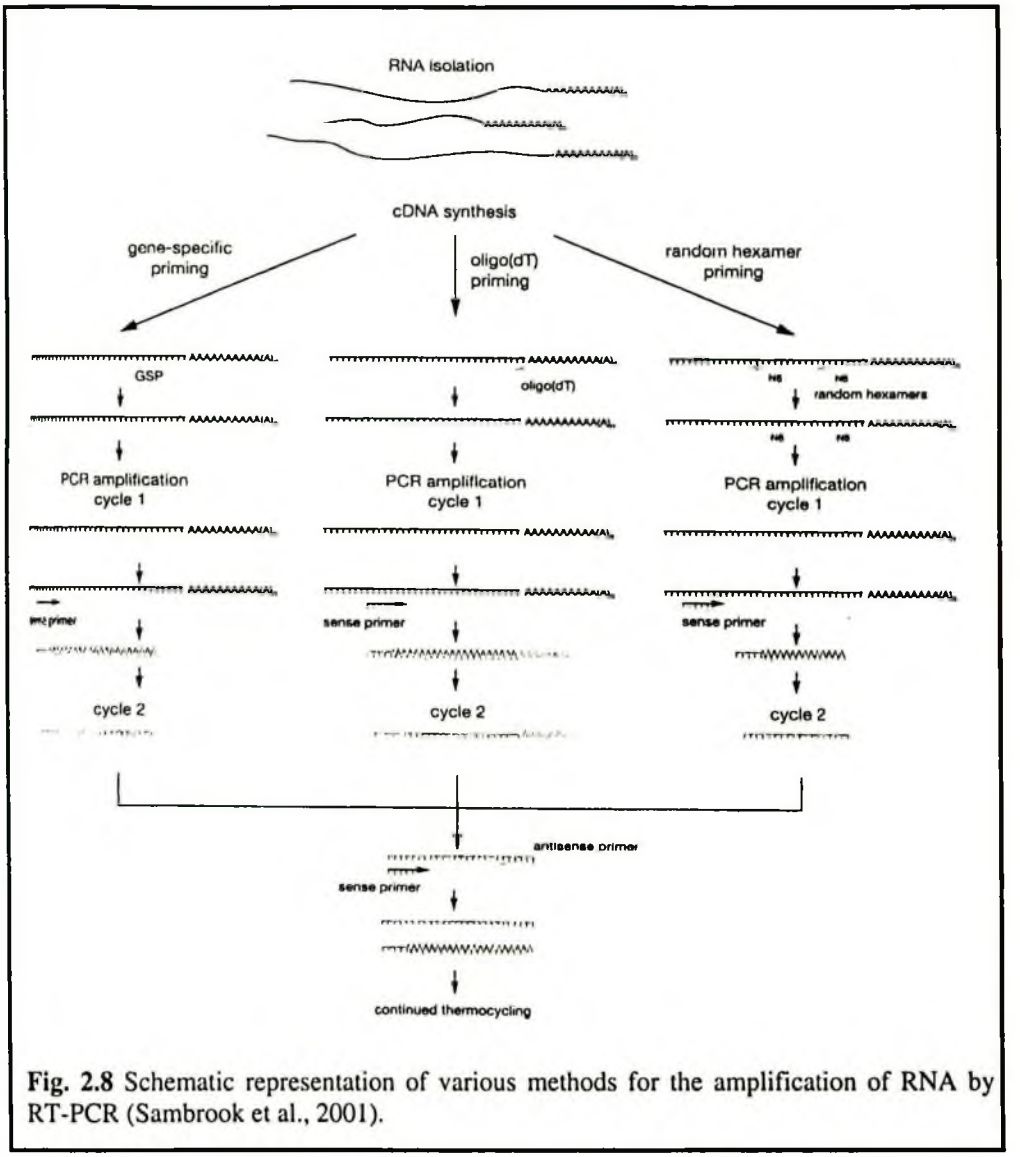

Fig. 2.8 Schematic representation of various methods for the amplification of RNA by RT-PCR (Sambrook et al., 2001).

2.11 Genes found to be linked with abiotic stress tolerance

2.11.1 Late embryogenesis abundant (lea) or lea related genes

Late-embryogenesis abundant (lea) genes encode a diverse group of proteins that accumulate to high levels during maturation phase of seed development (Baker et al., 1988) and anhydrobiotic plants, animals and microorganisms, in which their expression correlates with desiccation tolerance. However, their function has remained obscure for 20 years (Wise and Tuunacliffe, 2004). At least six different groups (1a, 1b, 2a, 2b, 3a, 3b etc.) of LEA proteins have been defined on the basis of expression pattern and sequence (Bray, 1993; Cuming, 1999; Bray, 2000). Many LEA proteins are induced by cold, osmotic stress or exogenous abscisic acid or can even be expressed constitutively (e.g. thux from Arabidopsis) (Welin, 1994).

The group3 LEA proteins are principally composed of up to 13 repetitions of an 11-mer amino acid (TAQAAKEKAGE) motif, forming an amphiphilic helical structure that was proposed to function as an ion scavenger upon cellular dehydration (Dure, 1993b). Group3 LEA proteins are abundant, mostly cytosolic proteins (Roberts et al., 1993), ubiquitous and conserved in plants. They have been variously proposed to protect cellular structure from the effects of water loss by acting as a hydration buffer, by sequestering ions in stressed tissues, by direct protection of other proteins or membranes or by renaturing unfolded proteins (Bray, 1993; Cuming, 1999). These suggestions for function have been made on a largely intuitive basis and are supported by relatively little evidence. An additional hypothesis is that LEA proteins might act as a 'molecular shield' simply decreasing the frequency of collisions between partially denatured proteins, thereby reducing the possibility of exposed hydrophobic regions seeding damaging aggregate formation (Wise and Tunnacliffe, 2004).

In a southern hybridization expreiment Moons et al., (1997) found the occurrence of a single or two strongly hybridizing bands and two or three additional less intense bands suggests the existence of a small rice oslea3 gene family. The weakly hybridizing bands might correspond to ricwsi18, exhibiting 75% DNA sequence identity to Oslea3 in the coding region, which is remarkably in contrast to the 56% aa sequence identity of the encoded proteins.

The plant hormone ABA is an important signal for changes in gene expression that are thought to have a protective function during periods of water deficit (Bray, 1993). Exogenous application of ABA and exposure to salt shock (150 mM NaCl) rapidly induces a de novo, abundant Oslea3 transcript accumulation in seedling roots, whereas application of jasmonic acid (9μM) does not induce oslea3 expression. Oslea3 expression was compared for the salt-tolerant variety Pokkali and the salt-sensitive cultivar Taichung N1. Higher maximal mRNA levels were found in roots of the tolerant variety, also declining less rapidly upon sustained salt-shock, concomitant with a delayed drop in shoot water content (Moons et al., 1997). The observed discrepancies in the salt-shock-induced oslea3 transcript accumulation in roots of Pokkali as compared to Taichung N1 seedlings, indicated differences in the regulation of the stress-induced expression in this tolerant and this sensitive variety (Moons et al., 1997).

Accumulation of LEA proteins in salinity-stressed rice seedlings may facilitate preservation of structural/functional integrity of cellular functions and aid subsequent recovery. Recovery from salt stress was consistently accompanied by degradation of the salt-induced LEA proteins. The marked accumulation of LEA/SSPs during salt stress related growth arrest and their mobilization during subsequent recovery from salinity stress clearly support such a role for LEA proteins in halotolerance as well as in seed development (Chourey et al., 2003).

Xu et al., 1996 produced transgenic rice plants expressing the barley HVA1 gene, driven by a constitutive promoter from the rice actin1 gene. This led to the constitutive accumulation of HVA1 protein in both leaves and roots of transgenic rice plants. The second-generation transgenic rice plants showed significantly increased tolerance to water deficit and salinity. Thus LEA genes hold considerable potential for use as molecular tools for genetic crop improvement toward stress tolerance.

2.11.2 Calmod (Z12828)

The protein product of the gene calmod, calmodulin is a Ca^{2+}-binding protein. In addition calmodulin is a part of the network of signal transduction pathways centered on calcium ions as second messenger in stress tolerance (Epstein, 1998).

2.11.3 SalT

Salt accumulates in rice leaf sheaths and roots in response to salt and drought but its role is not clear (Claes et al., 1990). When gel blot analysis of RNA and in-situ hybridization was performed on seedlings grown for 10 days in the presence of 1% NaCl, it was revealed that salT was expressed mainly in the younger tissues of the plant. In contrast, 6-week-old plants exhibited maximal salT mRNA accumulation in sheaths of older leaves. In addition, salT was normally expressed in rapidly dividing suspension-cultured cells, but not in quiescent ones. Altogether, these results may indicate that salt expression in each region of the plant is dependent on the metabolic activity of the cells as well as on whether or not they are stressed (Garcia et al., 1998).

2.11.4 SOD

SOD is considered crucial components in biological defense against oxidative stress (Bowler et al., 1992). Superoxide dismutases are metalloenzymes that, if protons are available, catalyze the disproportionation of two superoxide molecules into hydrogen peroxide and oxygen.

In higher plants, there are three known forms of the enzyme. Cu/Zn-, Mn- and Fe-SODs. These SOD activities are typically associated with specific subcellular locations such as mitochondria (Mn-SOD), plastids (Cu/Zn-SOD and/or Fe-SOD) and the cytosol (Cu/Zn-SOD) and display a differential expression pattern in response to environmental and chemical stresses (Perl-Treves and Galun, 1991; Tsang et al., 1991). Two nuclear genes (SodCc1 and SodCc2), which corresponded to the cytosolic Cu/Zn-SOD, DNAs, were also obtained in rice and the exon/intron organization of the SodCc2 gene was characterized (Sakamoto et al., 1992b).

Salinity causes a significant decrease of SOD activity in rice seedlings (Singha and Choudhuri, 1990; Dionisio-Sese and Tobita, 1998; Lin and Kao, 2000) and there is a significant difference between varieties. In salt-sensitive varieties SOD activity decreased, while in the tolerant landrace, Pokkali it increased (Dionisio-Sese and Tobita, 1998). The decrease in SOD activity in salinized salt-sensitive rice varieties could diminish the ability of the seedling to scavenge $O_2^{\cdot}$. radicals favoring accumulation of ROS, which could cause membrane damage (Dionisio-Sese and Tobita, 1998).

Chapter 3:

DNA Fingerprinting Of Traditional Landraces Using Microsatellite Primers

3.1 Materials and Methods

3.1.1 Plant genetic resources

3.1.1.1Collection of seeds

A total of 46 varieties composed of 31 traditional landraces, Pokkali, Nona Bokra, 8 salt-tolerant MVs and 5 salt-sensitive varieties (Table 3.1) were aimed for DNA fingerprinting study. Among the 31 landraces tested, 20 varieties were directly collected from the farmer's field or seed supplies; the rest from the BRRI gene bank. Seeds of BRRI and IRRI modern varieties were obtained from the BRRI breeding division.

Table 3.1 Properties of experimental rice varieties

Serial No.	Name of the variety	Location/ Origin	Plant type	Photo period sensitivity	Plant height (cm)	Days to maturity	Yield (t/ha)
1.	Binnatoa	Sonagazi, Noakhali	Intermediate	PI	119	90	1.2
2.	Boilam 3538	Sonagazi, Noakhali	Dwarf	PI	85	90	1.2
3.	Gheegoj 2413	Noakhali	Tall	PS	132	116	3.0
4.	Kajalshail 612	Sonagazi, Noakhali	Intermediate	PS	122	117	3.0
5.	Madhumaloti 614	Noakhali	Tall	PS	138	118	2.8
6.	Nonashail	Noakhali	Intermediate	PS	122	111	2.7
7.	Gunshi 3869	Barisal	Tall	PS	160	155	2.5
8.	Kaliboro 1281	Faridpur	Intermediate	PI	120	150	3.0
9.	Soloi 1713	Faridpur	Intermediate	PI	125	—	2.5
10.	Khaiaboro 4539	Habigonj	Intermediate	PI	125	140	3.0
11.	Hida	Rajshahi	—	—	—	—	—
12.	Razashail 1061	Narail	Intermediate	PS	119	95	3.0
13.	Ashfal	Khulna	Tall	PS	139	121	—
14.	Benapole	Khulna	Tall	PS	140	117	—
15.	Bajramuri	Khulna	—	—	—	—	—
16.	Dakshail	Khulna	Tall	PS	139	118	—
17.	Hoglapata	Khulna	Tall	PS	145	127	2.3
18.	Horkuch	Khulna	Tall	PS	145	119	2.3

19.	Jamainaru 2039	Khulna	Tall	PS	135	121	2.5
20.	Kachra 973	Khulna	Tall	PS	144	121	3.0
21.	Kalmilata	Khulna	Tall	PS	141	121	—
22.	Lakshmikajal	Khulna	Tall	PS	141	121	—
23.	Mohihi	Khulna	Intermediate	PS	128	120	—
24	Morich Shail	Khulna	Tall	PS	144	121	2.5
25.	Patnai Balam	Khulna	Tall	PS	133	127	—
26.	RaniSelute	Khulna	Tall	PS	144	127	—
27.	Capsule	Satkhira	Intermediate	PS	121	105	—
28.	Chini Kanai	Satkhira	Tall	PS	141	112	—
29.	Dhulubeej	Satkhira	—	—	—	—	—
30.	Jatai Balam	Satkhira	Intermediate	PS	123	117	—
31.	Moynamoti	Satkhira	Intermediate	PS	111	106	—
32.	Pokkali	Srilanka	Tall	PS	147	104	2.5
33.	BRRI dhan 40	BRRI	Intermediate	PS	110	123	4.5
34.	BRRI dhan 41	BRRI	Intermediate	PS	115	150 (L.C.)	5.0
35.	BRRI dhan 28	BRRI	Dwarf	PI	85	145 (L.C.)	6.0
36.	BRRI dhan 29	BRRI	Dwarf	PI	95	160 (L.C.)	7.5
37.	BRRI dhan 36	BRRI	Dwarf	PI	90	140 (L.C.)	5.0
38.	IR 50184	IRRI	Intermediate	PI	112	143 (L.C.)	—
39.	IR 51491AC	IRRI	Dwarf	PI	98	141 (L.C.)	4.0
40.	IR 52724	IRRI	Semidwarf	PI	103	140 (L.C.)	—
41.	IR 58440	IRRI	Semidwarf	PI	106	140 (L.C.)	—
42.	IR 60494	IRRI	Dwarf	PI	95	132 (L.C.)	—
43.	IR 65195	IRRI	Dwarf	PI	93	120 (L.C.)	—
44.	Nona Bokra	India	Tall	PS	150	155 (L.C.)	2.5

• PI = Photoperiod insensitive, • PS = Photoperiod sensitive, • L.C. = Life Cycle in days
• Source: Lisa et al., 2004

3.1.1.2 Varieties selected for DNA fingerprinting with microsatellite primers

Sixteen landraces of the coastal saline region, as well as Pokkali, IRRI derived tolerant and sensitive varieties IR65195, IR29, IR36 were genotyped and partially characterized by DNA fingerprinting.

Sl. No.	Variety	Sl. No.	Variety
1.	Jatai Balam	11.	Dhulubeej
2.	Capsule	12.	Moynamoti
3.	Chini Kanai	13.	Mohihi
4.	Lakshmikajal	14.	Horkuch
5.	RaniSelute	15.	Morichshail
6.	Benapole	16.	Hida
7.	Ashfal	17.	Pokkali
8.	Bajramuri	18.	IR29
9.	Kalmilata	19.	IR36
10.	Dakshail	20.	IR 65195

3.1.2 Isolation and quantification of plant genomic DNA

The primary objective of the isolation process was to recover the maximum yield of high molecular weight DNA devoid of protein and other restriction enzymes (Sambrook et al., 2001).

High molecular weight plant DNA, suitable for digestion with restriction endonuclease is difficult to isolate. CTAB method provides a less expensive procedure and is characterized by high yields of DNA from a small amount of tissue (Doyle and Doyle, 1990). The procedure followed for plant DNA isolation and quantification are given in Appendix C (see section C2.1, 2.2 and 2.3).

Phenol used during DNA isolation procedure must be equilibrated to a p^H of >7.8 because the DNA partitions into the organic phase at acidic p^H. The phenol was equilibrated according to the protocol modified from Sambrook et al. (2001). The procedure has been given in Appendix C (see section C1.1 and C1.2).

3.1.3 Rapid isolation and purification of high-activity Taq DNA polymerase

E. Coli strain taq-2 transformed with engineered plasmid P^{Taq} containing Taq gene, from thermophilic bacterium *thermus aquaticus*, which is expressed under control of tac promoter was used for the isolation of Taq DNA polymerase. This strain was kindly donated by a foreign lab. for restricted and self use only.

A modified protocol from Chien et al. (1976) was followed for the isolation and purification of Taq DNA polymerase which is given in Appendix C (see section C3.1, 3.2 and 3.3).

3.1.3.1 Checking enzymatic activity and optimization of stock dilution

3.1.3.1.1 Agarose gel run to detect any contaminated DNA

Different volumes (1µl, 2µl, 5µl etc) of isolated and purified Taq DNA pol. solution were loaded in the wells of an 1% agarose gel. After completion of electrophoresis, the gel was stained with EtBr solution and visualized under UV-illumination to detect the presence of any contaminating DNA band.

3.1.3.1.2 PCR with purified Taq DNA polymerase

1. Isolated and purified Taq DNA polymerase was diluted to 2X, 5X, 10X, 15X, 20X, 30X and 50X.
2. A standard PCR was carried out using Pokkali DNA (50 ng) as a template and a microsatellite primer RM23 (annealing temp. 55^0C, amplified product length 134-150bp). A single reaction was also carried out with commercial Taq DNA pol. as a check.
3. Amplified products were then subjected to agarose gel (2%) electrophoresis
4. The gel was stained in ethidium bromide (EtBr) solution for 15-20 minutes
5. Band pattern was observed under UV-illumination and photograph was taken.

3.1.4 Polymerase chain reaction (PCR) using microsatellite/SSR primers

Microsatellite primers used for the DNA fingerprinting, preperation of the master mixture, thermal cycling profile and other detailed procedures are given in the Appendix C (see section 4.1, 4.2 and 4.3).

3.1.4.1 Preparation of dNTPs mixture

10µl each of dATP, dGTP, dCTP and dTTP (their concentrations being 100mM each) were mixed in fresh, autoclaved eppendorf tube and the final volume was made 100µl by adding 960µl of autoclaved ultrapure water and dispensed as aliquots in tubes and stored at -20^0C. The concentration of each of the nucleotide in the above mixture was 1.0 mM.

3.1.4.2 Dilution of the template DNA

Since the isolated DNA from traditional rice landraces were highly concentrated and unsuitable for using in PCR reaction, those were diluted with TE solution (10mM Tris.HCl; 0.1mM EDTA, P^H 8.0) before use. Thus, the working concentration of the template DNA was 50ng/µl.

3.1.4.3 Microsatellite/SSR primers and their dilution

Detailed informations about the microsatellite primers used for DNA fingerprinting are given in the Appendix A. (see section A3.).

For the dilution of the primer equal volume of TE was added to the tube (provided by the manufacturer) as its molecular weight. Then 12 µl of the primer was added to 188 µl of TE. So, the final concentration of the primer was 60 ng/µl (~10µM).

3.1.4.4 Optimization of the annealing temperature of some primers

Following are the list of primers for what annealing temperature had to be optimized

Sl. No.	SSR primer	Sl. No.	SSR primer
1.	RM 01	12.	RM 218
2.	RM 14	13.	RM 219
3.	RM 24	14.	RM 220
4.	RM 34	15.	RM 221
5.	RM 35	16.	RM 227
6.	RM 51	17.	RM 228
7.	RM 80	18.	RM 237
8.	RM 143	19.	RM 240
9.	RM 209	20.	RM 243
10.	RM 210	21.	RM 246
11.	RM 217	22.	RM 257

For optimization of annealing temp. of the above mentioned primers a temp. gradient PCR was carried out, using mastercycler gradient of eppendorf, where G had been selected to $57^0C \pm 7$. The temperature gradient of the PCR machine was as follow:

• PCR tube holding no.	1	2	3	4	5	6	7	8	9	10	11
• Temp. selected (^0C)	50.0	50.5	51.6	53.0	54.7	56.6	58.5	60.4	62.0	63.3	64.2

3.1.4.5 Hot start PCR

Hot start is a method to optimize the yield of the desired amplified product in polymerase chain reactions and, simultaneously, to suppress nonspecific amplification. This is done by withholding an essential component of the PCR –Taq DNA polymerase or Mg^{2+}, for example –until the reaction mixture has been heated to a temperature that inhibits nonspecific priming and primer oligomerization.

There are several kinds of strategic variation/mode of hot start PCR. In this experiment, a mixture of DNA template, DMSO and water was denatured/heated to 94°C for 5 minutes and then master mixture was added just prior to restart the programmed profile designed for PCR.

3.1.5 Visualization of the amplified PCR product

The amplified PCR product, using microsatellite/SSR and gene specific primers, can be resolved and visualized with the help of agarose gel electrophoresis or polyacrylamide gel electrophoresis or sometimes long gels (sequencing gels). Type of gel selection generally depends on the length and nature of the amplified product.

3.1.5.1 Agarose gel electrophoresis

Using the technique of agarose gel electrophoresis amplified DNA fragments can be size fractionated by comparison with standard DNA markers. Depending on the size of the DNA band, different concentrations of agarose was used. For SSR markers 3-4% agarose was ideal.

The standard method was used to separate and identify DNA fragments through agarose gel electrophoresis (Sambrook et al., 2001). Detailed procedure has been given in Appendix C. (see section C5.1).

3.1.5.2 Denaturing sequencing gel electrophoresis

In some cases long gels or sequencing gels were used for the sake of better resolution of DNA bands generated by PCR amplification. Detailed protocol followed has been given in Appendix C. (see section C5.2).

3.1.5.3 Non-denaturing polyacrylamide gel electrophoresis

The resolution in agarose gel was reasonable but sometimes it might be necessary to run the amplified products (intact or digested) on a polyacrylamide gel for better resolution. Detailed protocol followed has been given in Appendix C. (see section C5.3).

3.1.6 Gel documentation for polymorphic band patterns

3.1.6.1 Gel processing after electrophoresis

After completion of electrophoresis, staining in EtBr solution and destaining in ddH_2O for a short moment, the agarose gel and 6-8% polyacrylamide gel become ready for visualization. But for denaturing polyacrylamide sequencing gel, it was allowed to dry completely before visualization and image taking.

3.1.6.2 Image capturing

Polymorphic band patterns visualized in the gel was photographed using Kodak electrophoresis documentation and analysis system. A special software was included in this system. Before taking photograph, agarose gel and 6-8% polyacrylamide gel was placed under UV-illumination. But denaturing polyacrylamide sequencing gel was photographed under visible light.

3.1.7 Scoring for polymorphic bands

For the microsatellite DNA fingerprinting of the *O.sativa* L. cultivars, polymorphisms were scored for the presence or absence of bands on agarose or polyacrylamide gels on a one or zero (1/0) basis and the data was analyzed using the program NTSYS-PC version 2.11 f (Rohlf, 2000). Monomorphic markers can be excluded from the analysis. The average proportion of alleles (bands on gels) that were shared between any two accessions was used to measure similarity using the Jaccard's similarity coefficient (Jaccard, 1908), which is suitable for qualitative data (Rohlf, 2000).

A model score sheet was as follows:

DNA fingerprinting score sheet for microsatellite primer.

Primer used : RM332 Gel type : 4% Agarose Date : 05/04/04

Sl. No.	Name of the landraces/cultivars	Scoring (3 levels)
1	Jatai Balam	1 0 0
2	Capsule	1 0 0
3	Chinikanai	0 0 1
4	Lakshmikajal	1 0 0
5	Raniselute	0 1 0
6	Benapole	0 1 0
7	Pokkali	0 1 0
8	IR 29	0 1 0
9	Ashfal	1 0 0
10	Bajramuri	0 1 0
11	Kalmilata	1 0 0
12	Dakshail	1 0 0
13	Dhulubeez	1 0 0
14	Moinamoti	0 1 0
15	Mohini	0 1 0
16	Pokkali	0 1 0
17	IR 36	1 0 0

N.B. A same scoring pattern was applied for the polymorphic bands generated using gene specific primers.

3.1.8 Cluster analysis

Cluster analysis on the marker was done based on the similarity matrix obtained using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973). A matrix of genetic distances (Nei, 1973) was computed and a complete linkage dendogram and distances was constructed. The efficiency of the UPGMA for estimation of genetic relatedness was determined by computing the cophenetic correlation coefficients (Mantel, 1967). Expected heterozygosity with respect to SSR/gene specific marker bands obtained for the different rice varieties was calculated from the sum of squares of allele frequencies (Nei, 1973) as follows:

$$H = \Sigma (1-\Sigma p^2_{ij})/n$$

Where, p_{ij} is the frequency of the jth allele for marker i and the summation extends over n alleles.

3.2 Results

3.2.1 Enzymatic activity checking of DNaseI treated Taq DNA polymerase

After treatment with DNaseI, isolated and purified Taq DNA polymerase was diluted to various combination and then a test PCR experiment was done with all diluted preparation. A reaction was also included with commercial Taq DNA polymerase as a control. 10X dilution showed better enzymatic activity. No amplification was observed in the control tube (Fig.3.1)

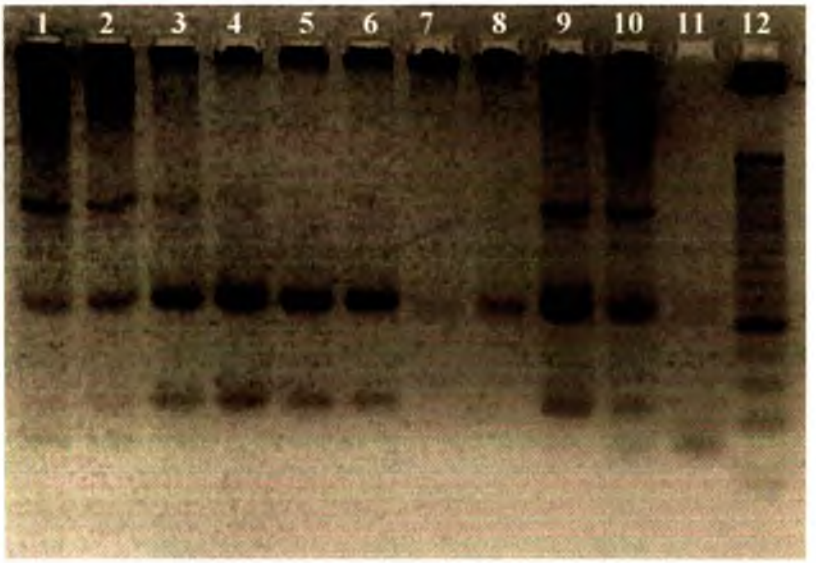

Fig.3.1 Enzymatic activity of isolated Taq DNA polymerase for different dilutions. Lane 1: 0Xd, Lane 2: 2Xd, Lane 3: 5Xd, Lane 4: 10Xd, Lane 5: 15Xd, Lane 6: 20Xd, Lane 7: 30Xd, Lane 8: 50Xd, Lane 9: commercial Taq DNA pol., Lane 10: Taq from another source, Lane 11: control, Lane 12: 25bp ladder.

Amplified band with expected size (134-150 bp) was found, with the rice microsatellite primer RM23, after gel electrophoresis. So the Taq DNA polymerase was isolated in a pure condition. To obtain a high level of transcription, under tac promoter of pTaq, IPTG was added to the cells at the mid log phase at 37^0C. At this stage the rate of growth of cells was maximum. DNaseI caused fragmentation of any bacterial DNA contaminated with isolated Taq DNA polymerase.

3.2.2 Annealing temperature optimization of some SSR primers

Annealing temperature optimization was carried out for 23 SSR (simple sequence repeat) primers using gradient PCR (Fig.3.2) and observed optimized temperatures were as follow:

SSR Primer	Optimized annealing temp. (^{0}C)	SSR Primer	Optimized annealing temp. (^{0}C)
1. RM 01	55.0	13. RM 219	58.0
2. RM 14	55.0	14. RM 220	55.0
3. RM 24	55.0	15. RM 221	55.0
4. RM 34	58.0	17. RM 227	55.0
5. RM 35	61.0	16. RM 228	55.0
6. RM 51	58.0	18. RM 237	55.0/61.0
7. RM 80	58.0	19. RM 240	55.0
8. RM 143	55.0	20. RM 243	55.0
9. RM 209	61.0	21. RM 244	55.0
10. RM 210	62.4	22. RM 246	61.0
11. RM 217	59.1	23. RM 257	55.0
12. RM 218	55.0		

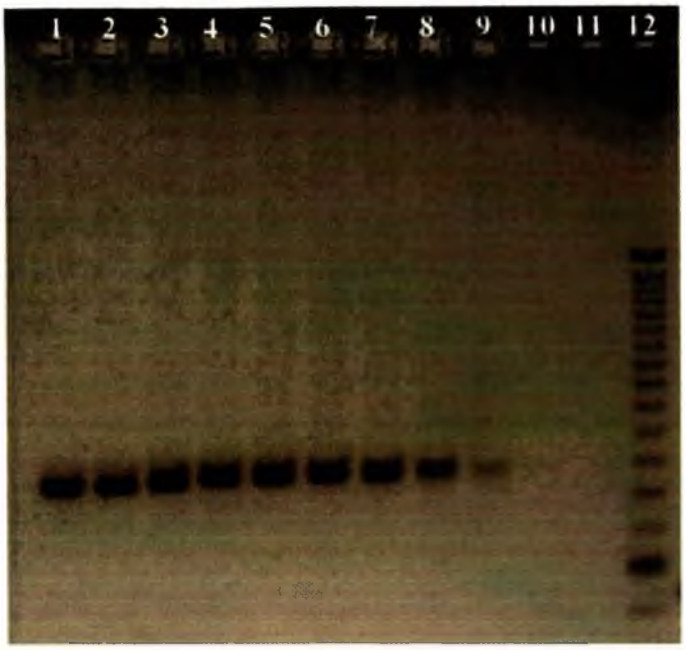

Fig. 3.2 Annealing temperature optimization of the SSR primer RM 221.

Lane 1: 50.0°C
Lane 2: 50.5°C
Lane 3: 51.6°C
Lane 4: 53.0°C
Lane 5: 54.7°C
Lane 6: 56.6°C
Lane 7: 58.5°C
Lane 8: 60.4°C
Lane 9: 62.0°C
Lane 10: 63.3°C
Lane 11: 64.2°C
Lane 12: 25 bp DNA ladder.

3.2.3 DNA fingerprinting of LRs using microsatellite primers

Using 60 microsatellite primers, 196 reproducible polymorphic bands or alleles were identified after amplification of the genomic DNA of selected 20 rice cultivars. The 60 microsatellite primers were evenly distributed over the 12 rice chromosomes (Appendix A2.). Agarose gel electrophoresis was adequate in 66% cases in detecting 2-4 alleles (Fig. 3.3a) Two alleles for 18%, 3 alleles for 42% and 4 for 8% primers were observed. For RM180, 7 alleles were observed in agarose gels similar to the reported range of sizes from 104 to 200 bp (Temnykh et al.,2000). Amplification with 32% of the selected microsatellite primers showed no polymorphism or only a few varieties gave amplification at a second level. In such cases, polyacrylamide gel electrophoresis was used to resolve amplified DNA resulting in bands of 3-7 different molecular sizes for the different primers (Fig. 3.3b).

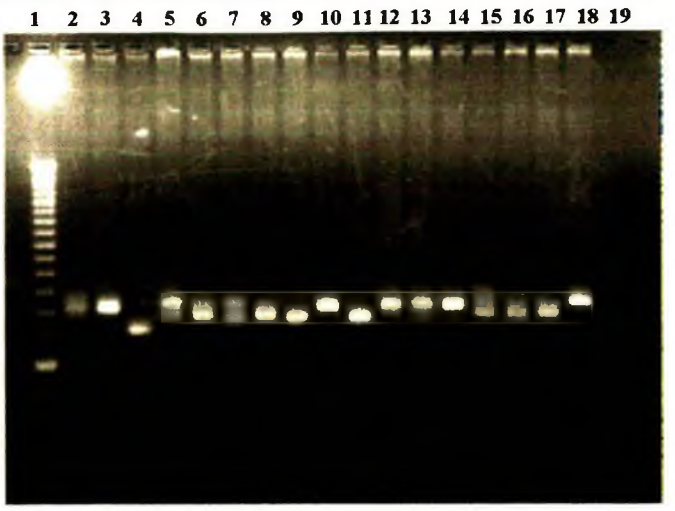

Fig. 3.3a Amplified DNA of traditional landraces, along with two MV, using microsatellite primer RM332 after resolution in 4% agrose gel.

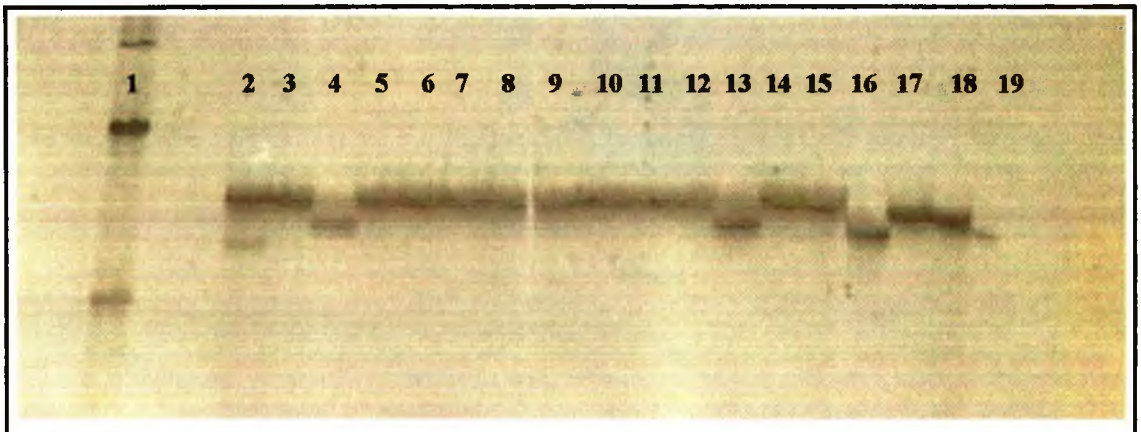

Fig. 3.3b: Amplified DNA of traditional rice cultivars from the southwest region using microsatellite primer RM172 after resolution in 6% polyacrylamide gel. Lane 1: Marker DNA, Lane 2: Jatai Balam, Lane 3: Capsule, Lane 4: Chinikanai, Lane 5: Lakshmikajal, Lane 6: Raniselute, Lane 7: Benapole, Lane 8: Pokkali, Lane 9: IR29, Lane 10: Ashfal, Lane 11: Bajramuri, Lane 12: Kalmilata, Lane 13: Dakshail, Lane 14: Dhulubeez, Lane 15: . Moynamoti, Lane 16: Mohini, Lane 17: Pokkali, Lane 18: IR36, Lane 19: Control.

Genetic relatedness between the 16 LRs as well as the traditional standard Pokkali, IRRI released modern varieties IR 65195 and sensitive control IR29 and IR36 based on their shared SSR alleles was computed using Jaccard's coefficient followed by clustering into a dendogram (Fig.3.4). 3 groups were apparent at the coefficient value of 0.284. The groups are named as G1, G2 (A and B) and G3. G1 (i.e. group 1) consisted of modern variety IR 65195, IR 36, salt tolerant benchmark Pokkali and Hida, an exceptional cultivar cultivated other than southwest coast. G2A, G2B and G3 contain all the photoperiod sensitive landraces from the highly saline southwest coastal regions of Satkhira and Khulna.

Comparison of the matrix of similarity generated with the cophenetic matrix of the dendogram showed a good fit (r=0.8), indicating the reliability of the groups within the tree diagram (Fig.3.4).

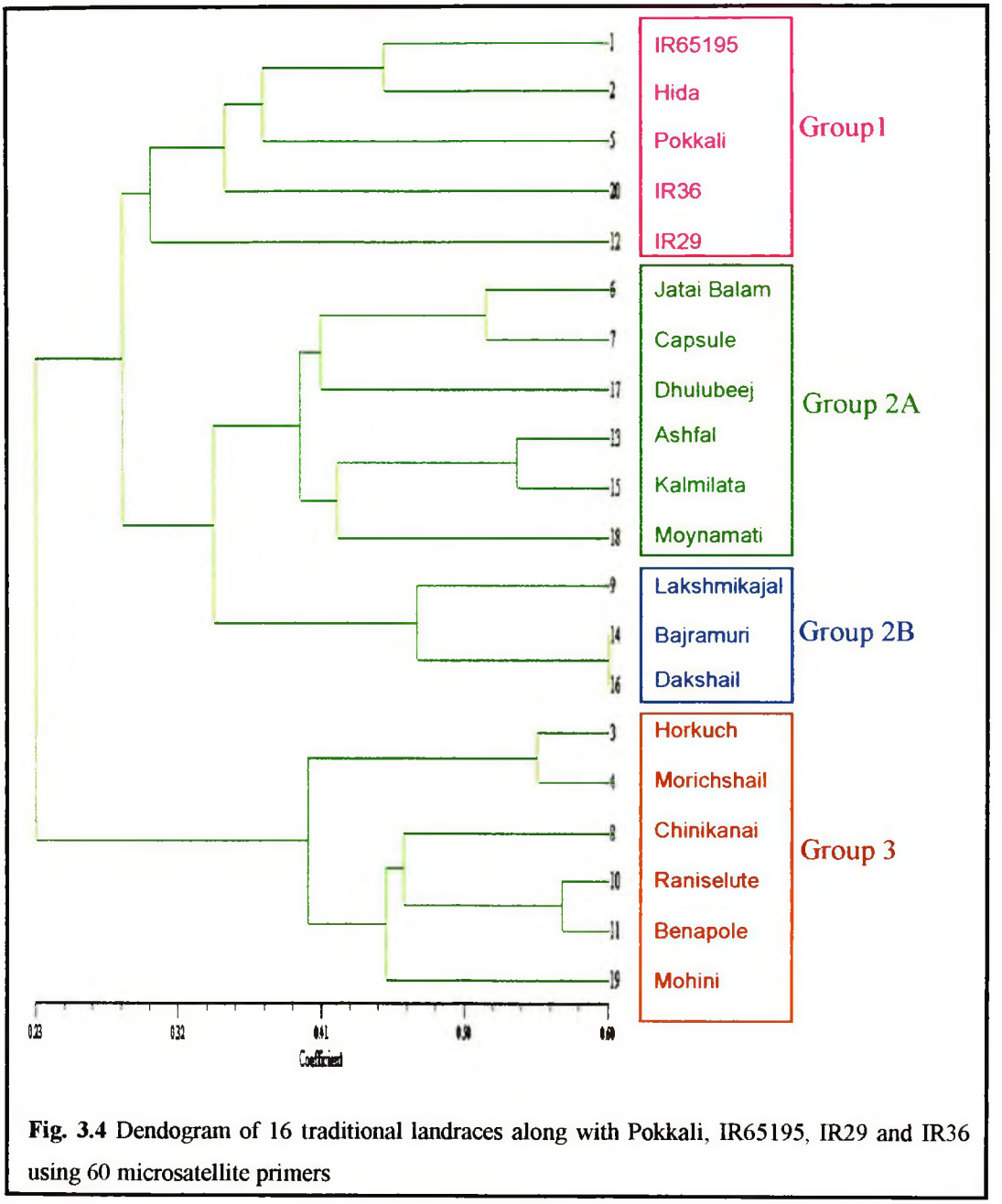

Fig. 3.4 Dendogram of 16 traditional landraces along with Pokkali, IR65195, IR29 and IR36 using 60 microsatellite primers

3.3 Discussion

Investigation of the relatedness of the landraces based on their shared SSR alleles separated cultivars into three groups (G1, G2, G3) with a sub grouping of G2 (G2A and G2B). IR36 and IR29 showed independent lineage and IR65195-3B-13-2-3, Hida and Pokkali grouped separately (G1) from other 15 landraces. Five cultivars, clustered into G1, might be genetically dissimilar to the traditional landraces collected from the highly saline southwest coastal regions of Satkhira and Khulna. Landraces of Satkhira and Khulna were clustered into group 2 (G2A and G2B) and group 3 (G3). Farmer popular landraces, Capsule, Ashfal and Lakshmikajal were grouped together (G2). Other popular landraces, Horkuch, Morichshail, Raniselute and Benapole were grouped into G3.

Lisa et al., (2004) observed that Capsule, Ashfal and Lakshmikajal had good salt tolerance score (73.3 to 83.3 percent survival) at the seedling stage and low shoot Na^+/K^+ ratio (0.86 to 1.18) at 12 dS/m. On the otherhand, Horkuch, Morichshail, Raniselute and Benapole clustered into G3 had moderate tolerance (~50 to 76.7 percent survival). Therefore, the groupings corresponds to physiological performance.

In order to breed for salt tolerant varieties, good plant type as well as tolerance is required. Considering plant type and the potential donors for salt tolerance Lakshmikajal, Morichshail, Ashfal and Raniselute should be used in breeding programs.

A combination of agarose as well as denaturing polyacrylamide was used to resolve microsatellite regions of the different rice cultivars from the coastal areas of Bangladesh. Using this approach, which was time-effective, adequate hoterozygosity was found as reported by Nagaraju et al., 2002. Although, the heterozygosity is greater when AFLP (Fuentes et al., 1999) or RAPD (Parsons et al., 1997) markers are used, one can be sure that all the regions of the 12 rice chromosomes have been represented with microsatellites.

The fingerprinting results indicated the linkage between LRs with salt tolerant and sensitive modern varieties. Hence this work will assist the breeders in selection of salt tolerant donors, in addition to Pokkali and Nona Bokra.

Chapter 4:

DNA Fingerprinting Of Traditional Landraces Using Primers Homologous To Stress-Related Genes

4.1 Materials and Methods

4.1.1 Rice varieties selected for DNA fingerprinting with gene specific primers

31 traditional landraces of the coastal saline region, as well as Pokkali, BonaBokra, IR29, BR29 and BR40 were genotyped and partially characterized by DNA fingerprinting with gene specific primers.

Sl. No.	Variety	Sl. No.	Variety
1.	Binnatoa	19.	Moynamoti
2.	Kajalshail 612	20.	NonaBokra
3.	Nonashail	21.	IR29
4.	Soloi 1713	22.	Boilam 3538
5.	Ashfal	23.	Gheegoj 2413
6.	Benapole	24.	Modhumaloti 614
7.	Bajramuri	25.	Gunshi 3869
8.	Dakshail	26.	Kaliboro 1281
9.	Hoglapata	27.	Khaiyaboro 4539
10.	Pokkali	28.	Hida
11.	BR29	29.	Rajashail 1061
12.	Horkuch	30.	Jamainaru2039
13.	Lakshmikajal	31.	Kachra 973
14.	Morichshail	32.	Kalmilata
15.	RaniSelute	33.	Mohihi
16.	Capsule	34.	PatnaiBalam
17.	Dhulubeej	35.	Chini Kanai
18.	Jatai Balam	36.	BR40

4.1.2 PCR using primers homologous to stress-related genes

Template DNA extracted and purified from 36 rice cultivars were amplified by polymerase chain reaction using primers homologous to stress-related genes. Amplified DNA fragments were then resolved using either agarose gel or polyacrylamide gel electrophoresis. Protocols for PCR and gel electrophoresis are given in Appendix C. (see section C4. and C5.).

4.1.2.1 Gene specific primers

Sl. No.	Primer/Gene	Primer sequence	Annealing temp. (°C)	Product size range (bp)	Type of gel used
1	Calmod 5′3′	Forward: cgcgcgcgcctgcgtcgccaatgg Reverse: cgatgcttcaacttacttggcc	55	1254	2.5% Agarose
2	LEA3 5′3′	Forward: gcttaggatcaatggcttcccacc Reverse: ccaaaggcaaatcattcacggcgtc	58	941	8% PAGE & 3% Agarose
3	SalT 5′3′	Forward: ccacgaagactatgacgctggtg Reverse: ctttgaccactgggaatcaagg	55	574	6% Sequencing gel & 8% PAGE
4	SOD (AB026724)	Forward: atgcaagccatcctcgc Reverse: ctacaacggggtcagcccaa	62	636	8% PAGE

Source: Rangan et al., 1999

4.1.2.2 Restriction digestion of PCR amplified products

Amplified products, using some gene specific primers, were subjected to restriction endonuclease digestion with an attempt to find a better polymorphic band pattern among the traditional rice varieties.

4.1.2.2.1 Restriction enzymes used for digestion

The desired sequence was downloaded from NCBI and EMBL/GenBank/DDBJ databases. DS gene product and nucleic acid and protein sequence manipulating software, OMIGA version 2.0 were used for finding a suitable restriction enzyme cutting site within the nucleotide sequence of amplified fragment (Fig. 4.1 a-d)

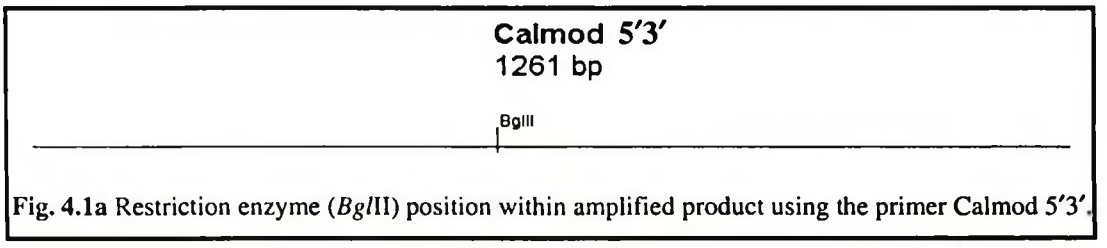

Fig. 4.1a Restriction enzyme (*Bgl*II) position within amplified product using the primer Calmod 5′3′.

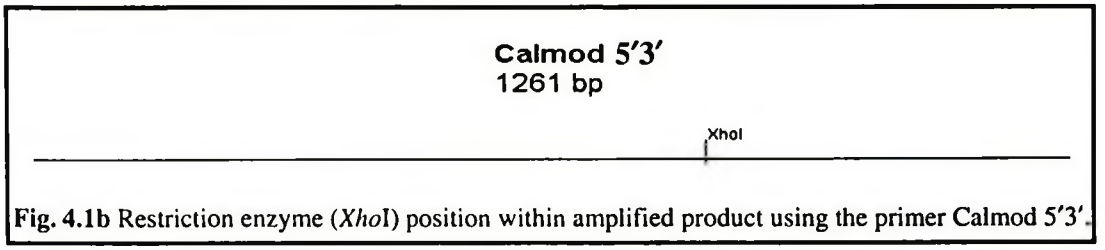

Fig. 4.1b Restriction enzyme (*Xho*I) position within amplified product using the primer Calmod 5′3′.

Fig. 4.1c Restriction enzyme (*Hae*III) position within amplified product using the primer LEA3 5′3′.

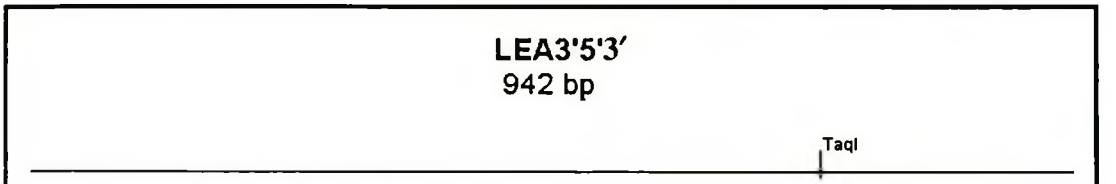

Fig. 4.1d Restriction enzyme (*Taq*I) position within amplified product using the primer LEA3 5′3′.

There were cutting sites of a number of enzymes and many of those had more than one cutting site. But enzymes, those had only one restriction site, were selected for digestion (Table 4.1).

Table 4.1 Amplified products using gene specefic primers and suitable restriction enzymes for digestion.

Sl. No.	Primer	Amplified product size (bp)	Restriction enzyme	Buffer	Optimal digestion temp. (^{0}C)	Length of fragments (bp)
1.	Calmod 5′3′	1254	*Bgl* II	RE act 3	37	567+693
			*Xho*I	RE act 2	37	818+ 442
2.	LEA3 5′3′	941	*Taq*I	RE act2	65	716+226
			*Hae*III	RE act2	37	335+379+101
3.	SalT 5′3′	574	*Eco*RI	RE act3	37	380+161
			*Taq*I	RE act2	65	447+94

4.1.2.2.2 Model digestion reaction (for 25 µl of reaction mixture)

- DNA (PCR amplified)..... 10-12 µl (corresponds to 800-1000 ng of amplified DNA)
- Buffer..... 2.5 µl (corresponds to 10% of total volume)
- Restriction Enzyme..... 0.3 µl (3 units of enzyme for 1 µg of DNA) (e.g. *Hae*III)
- PCR H_2O..... 12.2 -10.2µl

Total = 25.0 µl

4.1.2.2.3 Procedure

1. Amplified products (using any specific genomic DNA template) were collected/pooled from different tubes.
2. It was quantitated by loading 5 µl of the amplified product
3. ~ 10 µl of PCR product containing ~ 1 µg of amplified DNA was digested. The reaction was done using 2.5 µl of buffer specified for the restriction enzyme and with those 3 units of enzyme.
4. The reaction mixture was incubated at 37^0C for 3 hours
5. Electrophoresis of digested products were done in 2-4% agarose gel and also in 8% polyacrylamide gel for better resolution. The molecular weight marker DNA was also loaded in the left or right end of the gel.
6. After staining with EtBr, band patterns were visualized under UV-illumination and then photographed for polymorphism study.

N.B. 20-25 µl of digested reaction mixture was concentrated to 10 µl and mixed with 4 µl of loading dye. Then only 1-3 µl mixture (sample+dye) was loaded in 8% polyacrylamide gel.

4.2 Results

4.2.1 DNA fingerprinting using primers linked to stress-related genes

Polymorphic band patterns were observed from the amplification of DNA isolated from 36 cultivars using stress-related gene specific primers (i.e. Calmod, Lea, SalT, Sod). Polyacrylamide gel electrophoresis was adequate to resolve the amplified DNA resulting in bands of 2-4 different molecular sizes for the different primers. There was no difference between amplified products from the traditional landraces when primer homologous to the calmodulin gene was used (Figure not shown). Amplified DNA using primers homologous to lea3, Sod as well as salT genes showed distinct polymorphism (Table 4.2 and Fig. 4.2-4.4) between Pokkali, sensitive IR29 and BR29, as well as other landraces (section 4.1.1).

Table 4.2 Primers, homologous to stress-related genes, used to check polymorphism in rice landraces

Sl. No.	Primer locus	Optimize dTm and desired product size	Specific gel type used	No. of polymorphic bands/polymorphic status	Observation and comments
1.	SOD (super oxide dismutase, AB026724)	50.5°C, 636 bp	PAGE	Four	•10LRs,Nonabokra and sensitive BR29 same as Pokkali •Polymorphic
2.	SalT 5′3′ (Z25811)	55°C, 574bp	PAGE	Three	•4LRs, Nonabokra, BR40 and sensitive BR29 same as Pokkali • Polymorphic
3.	Lea3 5′3′ (AF046884)	58°C, 941 bp	PAGE & 3% Agarose (typing grade)	Two	• Results analyzed after restriction digestion (See Table 4.4) • Polymorphic
4.	Calmod 5′3′ (Z12828)	55°C 1254 bp	2.5% Agarose gel (typing grade)	One	• Monomorphic

PAGE : Polyacrylamide gel electrophoresis.

These analyses thus revealed that some of the salt tolerant landraces of the coastal region had unique polymorphic loci, quite distinct from Pokkali and Nonabokra. These differences may be related to translational control of the genes in question.

When the primer SOD (superoxide dismutase, AB026724) was used for the PCR based amplification of LRs DNA, 10 cultivars such as Ashfal, Benapole, Bajramuri, Dakshail, Horkuch, Lakshmikajal, Capsule, Dhulubeez, JataiBalam and Moynamoti showed band pattern similar to Pokkali (Fig.4.2). Among these, Ashfal, Benapole, Horkuch, Lakshmikajal and Capsule has already been identified as a potential donor for salt tolerance in the previous fingerprinting results using microsatellite primers. This result also confirm the findings of Lisa et al., 2004 for physiological and salt screening experiments.

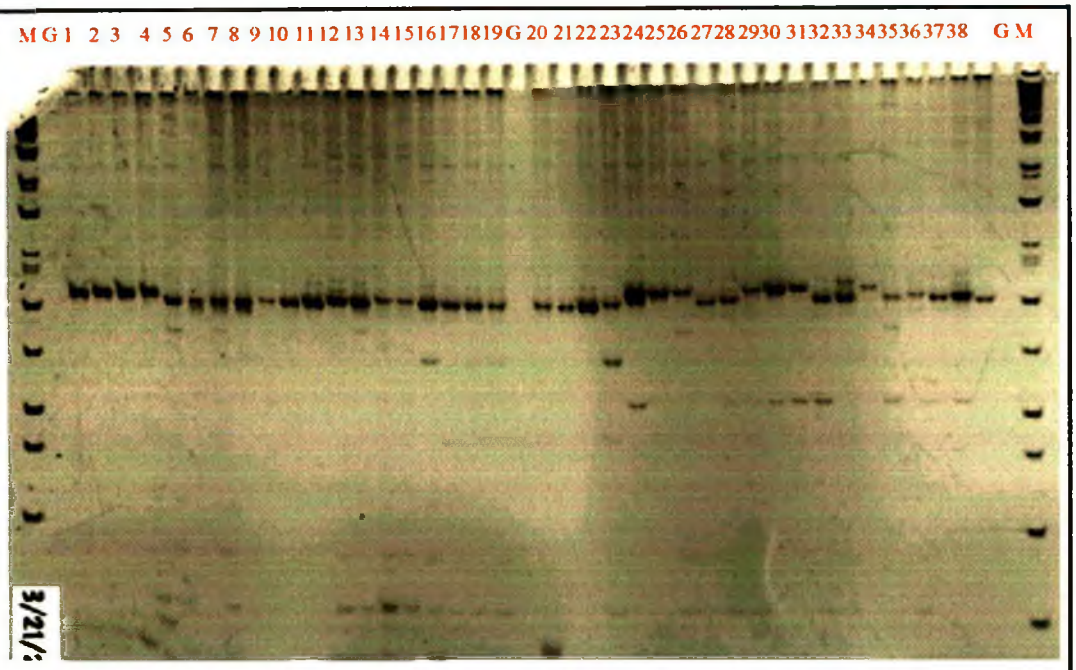

Fig. 4.2 DNA fingerprinting of 36 cultivars using the primer (SODAB026724) homologous to stress related gene Sod (superoxide dismutase). L 1: Binnatoa, L 2: Kajalshail 612, L3: Nonashail, L 4: Soloi 1713, L 5: Ashfal ,L 6: Benapole, L 7: Bajramuri, L 8: Dakshail, L 9: Hoglapata, L 10: Pokkali L 11: BR29, L 12: Horkuch, L 13: Lakshmikajal, L 14: Morichshail, L 15: RaniSelute, L 16: Capsule, L 17: Dhulubeej, L 18: Jatai Balam, L 19: Moynamoti, L 20: Pokkali, L 21: BR29, L 22: NonaBokra, L 23: IR29, L 24: Boilam 3538,L 25: Gheegoj 2413, L 26: Modhumaloti 614, L 27: Gunshi 3869, L 28: Kaliboro 1281, L 29: Khaiyaboro 4539, L 30: Hida, L 31: Rajashail 1061, L 32: Jamainaru2039, L 33: Kachra 973, L 34: Kalmilata, L 35: Mohihi, L 36: PatnaiBalam , L 37: Chini Kanai, L 38: BR40, G: Gap, M: DNA marker.

When the primer SalT 5′3′ homologous to coding as well as UTR was used, Ashfal, Jamainaru, Lakshmikajal, PatnaiBalam and Kajalshail612 formed one group but Benapole, Horkuch, Raniselute and Capsule in another group (Fig.4.3). The same grouping was also observed in salinity screening studies at seedling stage (Lisa et al.,2004). Therefore, there may be differences in translation of this salt responsive gene (salT). The functional nature of this protein remains to be identified (Claes et al., 1990).

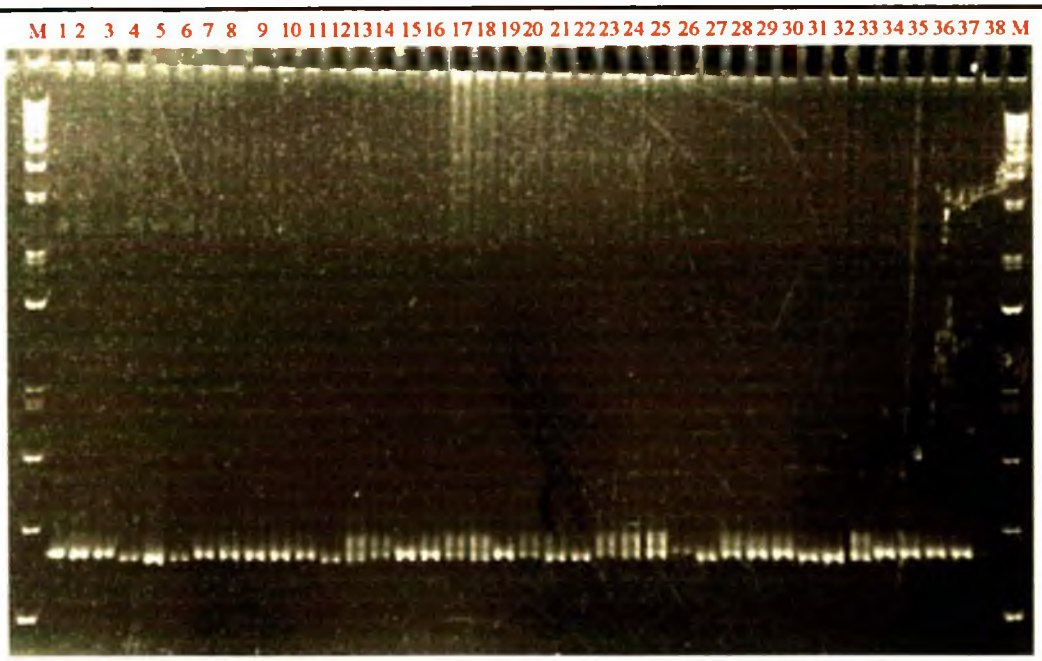

Fig. 4.3 DNA fingerprinting of 36 cultivars using the primer (SalT5′3′) homologous to stress related gene salT. L 1: Binnatoa, L 2: Boilam 3538, L3: Gheegoj 2413, L 4: Kajalshail 612, L 5: Modhumaloti 614 ,L 6: Nonashail, L 7: Gunshi 3869, L 8: Kaliboro 1281, L 9: Soloi 1713, L 10: Khaiyaboro 4539, L 11: Hida, L 12: Rajashail 1061, L 13: Ashfal, L 14: Benapole, L 15: Pokkali, L 16: BR29, L 17: Bajramuri , L 18: Dakshail , L 19: Hoglapata, L 20: Horkuch, L 21: Jamainaru2039 , L 22: Kachra 973, L 23: Kalmilata, L 24: Lakshmikajal,L 25: Mohilu , L 26: Morichshail, L 27: PatnaiBalam, L 28: RaniSelute, L 29: Capsule, L 30: Chini Kanai, L 31: Dhulubecj, L 32: : Jatai Balam, L 33: Moynamoti, L 34: Pokkali, L 35: BR29 , L 36: NonaBokra , L 37: IR29 , L 38: BR40 , G: Gap, M: DNA marker.

4.2.2 Restriction digestion of the amplified product using primers homologous to the gene lea3, Calmod and salT

Restriction digestion of amplified products using primers LEA35′3′, Calmod 5′3′ and SalT 5′3′were done to search further polymorphism among the cultivars. This is the first time search for restriction maps within the amplified products using rice DNA sequence databases and software OMIGA version 2. According to the data, the following restriction enzymes were selected for digestion purpose since recognition sequences were present in one or two position. (Table 4.3). Polyacrylamide gel electrophoresis was used for separating the digested fragments.

Table 4.3 Restriction digestion of the amplified product to find polymorphism.

Sl. No.	Primer locus(expected product size)	Selected restriction enzyme	Fragments generated	No. of polymorphic band(s)	Observation/ comment
1.	LEA35′3′ (941 bp)	*Taq*I	716 bp+226 bp	Two	Polymorphic
		*Hae*III	335 bp+397 bp + 101 bp	Three	Polymorphic
2.	Calmond5′3′ (1254 bp)	*Bgl*II	567 bp+693 bp	Zero	No polymorphism
		*Xho*I	818 bp+442 bp	Zero	No Polymorphism
3.	SalT 5′3′ (574 bp)	*Eco*RI	380 bp+161 bp	Zero	No polymorphism
		*Taq*I	447 bp+94 bp	Zero	No polymorphism

Two and three levels of polymorphisms were found when the amplified products, using primer LEA3 5′3′ were digested with *Taq*I (Fig. 4.4a) and *Hae*III (Fig. 4.4b) respectively (Table 4.4).

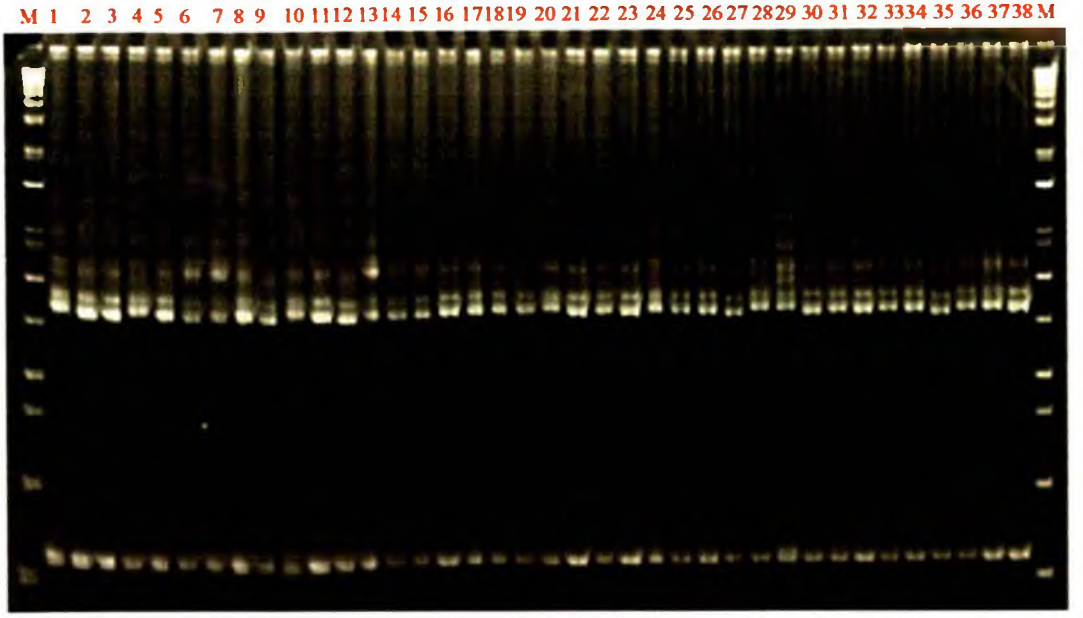

Fig. 4.4a Restriction digestion *(TaqI)* of the amplified product of traditional landraces using primer LEA3 5′3′ . L 1: Binnatoa, L 2: Kajalshail 612, L3: Nonashail, L 4: Soloi 1713, L 5: Ashfal ,L 6: Benapole, L 7: Bajramuri, L 8: Dakshail, L 9: Hoglapata, L 10: Pokkali L 11: BR29, L 12: Horkuch, L 13: Lakshmikajal, L 14: Morichshail, L 15: RaniSelute, L 16: Capsule, L 17: Dhulubeej, L 18: Jatai Balam, L 19: Moynamoti, L 20: Pokkali, L 21: BR29, L 22: NonaBokra, L 23: IR29, L 24: Boilam 3538, L 25: Gheegoj 2413, L 26: Modhumaloti 614, L 27: Gunshi 3869, L 28: Kaliboro 1281, L 29: Khaiyaboro 4539, L 30: Hida, L 31: Rajashail 1061, L 32: Jamainaru 2039, L 33: Kachra 973, L 34: Kalmilata, L 35: Mohihi, L 36: PatnaiBalam , L 37: Chini Kanai, L 38: BR40, M: DNA marker.

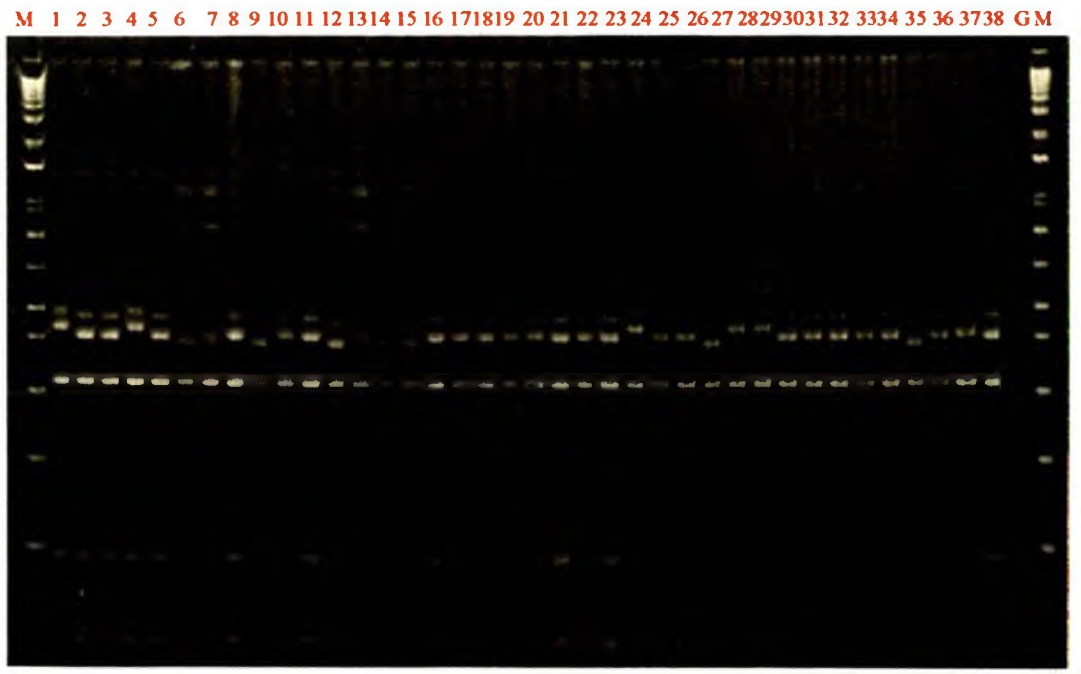

Fig. 4.4b Restriction digestion *(Hae*III*)* of the amplified product of traditional landraces using primer LEA3 5′3′. Loading sequence same as described in Fig: 4.4a. G:Gap.

Table 4.4 Digestion of LEA3 5′3′ amplified product with *Taq*I (Fig.4.4a) and *Hae*III (Fig.4.4b).

Restriction enzyme	Polymorphism level	Name of the Cultivar
*Taq*I	Upper (1 0)	Binnatoa, Soloi1713, Lakshmikajal, Murishail, Raniselute1061, Capsule, Dhulubeez, JataiBalam, Moynamoti, Boilam3538, Gheegoj2413, Modhumaloti614, Kaliboro1281, Khaiyaboro4539, Rajashail1061, Jamainaru2039, Kachra973, Kalmilata, Patnai Balam, Chinikanai, BR40, BR29, Pokkali.
	Lower (0 1)	Kajalshail612, Nonashail, Ashfal, Benapole, Bajramuri, Dakshail, Hoglapata, Horkuch, Nonabokra, Gunshi3869, Hida, Mohini, IR29.
*Hae*III	Upper (1 0 0)	Binnatoa, Soloi1713, Boilam3538, Kaliboro1281, Khaiyaboro4539.
	Middle (0 1 0)	Kajalshail612, Nonashail, Ashfal, Dakshail, Lakshmikajal, Capsule, Dhulubeez, Jataibalam, Moynamoti, Nonabokra, Gheegoj2413, Modhumaloti614, Hida, Rajashail1061, Jamainanu2039, Kachra973, Kalmilata, Patnaibalam, Chinikanai, BR40, Pokkali, BR29, IR29.
	Lower (0 0 1)	Benapole, Bajramuri, Hoglapata, Horkuch, Murishail, Raniselute1061, Gunshi3869, Mohini.

4.3 Discussions

The primers used (Calmod, Lea, SalT, Sod) were homologous to the coding region and part of the upstream as well as downstream region (5′ and 3′ UTR) of the lea3 and SalT genes. 5′-UTR sequences are defined as the mRNA region spanning from the Cap site to the starting codon (excluded), where as 3′-UTR sequences are defined as the mRNA region spanning from the stop codon (excluded) to the polyA starting site.

Since UTR regions are transcribed but not translated they are known to contain specific signals for efficient translation and mRNA stability which can respond to presence or absence of cofactors like iron, calcium etc. Primers corresponding to UTR rather than the actual coding region is expected to be polymorphic (Pesole et al., 2002).

18 LRs along with Pokkali, Nonabokra, IR29 and tolerant &sensitive BRRI varieties (BR40, BR29) showed middle level (0 1 0) polymorphic status when LEA3 5′3′ amplified DNAs of these cultivars were digested with *Hae*III . All most all (17) LRs showing this king of polymorphic status corresponds to the three groups (SW2, SW3 and Het) observed by Lisa et al., 2004 (see Appendix A4. and Table 4 of A5.)

Six LRs showed lower level (0 0 1) polymorphic status after *Hae*III digestion (see Appendix A4.) corresponds to the SW1 group observed by Lisa et al., 2004 (see Appendix A5.). Additional five LRs showed upper level (1 0 0) polymorphic status corresponds to the MNE1 and MNE2 groups of Lisa et al., 2004 (see Appendix A4. and A5.). Thus homology in grouping found after DNA fingerprinting with microsatellite primers and primer homologous to stress inducible gene lea3 proved reliable clustering among landraces.

DNA fingerprinting using gene-specific primers has recorded differences or polymorphism between various LRs. Therefore, expression analysis of these genes under salt stress might give a better indication about any positive role played by them in salt tolerance of the landraces.

Chapter 5:
Expression Study Of Some Stress-Linked Genes Under 100 mM Salt Stress

5.1 Materials and Methods

5.1.1 Study of specific gene expression

5.1.1.l Rice varieties selected for gene expression study

1. Pokkali (as a positive control)
2. IR29 (as a negative control)
3. Modhumaloti 614
4. Hoglapata
5. Boilam 3538
6. Dakshail
7. Kajalshail 612
8. Kaliboro 1281
9. Gheegoj 2413
10. Kalmilata

5.1.1.2 Application of salt stress on 18 days old rice seedlings

5.1.12.1 Materials used

i. Seedling floats

Floats were used to grow germinating seeds in the hydroponic system. A rectangular piece of 1.25 cm thick styrofoam size 28cm X 32cm was cut and then drilled holes with about 1 cm diameter in rows of 15. A piece of nylon net was attached to one side and stiched with nylon thread.

ii. Plastic tank/tray

iii. Concentrated H_2S0_4

iv. 1N NaOH

v. 1N HCl

vi. Stock nutrient solutions

a. Macronutrient

To prepare 1(one) liter stock solution, required amount of reagents (Table 5.1) were weighed and dissolved in ddH_2O. Finally it was made up to 1000 ml. All macronutrient stock solutions were prepared in this way and separately.

b. Micronutrients

To prepare 1(one) liter of micronutrient solution, each reagent listed in Table 5.1 was weighed accurately and dissolved separately in 100 ml ddH_2O. Then all solutions were mixed together and combined with 50 ml conc. H_2SO_4. Then the final volume was made up to 1(one) liter by adding ddH_2O. The final color of this solution was yellowish brown.

Table 5.1 Constituents of stock solution (Yoshida et al., 1976).

Element	Reagent/nutrient salt	Preparation (gm/L)
Macronutrient		
N	• Ammonium nitrate (NH_4NO_3)	91.4
P	• Sodium phosphate, mono basic monohydrate ($NaH_2PO_4.H_2O$)	35.6
K	• Potassium sulfate (K_2SO_4)	71.4
Ca	• Calcium chloride ($CaCl_2$)	88.6
Mg	• Magnesium sulfate, 7 hydrate ($MgSO_4.7H_2O$)	324.0
Micronutrient		
Mn	• Manganous chloride, 4 hydrate ($MnCl_3.4H_2O$)	1.5
Mo	• Ammonium molybdate, 24 hydrate $[(NH_4)_6 Mo_7 O_{24}.24H_2O]$	0.074
Zn	• Zinc sulfate, 7 hydrate ($ZnSO_4.7H_2O$)	0.035
B	• Boric acid (H_3BO_3)	0.934
Cu	• Cupric sulfate ($CuSO_4.5H_2O$)	0.031
Citric acid	• Citric acid, mono hydrate ($C_6H_8O_7, H_2O$)	11.9
Fe	• Ferric chloride, 6 hydrate ($FeCl_3.6H_2O$)	7.7

vii. Nutrient culture solution

The following reagent stocks were necessary for the preparation of culture solution (Table 5.2). To prepare every 4(four) liter of culture solution, 5 ml each of the stock solution was added to 1(one) liter of water and the volume was made up to 4(four) liter with tap H_2O. The P^H of the culture solution was adjusted at 5 using 1N HCl or 1N NaOH whichever necessary.

Table 5.2 Composition of nutrient culture solution (Yoshida et al., 1976)

Element	Reagent/ nutrient salt	Stock solution added for 4 L of culture solution (ml)	Conc. of element in nutrient solution (ppm)
Macron utrient			
N	• NH_4NO_3	5	40
P	• $NaH_2PO_4.2H_2O$	5	40
K	• K_2SO_4	5	40
Ca	• $CaCl_2$	5	40
Mg	• $MgSO_4.7H_2O$	5	40
Micron utrient			
Mn	• $MnCl_3.4H_2O$	5	0.05
Mo	• $(NH_4)_6\ Mo_7\ O_{24}.24H_2O$	5	0.05
Zn	• $ZnSO_4.5H_2O$	5	0.01
B	• H_3BO_3	5	0.02
Cu	• $CuSO_4.5H_2O$	5	0.01
Citric acid	• $C_6H_8O_7.H_2O$	5	-
Fe	• $FeCl_3.6H_2O$	5	2.00

5.1.1.2.2 Procedure

1. The seeds of each variety/cultivar were dried very well in the sunshine (or heat shocked at 50^0C for 24h to break the dormancy). The seeds of each variety were placed in petridishes with moisture filter papers and incubated at 37^0C for 3-4 days to germinate.
2. After germination , the seeds were transplanted into the hole of styrofoam with net and kept on water in stainless steel trays for one day.
3. The styrofoam containing seedlings were transferred in sufficient amount of nutrient culture solution containing plastic trays. This experiment was done in a net house covered by plastic sheet for protection from rain and birds.
4. The nutrient culture solution was renewed every 3 days and maintained at a P^H 5.0-5.1daily by adding acid or alkali.
5. Due to evaporation and transpiration there was loss of solution volume in the trays and the optimum level was maintained with tap water in every two days. The seedlings were grown here for 16-18 days.
6. 100 mM NaCl stress (105.19 g NaCl in 18 liter solution) was applied to the seedlings.
7. Shoot and root samples of both stressed and without stressed seedling were collected at different timings (i.e. 0h, 1h, 4h, 24h, 6 days) after applying salt stress.
8. The visual symptoms of salt injury or toxicity of different varieties were recorded at different timings.

5.1.2 Isolation of total RNA after 100 mM salt stress using TRIZOL reagent

Total RNA from root and shoot samples, collected at different time points after applying 100 mM NaCl to 18 days old rice seedings and then quantified using spectrophotometer and gel estimation. Detailed prodecures for total RNA isolation and quantification are given in Appendix C. (see section C6.).

5.1.3 One-step RT-PCR with gene specific primers (GSP)

5.1.3.1 Gene specific cDNA primers

Sl. No.	Primer/Gene	Primer sequence	Annealing temp. (°C)	Product size range (bp)	Type of gel used
1	Calmod NC	Forward: atggcggaccagctcaccgacga Reverse: caccatcaacatcggcctgaccg	68	1178	2.5 % Agarose
2	LEA3 NC	Forward: ctaccgcgccggcgagacca Reverse: tccctcgccgtcgtctccgt	67	571	3% Agarose
3	SalT NC	Forward: atgacgctggtgaagattggcc Reverse: ggtggacgtagatgccaattgc	60	433	3% Agarose
4	SOD (Cc2)A (designed)	Forward: gtgtcaagggcaccatcttt Reverse: ctaaccctggagtccgatga	53.9	1134	2.5% Agarose
5	SOD (Cc2)B (designed)	Forward: ggcaccacaagatgagaacc Reverse: tcaggatcagcatggacaac	54	424	3% Agarose

Source: Rangan et al., 1999

5.1.3.2Designing of primers

Two primers, namely SOD (Cc2)A and SOD (Cc2)B were designed for the amplification of expressed as well as intron sequences of the gene for copper/zinc-superoxide dismutase. SOD(Cc2) gene organization was found using NCBI and RGP databases and that was-

- Gene- 2129....2204, 2289....2390, 2479....2574, 2737....2768, 2875....2950, 3087....3140, 3277....3299
- Total length–3522 bp, Exon-7, Intron-7, Non-coding exon-1497-1544.

The primers were designed using web based software primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi) and the designed primers were checked using the software OMIGA version 2.0.

Data placed in primer designing software were as follows:

- %GC = 50- 60 (50-55-60)
- Primer length (bp) = 20-22(19-20-21)
- Tm = 55-60-65, Maximum Tm difference = 2°C
- No. of return = 50
- Self <4, 3′ <2, any 3-1
- Organism- rice

Finally, suitable primers were picked up among the rank of potential primers.

5.1.3.3 RT-PCR (Reverse transcriptase-Polymerase chain reaction)

The superscript ™ one-step with platinum ® Taq system was designed for the convenient, sensitive and reproducible detection and analysis of RNA molecules by RT-PCR. Both cDNA synthesis and PCR were performed in a single tube. Using gene-specific primers and target RNAs from either total RNA or mRNA. RT/ platinum ® Taq mix was a mixture of recombinant Taq DNA polymerase complexed with proprietary antibody that inhibits polymerase activity and superscript ™II H-reverse transcriptase. The activity of Taq DNA polymerase was blocked at ambient temperature but was regained after the denaturation step in PCR cycling at 94^0C. This reagent provided an automatic "hot start" for Taq DNA polymerase in PCR.

Protocols followed for RT-PCR experiment are given in Appendix C. (see section C7.).

5.1.3.3.1 Important parameters for consideration during RT-PCR

- High quality intact RNA was made available for successful full-length cDNA synthesis.
- RNA were devoid of any RNase contamination and aseptic conditions were maintained.
- A final primer concentration of 0.2 μM is generally optimal. But for best results, a primer titration using 0.15-0.5 μM wasdone.
- Primers were not self-complementary or complementary to each other at the 3′ ends.
- 1.2 mM final conc. of magnesium in the reaction mix works well for most targets. Therefore, the magnesium conc. was further optimized (1.2-2.0 mM) with $MgSO_4$ solution when required.
- The annealing temperature was 10^0C below the melting temperature of the primers used. The optimal temperature for reverse transcription was depend on primer and target sequences.

5.2 Results

5.2.1 Recovery responses of different traditional landraces at seedling stage along with Pokkali and IR29

Wilting of the leaves of all varieties were visualized within one hour after applying salt stress of 100 mM (Fig. 5.1). Also the different traditional landraces showed different type of recovery response .

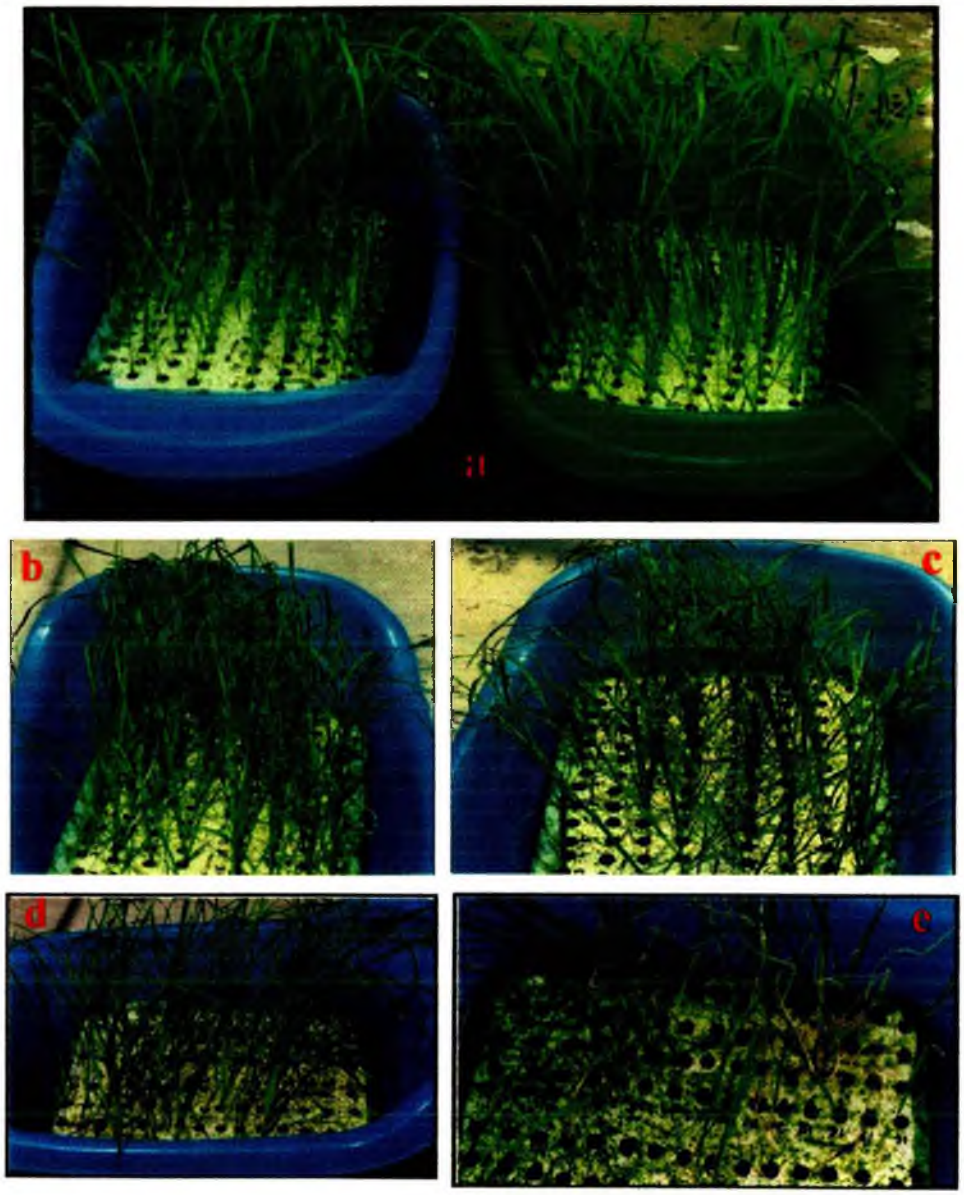

Fig. 5.1 Salt stress (100 mM) responces of some selected traditional landraces

a) Control and stressed sample at 0 hr

b) Stressed sample after 1 hr

c) Stressed sample after 4 hr

d) Stressed sample after 24 hr

e) Stressed sample after 6 days

Salt tolerant landraces adapt themselves in order to survive in presence of salt stress using its own physiological and molecular mechanism. Salt tolerant Pokkali leaves exhibited immediate rolling but recover turgor within 4 h of salt stress and alive at 6 days. On the other hand, IR29 took ~24 hrs to show leaf rolling but failed to recover turgor and thus remained wilted and eventually died at 6 days. Among the eight test landraces, Madhumaloti614, Boilom3538 and Gheegoj2413 showed quick recovery within 4 hr of salt stress, similar to Pokkali. In addition to these varieties, Hoglapata, Dakshail and Kajalshail 612 showed recovery responses within 24 hr after salt stress. Kaliboro 1281 showed very poor recovery response from the beginning and remain wilted although did not die upto 6(six) days of salt stress. Madhumaloti614, Boilam3538, Kajalshail612 and Kalmilata were able to recover.

5.2.2 RNA isolation and quantitation

Total RNA isolated from leaf and root samples of the selected traditional landraces (section 5.1.1.1) at the seedling stage before and after application of salt stress (1 hr to 6 days). After taking absorbance reading, all RNA samples were diluted to a concentration of 0.125 μg/μl. All samples were also, quantitated based on visual estimation on 1.5% denaturing agarose gels (Fig. 5.2).

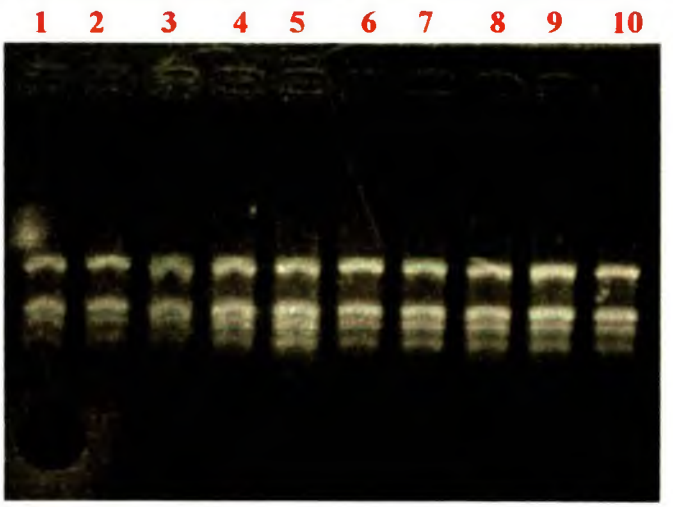

Fig. 5. 2 Shoot RNA samples after electrophoresis on 1.5% agarose gel. L 1: Pokkali , L2: IR29, L3: Modhumaloti 614, L4: Hoglapata, L5: Boilam 3538, L6: Dakshail, L7: Kajalshail 612, L8: Kaliboro 1281, L9: Gheegoj 2413, L10: Kalmilata

5.2.3 Expression of oslea3 gene in seedling roots and leaves of a salt tolerant, Pokkali and a salt sensitive variety, IR29 exposed to salt stress

Salinity stress triggers the expression of several osmoresponsive genes and proteins in rice tissues (Claes et al., 1990). Moons et al., (1997) have reported the salt inducible expression of *Oryza sativa* lea3 gene. Therefore the expression of this gene was monitored in rice landraces and using Pokkali and IR29 as a tolerant and sensitive control respectively.

Transcriptome was changed when 100 mM (~ 12 ds/m) salt stress was applied to the 18 days-old seedling of Pokkali and IR29 in hydroponic system. Expression of a salt inducible gene oslea3 was monitored, at different timings after stress, by a one step RT-PCR techniques. A maximal expression of oslea3 was found in root after 4 hr of salt shock. It was shown that expression started after 1 hr (it might be earlier, Kawasaki et at., 2001), peaked at 4 hrs and became down regulated on or before 6 days (Fig.5.3).

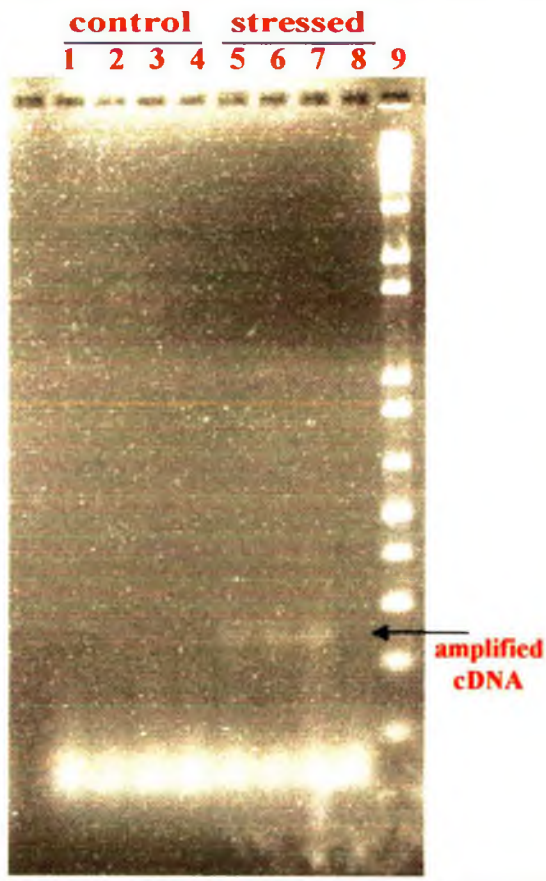

Fig.5.3 RT-PCR of Pokkali root RNA using LEA3 NC primer.

Lane1: 0h, control
Lane2: 1h, control
Lane3: 4h, control
Lane4: 24h, control
Lane5: 1h, stressed
Lane6: 4h, stressed
Lane7: 24h, stressed
Lane8: 6d, stressed
Lane9: 1kb plus DNA ladder.

On the other hand, expression in the leaf remained upregulated even on/after 6 days (Fig. 5.4).

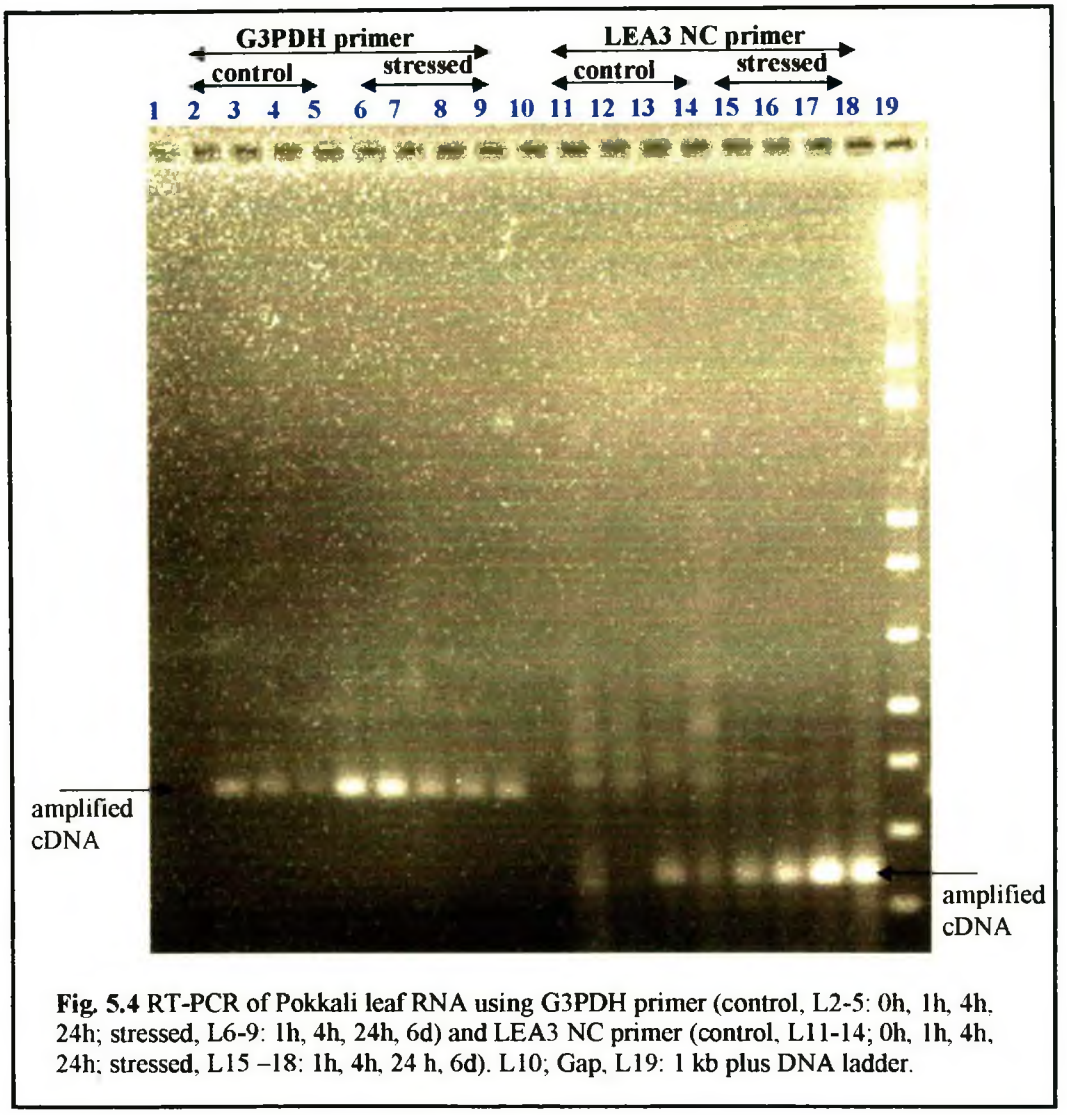

Fig. 5.4 RT-PCR of Pokkali leaf RNA using G3PDH primer (control, L2-5: 0h, 1h, 4h, 24h; stressed, L6-9: 1h, 4h, 24h, 6d) and LEA3 NC primer (control, L11-14; 0h, 1h, 4h, 24h; stressed, L15 –18: 1h, 4h, 24 h, 6d). L10; Gap, L19: 1 kb plus DNA ladder.

These results confirmed the report that a tolerant variety such as Pokkali delayed drop in shoot water content (Moons at el., 1997). It is possible that LEA protein might help in retention of water.

For the sensitive control, IR29, no expression/upregulation of the lea3 gene was detected after salt stress at any time (Fig. 5.5)

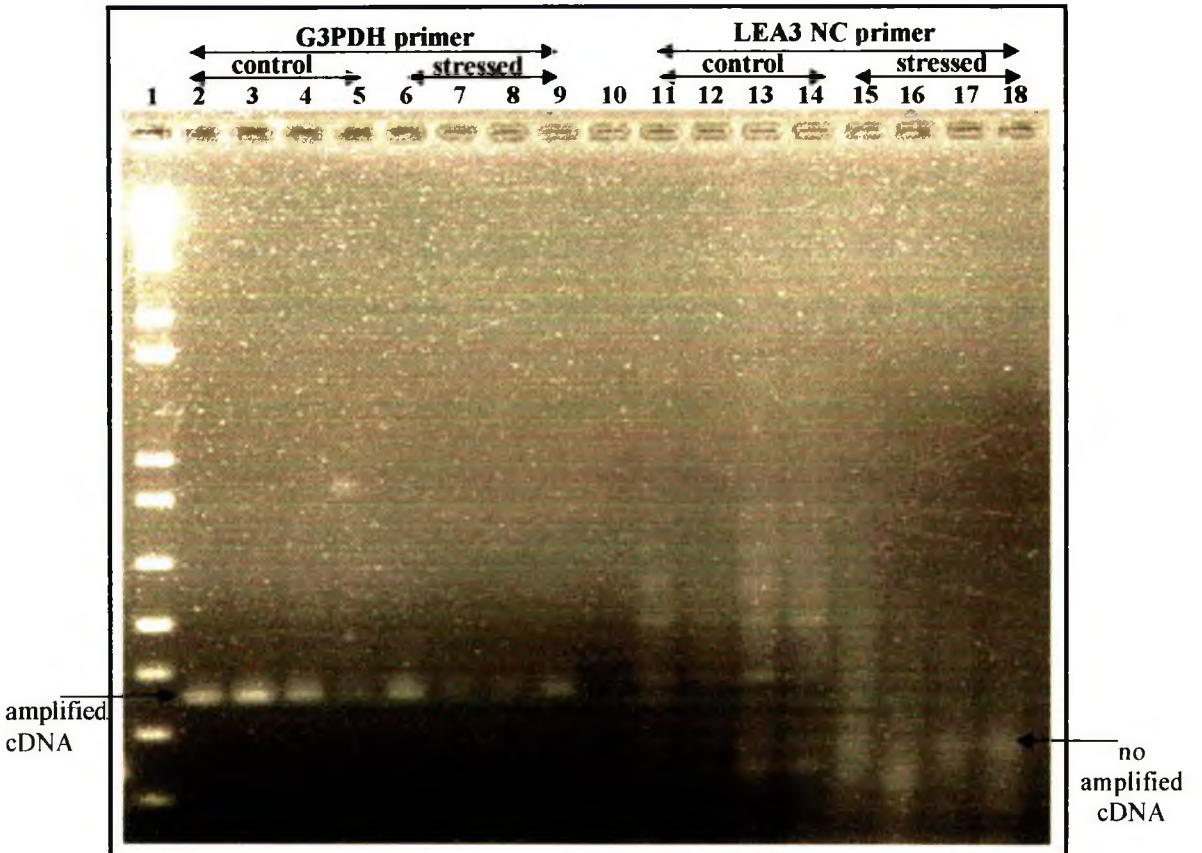

Fig. 5.5 RT-PCR of IR29 shoot RNA using G3PDH primer (control, L2-5: 0h, 1h, 4h, 24h; stressed, L6-9: 1h, 4h, 24h, 6d). L1: 1Kb plus DNA ladder; L10:Gap. LEA3 NC primer (control, L11-14: 0h, 1h, 4h, 24h; stressed, L15–18: 1h, 4h, 24h, 6d).

These results indicated the differences in salt-stress responsive regulation of oslea3 expression in Pokkali and IR29.

5.2.4 Expression of oslea3 gene in some traditional landraces

A comparative analysis of oslea3 expression in root and leaf of eight traditional landraces compared to Pokkali and IR29 (Table 5.3). The lanraces showed a variable expression patterns both for root and leaf RNA samples Maximal expression was noted at 24 hrs which decreased at on/before 6 days for any variety at random. Therefore these two time points were used for expression assay.

Table 5.3 Expression profile of oslea3 gene in leaf and root of some landraces at different time point.

Sl. No.	Variety/landrace	Shoot/leaf, stressed for		Root, stressed for		
		24 hr	6 days	4 hr	24 hr	6days
1	Pokkali	++	++	+++	+	0
2	IR29	0	0	0	0	0
3	Madhumaloti 614	0	+++	++	0	0
4	Hoglapata	0	0	0	+	0
5	Boilam 3538	++	++	++	+	0
6	Dakshail	+	0	0	0	0
7	Kajalshail 612	++	+++	++	+++	+
8	Kaliboro 1281	+++	no sample	+	++	no sample
9	Gheegoj 2413	+	+	0	+++	+
10	Kalmilata	0	0	++	++	0

N.B. '+++' indicate high expression; '++' indicate medium expression; '+' indicate low expression; '0' indicate very low/ insufficient expression

5.2.4.1Expression in root

In case of roots, 4 hrs stress was also checked for expression studies. Root tissues of the cultivars Madhumaloti 614, Boilam 3538 and Kalmilata showed peak lea3 expression at 4 hrs of stress which coincided with the expression pattern of Pokkali (Fig. 5.6). In these LRs, expression of lea3 was reduced at 24hrs and on /before 6 days. For the root tissues of Kajalshail 612 and Gheegoj 2413, expression was noticed even after 6 days of salt stress (Fig. 5.7). For the other two landraces, Hoglapata and Dakshail, insignificant expression was found for all 3 timings (4h, 24h, 6d). A medium level of expression was observed at 24h of salt stress in case of kaliboro1281.

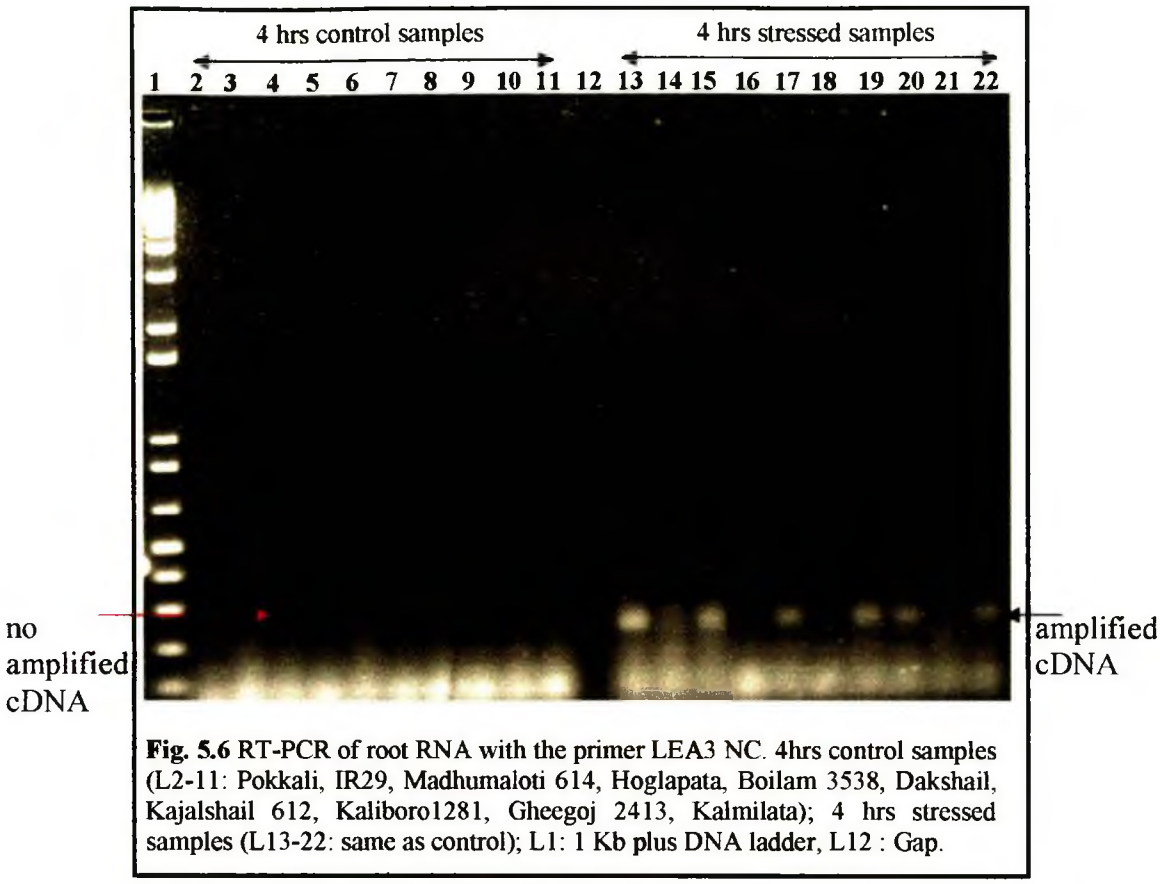

Fig. 5.6 RT-PCR of root RNA with the primer LEA3 NC. 4hrs control samples (L2-11: Pokkali, IR29, Madhumaloti 614, Hoglapata, Boilam 3538, Dakshail, Kajalshail 612, Kaliboro1281, Gheegoj 2413, Kalmilata); 4 hrs stressed samples (L13-22: same as control); L1: 1 Kb plus DNA ladder, L12 : Gap.

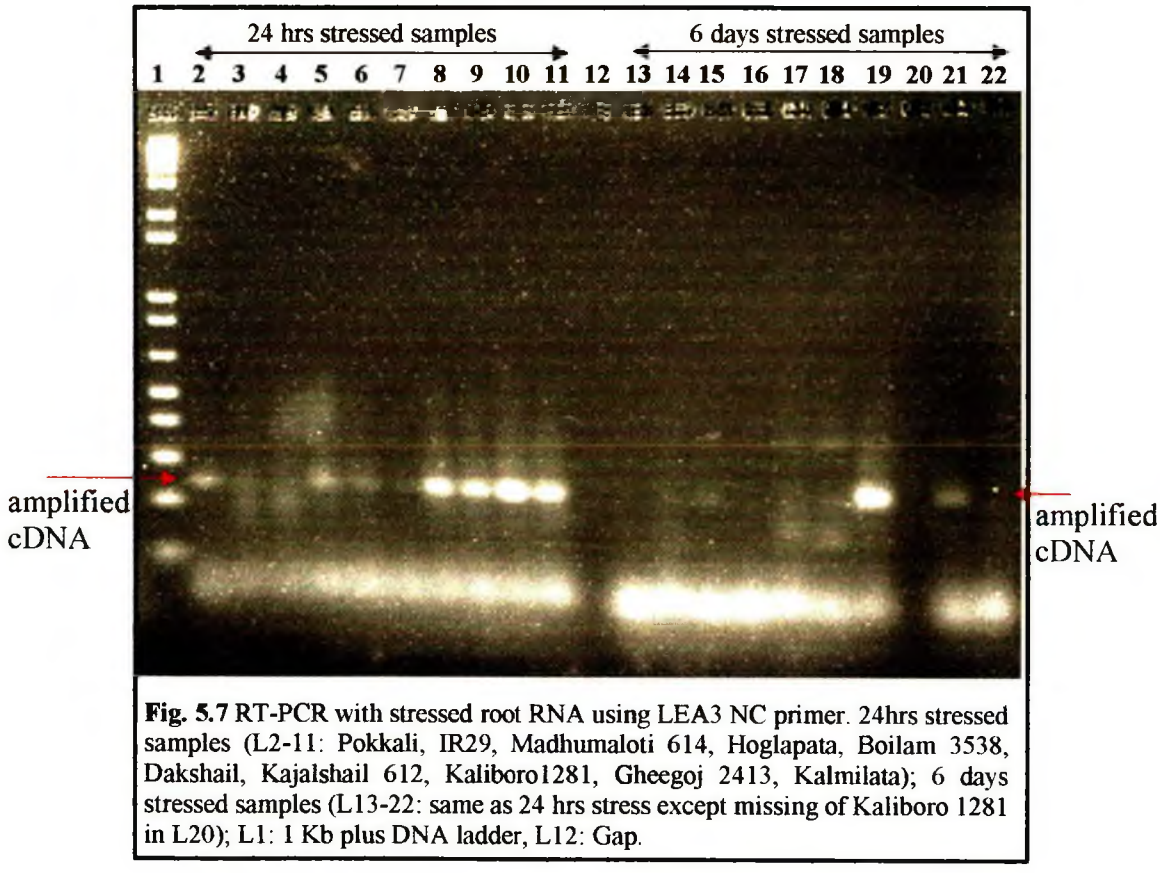

Fig. 5.7 RT-PCR with stressed root RNA using LEA3 NC primer. 24hrs stressed samples (L2-11: Pokkali, IR29, Madhumaloti 614, Hoglapata, Boilam 3538, Dakshail, Kajalshail 612, Kaliboro1281, Gheegoj 2413, Kalmilata); 6 days stressed samples (L13-22: same as 24 hrs stress except missing of Kaliboro 1281 in L20); L1: 1 Kb plus DNA ladder, L12: Gap.

5.2.4.2 Expression in leaf/shoot

For three landraces, Madhumaloti 614, Boilam 3538 and Kajalshail 612, a high level of expression of lea3 gene persisted in leaf after 6 days of salt stress which coincided with the expression pattern of Pokkali. For other three varieties Hoglapata, Dakshail and Gheegoj 2413, insignificant expression was found (Fig.5.8) Kaliboro 1281 showed a high expression at 24 hrs of salt stress (unfortunately 6 days sample was missing). Finally, Kalmilata showed no expression at 24 hr/6d of salt stress which contrasts with the expression of the lea3 gene in root tissue of this plant.

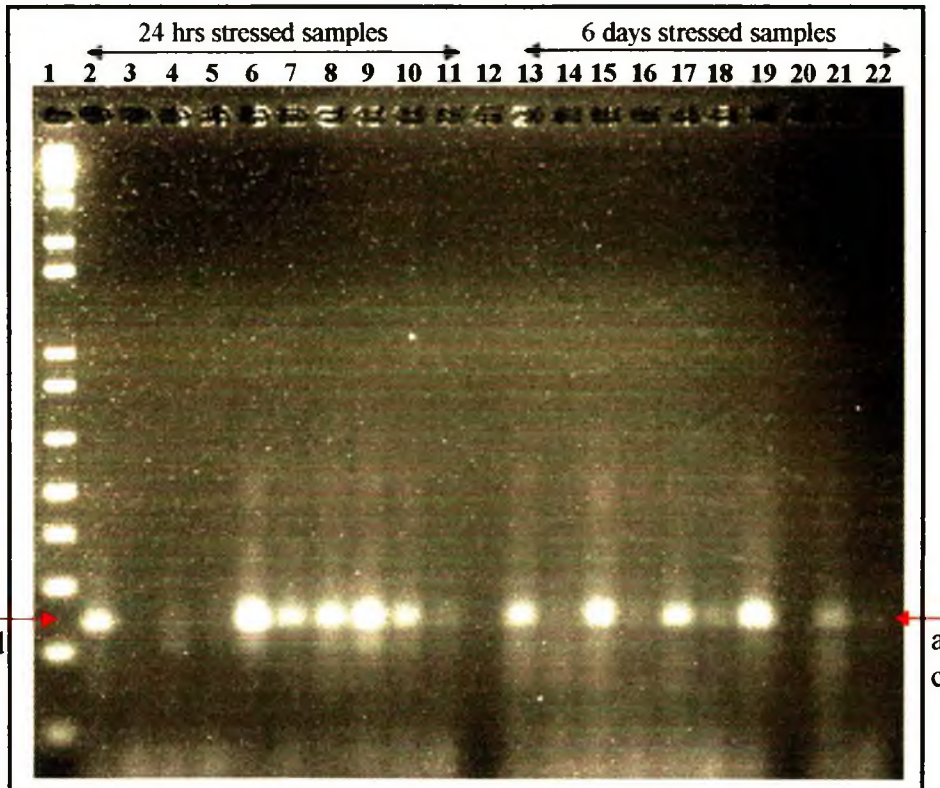

Fig. 5.8 RT-PCR with stressed leaf RNA samples using LEA3 NC primer. 24hrs stressed samples (L2-11: Pokkali, IR29, Madhumaloti 614, Hoglapata, Boilam 3538, Dakshail, Kajalshail 612, Kaliboro1281, Gheegoj 2413, Kalmilata); 6 days stressed samples (L13-22: same as 24 hrs stress except missing of Kaliboro 1281 in L20); L1: 1 Kb plus DNA ladder, L12 : Gap.

5.2.4.3 Constitutive expression of G3PDH gene

During the above mentioned expression studies with the lea3 gene, a control RT-PCR was also done with the primer for G3PDH gene. The expression of this house keeping protein glyceraldehyde-3-phosphate dehydrogenase did not vary under most conditions (Fig. 5.9).

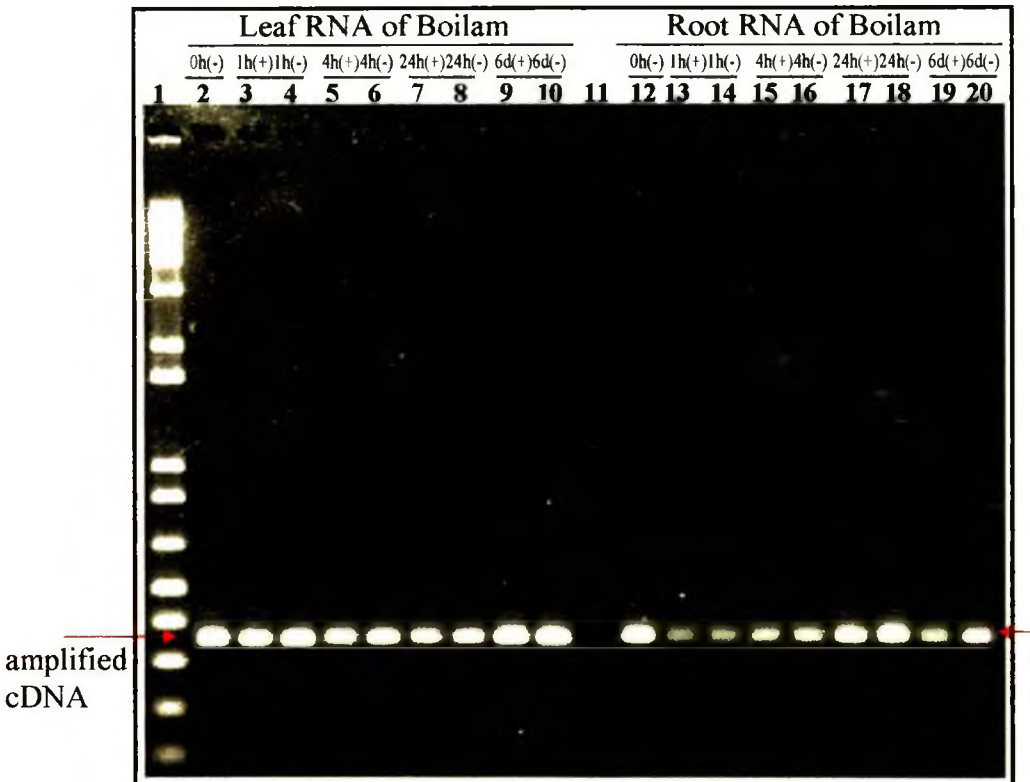

Fig. 5.9 RT-PCR using the primer G3PDH. Leaf RNA of Boilam [(L2-10: 0h(-), 1h (+), 1h (-), 4h (+), 4h (-), 24h (+), 24h (-), 6d (+), 6d (-)]; Root RNA of Boilam (L12-20: same as leaf). L1 : 1 kb plus DNA ladder; L11: Gap. N.B. '—' stand for control samples and '+' stand for stressed samples.

5.2.5 Expression of Calmod, salT and Sod genes in roots and leaves of selected traditional landraces at seedling stage

RT-PCR experiments were also carried out using the gene specific primers for Sod, Calmod and salT gene (Table 5.4 and Fig. 5.10).

Table 5.4 Expressional study with some gene specific primers

Sl. No.	Primer	RNA sample	Duration of stress
1	Sod(Cc2) A	• Leaf (Boilam 3538)	• Control: 0h, 1h, 4h, 24h,6d • Stress: 1h, 4h, 24h, 6d
2	Sod(Cc2) B	•Leaf (Boilam 3538)	• Control: 0h, 1h, 4h, 24h,6d • Stress: 1h, 4h, 24h, 6d
3	SalT NC	• Root (Boilam 3538)	• Control: 0h, 1h, 4h, 24h, 6d • Stress : 1h, 4h, 24h, 6d
		• Root (all selected varieties)	• Control: 4hr • Stress : 4hr (Fig. 5.10)
4	Calmod NC	• Root (Kajalshail 612)	• Control: 0h, 1h, 4h, 24h, 6d • Stress : 1h, 4h, 24h, 6d

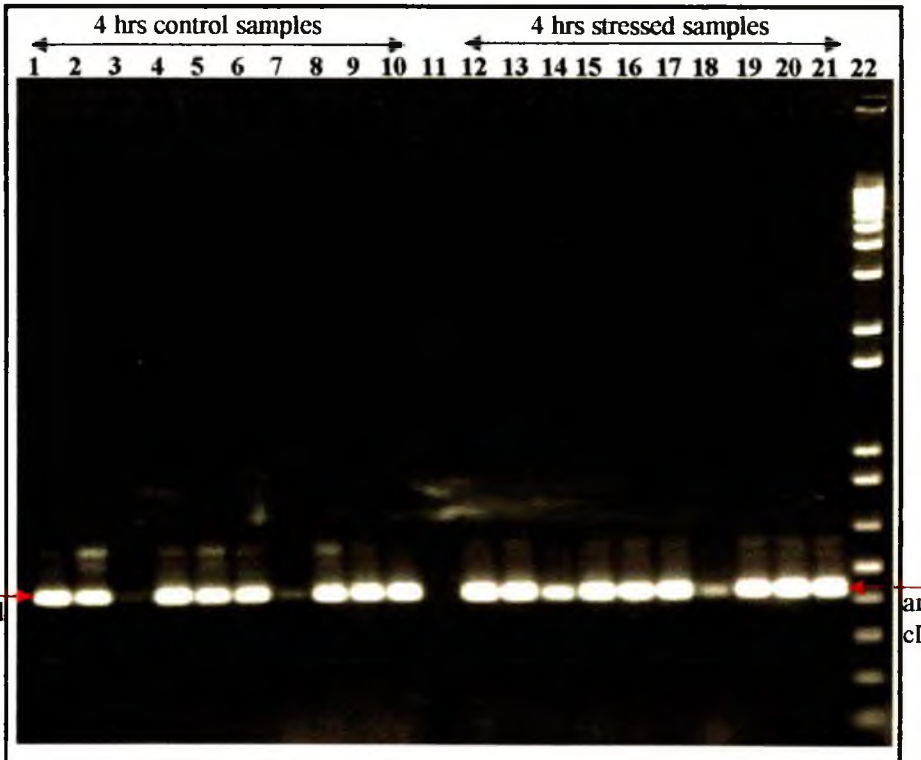

Fig.5.10 RT-PCR with 4hr control and stressed root RNA samples using the primer SalT NC. Control (L1-10: Pokkali, IR29, Modhumaloti 614, Hoglapata, Boilam 3538, Dakshail, Kajalshail 612, Kaliboro 1281, Gheegoj 2413, Kalmilata); stressed (L12-21: same as control). L11: Gap; L22: 1 kb plus DNA ladder.

There were no significant differences for control and salt stress condition at any time point. The result indicated no correlation between expression profile and differences in salt tolerance levels among the traditional landraces.

5.3 Discussions

i) The salt stress induced oslea3 transcript gradually declined upon prolonged salt shock at 6 days only in roots.

In case of Pokkali, recovery from salt stress (e.g. regaining of turgor) seems to be related to the degradation of the salt-stress induced LEA proteins. On the otherhand, excess salt in the xylem (apoplastic space) results in water loss from the cell in case of IR29 where no lea3 gene expression was observed and wilting-induced damage eventually caused death.

ii) Expression of lea3 gene for longer period in the leaf of tolerant Pokkali compared to the root may indicate that acclimatization provided by LEA proteins under salinity stress takes more time particularly as the leaf has a more functionally important role e.g. for photosynthesis.

iii) The expression pattern of the house keeping gene G3PDH was nearly equal for most samples under control and stress conditions. Minor differences in G3PDH expression were observed perhaps due to pipetting errors. However the expression of lea3 gene that was observed holds true even after taking these minor differences into account.

vi) The present research was carried out in an open net house with a humidity of ~75%. So this gene expression study may need to be repeated at different times throughout the year, at different temperature and humidity in order to have more conclusive result.

Chapter 6: Conclusion

Conclusion

DNA fingerprinting of 31 traditional landraces, from the coastal belt of Bangladesh, using 60 microsatellite primers and primers linked to stress-related genes (salT, lea etc) identified ten potential cultivars as donors for salt tolerance into modern varieties. These are Benapole, HorKuch, MorichShail, Raniselute, Ashfal, Capsule, Lakshmikajal, KajalShail, Jamainaru and PatnaiBalam. During participatory breeding program, breeders/farmers can select any suitable donor from any one in the groups identified, since these are all low yielding, for introduction into farmer popular modern high yielding varieties. These well-adapted germplasm can therefore be exploited in breeding purpose to develop salt tolerant high yielding varieties resulting in widening of the genetic base for salt tolerance traits.

There may be a general correlation between oslea3 expression and the differences in salt tolerance capacity among the various traditional landraces. lea3 genes have been suggested to be involved in tolerance of cells to dehydration. Expression pattern of lea3 gene in leaf of Madhumaloti 614, Boilam 3538 and Kajalshail 612 was similar to that in salt tolerant Pokkali. In addition, Madhumaloti 614, Boilam 3538 and Kalmilata showed better performance than other varieties and showed similar lea3 gene expression pattern in their roots compared to Pokkali. Therefore these LRs may show the same mechanism of tolerance to stress as Pokkali in the case of lea3 gene expression.

Chapter 7: References

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Appendices

Appendices Index

Appendix A

A1. Selected genes reported to enhance salt tolerance in transformed plants a
A2. Distribution of microsatellite primers over the 12 (twelve) rice chromosomes b
A3. Microsatellite primers; their sequences, annealing temperatures and amplified product size. Also number of polymorphic bands generated. c-f
A4. Homology in grouping of landraces based on their DNA fingerprinting done by microsatellite and stress-related gene (i.e. lea3) linked primer. g
A5. Published article of Lisa at al., (2004) in *Plant and Soil*, Kluwer Academic Publishers, Netherlands. h-w

Appendix B

Solution preparation x

Appendix C

y-ww

C1. Equilibration of phenol y
C2. Isolation and quantification of plant genomic DNA z
C3. Rapid isolation and purification of high-activity Taq DNA polymerase cc-gg
C4. Polymerase chain reaction (PCR) using microsatellite/SSR and gene specific primers. gg-ii
C5. Electrophoresis of PCR amplified product ii-rr

C5.1 Agarose gel electrophoresis ii-kk
C5.2 Denaturing sequencing gel electrophoresis kk-pp
C5.3 Non-denaturing polyacrylamide gel electrophoresis pp-rr

C6. Extraction and quantification of total RNA rr-uu
C7. One step RT-PCR with gene specific primers vv-ww

Appendix D

Searched internet websites ww

Appendix E

E1. Chemicals used and their origin xx
E2. Instruments used and their origin yy

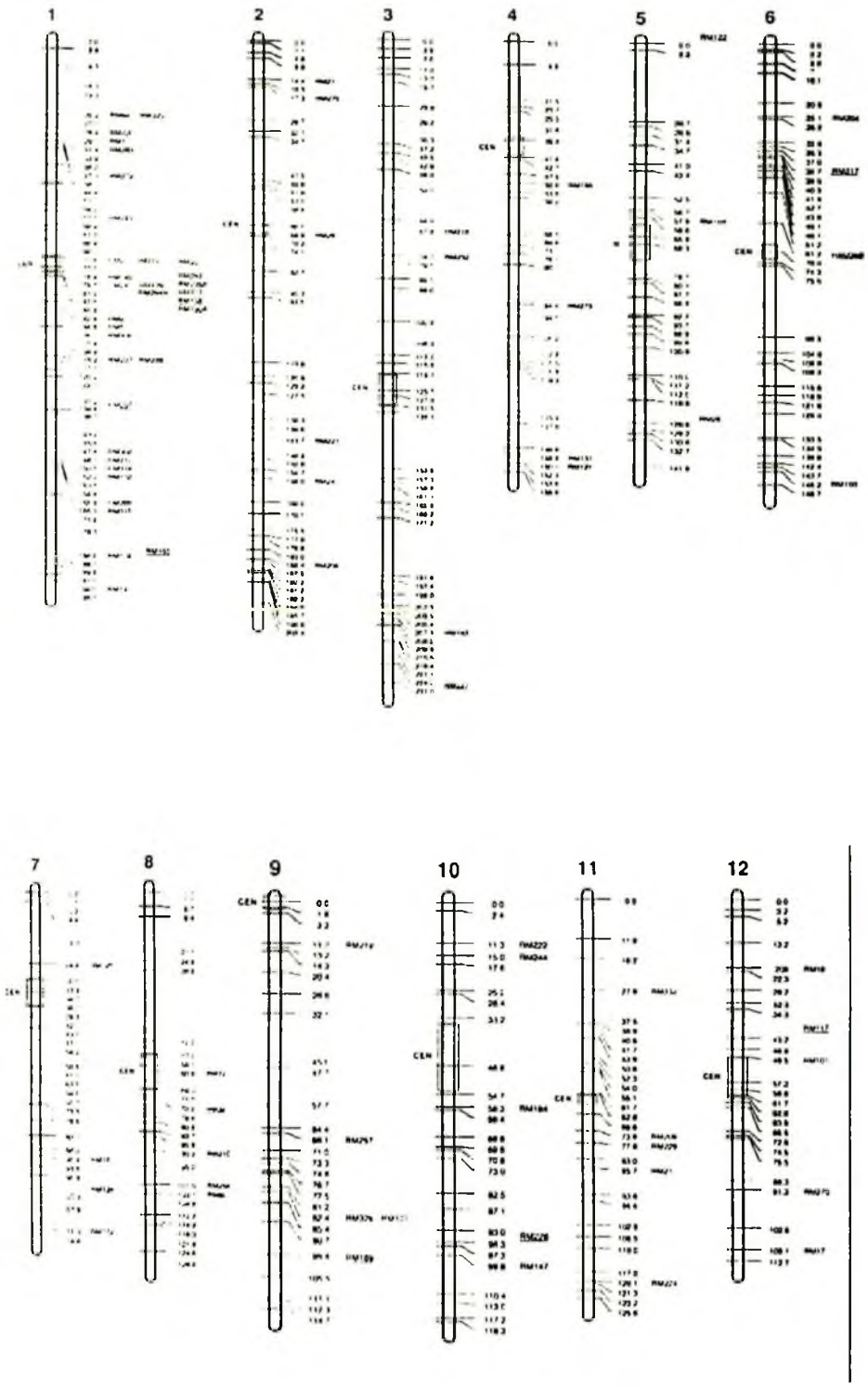
Appendix A

A1. Selected genes reported to enhance salt tolerance in transformed plants

Gene	Form	Plant	Tolerance	Reference
Ion homeostasis				
HAL1	Sense	Tomato	Salt	Gisbert et al. (2000)
NHX1	Sense	Tobacco	Salt	Apse et al. (1999)
Protection				
Barley HVA1	Sense	Rice	Water deficit, Salt	Xu et al. (1996)
Mn-SOD	Sense	Rice	Salt	Tanaka et al. (1999)
Arginine decarboxylase	Sense	Rice	Salt	Roy and Wu (2001)
Osmoprotectant				
Osmotin	Sense	Potato	Salt	Evers et al. (1999)
P5CS	Sense	Rice	Salt	Zhu et al. (1998)
Mannitol-1-phosphate dehydrogenase	Sense	Tobacco	Salt	Tarczynski et al. (1993)
Trehalose-6-phosphate synthase	Sense	Melon	Salt, drought	Serrano et al. (1999)
BetA (CDH)	Sense	Tobacco	Salt	Lilius et al. (1996)
Regulator				
Calcineurin	Sense	Tobacco	Salt	Pardo et al. (1998)
AtGSK1 Kinase	Sense	Arabidopsis	Salt	Piao et al. (2001)
DREB1	Sense	Arabidopsis	Drought, salt, cold	Kasuga et al. (1999)
Alfin1	Sense	Alfala	Salt	Winicov (2000), Winicov and Bastola (1999)

Source : Bennett and Khush, 2003

A2. Distribution of microsatellite primers over the 12 (twelve) rice chromosomes



Source : Temnykh et al., 2000

A3. Microsatellite primers; their sequences, annealing temperatures and amplified product size. Also number of polymorphic bands generated.

Sl. No.	Primer	Chromosome no.	SSR Motif	Primer sequence	Annealing temp (°C)	Product size range (bp)	Type of gel used	No. of polymorphic bands
1	RM05	1	$(GA)_{15}$	Forward: tgcaacttctagctgctcga Reverse: gcatccgatcttgatggg	55	108-130	A	4
2	RM09	1	$(GA)_{15}$	Forward: ggtgccattgtcgtcctc Reverse: acggccctcatcaccttc	55	124-194	A	4
3	RM14	1	$(GA)_{17}$	Forward: ccgaggagaggagttcgac Reverse: gtgccaatttcctcgaaaaa	55	174-191	A	3
4	RM17	12	$(GA)_{21}$	Forward: tgccctgttatttttcttctctc Reverse: ggtgatcctttcccatttca	55	162-184	A	3
5	RM18	7	$(GA)_{15}$	Forward: ttccctctcatgagctccat Reverse: gagtgcctggcgctgtac	55	151-165	A	3
6	RM19	12	$(ATC)_{10}$	Forward: caaaaacagagcagatgac Reverse: ctcaagatggacgccaaga	55	219-252	A	3
7	RM21	11	-	-	55	-	A	4
8	RM24	1	$(GA)_{29}$	Forward: gaagtgtgatcactgtaacc Reverse: tacagtggacggcgaagtcg	55.5	152-198	A	4
9	RM26	5	$(GA)_{15}$	Forward: gagtcgacgagcggcaga Reverse: ctgcgagcgacggtaaca	55	102-112	P	4
10	RM29	2	$(GA)_{7}$	Forward: cagggacccacctgtcatac Reverse: aacgttggtcatatcggtgg	55	188-200	A	2
11	RM31	5	$(GA)_{15}$	Forward: gatcacgatccactggagct Reverse: aagtccattactctcctccc	55	141-153	A	3
12	RM32	8	$(TC)_3A(CT)_9(TC)_5$	Forward: gtctacgtggtgtacacgtgg Reverse: tgcggcctgccgtttgtgag	55	~168	A	3
13	RM72	8	$(TAT)_5C(ATT)_{15}$	Forward: ccggcgataaaacaatgag Reverse: gcatcggtcctaactaaggg	55	152-198	P	7
14	RM80	8	$(TCT)_{25}$	Forward: ttgaaggcgctgaaggag Reverse: catcaacctcgtcttcaccg	59.1/58	116-173	A	3

15	RM101	12	$(CT)_{37}$	Forward: gtgaatggtcaagtgacttaggtggc Reverse: acacaacatgttccctcccatgc	55	258-334	A	2
16	RM103	6	$(GAA)_5$	Forward: cttccaattcaggccggctggc Reverse: cgccacagctgaccatgcatgc	55	334-340	P	4
17	RM107	9	$(GA)_7$	Forward: agatcgaagcatcgcgcccgag Reverse: actgcgtcctctgggttcccgg	55/67	180-189	P	4
18	RM117	12	$(AG)_7$	Forward: cgatccattcctgctgctcgcg Reverse: cgcccccatgcatgagaagacg	55	203-207	P	3
19	RM122	5	$TAA(GA)_7A(GA)_2A(GA)_{11}TTGC$	Forward: gagtcgatgtaatgtcatcagtgc Reverse: gaaggaggtatcgctttgttggac	55	227-233	A	3
20	RM124	4	$(TC)_{10}$	Forward: atcgtctgcgttgcggctgctg Reverse: catggatcaccgagctcccccc	67	265-271	P	3
21	RM125	7	$(GCT)_8$	Forward: atcagcagccatggcagcgacc Reverse: aggggatcatgtgccgaaggcc	55	124-136	A	3
22	RM131	4	$(CT)_9$	Forward: tcctccctcccttcgcccactg Reverse: cgatgttcgccatggctgctcc	61	209-217	P	4
23	RM134	7	$(CCA)_7$	Forward: acaaggccgcgagaggattccg Reverse: gctctccggtggctccgattgg	55	84-93	A	2
24	RM140	1	$(CT)_{12}$	Forward: tgcctcttccctggctcccctg Reverse: ggcatgccgaatgaaatgcatg	55	259-263	P	4
25	RM143	3	$(CGG)_7$	Forward: gtcccgaaccctagcccgaggg Reverse: agaggccctccacatggcgacc	55.1/67	195-207	P	2
26	RM150	6	$(CGT)_6(CGG)_5$	Forward: cacgacgacgacgagcagcagc Reverse: gctcgagggagagcgacctgcc	61	n.a	A	3
27	RM169	5	$(GA)_{12}$	Forward: tggctggctccgtgggtagctg Reverse: tcccgttgccgttcatccctcc	67	164-194	A	3
28	RM171	10	$(GATG)_5$	Forward: aacgcgaggacacgtacttac Reverse: acgagatacgtacgcctttg	55	318-343	A	3
29	RM172	7	$(AGG)_6$	Forward: tgcagctgcgccacagccatag Reverse: caaccacgacaccgccgtgttg	55	159-165	P	4
30	RM180	7	$(ATT)_{10}$	Forward: tacatcggcttaggtgtagcaacacg Reverse: acttgctctacttgtggtgagggactg	55	107-204	A	7

31	RM184	10	$(CA)_7$	Forward: atcccattcgccaaaaccggcc Reverse: tgacacttggagagcggtgtgg	55	207-221	A	2
32	RM185	4	$(AGG)_9$	Forward: agttgttgggagggagaaaggcc Reverse: aggaggcgcaggcgatgtcctc	61	194-197	P	2
33	RM189	9	$(AG)_{11}$	Forward: cgtcttccccaacgctaaaa Reverse: cgccgggcttcgcttc	61	~126	P	4
34	RM204	6	$(GA)_{44}$	Forward: gtgactgacttggtcataggg Reverse: gctagccatgctctcgtacc	55	106-194	A	4
35	RM208	2	$(GA)_{17}$	Forward: tctgcaagccttgtctgatg Reverse: taagtcgatcattgtgtggacc	55	166-180	A	3
36	RM209	11	$(GA)_{18}$	Forward: atatgagttgctgtcgtgcg Reverse: caacttgcatcctcccctcc	61	127-165	A	3
37	RM210	8	$(GA)_{23}$	Forward: tcacattcggtggcattc Reverse: cgaggatggttgttcacttg	62.4	141-163	A	2
38	RM211	2	$(GA)_{18}$	Forward: ccgatctcatcaaccaactg Reverse: cttcacgaggatctcaaagg	55	144-163	A	3
39	RM217	6	$(GA)_{20}$	Forward: atcgcagcaatgcctcgt Reverse: gggtgtgaacaaagacac	59.1/60.7	120-166	A	3
40	RM218	3	$(GA)_{24}$	Forward: tggtcaaaccaaggtccttc Reverse: gacatacattctacccccgg	55.2	120-148	A	3
41	RM219	9	$(GA)_{17}$	Forward: cgtcggatgatgtaaagcct Reverse: catatcggcattcgcctg	57.6/58	192-222	A	3
42	RM221	2	$(GA)_{10}$	Forward: acatgtcagcatgccacatc Reverse: tgcaagaatctgacccgg	55.1	187-193	P	3
43	RM222	10	$(GA)_{18}$	Forward: cttaaatgggccacatgcg Reverse: caaagcttccggccaaaag	55	199-215	P	6
44	RM224	11	$(GA)_{13}$	Forward: atcgatcgatcttcacgagg Reverse: tgctataaaaggcattcggg	55	124-158	A	3
45	RM227	3	$(GA)_{10}$	Forward: acctttcgtcataaagacgag Reverse: gattggagagaaaagaagcc	55	99-107	A	2
46	RM228	10	$(GA)_{36}$	Forward: ctggccattagtccttgg Reverse: gcttgcggctctgcttac	55	108-154	A	3
47	RM229	11	$(GA)_{11}$	Forward: cactcacacgaacgactgac Reverse: cgcaggttcttgtgaaatgt	59.1/60.5	106-131	P	3

48	RM232	3	$(GA)_{24}$	Forward: ccggtatccttcgatattgc Reverse: ccgacttttcctcctgacg	55	142-166	A	3
49	RM233	5	—	Forward: ccaaatgaacctacatgttg Reverse: gcattgcagacagctattga	55	—	A	3
50	RM238	6	—	Forward: gatggaaagcacgtgcacta Reverse: acaggcaatccgtagactcg	55	—	A	2
51	RM244	10	$(GA)_{8}$	Forward: ccgactgttcgtccttatca Reverse: ctgctctcgggtgaacgt	55	157-165	P	3
52	RM256	8	$(GA)_{21}$	Forward: gacagggagtgattgaaggc Reverse: gttgatttcgccaagggc	55	107-139	A	2
53	RM257	9	$(GA)_{24}$	Forward: cagttccgagcaagagtactc Reverse: ggatcggacgtggcatatg	55.1	121-173	A	3
54	RM273	4	$(GA)_{11}$	Forward: gaagccgtcgtgaagttacc Reverse: gtttcctacctgatcgcgac	55	199-207	P	3
55	RM279	2	$(GA)_{16}$	Forward: gcgggagagggatctcct Reverse: ggctaggagttaacctcgcg	55	148-174	A	3
56	RM270	12	$(GA)_{13}$	Forward: ggccgttggttctaaaatc Reverse: tgcgcagtatcatcggcgag	55	104-117	A	3
57	RM293	3	$(GT)_{20}$	Forward: tcgttgggaggtatggtacc Reverse: ctttatctgatccttgggaagg	55	202-211	P	5
58	RM303	4	$(GT)_{7}$)(ATGT)^{6}	Forward: gcatggccaaatattaaagg Reverse: ggttggaaatagaagttcggt	55	143-205	A	3
59	RM328	9	$(CAT)_{5}$	Forward: catagtggagtatgcagctgc Reverse: ccttctcccagtcgtatctg	55	172-181	A	3
60	RM332	11	$(CTT)_{5-12}(CTT)_{14}$	Forward: gcgaaggcgaaggtgaag Reverse: catgagtgatctcactcaccc	55	162-183	A	2

- Source : Temnykh et al., 2000 and rice genes database

- A = 4% Typing grade agarose gel electrophoresis; P = 6% Denaturing polyacrylamide gel electrophoresis.

A4. Homology in grouping of landraces based on their DNA fingerprinting done by microsatellite and stress-related gene (i.e. lea3) linked primer.

Microsatellite fingerprinting (Lisa et al., 2004, Table 4)		Polymorphic levels found after *Hae*III digestion of LEA3 5′3′ amplified product		
Group	**Landraces**	**Upper level (1 0 0)**	**Middle level (0 1 0)**	**Lower level (0 0 1)**
SW1	Benapole Hoglapata Horkuch Mohini Morichshail Raniselute Chinikanai	–	Chinikanai	Benapole Hoglapata Horkuch Mohini Morichshail Raniselute
SW2	Ashfal Kalmilata Capsule Dhulubeez JataiBalam Moynamoti	–	Ashfal Kalmilata Capsule Dhulubeez JataiBalam Moynamoti	–
SW3	Bajramuri Dakshail Lakshmikajal	–	Dakshail Lakshmikajal	Bajramuri
MNE1	Kaliboro 1281 Soloi1 713	Kaliboro 1281 Soloi1 713	–	–
MNE2	Binnatoa Boilam 3538 Khaiyaboro 4539	Binnatoa Boilam 3538 Khaiyaboro 4539	–	–
Het	Gheegoj 2413 Kajalshail 612 Modhumaloti 614 Nonashail Hida Rajashail 1061 Jamainaru 2039 Kachra 973 PatnaiBalam	–	Gheegoj 2413 Kajalshail 612 Modhumaloti 614 Nonashail Hida Rajashail 1061 Jamainaru 2039 Kachra 973 PatnaiBalam	–

A5. Published article of Lisa at al., (2004) in *Plant and Soil*, Kluwer Academic Publishers, Netherlands.

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Genetic variation in microsatellite DNA, physiology and morphology of coastal saline rice (*Oryza sativa* L.) landraces of Bangladesh

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Abstract

Genetic variation of *Oryza sativa* L. landraces (LRs) collected from the saline coastal belt of Bangladesh, modern varieties (MVs), as well as Pokkali, Nona Bokra and salt tolerant modern varieties (SMVs) derived from the last two were analyzed with 60 evenly distributed rice microsatellite DNA markers. A total of 196 reproducible polymorphic alleles were identified from the band loci. Heterozygosity among the 31 LRs was found to be 0.57, 0.46 in the 5 MVs and 0.40 in the 8 SMVs. Computation of genetic similarity with this data, using Jaccard's coefficient followed by UPGMA clustering, divided the landraces into 6 distinct groups. Three groups were composed of LRs only from the highly saline southwest. Two groups consisted of LRs from the mild to moderately saline mid-east and northeast coasts. The sixth group was heterogeneous, with LRs from the northeast, LRs from the southwest and Nona Bokra. Pokkali and Gunshi, a LR of the southwest, branched out individually. When all the 46 *O. sativa* L. cultivars were clustered together, most of the MVs and SMVs were found to be linked within the heterogeneous group. The measure of seedling Na and K concentration, Na/K ratios, affected leaf area as well as survival under salinity stress in hydroponics identified 6 LRs from the highly saline southwest as the most tolerant. These group with Pokkali when UPGMA clustering using the Pearson product-moment correlation coefficient suitable for the quantitative physiological data on seedling saline stress was computed. Morphological observations of plant type and height, days to maturity and yield components in non-saline soil indicated low variability among the different LRs. When yield performance as well as tolerance scores were considered, 7 LRs from the southwest and 1 LR from the mid-northeast show potential as donors for breeding salt tolerant rice. The microsatellite fingerprinting analysis thus revealed that some of the salt tolerant landraces of the coastal region have unique polymorphic loci, quite distinct from the popular salt tolerance donor Pokkali. The similarity matrices between the *O. sativa* L. cultivars chosen for the study can be used as a valuable tool for the proper choice of parents for mapping or breeding purposes.

Abbreviations: BARC – Bangladesh Agricultural Research Council; BRRI – Bangladesh Rice Research Institute; CTAB – Hexadecyltrimethyl ammonium bromide; DMRT – Duncan's multiple range test; DU – University of Dhaka; Het – heterogeneous; IRIS – International Rice Information System; IRRI – International Rice Research Institute; LRs – land races; MNE – mid & north east; MVs – modern varieties; SMVs – salt tolerant modern varieties; SRDI – soil resources Development Institute; SSR – simple sequence repeat; SW – south west.

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Introduction

The Sundarban forest area spans one-third of the southern portion of 3 districts in Bangladesh and is known to be highly saline. From the west to east, these are respectively, Satkhira, Khulna and Bagerhat. Salinity levels gradually decline from west to east; 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 Patuakhali, Barguna, Barisal and Pirojpur. These areas are slightly saline (2–4 dS/m), with some pockets being non-saline. The coastline extends northeastwards, where there are the districts of Lakshmipur, Noakhali and Feni. The salinity in Noakhali is moderate (4–8 dS/m), and extends to larger areas compared to Lakshmipur and Feni. The coastal line moves southwards into Chittagong and Cox's Bazaar, where there are pockets of high salinity as well as mild levels (Karim and Iqbal, 2001). In general, coastal salinity levels start gradually increasing from November at the beginning of the dry season and peaks in March until start of the annual rains, from April till October. Variations in the salinity levels from the west to the east coast are due to rivers flowing into the basin from the north and through into the Bay of Bengal (Karim and Iqbal, 2001). In the moderate to highly saline southwest coastal areas, farmers can only grow a single rice (*O. sativa* L.) crop during the monsoon season when the salinity levels are relatively low. In the rainfed lowlands of Satkhira and Khulna in the southwest, landraces still account for 80% of the top 20 rice varieties, despite introduction of modern varieties (Bose et al., 2001). Soil salinity in this region can be moderate even during the monsoon season, if there is lower than average or early rainfall. The popular landraces of this region are therefore well adapted to the prevailing soil stresses, including soil salinity. In the slight to moderate saline northeastern coast, farmers grow Aus rice, which is planted in the dry season in late March, when soil and canal water salinity levels are known to be moderate (Panaullah, 1993). Landraces in the eastern coast of Noakhali and Feni form 60% of the top 20 varieties grown there in the dry season (Bose et al., 2001). These varieties should therefore possess some resistance to salt stress, particularly at the seedling stage.

Landraces have been shown to be excellent sources of genes for novel alleles (Evenson and Gollin, 1997; Guevarra et al., 2001; Hoisington et al., 1999; Jackson, 1999; Tanksley and McCouch, 1997). More than 4000 traditional Bangladesh rice accessions or landraces have been collected and registered at a rice gene bank in the Bangladesh Rice Research Institute (BRRI) for medium-term storage and an identical set is held in trust at IRRI for longer storage (Bashar and Sarker, 1997; Jackson, 1999). Core selections of rice germplasm from many countries, including Bangladesh are being analyzed at IRRI for identification of new alleles and molecular markers for future use in enhancing the productivity of rice cultivars (IRRI; Medium Term Plan 2002–4). There has however been no systematic study of the traditional cultivars from Bangladesh. This paper is an effort at identifying traditional landraces from the coastal saline zones of Bangladesh, which could be donors for salt tolerance traits.

Breeding for salt stress tolerance in rice has been moderately successful (Mishra et al., 2001; Senadhira et al., 2002). However, the genetic base for salinity tolerance in internationally released cultivars has originated mainly from two common salt tolerance donors, Pokkali and Nona Bokra, particularly in case of improved genotypes released for coastal salinity (Gregorio et al., 2002). Initial breeding programs using these donors resulted in moderately salt tolerant, improved cultivars. More complex crosses using a somaclonal variant of Pokkali, TCCP 266-2-49-B-B-3, which had better agronomical properties, resulted in improved cultivars with salt tolerance levels comparable to their donor parents (Gregorio et al., 2002) (www.cgiar.org/irri/iris). BRRI has released two moderately salt tolerant modern varieties, photosensitive BRRIdhan 40 and BRRIdhan 41, originating from advanced IRRI lines and suitable for growth in the coastal areas of Bangladesh only in the monsoon season (BRRI, 2003). Therefore, there is a need to widen the genetic base for donors of salt tolerance traits for adaptability to different agroecological conditions common in most coastal areas. Bangladesh coastal areas are affected by both tidal saline intrusion as well as water stagnation because of lowlands in the monsoon season (July to October), upward or lateral movement of saline ground water during the dry season (November to May) and willful inundation with brackish water for shrimp cultivation (Karim and Iqbal, 2001). Soil profiles in the coastal areas of Bangladesh have an excess of magnesium, calcium, and sulphate and are generally deficient in zinc (Panaullah, 1993; Karim and Iqbal, 2001). Characterization of the landraces adapted to the variable nature of the saline coastal region of Bangladesh will help

identify useful donors for new salt tolerance traits into modern varieties or indicate approaches for improving the yields of the landraces. Any improved rice variety will make major impacts in the livelihoods of the resource-poor farmers of the region, since it has been shown that introduction of modern rice varieties resulted in the production of staple food for 46% of the Bangladesh population between the years 1985–1997 (Hossain, 1998).

Use of simple sequence repeats or SSRs as genetic markers for rice germplasm assessment has gained popularity because of the technical efficiency of its use, its codominance and multi-allelic, highly polymorphic nature as well as its uniform distribution throughout the genome (McCouch et al., 1997; Temnykh et al., 2000). SSRs have been found to be of particular value in analysis of closely related rice genotypes (Nagaraju et al., 2002; Thanh et al., 1999). This manuscript explores variation in 31 landraces at the molecular, physiological and morphological levels along with 15 released rice varieties, seeking to identify locations of prevalence of these landraces as well as sources of tolerance distinct from Pokkali and Nona Bokra, the standards presently used by breeders.

Materials and methods

Collection of Oryza sativa *L., landraces from coastal Bangladesh*

Seeds were collected from farmer's fields in the saline-prone districts of Satkhira and Khulna in the southwest as well as from districts of Feni and Noakhali in the northeastern coast of Bangladesh. Farmers grow the rice (*O. sativa* L.) crop in the southwest during the monsoon season, from June till the end of December. Seeds and soil samples were collected from the southwest in the first week of December 2001 at crop maturity. Samples from various fields within a designated location as well as several locations within the two districts were collected and labeled separately. Seeds collected from each location were planted separately and plants visually scored for morphological uniformity. Leaves from plants of one location were used for isolation of DNA. For the northeastern coast, soil samples were collected as above from the farmer's field in April of 2002. Since farmers plant seeds at this time, seeds were collected from their stock. Rice (*O. sativa* L.) landraces grown in the coastal areas were also obtained from the gene bank at the Bangladesh Rice Research Institute (BRRI). These lines were collected in 1973 and were assigned accession numbers when entered into the gene bank. This information was not provided until after the fingerprinting to ensure unbiased molecular evaluation. Out of the 31 landraces tested, 20 varieties were directly from the farmer's field or seed supplies; the rest from the BRRI gene bank. The total list of 31 LRs along with their location is in Table 4. Seeds of Pokkali, Nona Bokra, 8 salt tolerant MVs derived from these and 5 salt-sensitive BRRI and IRRI modern varieties were obtained from the BRRI breeding division. The SMVs were IRRI varieties, IR50184-3B-18-2B-1, IR51491-AC5-4, IR52724-2B-6-2B-1-1, IR58440-3B-9-2-2, IR60494-2B-18-3-2-3, IR65195-3B-13-2-3, and BRRI varieties, BRRIdhan 40, BRRIdhan 41. The MVs were IRRI varieties, IR29, IR36 and BRRI varieties, BRRIdhan 28, BRRIdhan 29 and BRRIdhan 36. Origin of the BRRI varieties is given in Table 1A and salt tolerance donors of the SMVs summarized in Table 1B.

DNA isolation

Leaves (0.5–1 gm) of the 46 *O. sativa* L. cultivars were obtained from a pooled sample containing at least 10 seedlings (Parsons et al., 1997). These were cut finely, crushed to powder in liquid nitrogen and subjected to DNA isolation using the CTAB method (Doyle and Doyle, 1990). Slight modifications to the protocol included use of sodium acetate instead of ammonium acetate to precipitate DNA after RNase treatment and additional phenol:chloroform and chloroform purification steps after preliminary DNA isolation.

Amplification of DNA using SSR primers

A total of 60 microsatellite primers, 5 primers from each chromosome were used to amplify DNA from the leaves of the *O. sativa* L. cultivars. List of the primers used, chromosome number and the maximum number of alleles obtained is given in Table 2. The PCR reactions conducted in a volume of 25 μl contained 10 mM Tris-HCl (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM dNTPs, 60 ng/μl primer, 1 unit Taq DNA Polymerase (Life Technologies, USA) and 75–100 ng Template DNA. Amplification reaction consists of preheating for 5 min at 94 °C and of 35 cycles of 1 min at 94 °C (denaturation) 1 min at 55 °C–61 °C (annealing) and 3 min at 72 °C (elongation) followed by 7 min

Table 1A. Origin of BRRI-derived varieties

Sl. no.	Variety	Origin
1	BRRIdhan 28	IR28 × Purbachi
2	BRRIdhan 29	BG90-2* × BR10; BR10 → IR5 × IR20
3	BRRIdhan 36	IR54791
4	BRRIdhan 40	IR4595-4-1-13 × BR10;
5	BRRIdhan 41	BR5828 → BR23* × BR1185*;

*Origin at IRIS site: www.iris.irri.org.

Table 1B. Salt tolerance trait donors of the SMVs

Sl. no.	Variety	Salt tolerance donors
1	IR50184-3B-18-2B-1	Pokkali & SR26B
2	IR51491-AC5-4	Pokkali & Pokkali
3	IR52724-2B-6-2B-1-1	Pokkali
4	IR58440-3B-9-2-2	Pokkali & Pokkali
5	IR60494-2B-18-3-2-3	Nona Bokra & Pokkali
6	IR65195-3B-B-2-3	Nona Bokra & TCCP266-B-B-B-10-3-1

Source: www.iris.irri.org.

at 72 °C in a Mastercycler Gradient PCR system (EPPENDORF) (Panaud et al., 1996). Amplified products were separated in 4% Typing Grade agarose gel (Life Technologies, USA.), containing 0.5 ng/ml EtBr (Ethidium Bromide). Separated PCR products were visualized under UV light and photographed using Kodak Electrophoresis Documentation & Analysis System, when 2–4 polymorphic bands were visualized. When no polymorphism was observed in agarose gels, the products were separated on long denaturing 6% polyacrylamide gels with a thickness of 0.4 mm (http://www.irri.org/GRC/GRChome/IRG manual/section8.pdf). Gels were pre-run, samples were denatured in loading buffer and electrophoresed for about 2 h or until the bromophenol blue remained visible. The molecular weight markers used, were 25-bp ladder (Life Technologies). Gels were stained with silver nitrate, according to the protocol by Promega. After staining, gels were dried in air and photographed.

Data analysis

For the microsatellite DNA fingerprinting of the of the *O. sativa* L. cultivars, polymorphisms were scored for the presence or absence of bands on agarose or polyacrylamide gels on a 1/0 basis and the data analyzed using the NTSYS-PC version 2.11f (Rohlf, 2000). The average proportion of alleles (bands on gels) that were shared between any two accessions was used to measure similarity using the Jaccard's similarity coefficient (Jaccard, 1908), which is suitable for qualitative data (Rohlf, 2000). Data on screening of seedlings for tolerance to salinity, such as, survival, area of leaf affected, reduction in leaf height, Na and K concentration and Na/K ratio under saline stress in hydroponics, were subjected to measure of similarity using the Pearson product-moment correlation coefficient (Pearson, 1896), suitable for quantitative data (Ludbrook, 2002). Cluster analysis on the marker and physiological data was done separately based on the similarity matrix obtained in each case, using the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973). The efficiency of the UPGMA for estimation of genetic relatedness in each case was determined by computing the cophenetic correlation coefficients (Mantel, 1967). Expected Heterozygosity with respect to SSR marker bands obtained for the different rice varieties was calculated from the sum of squares of allele frequencies (Nei, 1973) as follows:

$$H = \sum(1 - \sum P_{ij}^2)/n,$$

where, P_{ij} is the frequency of the jth allele for marker i and the summation extends over n alleles.

Table 2. List of the total microsatellite primers used, type of gel and the polymorphism in band levels obtained for the 46 rice cultivars

Serial no.	Primer	Chr. no.	No. of polymorph. bands	Type of gel used	Serial no.	Primer	Chr. no.	No. of polymorph. bands	Type of gel used
1	RM5	1	4	A	31	RM184	10	2	A
2	RM9	1	4	A	32	RM185	4	2	A
3	RM14	1	3	A	33	RM189	9	4	P
4	RM17	12	3	A	34	RM204	6	4	A
5	RM18	7	3	A	35	RM208	2	3	A
6	RM19	12	3	A	36	RM209	11	3	A
7	RM21	11	4	A	37	RM210	8	2	A
8	RM24	1	4	A	38	RM211	2	3	A
9	RM26	5	4	P	39	RM217	6	3	A
10	RM29	2	2	A	40	RM218	3	3	A
11	RM31	5	3	A	41	RM219	9	3	A
12	RM32	8	3	A	42	RM221	2	3	P
13	RM72	8	7	P	43	RM222	10	6	P
14	RM80	8	3	A	44	RM224	11	3	A
15	RM101	12	2	A	45	RM227	3	2	A
16	RM103	6	4	P	46	RM228	10	3	A
17	RM107	9	4	P	47	RM229	11	3	P
18	RM117	12	3	P	48	RM232	3	3	A
19	RM122	5	3	A	49	RM233	5	3	A
20	RM124	4	3	P	50	RM238	6	2	A
21	RM125	7	3	A	51	RM244	10	3	P
22	RM131	4	4	P	52	RM256	8	2	A
23	RM134	7	2	A	53	RM257	9	3	P
24	RM140	1	4	P	54	RM273	4	3	P
25	RM143	3	2	A	55	RM279	2	3	A
26	RM150	6	3	A	56	R M270	12	3	P
27	RM169	5	3	A	57	RM293	3	5	P
28	RM171	10	3	A	58	RM303	4	3	A
29	RM172	7	4	P	59	RM328	9	3	A
30	RM180	7	7	A	60	RM332	11	2	A

A = 4% typing grade agarose gel electrophoresis; P = 6% denaturing polyacrylamide gel electrophoresis.

Na/K evaluation for assessment of salinity tolerance at the seedling stage

Sodium and potassium uptake by the shoots, Na/K ratio and seedling survival rates are well correlated with tolerance of the rice (*O. sativa* L.) plant to salinity stress (Bohra and Dorffling, 1993; IRRI, 1996, Lee et al., 2003). Therefore seedling survival and K and Na concentrations at seedling stage under control and stressed conditions, was measured in hydroponic systems as follows. Seeds of the LRs (20 per cultivar, 10 each for the control and stressed conditions) were germinated in 3 separate sets on netted, floating styrofoam in a completely randomized design. The size of the float was 28 × 32 × 1.25 cm with a hundred holes. The germinated seedlings were allowed to grow in 10 liters normal Yoshida culture solution/per float until the seedlings reached the 4-leaf stage (14–18 days from germination). The ECe of the culture solution was increased to 6 dS/m for the next two days and then to 12 dS/m until 90% of the leaves of the sensitive control were damaged (23–27 days after stress application). This was done by replacing the culture solution with Yoshida solution containing, NaCl (Ponnemperuma, 1977). The pH of the non-aerated Yoshida culture solution was adjusted to 5.0 every day and changed every five

days. BRRIdhan 29 and Pokkali were used as the sensitive and tolerant controls respectively. Data for percent survival and total leaf area affected was recorded according to the Standard Evaluation System of Rice at IRRI (IRRI, 1996; Gregorio et al., 1997). The percent reduction in shoot and root length and dry weight compared to controls were recorded for each seedling. For the measurement of sodium and potassium concentration in shoot and root at 0 dS/m and 12 dS/m, plants were washed in flowing tap water for 30 sec and 10 oven-dried plants from each replicate were pooled, ground and analyzed by a flame photometer (Jenway, UK) after 48 h of extraction with 1N HCl following the procedure described by Yoshida et al. (1976). Concentrations were expressed as per cent of dry weight. Sodium to potassium ratio was also determined. All the data were analyzed statistically and means were separated using DMRT when a significant F-value was obtained. Regression analysis was carried out, by assuming the Leaf Na/K ratio or Na concentration as the independent variable and leaf area affected, and percent reduction in shoot height or dry weight as dependent variables. The screening was done in a screen house, in March–April, July–August and October to November, 2002, so that variation due to changes in humidity would not affect the outcome of the screening tests (Asch et al., 1995). The temperatures and relative humidity in the screen-house varied between 30–37 °C and 50–80%, respectively.

Field observation

For the *O. sativa* L. landraces, 20 seedlings (25 day-old) of each cultivar were transplanted (July to November, 2002) in a 3 m × 2 row plot at the BRRI research fields, in Gazipur, Dhaka. Fertilizer applied to the soil during transplanting was half of that normally provided for MVs. Amounts of urea, TSP, MP and Gypsum were respectively, 80, 50, 60 and 35 kg/ha. The concentrations of N, P, K and S in Kg/ha were therefore, 36.3, 10.1, 30.3 and 6.36, respectively. Soil ECe value was 2 dS/m. Individual plants were 15 cm apart within rows and there was a distance of 25 cm between rows. Five plants from each row were selected randomly for measurement of morphological characteristics. Variation among the 33 *O. sativa* L. cultivars, including Pokkali and BRRIdhan 40, in plant height, total tiller number, panicles per plant, panicle length, flag leaf length, spikelets per panicle, per cent fertility, 1000 grain weight and grain yield per plant were evaluated using Duncan's multiple range test. Only data for 4 yield components as well as days required to maturity after seeding has been reported in the results section.

Analysis of ionic composition of soil from collection sites

Soil at a depth of 10 cm from 3 locations in each harvested field where the farmer had grown a particular landrace was collected. Soil samples were collected from Satkhira and Khulna in the first week of December, when the soil salinity is low because this period coincides with the end of the monsoon season. Soil samples from Feni and Noakhali were collected in April, where the soil salinity may be moderate in certain regions at this time of the year (Panaullah, 1993). The 3 samples from each field were dried separately, powdered and then mixed in equal proportions before analysis. Soil pH was measured in a 1:2.5 soil-water ratio and for ECe, water extract in a 1:1 ratio was used. Na, K, Ca, Mg, P, S, B and Zn were extracted and analyzed at the Regional Laboratory of the Soil Resources Development Institute (SRDI) in Comilla. Extraction of Na and K was according to the method of Helmke and Sparks (1996); Ca and Mg according to Suarez (1996); Cu and Zn according to Reed and Martens (1996); Fe according to Loeppert and Inskeep (1996); Mn according to Gambrell (1996); B according to Keren (1996); P according to Kuo (1996) and S according to Tabatabai (1996). All the ions were analyzed by atomic absorption spectrometry, except S and B, which were analyzed colorimetrically (Page et al., 1982).

Results

A total of 196 reproducible polymorphic bands or alleles were identified using 60 microsatellite primers after amplification of the DNA from the 46 rice genotypes. The 60 microsatellite primers were evenly distributed over the 12 rice chromosomes (Table 2). In 66% cases, agarose gel electrophoresis was adequate in detecting polymorphism showing 2–4 alleles across the various rice varieties (Figure 1). Two alleles were observed for 18% of the primers, 3 alleles for 42% and 4 for 8%. When RM180 was used, 7 alleles were observed in agarose gels similar to the reported range of sizes from 104 to 200 bp (Temnykh et al., 2000). Therefore for 32% of the primers, amplified bands were monomorphic or only a few varieties showed

amplification at a second level. In these cases, polyacrylamide gel electrophoresis was used to resolve amplified DNA resulting in bands of 3–7 different molecular sizes for the different primers (Figure 2). Genetic diversity or Heterozygosity was found to be 0.57 for the landraces (LRs) of the coastal region, while that of modern varieties (MVs) was 0.46 and salt tolerant modern varieties (SMVs) was 0.40.

Genetic relatedness between the 31 LRs as well as the traditional standards, Pokkali and Nona Bokra based on their shared SSR alleles was computed using Jaccard's coefficient followed by clustering into a dendrogram (Figure 3a). Six groups are apparent, at the coefficient value of 0.366. Three are named SW1, SW2 and SW3, all of which contain photoperiod sensitive landraces from the highly saline southwest coastal regions of Satkhira and Khulna. Two more groups, called MNE1 and MNE2 have photoperiod insensitive landraces from the mildly saline coast of the mid northeast, Noakhali, as well as non-saline Faridpur and Habigonj, further north from the above coastal region. The 6th group of the dendrogram consists of landraces from all the different coastal regions as well as Nona Bokra and is called the Het group. The internationally well-known standard for saline tolerant rice, Pokkali as well as Gunshi, a landrace grown in the mildly saline Barisal, did not group with any of the 30 other landraces. Comparison of the matrix of similarity generated with the cophenetic matrix of the dendrogram showed a good fit ($r = 0.79$), indicating the reliability of the groups within the tree diagram (Figure 3a).

When the genetic similarity of all 46 cultivars, including the 31 LRs, 8 SMVs, 5 MVs as well as Pokkali and Nona Bokra, was computed as above and subjected to clustering using the Jaccard's coefficient, all the MVs and SMVs were found within the Het group (Figure 3b), except that IR65195-3B-13-2-3, Hida and Pokkali became grouped separately and IR29 and IR36 did not group with any other cultivar.

Seedlings of 6 landraces scored well in comparison to Pokkali under 12 dS/m saline stress in hydroponic systems. These had high survival percent, low percent leaf area affected, low reduction in shoot height, low Na concentration and low Na/K ratios in shoots (Table 3). These cultivars are Ashfal, Benapole, Jamainaru, Lakshmikajol, Patnai Balam and Horkuch, all farmer popular landraces of the Khulna region (Tables 3 and 4). All 6 cluster very closely with Pokkali in the dendrogram generated from the similarity matrix of scores from the above seedling screening tests using the Pearson Correlation coefficient (Figure 4). Capsule, Morichshail and Kajalshail also performed moderately well in above stress tests at the seedling stage. Capsule grouped closely with Pokkali, while Morichshail and Kajalshail fuse at a slightly higher level. All the landraces popular in the southwest districts cluster with Pokkali when seedling stress scores were considered. In addition, Binnatoa, Kajalshail and Gunshi also group with Pokkali. Binnatoa and Kajalshail are grown in the mid-northeast. Gunshi grows in the mildly salt stressed Barisal in the mideast. All the cultivars of the mid northeast cluster with salt sensitive MV, BRRIdhan 29. Chinikanai, which is popular in the southwest also grouped with BRRIdhan 29. Dakshail and Mohini, also popular in the southwest form a group of their own with Khaiyaboro, a cultivar grown in non-saline areas away from the coast.

Morphological observations of the 31 traditional landraces in the field, in normal unstressed soil, show these to be of typical, moderate to tall plant-type, with spread-out tillers (Table 4). Jatai Balam, Patnai Balam, Morichshail and Kachra show good yields when compared to the SMV control, BRRIdhan 40 (Table 4). Gheegoj, Jamainaru, Lakshmikajol, Mohini, Hoglapata and Horkuch also show reasonable yields (Table 4).

Considering the seedling screening under saline stress as well as the morphological observation of the mature plants in non-stressed condition, it can be concluded that Jamainaru, Lakshmikajal, Patnai Balam, Horkuch and Morichshail may prove to be good donors for salt tolerance traits. Ashfal has a very good tolerance score but moderate yield. Kajalshail and Raniselute gave moderate tolerance scores but have only average yields. These 8 landraces may have good potential as donors for salt tolerance traits. All of them, except for Kajalshail are grown popularly in the saline southwest district of Khulna, while Kajalshail is popular in mildly saline coastal areas in Noakhali (Table 5).

Ionic composition of the soil from the fields where the landraces had been grown, was analyzed. Results for one field per landrace has only been reported (Table 5). The composition of the soil samples collected from the southwest, as well as, the northeast was generally similar in that calcium and magnesium concentrations were very high, iron high, phosphorous and zinc low, and levels of sulphate and boron toxically high. Differences included low potassium in the

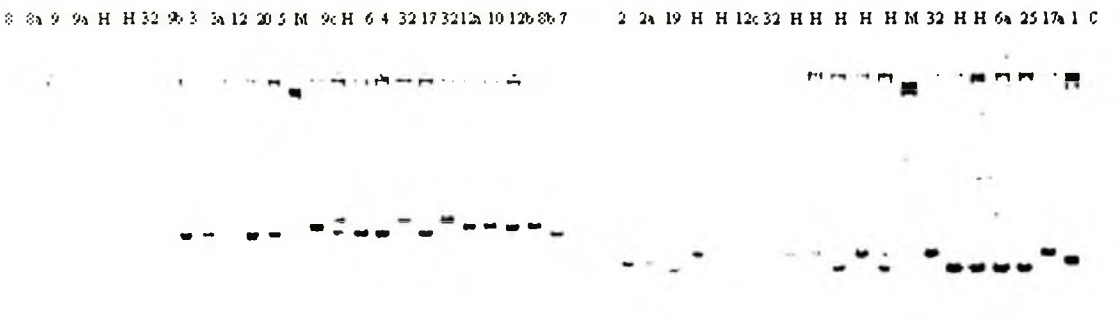

Figure 1. Amplified DNA of traditional rice cultivars using microsatellite primer RM17 after resolution in 4% agarose gel. The lanes are numbered according to the serial followed in Table 3. Lanes which are not numbered show amplified DNA from modern varieties (H). Lane M and C are 25 bp DNA ladder and PCR control respectively. a,b,c denote different accessions of the same landrace.

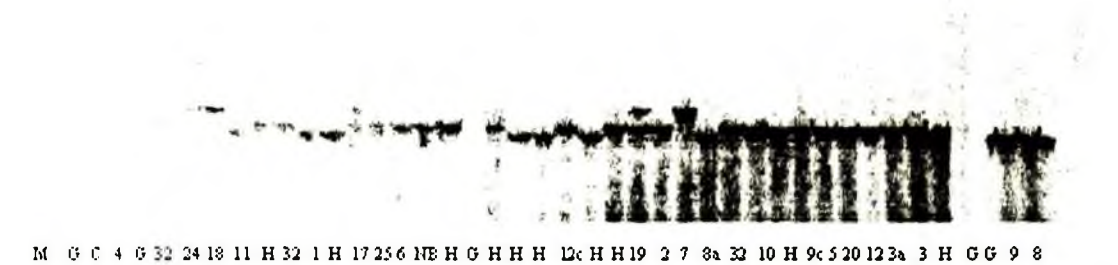

Figure 2. Amplified DNA of traditional rice cultivars from the southwest region using microsatellite primer RM124 after resolution in 6% polyacrylamide gel. The lanes are numbered according to the serial followed in Table 3. Lane G, M and C show gap, 25 bp DNA ladder and PCR control respectively. Lane NB denotes Nona Bokra.

mid-northeast and high in the southwest and higher level of sulphate toxicity in the former areas.

Discussion

Thirty-one landraces of the coastal saline region were genotyped and partially characterized, twenty of which were collected directly from the farmer's fields. Farmer's preference for these landraces, despite introduction of modern varieties (Bose and Hossain, 2003) suggests greater adaptability of these landraces in the variable coastal environment, which includes unpredictable rainfall, variable water stagnation in low lands, depending upon the season, sea-water intrusion due to upstream withdrawal of water, again depending upon the amount of rainfall in the upper riparian areas in our neighboring country. The landraces are also adapted to the ion toxicities as well as deficiencies of the coastal soils. Investigation of the relatedness of the landraces based on their SSR alleles, separated cultivars growing in the southwest (SW1, SW2 and SW3) from those in the mid-northeast (MNE1 and MNE2). Due to lower levels of saline stress in the mid-northeast, farmers grow Aus or dry season photoperiod insensitive varieties, which are seeded in April. These are the ones forming the groups MNE1 and MNE2. Therefore genetic variation relates to adaptability of the cultivars in distinct coastal regions. The southwest coastal soil has a higher amount of Ca, K and Fe compared to the mid-northeast, while its sulfur toxicity is less (Table 5). Its P and organic content is higher and drainage poor due to prevalence of clayey soil, in contrast to poor organic matter and clay loam soil in the mid-northeast (Karim and Iqbal, 2001). Pokkali and Gunshi did not group with any of the landraces. Therefore, these two have distinct genetic identity.

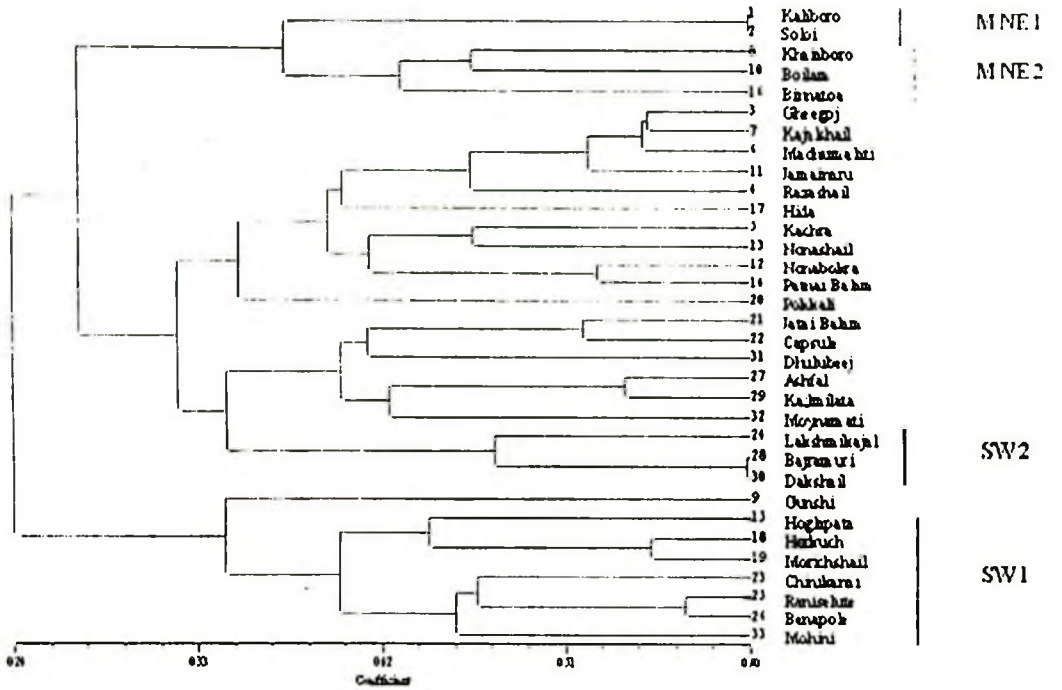

Figure 3a. Dendrogram of 31 traditional landraces along with Nona Bokra and Pokkali.

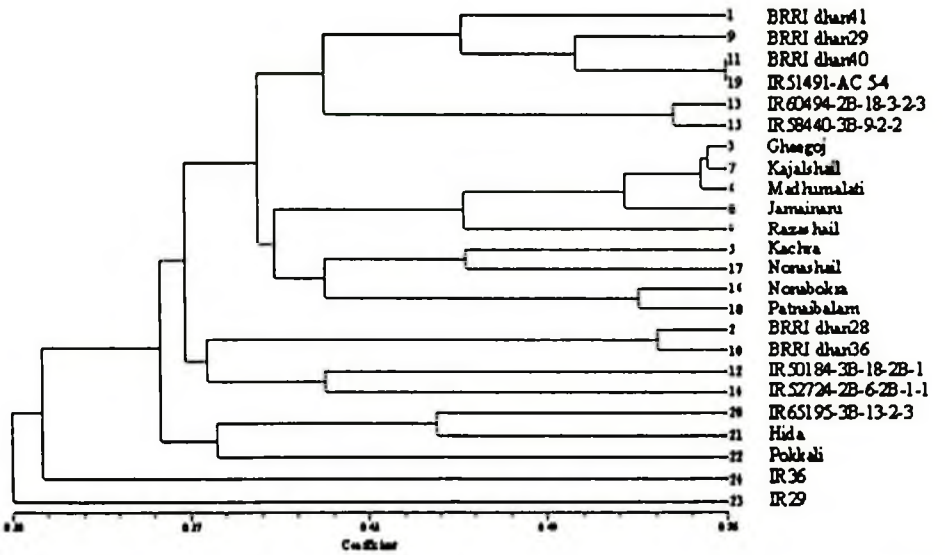

Figure 3b. Dendrogram of modern varieties and salt tolerant modern varieties along with cultivars of the heterogenous group (Het).

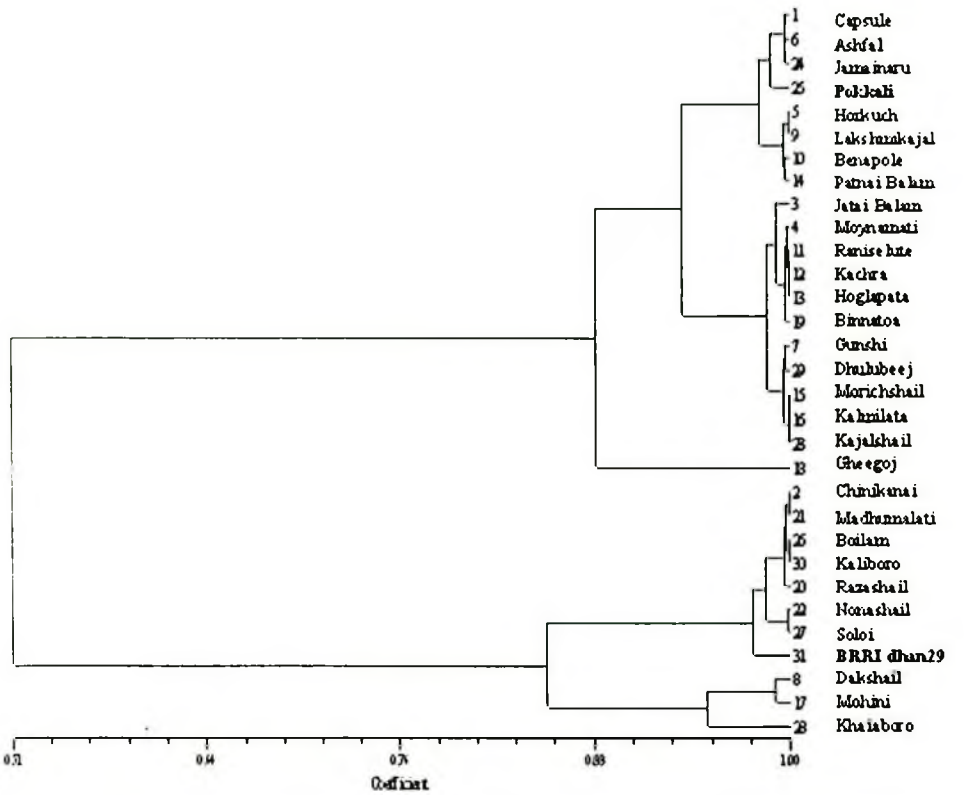

Figure 4. Dendrogram from the data of salinity screening of 29 landraces along with Pokkali and BRRI dhan29.

Three landraces from the southwest, 4 from the mid-northeast, 2 from non-coastal areas as well as Nona Bokra cluster together in a group named Het (heterogeneous). All the total 46 fingerprinted cultivars, including the modern varieties (MVs) as well as the salt tolerant modern varieties, clustered within the Het group (cophenetic correlation $r = 0.8$), with 3 exceptions. IR36 and IR29 showed independent lineage, and IR65195-3B-13-2-3, Hida and Pokkali grouped separately, although Hida was part of the Het in the dendrogram of the landraces only. Hida is actually an lowland variety, grown in Rajshahi in the North, which was mixed in as a blind sample from the BRRI germplasm bank. That is why screening tests as well as field observations were not undertaken. IR65195-3B-13-2-3 however has both Pokkali and Nona Bokra as salt tolerance donors, since TCCP266-B-B-B-10-3-1 is a somaclonal mutant of Pokkali (Gregorio et al., 2002). With the exception of two landraces, Kajalshail and Nonashail, all the cultivars in the Het group, are a part of international collections, since they have their corresponding IRGC or IRTP numbers (Table 4). All the landraces of the Het group are also popularly grown in many areas of the coast. Proportion of Razashail, is 1.2% of the total planted monsoon rice, including modern varieties (Bose and Hossain, 2003). Patnai Balam is next forming 0.4%, Kajalshail 0.3%, while Gheegoj and Hida are grown in 0.2% of total monsoon-rice planting area (Bose and Hossain, 2003).

Evaluation of the physiological response under seedling saline stress identified 6 landraces whose performance was not significantly different from Pokkali, the benchmark, or reference cultivar for assessment of tolerance. These six cultivars grouped with Pokkali in a dendrogram based on computation of similarity based on the above physiological response, using the Pearson's product moment coefficient. All the cultivars that grouped with Pokkali had low Na/K scores (0.81–1.05) while that of Pokkali was 1.03. Low Na/K ratios at the seedling stage are associated with tolerance due to Na exclusion as well as partitioning of Na into older leaves (Flowers and Yeo, 1995; Moradi

Table 3. Percent survival, area leaf affected, shoot and root reduction in height, shoot sodium and potassium concentration and ratio in hydroponically-grown 45-day-old seedlings of 31 landraces, 27 days after saline stress application at 12 dS/m

Serial	Variety name	Percent survival	Percent leaf area affected	Percent shoot length reduction	Percent leaf Na at 12 dS/m	Percent leaf K at 12 dS/m	Shoot Na/K ratio at 12 dS/m
1*	Binnatoa	60.0 c-i	50.0 e-j	9.5 abc	1.98 a	1.35 bc	1.47 a-d
2*	Boilam 3538	20.0 kl	80.0 m-q	25.5 ijk	2.78 a-h	1.28 c	2.00 a-d
3	Gheegoj 2413	33.3 jk	70.0 k-o	21.0 f-j	3.03 a-i	1.98 abc	2.06 a-d
4	Kajalshail 612	66.7 b-g	40.0 b-g	9.5 abc	2.96 a-i	2.20 abc	1.4 a-d
5	Madhumalati 614	15.0 kl	85.0 opq	21.8 g-j	3.24 c-i	1.94 abc	1.69 a-d
6	Nonashail	8.3 l	86.7 opq	33.5 l	3.27 d-i	1.73 abc	2.52 de
7	Gunshi 3869	63.3 b-h	40.0 b-g	16.5 c-h	3.06 a-i	2.28 abc	1.35 a-d
8*	Kaliboro 1281	18.33 kl	81.7 n-q	24.7 ijk	3.56 ghi	1.63 abc	1.81 a-d
9*	Soloi 1713	10.0 l	91.7 pq	31.2 kl	3.29 d-i	1.577 abc	2.16 bcd
10*	Khaiaboro 4539	40.0 ij	76.7 l-p	28.1 f-j	3.46 e-i	1.887 abc	1.79 a-d
11	Hida	–	–	–	–	–	–
12	Razashail 1061	20.0 kl	83.3 n-q	20.0 e-j	3.50 f-i	1.77 abc	2.28 cd
13	Ashfal	80.0 abc	26.7 abc	6.9 ab	2.39 a-e	2.67 a	0.91 ab
14	Benapole	76.7 a-d	30.0 a-d	4.7 a	2.49 a-g	2.47 abc	1.03 abc
15	Bajramuri	–	–	–	–	–	–
16	Dakshail	46.7 g-j	56.7 g-k	8.3 abc	3.11 b-i	1.82 abc	1.85 a-d
17	Hoglapata	56.7 d-i	43.3 c-h	10.3 a-d	3.03 a-i	2.01 abc	1.66 a-d
18	Horkuch	70.0 a-f	33.3 a-e	7.4 ab	2.59 a-h	2.61 ab	1.05 abc
19	Jamainaru 2039	80.0 abc	23.3 ab	9.0 abc	2.17 abc	2.68 a	0.81 a
20	Kachra 973	60.0 c-i	46.7 d-i	11.4 a-d	3.08 b-i	2.16 abc	1.5 a-d
21	Kalmilata	63.3 b-h	40.0 b-g	10.7 a-d	2.71 a-h	2.37 abc	1.17 abc
22	Lakshmikajol	73.3 a-e	36.7 b-f	7.5 ab	2.81a-h	2.45 abc	1.18 abc
23	Mohini	43.33 hij	63.3 i-m	13.02 a-f	3.41 e-i	1.633 abc	2.25 cd
24	Morichshail	63.3 b-h	40.0 b-g	10.5 a-d	2.97 a-i	2.26 abc	1.4 a-d
25	Patnai Balam	70.0 a-f	30.0 a-d	8.3 abc	2.387 a-e	2.66 a	0.89 ab
26	Raniselute	50.0 f-j	40.0 b-g	9.2 abc	3.32 e-i	2.04 abc	1.82 a-d
27	Capsule	83.3 ab	26.7 abc	13.3 b-f	2.20 a-d	2.56 abc	0.86 a
28	Chinikanai	16.7 kl	86.7 opq	24.3 h-k	2.83 a-i	1.83 abc	1.65 a-d
29	Dhulubeej	60.0 c-i	40.0 b-g	13.0 a-f	2.15 ab	1.81 abc	1.19 abc
30	Jatai Balam	53.3 e-j	50.0 e-j	12.5 a-e	2.47 a-f	2.51 abc	1.12 abc
31	Moynamoti	60.0 c-i	43.3 c-h	11.0 a-d	3.07 b-i	2.15 abc	1.48 a-d
32	Pokkali	90.0 a	16.0 a	7.8 ab	2.19 abc	2.2 abc	1.03 abc
33	BRRIdhan 29	5.7 l	96.0 q	14.39 b-g	3.90 i	1.48 abc	3.40 ef

Cultivars marked with an asterisk are photoperiod insensitive. Means followed by a common letter are not significantly different at the 5% level by DMRT.

et al., 2003). The capacity of plants to maintain low Na/K ratio has been correlated with their capacity for salt tolerance as reviewed by Maathuis and Amtmann (1999) and Tester and Davenport (2003). Under IRRI's standard system of evaluation (IRRI, 1996; Gregorio et al., 1997), all 6 landraces scored between 3–5, which was graded according to the percent leaf area affected after about a 25-day period of salt stress and when the age of the seedlings was 45 days. Under these conditions, the score of the tolerant check, Pokkali, was less than 3 and that of the sensitive check BRRIdhan 29, around 9. We found a significant dependence of the percent leaf area affected and tolerance score on Na/K ratio (coefficient of determination r^2 = 0.69 and 0.63, respectively) for all the landraces after regression analysis. Sodium concentrations were expressed as percent of dry weight and these were found to be higher than the reported val-

Table 4. Location, SSR group identification, days to maturity and mean of yield components of 31 traditional rice cultivars grown from August to November in normal soil at the BRRI field station

Sl no.	Cultivar name	Origin	IRGC entry with same name	Days to mat.	Plant height	Panicles /plant	Spikelets /panicle	Percent fertile grains	1000 grain weight (gm)
1*	Binnatoa	Noakhali, MNE2		90	119 l	7.0 ghi	38.51 m	81.64 ab	22.9 ef
2*	Boilam 3538	Noakhali, MNE2	37389	-		–	–		–
3	Gheegoj 2413	Noakhali, Het	IRTP 13300	116	132 efg	12.8 ab	101.73 d-i	77.01 a-e	27.48 bcd
4	Kajalshail 612	Noakhali, Het		117	122 hi	15.0 a	73.13 jkl	68.12 c-g	27.91 a-d
5	Madhumalati 614	Noakhali, Het	46274	118	138 cde	13.0 ab	106.32 c-h	62.52 f-l	20.56 g
6	Nonashail	Noakhali, Het		111	122 hi	11.2 b-f	63.51 l	68.0 c-g	28.43 abc
7	Gunshi 3869	Barisal	37093	–	–	–	–		–
8*	Kaliboro 1281	Faridpur[1], MNE1	43880	–		–	–		–
9*	Soloi 1713	Faridpur[1], MNE1	37598	–		–	–		–
10*	Khaiaboro 4539	Habigonj[1], MNE2	IRTP 4216	–		–	–		–
11	Hida	Rajshahi, Het	26471	–		–	–		–
12	Razashail 1061	Narail[1], Het	37577	95	119 i	8.0 f-l	79.88 i-l	67.19 f-i	15.74 h
13	Ashfal	Khulna, SW2		121	139 bcd	7.0 ghi	117.36 b-e	73.1 a-g	26.64 cd
14	Benapole	Khulna, SW1		117	140 a-d	9.2 d-h	89.22 g-k	51.36 ijk	24.03 e
15	Bajramuri	Khulna, SW3		–		–	–		–
16	Dakshail	Khulna, SW3		118	139 b-e	6.6 hi	126.96 bc	48.58 jk	16.79 h
17	Hoglapata	Khulna, SW1		127	145 abc	10.4 b-f	92.31 e-j	81.78 ab	28.07 a-d
18	Horkuch	Khulna, SW1		119	145 abc	11.2 b-f	82.1 h-l	74.69 a-f	28.36 abc
19	Jamainaru 2039	Khulna, Het	37116	121	135 def	12.2 a-d	114.65 b-f	71.12 b-g	28.85 abc
20	Kachra 973	Khulna, Het	31821	121	144 abc	12.6 abc	75.76 jkl	86.53 a	29.47 ab
21	Kalmilata	Khulna, SW2		121	141 a-d	10.4 b-f	91.33 f-j	77.97 a-d	26.76 cd
22	Lakshmikajal	Khulna, SW3		121	141 a-d	11.4 b-e	97.9 d-j	77.32 a-e	26.07 d
23	Mohini	Khulna, SW1		120	128 fgh	10.2 b-g	78.284 i-l	83.17 ab	30.02 a
24	Morichshail	Khulna, SW1		121	144 abc	10.6 b-f	114.51 b-f	82.16 ab	28.19 a-d
25	Patnai Balam	Khulna, Het	31914	127	133 def	9.4 c-h	134.24 ab	84.32 ab	22.87 ef
26	Raniselute	Khulna, SW1		127	144 abc	10.4 b-f	92.67 e-j	81.49 ab	26.68 cd
27	Capsule	Satkhira, SW2		105	121 hi	9.8 b-h	65.45 kl	53.48 h-k	22.15 efg
28	Chinikanai	Satkhira, SW1	49041	112	141 a-d	5.8 l	130.85 abc	72.76 b-g	12.40 l
29	Dhulubeej	Satkhira, SW2		–		–	–		–
30	Jatai Balam	Satkhira, SW2		117	123 hi	12.8 ab	121.1 bcd	81.02 abc	28.4 abc
31	Moynamoti	Satkhira, SW2		106	111 j	10.6 b-f	89.18 g-k	64.69 e-h	21.35 fg
32	Pokkali	Sri Lanka	IRTP 10169	104	147 a	6.6 hi	151.42 a	54.59 hij	27.67 bcd
33	BRRIdhan 40	SMV		123	110 j	11.0 b-f	148.3 a	81.0 abc	24.0 e

Cultivars marked with an asterisk * are photoperiod insensitive. Means followed by a common letter are not significantly different at the 5% level by DMRT. SMV: Salt tolerant modern variety. SW1, SW2, SW3, MNE1, MNE2 and Het refer to the groups in the dendrogram in Figure 3a.

[1]Non-saline coastal areas.

ues for Pokkali (Lee et al., 2003), so that our Na/K ratios were proportionately higher for all cultivars. The higher concentration of Na can be explained by the fact that the sensitive control BRRIdhan 29 needs 5–7 days longer to score 9 compared to IR29, the sensitive standard used by IRRI. Since BRRIdhan 29 is a farmer-accepted cultivar in the non-saline coastal region, we chose it as the sensitive check.

Field observation of the morphology of the landraces in unstressed soil at the BRRI field station, identified plants with acceptable plant type and yield parameters. The morphological observations could not be made with replicated plots due to lack of seeds, particularly the ones collected from the germplasm bank at BRRI. Since the size of the manually prepared field, where the landraces were planted was small, about

Table 5. Ionic composition, electrical conductivity and pH in the 1st week of April, 2002, of soil in area of Landrace collection

Region of plantation	Landrace	pH	ECe dS/m	Ca	Mg	K	Na	P	S	B	Fe	Zn
				meq/100 g soil						μg/g soil		
Sonagazi, Feni	Razashail Binnatoa*	4.6	5.87	4.2	4.1	0.19	2.7	2.50	157	0.71	29	2.5
Char Jubilee, Noakhali	Gheegoj	7.1	0.66	6.9	4.6	0.28	2.0	2.90	156	0.42	12	1.46
Char Laurance, Noakhali	Kajalshail	5.5	5.62	9.9	5.7	0.16	3.4	2.70	299	0.83	30	3.32
Char Laurance, Noakhali	Boilam*	5.6	3.14	10.0	5.5	0.15	3.4	1.81	259	0.86	24	2.44
Char Jabbar, Lakshmipur	Kajalshail	5.5	1.32	7.0	6.0	0.31	2.5	3.40	163	0.68	32	1.72
Char Jabbar, Lakshmipur	Gheegoj	5.8	1.90	8.4	7.3	0.30	3.2	2.34	186	0.53	19	3.68
Bigordana, Deluti Union, Paikgacha, Khulna	Jatai Balam	5.3	1.74	7.8	6.4	0.54	3.3	1.17	83	0.87	68	1.46
As above	Benapole	5.2	2.81	10.8	6.2	0.75	4.0	26.90	142	0.97	109	2.06
As above	Patnai Balam, Lakhsmikajol	4.7	0.83	9.4	6.3	0.42	2.9	1.23	134	1.24	121	3.26
Sener Ber, Deluti Union, Paikgacha, Khluna	Morichshail	5.2	0.68	6.6	6.5	0.70	3.4	2.32	87	0.85	102	1.82
As above	Bajramuri, Dakshail, Hoglapata, Kalmilata	7.6	3.39	27.8	9.5	0.87	5.3	19.19	145	1.27	28	2.06
As above	Kachra, Mohini, Raniselute	6.9	0.83	13.2	5.9	0.58	3.3	5.70	79	0.67	32	1.44
Sreefaltala, Soladana Union, Paikgacha, Khulna	Ashfal	7.8	2.98	35.4	9.6	0.86	4.3	9.65	145	0.87	17	1.26
As above	Horkuch	7.9	2.4	33.1	9.2	0.93	5.2	9.50	112	0.91	18	1.58
Hazrakhali, Mariali Union, Ashashuni, Satkhira	Dhulubeez	4.9	1.24	5.8	5.5	0.60	3.6	1.94	103	0.86	52	1.86
As above	Moynamoti	4.7	4.14	6.3	6.0	0.85	5.6	2.31	177	1.26	62	5.58
As above	Patnai Balam	5.4	2.07	7.1	7.0	0.90	4.1	4.36	120	0.75	61	3.3
As above	Jatai Balam	6.9	6.04	6.7	6.3	1.10	5.2	2.74	89	0.47	10	1.10
As above	Chinikanai	4.4	5.79	6.4	6.1	0.50	3.0	2.70	100	0.52	87	2.08
As above	Capsule	4.5	3.72	4.8	4.5	0.76	4.0	1.08	107	0.64	61	2.64

*These landraces grown in the Aus season, from April to July.

23 m^2, variability in field conditions was deemed to be insignificant.

Combining good tolerance scores with good plant-type, identified the following 8 landraces to be potential donors for salt tolerance traits in breeding programs, even though the variability among the landraces was too low to allow clustering. These are Jamainaru, Lakshmikajal, Patnai Balam, Horkuch, Morichshail, Ashfal, Raniselute and Kajalshail. The former 7 are all landraces from the southwest Khulna region, whereas Kajalshail is popularly grown in the mid northeast Noakhali region. All of these cultivars however, need a 10-day longer period for maturity compared to Pokkali, which may become even longer due to saline stress. If saline stress occurs in the first 60 days and is then removed, then the number of panicles per square meter is directly affected which is one of the components of yield (Bennett and Khush, 2003). Therefore landraces that show good performance at the seedling stage are expected to contribute more towards this component of yield. Na/K ratio, have been also shown to be negatively correlated with yield in the fully-grown plant by Mishra and coworkers (2001). In mature plants, it has been reported that tolerant cul-

tivars were able to efficiently exclude sodium from the reproductive parts (Khatun et al., 1995; Moradi et al., 2003). Therefore any genotypes showing the ability to exclude Na at the seedling stage are expected to do so when more mature. Asch et al. (2000) have shown that salinity-induced yield loss can be predicted with a high degree of confidence through Na/K ratio of seedlings when 60 days old. Yield component such as 1000-grain weight has been shown to be the least affected by salinity stress (Moradi et al., 2003). Therefore landraces with good scores for this yield component in unstressed soil and having good scores for tolerance under seedling stress are expected to be good donors for salt tolerance traits.

In breeding programs for production of salt tolerant rice, the potential donor LRs, which grouped into Het can be crossed with SMVs within or outside the Het group (Figures 3a and b), depending on the objective of an area-specific improved cultivar or one with broader adaptability. The LRs from the SW1, 2 or 3 groups could be crossed with SMVs like IR50184-3B-18-2B-1 which has Pokkali and SR26B as well as good yields or IR60494-2B-18-3-2-3 which has Nona Bokra and Pokkali. The above 8 landraces were also found to be Heterozygous to Pokkali with respect to the rice microsatellite marker from chromosome 1, RM140. This marker has been found to be linked to the major QTL for salt tolerance and low Na/K ratio in Pokkali (Elahi et al., 2003). Other markers are being tested for heterozygosity, within the linked region.

The MNE group contained varieties that are grown in the northeastern coast as well as in non-saline, Narail and Faridpur, in east and central Bangladesh respectively, and Comilla and Habiganj, in the northeast. Seeds for all these landraces were from the BRRI germplasm bank and were from a collection of 1973 provided as blind samples, until after the fingerprinting was completed. It turns out that cultivars with the same name are grown in the non-saline areas above as well as the northeastern coastal areas (Bose and Hossain, 2003). The common factors about these landraces are that they grow in areas, which are non-saline and where the soil has low to medium phosphorus and potassium content. We determined the heterozygosity between landraces with the same name but grown in different locations (results not shown). In all cases, the heterozygosity was less than 0.1, which was not enough to separate them, when a dendrogram was attempted.

Farmer popularity of the cultivars that performed well were noted from unpublished results of scientists from IRRI and Bangladesh (Bose and Hossain, 2003). These data were collected from the department of extension (DAE) survey under the Ministry of Agriculture in 1996/97. Jamainaru was grown in 3.3% area in Bagerhat and was amongst the top 10 rice varieties grown in this area. The seeds that we used were from a 1973 BRRI gene bank collection, where the area of collection was noted as Khulna. The DAE survey showed the area under Patnai Balam cultivation in Satkhira to be 7.6% and in Khulna 3.6%, also ranking among the top 10 rice varieties to be grown. Our collections of Patnai Balam were also from farmers in Khulna as well as Satkhira. Raniselute and Morichshail, both collected from Khulna were cited to be cultivated only in 1.5% and 1% area of Khulna, respectively. Our Horkuch collection was from Khulna. However the DAE survey showed that it was grown only in 0.4% area in Satkhira. Although we collected Lakshmikajal from Khulna, the DAE survey observation was that it is grown only in 3.6% area of Lakshmipur in the northeast coast, again representing one of the top 20 rice of this area. Discrepancies between location of cultivation area may indicate shifting farmer preferences over different years. Landraces, which remain popular over years may therefore indicate their greater adaptability.

We have checked the names of the LRs against the gene bank collections at IRRI, listed in the site www.cgiar.org/irri/iris. Any entry was recorded and noted in Table 4. Jamainaru, grouped within the Het family, is mentioned as a photoperiod insensitive variety. We however found Jamainaru to be responsive to daylength hours and therefore, photoperiod sensitive. We have fingerprinted a LR collected from the BRRI gene bank called Patnai; however we collected a LR called Patnai Balam from farmers in Satkhira. Incidentally both these LRs have the IRGC entries 31913 and 31914, respectively. On fingerprinting, we found that the Heterozygosity between these two is less than 0.1, which indicated that these cannot be separated as different cultivars. We have included only the fingerprinting data of Patnaibalam in the dendrogram in Figure 3a.

A combination of agarose as well as denaturing polyacrylamide was used to resolve microsatellite regions of the different rice cultivars from the coastal areas of Bangladesh. Using this approach, which was time-effective, adequate heterozygosity was found as reported by other authors as well (Nagaraju et al., 2002). Although the heterozygosity is greater when

AFLP (Fuentes et al., 1999) or RAPD (Parsons et al., 1997) markers are used, one can be sure that all the regions of the 12 rice chromosomes have been represented with microsatellites. Moreover genetic distance between two reference varieties like Pokkali and Nona Bokra (Singh et al., 1999) was similar to what we found in our approach as described in the discussion above. Seven cultivars, which are grown exclusively in the highly saline zones and one for the moderately saline mid-northeast, were identified with superior salinity tolerance levels at the seedling stage as well as having good agronomic properties. These landraces will be taken up for further study and their yield parameters under moderate saline stress determined. The fingerprinting results of these cultivars also indicate linkages between these and other modern varieties, including some salt tolerant modern varieties. Hence this work will assist the breeders in selection of salt tolerant donors, which are different from the traditional ones, Pokkali and Nona Bokra.

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Appendix B

- **Acrylamide/bis-acrylamide(40%):** 190 g Acrylamide, 10 g Bis-acrylamide; dissolve in 500 ml autoclaved ddH_2O and store in dark bottle.
- **APS (10%):** 1 g Ammonium persulfate dissolve in 10 ml autoclaved ddH_2O and store in dark bottle.
- **Ethidium bromide:** Prepare as a stock solution of 10 mg/ml in H_2O and store at room temp. in dark bottle.
- **0.5M EDTA:** 18.61g of EDTA (MW. 372.2) dissolve in 40 ml of dd H_2O with the help of magnetic stirrer, adjust P^H to 8.0 by NaOH pellet, make the volume 50 ml and autoclave.
- **Gel loading buffer (6X):**
    i. **for agarose gel:** 0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 30% Glycerol; store at 4^0C.
    ii. **for sequencing gel:** 0.05% Bromophenol blue, 40% Sucrose, 0.5% SDS, 0.1 M EDTA; store at 4^0C.
- **3M Na-acetate (P^H 5.2):** 40.81g of sodium acetate dissolve in 40 ml of water, adjust P^H to 5.2 with glacial acetic acid; make volume 100 ml and sterilize by filtration.
- **RNase solution (DNase free):** Dissolve pancreatic RNase (RNaseA) at a conc. of 10 mg/ml in 0.01M sodium acetate (P^H 5.2), allow to cool slowly to room temp. after heating to 100^0C for 15 minutes, adjust P^H by adding 0.1 volume of 1.0M Tris.HCl (P^H 7.4); make aliquots and store at -20^0C.
- **1M of Tris.HCl:** 12.11g of Tris.HCl (MW 121.14) dissolve in 80 ml of ddH_2O with the help of magnetic stirrer, adjust P^H 8.0 by concentrated HCl, make volume 100 ml and autoclave.
- **TE buffer:** 10 mM Tris.HCl , 0.1 mM EDTA; P^H 8.0
- **50 X TAE Buffer:** 242 g Tris base, 57.1 ml Glacial acetic acid, 100 ml 0.5M EDTA (p^H8.0); make volume 1000 ml with ddH_2O amd autoclave.
- **1 X TAE Buffer:** 40 mM Tris acetate, 1mM EDTA (p^H8.0); make 1 L 1 X TAE buffer by mixing 20 ml of 50 X TAE buffer solution and 980 ml of ddH_2O.
- **10 X TBE Buffer:** 108 g Tris base, 9.3 g EDTA$(Na)_2$, 55 g Boric acid; make volume 1000 ml with ddH_2O and autoclave.

Appendix C

C1. Equilibration of phenol

C1.1 Materials used

i. Distilled phenol
ii. Hydroxyquinoline
iii. 0.5M Tris.HCl (P^H 8.0)
iv. 0.1M Tris.HCl (P^H 8.0)
v. β-mercaptoethanol
vi. P^H paper

C1.2 Procedure

1. Crystalline phenol was redistilled at 160^{0}C to remove oxidation products (i.e. quinones etc.) and liquefied phenol was stored at –20^{0}C
2. The phenol was removed from the freezer, allowed it to warm to room temperature and then it was melted at 68^{0}C.
3. Hydroxyquinoline (antioxidant) was added to a final concentration of 0.1%.
4. An equal volume of buffer (0.5M Tris.HCl, P^H 8.0) was added to the metled phenol at room temperature.
5. The mixture was stirred on a magnetic stirrer for 15 minutes.
6. The stirrer was turned off and when the two phases had separated, as much as possible of the upper (aqueous) phase was aspirated using a glass pipette attached to a vacuum line equipped with appropriate traps.
7. An equal volume of 0.1M Tris.HCl (P^H8.0) was added to the phenol.
8. The mixture was stirred on a magnetic stirrer for 15 minutes.
9 The stirrer was turn off and the upper aqueous phase was removed.
10. The extractions was repeated until the P^H of the phenolic phase was>7.8 as measured with P^H paper.
11. After the phenol was equilibrated and the final aqueous phase had been removed, 0.1 volume of 0.1M Tris.HCl (p^H 8.0) containing 0.2% β-mercaptoethanol was added.
12. The phenol solution was stored in a light-tight bottle at 4^{0}C for periods of up to 1 month.

C2. Isolation and quantification of plant genomic DNA

Leaves of each rice variety were cut finely, crushed to powder in liquid nitrogen and the DNA was isolated using CTAB method of Doyle and Doyle (Doyle and Doyle, 1990). Slight modifications to the protocol included use of sodium acetate instead of ammonium acetate to precipitate DNA after RNase treatment and additional phenol: chloroform and chloroform purification steps after preliminary DNA isolation.

C2.1 Materials used

i. Liquid Nitrogen (collected from BOC)
ii. 1M of Tris.HCl
iii. 0.5M EDTA
iv. Buffer saturated phenol
v. Chloroform: Isoamylalcohol (24:1, v/v)
vi. TE buffer
vii. 3M Na-acetate (P^H 5.2)
viii. RNase solution (10mg/ml)
ix. CTAB isolation buffer (per 100 ml)

- CTAB 2.0 g
- 5M NaCl 28.0ml
- 1M Tris. HCl (P^H 8.0) 10.0ml
- 0.5 M EDTA (P^H 8.0) 4.0 ml
- 2-mercaptoethanol 0.2 ml

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

x. Ethanol (70% and 99%)
xi. Isopropanol
xii. 5M NaCl

C2.2 Procedure

1. 5 to 7.5 ml of CTAB isolation buffer was preheated at 60°C water bath in a screw cap tube
2. 1g of fresh tissue (i.e. leaves) was ground to a powder in a mortar containing liquid nitrogen
3. The powder was scraped directly into preheated buffer and swirled gently to mix.
4. The sample was incubated at 60°C for 30 minutes with optional occasional gentle swirling.
5. The mixture was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1)mixing thoroughly and centrifuged at 4000 rpm for 10 minutes at room temperature.
6. The aquous phase was removed with a pipette and transferred to a clean screw capped tube.
7. Two third volume of ice-cold isopropanol was added and mixed gently to precipitate the DNA (if needed keep it for overnight at -20°C)
8. The solution was centrifuged at 4000 rpm for 10 minutes, supernatant discarded and the precipitate was washed twice with 3-5 ml 70% ethanol (ice-cold)
9. Pellet was allowed to dry and resuspended in minimal amount of TE buffer (in eppendorf tube)
10. DNase free RNase added to a final concentration of 10 μg/ml and incubated at 37°C for 10-30 minutes
11. Equal volume of phenol: chloroform: IAA (25:24:1) was added (2X)
    (**N.B.** sometimes an additional step with chloroform: IAA was needed)
12. Centrifuged at 14000 rpm for 10 minutes and supernatant was collected.
13. One-tenth of 3M Na-acetate and double volume of 99% ethanol (ice-cold) was added and kept on ice for 30 to 60 minutes.
14. Centrifuged at 14000 rpm for 10 minutes and supernatant was removed
15. DNA was washed with 1 ml 70% ethanol (rinse vigorously) [2X]
16. Supernatant was removed and allowed to dry the pellet completely
17. Pellet was dissolved in minimal amount of TE and stored at -20°C

C2.3 Quantitation of DNA

Once DNA is prepared from leaf tissue it is important that the quality of DNA be checked for obtaining good results and for long-term storage. It is also important to know that one should know exactly how much DNA is available for the experiment and the conc. of DNA in known volume of TE buffer.

C2.3.1 By checking the OD using spectrophotometer

Measurement of the UV radiation absorbed by the nucleic acid bases offers an accurate means of quantifying low molecular weight DNA concentration in a sample. Steps were as follows:

1. The spectrophotometer settings were selected at 260 nm and 280 nm
2. Cuvettes were washed with ddH_2O, TE buffer and dry
3. Calibration was carried out using TE buffer
4. 5 µl of the purified DNA sample was added in 995 µl of TE solution in cuvette. It was mixed well, cuvette placed in the compartment and absorbance reading was taken from the display.
5. Concentration of DNA was calculated using the formula

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

$= OD_{260} \times 50 \times 200$ µg/ml

$= OD_{260} \times 50 \times 200/1000$ µg/µl

$= OD_{260} \times 10$ µg/ µl

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

(OD_{260} = Absorbance at 260 nm)

C2.3.2 By comparing with λDNA standard

1. Stock DNA preparations were diluted by 10X and 20X
2. 1-2 µl of diluted (10X, 20X) DNA samples followed by 25,50,100,200ng of λDNA standard were loaded in the wells of 0.8% agarose gel
3. Electrophoresis and staining with EtBr was carried out
4. DNA concentration was estimated by visually comparing the fluorescence of any of the standard with the fluorescence of diluted sample DNA.

C3. Rapid isolation and purification of high-activity Taq DNA polymerase

Taq DNA polymerase was isolated from *E. Coli* strain taq-2 transformed with the plasmid P^{Taq} following protocol modified from Chien et al. (1976)

C3.1 Materials used

All the chemicals used for the preparation of the following reagents were of Molecular Biology grade and water used was ultra-pure.

i. Buffer A

Components	MW	Concentration
• Tris	121.14 g	50 mM
• Dextrose	180.16 g	50 mM
• EDTA	372.2 g	1 mM

100ml of bufferA was prepared by dissolving 0.605g of Tris, 0.9008g of dextrose and 0.037g of EDTA in distilled-deionized water. After adjusting P^H to 7.9 by concentrated HCl, the final volume was made 100 ml. The resulting buffer was filter-sterilized by using 0.22μm millipore filter and was stored at 4^0C.

ii. Pre-lysis buffer

Pre-lysis buffer was prepared by adding lysozyme (crystalline state) at the concentration 4mg/ml to the bufferA. This buffer was prepared just before use.

iii. Lysis buffer

Components	MW	Concentration
• Tris	121.14 g	10 mM
• KCl	74.5 g	50 mM
• EDTA	372.2 g	1 mM
• PMSF	174.2 g	1 mM
• Tween (liquid)	—	0.5% (v/v)
• Nonidet (liquid)	—	0.5% (v/v)

50 ml of lysis buffer was prepared first by dissolving 0.0085g of PMSF in minimal volume of isopropanol (~1 ml). Afterwards, 0.0605g of Tris, 0.1862g of KCl, 0.0185g of EDTA were added. After dissolving in ~40ml of distilled-deionized water, 0.25ml each of Tween and Nonidet were added. After adjusting P^H to 7.9, with 0.001 N HCl, final volume of 50 ml was made by adding water. Lysis buffer was sterilized by filtration and stored at 4^0C.

iv. Storage buffer

Components	MW	Concentration
• Tris	121.14 g	50 mM
• KCl	74.5 g	50 mM
• EDTA	372.2 g	0.1 mM
• DTT	154.2 g	1 mM
• PMSF	174.2 g	0.5 mM
• Glycerol	—	50%

200ml of storage buffer was prepared first by dissolving 0.01742g of PMSF in minimal volume of isopropanol (~1ml). Afterwords, 1.21g of Tris, 0.745g of KCl, 0.0074g of EDTA and 0.03084g of DTT were added. After adjusting P^H to 7.9, final volume of 75ml was made by adding water.

Meanwhile, 80% glycerol was prepared from commercially available glycerol (98%) and autoclaved. 125 ml of the 80% glycerol was added to 75 ml of the mixture of storage buffer and stored at 4°C.

v. IPTG

vi. Sorvall centrifuge tubes

vii. Pyrex flask (100 ml)

viii. Dialysis bag

C3.2 Detailed Isolation Procedure

1. 5 ml of LB media containing ampicillin (100 μg/ml) was inoculated with *E Coli* strain Taq-2 transformed with pTaq plasmid containing taq gene expressed under control of tac promoter

2. Overnight incubation was accomplished at 37°C
3. 125 µl of an overnight culture was added to 250ml LB media with ampicillin (100 µg/ml)
4. The culture was allowed to grow for 11 hours at 37°C with continuous shaking
5. 31.25 mg of IPTG (125mg/ml) were added after 11 hours of incubation and IPTG-induced culture was allowed to grow for 12hours at 37°C with continuous shaking
6. Cells were harvested by centrifugation in 5 separate centrifuge tubes each containing ~50 ml of the culture broth at 4000 rpm at 4°C for 20 min. and the supernatant was discarded
7. 5.0 ml of buffer A was added to each of 5 tubes and cell pellet was mixed well without vortexing
8. Centrifuged at 4000 rpm for 20 minutes at 4°C and the supernatant was discarded
9. The cells were resuspended in pre-lysis buffer by adding 2.5 ml of pre-lysis buffer to each tube (buffer A+ 4.0 mg/ml lysozyme) and they were kept at room temp. for 15 minutes
10. 2.5 ml of lysis buffer was added to each tube and mixed carefully. Thus, the total volume was 5.0 ml in each tube.
11. All the samples were pooled in pyrex flask (100 ml) and incubated at 75°C for 1 hour.
12. Lysis mixture was transferred to one centrifuge tube (the total volume was 5 × 5 ml = 25 ml) and centrifuged at 8000 rpm for 25 minutes at 4°C
13. Again centrifuged at 11,000 rpm for 20minutes at 4°C and clarified lysate was transferred to clean pyrex flask (100 ml)
14. 7.5g of powdered ammonium sulfate was added to 25 ml of lysate slowly with stirring the mixture rapidly at room temperature
15. Centrifugation was carried out at 11,000 rpm for 20 minutes at 4°C and the supernatant was discarded

16. The protein was resuspended by adding 5.0 ml of buffer A with gentle shaking.
17. Resuspended protein was transferred to eppendorf tubes and centrifuged at 13000 rpm for 5 minutes at 4°C
18. The supernatant of protein solution was collected and transferred into autoclaved dialysis bag
19. The protein was dialyzed for 12 hours in storage buffer at 4°C
20. Dialysis was repeated (2 to 3 times) every after 12 hours in new storage buffer under identical condition
21. The dialyzed protein was aliquoted in fresh, autoclaved microfuge tubes
22. Equal volume of storage buffer was added in each tube so that protein: storage buffer was in the ratio of 1:1
23. The aliquots were stored at -80^0C.

C3.3 Purification of isolated Taq DNA polymerase by DNaseI treatment

As the isolated Taq DNA polymerase might be contaminated with bacterial DNA, hence it was allowed to digest with DNaseI in order to purify according to the following protocol modified from Sambrook et al., (2001).

C3.3.1 Materials used

i. 10XDNase Mn^{++} digestion buffer

- Tris.HCl 500 mM (p^H 7.6)
- $MnCl_2$ 100 mM
- EDTA 50 mM (p^H 8.0)

ii. DNaseI stock solution

iii. Isolated Taq DNA pol. solution

iv. EDTA (50 mM p^H 8.0)

* Target DNA (i.e. Taq DNA pol. solution)	900 µl
* 10XDNase /Mn++ digestion buffer	100 µl
* DNaseI	5 µl

2. Reaction mixture was shacked by hand
3. Reaction mixture was incubated at 37°C for 15-20 minutes
4. Digestion reaction was stopped with 50 mM EDTA (p^H 8.0)
5. The tube containing Taq DNA pol. was then inactivated at 80°C for several minutes (~5 min.) to inactivate the DNaseI.

C4. Polymerase chain reaction (PCR) using microsatellite/SSR and gene specific primers

C4.1 Materials used

i. 10 X PCR reaction buffer (500mM KCl; 100mM Tris.HCl, P^H 8.3; 0.1% gelatin)
ii. 50 mM $MgCl_2$
iii. dNTPs (100 mM; sodium salt, P^H 7.35)
iv. Taq DNA polymerase (self isolated)
v. Forward and reverse primers (SSR/gene specific)
vi. Plant genomic DNA as a template
vii. DMSO (20%)
viii. Autoclaved ultra pure water
ix. TE buffer for diluting template DNA

C4.2 Procedure

1. Master mixture containing buffer, dNTPs, Mg^{2+}, specific primer and Taq DNA polymerase was prepared for 9 reactions (in practice for 10 to avoid shortage of the master mix, due to pipetting losses) as for example, in a sterile 0.5ml microfuge tube in the following order:

Component	1 reaction (µl)	10 reactions (µl)	Final concentration
* PCR buffer (10X)	1.5	15	Tris, 10 mM; KCl, 50 mM; gelatin, 0.01%
* dNTPs (1 mM)	1.5	15	100µM (0.1 mM)
* Mg^{2+} (50 mM)	0.5	5	1.67 mM
* Forward primer (60 ng/µl)	0.3	3	-1.2 ng/µl or 200 nM
* Reverse primer (60 ng/µl)	0.3	3	-1.2 ng/µl or 200 nM
* Taq DNA polymerase(10X diluted)	0.3	3	-
Total=	4.4	44	

2. After thorough mixing and momentary spin, 4.4 µl of the above master mixture was dispensed to thin walled PCR tubes (labeled for proper identification) containing genomic DNA. The volume was made 15 µl by adding varying amounts of sterilized ultra-pure water.
3. Genomic DNA was dispensed in the above labeled tubes prior to adding the master mixture as follows:

	DNA (50ng/µl)	DMSO (20%)	ddH_20 (adjustable)	Total volume
* Sample tube	1.0 µl	2.0µl	7.6µl	15 µl
* Control tube	0.0 µl	2.0µl	8.6µl	15 µl

[**N.B.** Control tube is required to detect any kind of contamination, which causes false positive amplification]

4. Finally, the tubes were subjected to momentary spin and transferred to thermo-cycler for the amplification reaction.

C4.3 Thermal cycling profile used in PCR

The thermal cycling profiles that were programmed to amplify the gene by polymerase chain reaction (PCR) for 35 to 40 cycles as follows:

Step	Temperature	Time	No. of cycles
* Initial denaturation	94°C	5 minutes	1
* Denaturation	94°C	1 minute	35 to 40
* Annealing	55° C(variable)	1 minute (±)	35 to 40
* Elongation	72°C	3 minutes (±)	35 to 40
* Final elongation	72°C	7 minutes	1

NB. For different primers different annealing temperature was employed.

C5. Electrophoresis of PCR amplified product

The PCR amplified product, using microsatellite/SSR and gene specific primers, were resolved and visualized with the help of agarose gel electrophoresis or polyacrylamide gel electrophoresis or sometimes long gels (i.e. sequencing gels).

C5.1 Agarose gel electrophoresis

Using the technique of agarose gel electrophoresis amplified DNA fragments were size fractionated by comparison with standard DNA markers. Depending on the size of the DNA band, different concentrations of agarose was used. For SSR markers 3-4% agarose was ideal. This was done by the protocol of Sambrook et al., (2001).

C5.1.1 Materials used

i. Ultrapure agarose (typing grade)
ii. 1 X TAE Electrophoresis buffer
iii. Gel loading buffer (6X)
iv. Ethidium bromide
v. DNA size standards/ DNA markers
vi. Horizontal electrophoresis tank and power supply device

C5.1.2 Procedure

1. The open ends of a clean, dry plastic tray was sealed with tape and placed on a horizontal section of the bench.
2. Sufficient electrophoresis buffer (usually 1XTAE or 0.5XTBE) was prepared to fill the electrophoresis tank and to cast the gel.
3. A solution of agarose in electrophoresis buffer was prepared in an Erlenmeyer flask at a concentration appropriate for separating the particular size fragments expected in the DNA sample.
4. The flask was placed in the microwave oven on high temperature (i.e.60^0C-80^0C) for 2-3 minutes or until the agarose dissolves.
5. Using insulated gloves, the flask was transferred into a water bath at 55^0C. The gel solution was mixed thoroughly by gentle swirling when the melted gel had cooled.
6. The cooled gel solution was poured into the gel tray. An appropriate comb was placed for forming the sample slots in the gel assuring that there were no bubbles around the combs (a pipette tip was used to remove if there was any bubble)
7. The gel was allowed to set completely (30-45 minutes at room temperature), then poured a small amount of electrophoresis buffer on the top of the gel and the comb was removed carefully. The electrophoresis buffer was poured off and the tape was removed carefully. The gel was mounted in the electrophoresis tank.
8. Electrophoresis buffer (1XTAE) was adjusted sufficiently to cover the gel to a depth of ~ 1 mm.
9. The samples of DNA was mixed with 0.20 volume of the desired 6X gel-loading buffer and loaded slowly into the slots of the submerged gel using a disposable micropipette. Size standard which will depend on the type of marker being analyzed was loaded into slots on both the right and left sides of the gel.
10. The lid of the gel tank was closed and the electrical leads was attached to the power supply device so that the DNA will migrate toward the positive anode (red lead).

11. A voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes) was applied.
12. The electric current was turn off when the DNA samples or dyes had migrated a sufficient distance through the gel and the electrical leads and lid from the gel tank was removed.
13. The gel was stained by immersing it in electrophoresis buffer or H_2O containing ethidium bromide (0.5µg/ml) for 30-45 minutes at room temperature.
14. Photograph of the gel was taken under UV illumination.

C5.2 Denaturing sequencing gel electrophoresis

C5.2.1 Materials used

i. APS (10%)

ii. Acrylamide/bis-acrylamide (40%)

iii. Acrylamide gel solution (6%)

• 40% Acrylamide/bis-acrylamide solution	150 ml
• Urea	420 g
• 5XTBE	200 ml
• The mixture was dissolved and filtered	
• Autoclaved ddH_2O to make volume	1000 ml
• TEMED	500µl
• 10% APS	6 ml

iv. Binding solution

• Ethanal, 95%	1 ml
• Acetic acid (0.5%)	5 µl
• Bind silane $^{(R)}$	3 µl

v. Developer solution

- Na_2CO_3 60 g
- For polyacrylamide gel Dissolve in 2000 ml ddH_2O and cool at 4^0C
- HCHO (37%), added just before use 3 ml
- $Na_2S_2O_3$. $5H_2O$ (10 mg/ml), added just before use 400 µl

vi. Fix/stop solution (freshly prepared)

- Glacial acetic acid 200 ml
- ddH_2O for polyacrylamide gel 1800 ml

vii. Silver stain solution

- $AgNO_3$ (promega(R) kit) 2 g
- HCHO (37%), added just before use 3 ml
- ddH_2O to make volume 2000 ml

viii. Gel loading dye

ix. Sigma Coat(R)

x. 1M NaOH (for gel removing)

C5.2.2 Procedure

C5.2.2.1 Assembling the glass plate sandwich

(Adapted from Sequi-Gen(R) GT Nucleic Acid Electrophoresis Cell Instructional Manual)

1. Both Sequi-Gen(R) GT integral plate chamber (IPC) glass plate and outer glass plate was cleaned thoroughly with liquid soap. Plates were rinsed with ddH_2O. [**N.B.** Always weared gloves while handling the glass plates because fingerprint would cause bubbles to form during gel casting.]
2. The outer glass plate was placed flat on a lab table and wiped the entire surface of the outer glass plate with fresh binding solution. It was spreaded evenly with kimwipe (R) facial tissue and allowed to dry for 5 minutes.
3. The IPC was placed flat on the lab. table with the glass plate facing upward. 1ml of sigmacote (R) was applied and spreaded evenly over the entire surface of the

glass plate with kimwipe(R) /facial tissue. Then it was allowed to dry for 2 min. and cleaned/washed with absolute ethanol rapidly (2 times).

4. One 0.4 mm side spacer was positioned along each edge of the IPC glass plate. The bottom edges of the spacer and the IPC glass plate was needed to be flush and the long edge of the spacer needed to be next to the plastic lip of the IPC panel.
5. The front of the outer plate was placed on to the IPC and spacers with the coated surface facing down.
6. The IPC/outer glass plate sandwich was stood on the lab. table with the outer glass plate facing away from me.
7. The clamps was slided over the sides of the IPC assembly. The lever of the clamp was needed to be on the IPC panel side of the assembly and facing away from the unit and perpendicular to the IPC panel for the clamps to slide easily onto the assembly. The clamps to the IPC/outer glass plate sandwich was secured by moving the lever towards the IPC panel.
8. The assembly was placed in a flat surface with the IPC panel facing up. It was checked if the glass plates and the spacers were flush.
9. The precision caster base was placed on the lab table with the open cavity facing up. The gray precision gasket was placed into the base. The cam pegs in the precision caster must be pulled out to accommodate the apparatus.
10. Bottom edge of the IPC assembly was placed into the precision caster base with the bottom edge of the assembly vesting against the gray gasket of the precision caster base.
11. Once vesting on the gasket, the cam pegs was used to connect the base to the clamps. Each cam peg was pushed into the corresponding hole on the clamp with the lever in the up position. Slight downward pressure applied to the top of the IPC assemble might be required to engage each cam peg.
12. The IPC assembly was laid flat on the lab. table with the drain port facing up and the precision caster base facing towards me. It was made sure that the assembly was level to prevent gel leakage.

C5.2.2.2 Gel preparation

1. The gel was prepared by mixing 100 ml 6% acrylamide solution, 50µl TEMED and 600 µl 10% APS. It was mixed gently by swirling.
2. A 100 ml syringe was filled slowly with the solution and attached the tubing assembly. The bubbles from the syringe was removed by forcing some of the gel out.
3. When all the bubbles were removed from the tubing, the lower taper was placed into the injection port of the precision caster base. The gel solution was injecting slowly on to the glass plate sandwich and the tubing was not removed until the gel had polymerized.
4. The flat edge of a shark tooth comb was inserted 5 mm past the edge of the outer glass plate. To hold it in place it was clamped with a large metal binder.
5. The gel was allowed to ploymerize for 30-60 min. The tubing and the precision caster base was removed from the assembly. The caster base and gasket of polymerized gel solution was cleaned with tap H_2O, followed by a ddH_2O rinse.

C5.2.2.3 Pre-electrophoresis

1. The lower buffer chamber was filled with 350-500 ml 1XTBE buffer.
2. The gel assembly was gently lowered to the universal base and the stabilizer bar was inserted.
3. The upper buffer chamber was filled with 1400 ml 1XTBE buffer. The level of the buffer needed to be about 1cm from the top of the fill spout at all times during the run. Gel electrophoresis buffer could be heated to 50^0C in a microwave oven before adding buffer into the upper buffer chamber. This would reduced the time needed to bring the gel to the appropriate run temperature before sample loading and would greatly reduced pre-electrophoresis run time.
4. The comb was removed from between the glass plates. The well area was cleaned using syringe. It was made sure to remove air bubbles and unpolymerized acrylamide.
5. A gel temperature indicator was adhered onto the outside of the outer glass plate, somewhere may the center to monitor the gel temperature during electrophoresis.

6. The top and bottom safety covers were attached. Also electrode wires were attached to the power supply and the gel was pre-run at 120 W to achieve a gel surface temperature of approximately 45-50^{0}C pre-electrophoresis prior to sample loading would create a uniform gel temperature and bring the gel temperature to the recommended run temperature. This would help to eliminate any smile pattern from developing early in the run.

C5.2.2.4 Loading the DNA samples and gel electrophoresis

1. DNA samples were denatured by heating in a thermal cycler at 95^{0}C for 5 min. and immediate chilling on ice.
2. After the pre-run the power supply was turn off and the top safety cover was removed. The well area was cleaned again.
3. The teeth of the shark tooth comb was inserted carefully into the gel 5.1 mm deep.
4. 6 µl for 46 wells or 4 µl for 72 wells of each sample was loaded into the wells. It was required not to exceed 20 min, for loading of samples, to prevent cooling of the gel and to maintain the denatured state of DNA.
5. Top safety cover was attached. Power supply was turn on and the gel ran at 120 W maintaining the temperature at 50^{0}C for 1 hr. Running time would vary depending upon the primer used. Generally, the run was stoped after the bromophenol blue (leading dye) reached the bottom of the gel.

C5.2.2.5 Disassembly

1. After electrophoresis, the power supply was turn off and both safety covers were removed.
2. The upper buffer chamber was partially emptied by inserting the drain port connector into the drain port on the IPC. Buffer needed to be drained out immediately form the IPC.
3. After the upper chamber was emptied to the level of the drain port, the stabilizer bar was pulled out and the IPC assembly was removed. The bottom edge of the IPC assembly was blotted onto the absorbent paper.
4. The remaining upper buffer was poured carefully out of the IPC assembly into a container. Also, the lower buffer from the universal base was carefully

drained out into a container. It was suggested not to store buffers in an IPC and not to add buffer to an IPC unless the clamps were in place.

5. The clamps were removed from the IPC assembly by first pulling the levers away from the IPC and then sliding the clamps off the IPC assembly.
6. The IPC assembly was laid flat on a lab. table with the outer glass plate facing up. The glass plate was carefully separated by pulling up gently near the top of the outer plate. The gel usually come apart from the IPC and become strongly affixed to the outer glass plate. The comb and side spacers were removed.

C5.2.2.6 Staining

(Adapted from promega's silver sequence$^{(R)}$ DNA sequencing system Technical Manual Rev. 8/96)

1. Outer glass plate with the gel was placed in a plexiglass tray with fix/stop solution for 20 min. with continuous shaking. It is suggested not to discard the fix/stop solution and do this step inside a fume hood.
2. The gel was washed thrice for 2 min each in a tray with ddH_2O with continuous shaking.
3. The gel was stained with silver stain solution for 30 min. in a tray with continuous shaking.
4. The gel was rinsed in a tray with ddH_2O for 10 seconds.
5. The gel was transferred to a tray with developer solution (pre-cooled to 4-10^{0}C) for 2-5 min. (or as soon as the bands appear) with shaking.
6. The gel was returned to the fix/stop solution for 5-6 min.
7. The gel was rinsed in a tray with ddH_2O for 2-3 min. and allowed the gel to dry at room temperature or at 50^{0}C.

C5.3 Non-denaturing polyacrylamide gel electrophoresis

The resolution in agarose gel is reasonable but sometimes it may be necessary to run the amplified products (intact or digested) on a polyacrylamide gel for better one. This was done according to following protocol modified from Santos, F. (1996).

C5.3.1 Materials used

i. Acrylamide/bis-acrylamide (40%)

ii. Ammonium persulfate (APS, 10%)

iii. 5XTBE Buffer (for gel preperation)

iv. 1X TBE buffer (for gel electrophoresis)

v. Gel loading dye

vi. Ethidium bromide (10 mg/ml)

vii. Composition of 8% polyacrylamide gel (for two gels)

• 40% acrylamide	20 ml
• 5XTBE	20 ml
• ddH_2O, volume to	100 ml
• 10% APS	700 µl
• TEMED	80 µl

C5.3.2 Procedure

1. Both glass plates were cleaned carefully by 99% ethanol and were assembled, placing both cleaned surface inside, with spacers (~1.5 mm thick) and elastic rubber as a sealer surrounding the edges of glass plate.
2. The assembly was leveled and checked for leakage with ddH_2O.
3. The gel was poured in the gel case and the comb was assembled for wells formation.
4. The gel was allowed to solidify for ~ 1 hour
5. The combs were removed and sandwich glass plate/gel were attached with the electrophoresis apparatus.
6. The PCR product was mixed with appropriate volume of loading buffer and denatured by incubation for 5 minutes in 95^0C.
7. 2-4 µl of the mixture was generally loaded onto 6% denaturing polyacrylamide gel very carefully to prevent cross contamination.
8. Electrophoresis was carried out in 1XTBE buffer at 100 W for 2 hours or upto the time when the bromophenol blue traveled a satisfactory distance.
9. The electric current was turn off and both glass plates were disassembled. Elastic rubber and spacers were removed.

10. The gel was stained by immersing it in electrophoresis buffer or ddH_2O containing ethidium bromide (0.5 μg/ml) for 30-45 minutes at room temperature.
11. Photograph of the gel was taken under UV illumination.

C6. Extraction and quantification of total RNA

Total RNA was extracted from 18 days old seedlings of some selected traditional landraces after 100 mM NaCl stress, for different timings, using TRIZOL reagent.

C6.1 Materials used

i. Chloroform
ii. Isopropanol
iii. 2M NaCl (DEPC treated)
iv. 75% ethanol (made by DEPC treated water)
v. Ultrapure water (0.1% DEPC treated for 1 hr at 37^0C and then autoclaved)
vi. DEPC treated glassware and plastic ware
vii. Liquid nitrogen.

C6.2 Procedure

1. Frozen plant tissue (leaf/root of rice plant) was ground to a powder in liquid nitrogen using mortar and pestle.
2. 1 ml of TRIZOL/100-200 mg of ground tissue was added and homogenized thoroughly.
3. Samples were incubated at room temperature for 5 min. or more (until all samples were homogenized)
4. Centrifuged at 12,000xg for 10 min. in the cold (4^0C) and supernatant was transferred to a clean tube
5. 0.2 ml of chloroform (without IAA) was added for each 1 ml of TRIZOL used initially and vigorously shaked by hand or vortexing for 15 second.
6. Incubated at room temperature for 2 to 3 minutes.
7. Centrifuged at 12,000xg for 15 minutes in the cold (4^0C).
8. Aqueous phase was transferred to a clean tube (**N.B.** It should be about 60% of the initial volume of TRIZOL)

9. 0.25 ml of isopropanol and then 0.25 ml of 2 M NaCl (DEPC treated) was added per 1 ml of TRIZOL used initially.
10. Mixed by inversion and incubated for 10 min or more at room temperature.
11. Centrifuged at 12,000 xg for 10 minutes in the cold (4^0C)
12. Supernatant was removed and pellet was washed with 75% ethanol, adding 1 ml for each ml of TRIZOL used initially and vortexing well.
13. Centrifuged at 7,500 xg for 5 minutes (or 12000 xg for 2 minutes) in the cold (4^OC)
14. Supernatant was removed, pellet was briefly dried (avoiding hardening of the pellet by over-drying)
15. DEPC-treated water was added (~1000µl for 500 mg of tissue)
16. Vortexing for 10 to 30 sec. and incubated at 60^0C for 5 minute.
17. Again vortexing, pellet was broken, if necessary (probably gelatinous and transparent if RNA was still not dissolved)
18. It necessary, incubated again for 5 min at 60^0C with further vortexing
19. Centrifuged at 12,000 xg for 5 min. at room temperature if the transparent gelatinous pellet was gone but small white 'flakes' were still present.
20. Supernatant (RNA) was transferred to a clean tube and stored at -80^0C.

C6.3 Quantitation of RNA

For quantitating the amount of RNA, spectrophotometric readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid (RNA) in the sample. An O.D. of 1 corresponded to ~ 40 µg/ml for RNA. The ratio between the readings at 260 nm and 280 nm (OD_{260} : OD_{280}) provided an estimation of the purity of the RNA. Pure preparations of RNA should have a ratio (OD_{260} : OD_{280}) of 2.0. If the RNA sample is contaminated with protein or phenol, the ratio will be less than the above values and accurate quantitation of RNA will not be possible.

C6.4 Electrophoresis of RNA

Samples of RNA were denatured by treating with formamide and separated through electrophoresis in agarose gel containing 2.2 M formaldehyde as done by Sambrook et al., (2001).

C6.4.1 Materials used

All reagents used in this protocol were prepared with DEPC- treated H_2O.

1. Buffers and solutions

   i. Ethidium bromide (200 µg/ml)
   prepared in DEPC treated H_2O

   ii. Formaldehyde (37-40% W/V solution, deionized)

   iii. Formamide (distilled-deionized)

   iv. 10X Formaldehyde gel loading buffer
   - 50% glycerol (diluted in DEPC-treated H_2O)
   - 10 mM EDTA (P^H 8.0)
   - 0.25% (w/v) xylene cyanol FF

   v. 10X MOPS electrophoresis buffer
   - 0.2 M MOPS (P^H 7.0)
   - 20 mM Sodium acetate
   - 10 mM EDTA (p^H 8.0)

   41.8 g of MOPS was dissolved in 700 ml of sterile DEPC- treated H_2O and the P^H adjusted to 7.0 with 2N NaOH. 20 ml of DEPC-treated 1 M sodium acetate and 20 ml of DEPC-treated 0.5 M EDTA (P^H8.0) was added and the volume of the solution adjusted to 1 liter with DEPC-treated H_2O. Finally the solution was sterilized by fitration through a 0.45 millipore filter and stored at room temperature protected from light.

2. Gels

   1.5 g of agarose was added to 72 ml of sterile H_2O and dissolved by boiling in a microwave oven. After cooling the solution at 55^0C, 10 ml of 10X MOPS electrophoresis buffer and 18 ml of deionized formaldehyde was added. In a chemical fumehood, an agarose gel was casted with slots formed by a 3mm comb and allowed the gel to set for at least 1 hour at room temperature. As soon as the agarose had set, the gel was covered with saran wrap until the samples were ready to be loaded.

   N.B. A 1.5% agarose gel is suitable for resolving RNAs in the 0.5-8.0 kb size range. Larger RNAs should be separated on gels cast with 1.0% or 1.2% agarose.

3. RNA samples
4. Horizontal electrophoresis apparatus

   Electrophoresis tanks and combs was cleaned with detergent solution, rinsed in H_2O, dried with ethanol and then filled with a solution of 3% H_2O_2. After 10 minutes at room temperature, the electrophoresis tanks and combs were thoroughly rinsed with H_2O treated with 0.1% DEPC.
5. Water bath pre-set to 55^0C

C6.4.2 Procedure

1. The denaturation reaction was set up in sterile microfuge tubes as followed:
   - RNA (up to 20μg) 2.0 μl
   - 10X MOPS electrophoresis buffer 2.0 μl
   - Formaldehyde 4.0 μl
   - Formamide 10.0 μl
   - Ethidium bromide (200 μg/ml) 1.0 μl

2 The tops of the microfuge tubes was closed and incubated the RNA solutions for 60 minutes at 55^0C (or 10 minutes at 85^0C). The samples were chilled for 10 minutes in ice water and then centrifuged them for 5 seconds to deposit all of the fluid in the bottom of the microfuge tubes.

3. 2μl of 10X formaldehyde gel-loading buffer was added to each sample and returned the tubes to an ice bucket.

4 The agarose formaldehyde gel was installed in a horizontal electrophoresis box and sufficient 1X MOPS electrophoresis buffer was added to cover the gel to a depth of ~ 1mm. After running the gel for 5 minutes at 5 V/cm, RNA samples were loaded into the wells of the gel.

5 The gel submerged in 1X MOPS electrophoresis buffer was run at 4-5V/cm until the bromophenol blue had migrated ~ 8 cm (4-5 hours).

6 The RNAs was visualized by placing the gel on a piece of saran wrap on a UV transilluminator and photograph was taken.

C7. One step RT-PCR with gene specific primers

The superscript ™ one-step with platinum ® Taq system, manufactured by Invitrogen, USA, was used for RT-PCR experiments.

C7.1 Kit components

i. RT/platinum ® Taq Mix

ii. 2X Reaction Mix. (a buffer containing 0.4 mM of each dNTP, 2.4 mM $MgSO_4$).

iii. 5 mM Magnesium sulfate

iv. 50 mM Magnesium sulfate

C7.2 Procedure

1. The thermal cycler (BIO-RAD/EPPENDORF) was programmed as follow so that cDNA synthesis was followed immediately with PCR amplification automatically.

A: cDNA synthesis and pre-denaturation	B: PCR amplification	C: Final extension
1 cycle of : • 50°C for 50 min. • 94°C for 2 min.	35 cycles of: • Denaturation, 94°C for 30 sec. • Annealing, 52-68°C for 45 sec. • Extension, 72°C for 1.15 min.	1 cycle of: • 72°C for 10 min.

2. The following components were added to the microcentrifuge tubes placed on ice. Reaction cocktails was made when multiple reactions were being assembled.

Components	Volume /25 µl	Final concentration
• 2X Reaction Mix	12.5 µl	1X
•Template RNA (0.125 µg/µl)	4.0 µl	500 ng or 0.5 µg
• Sense primer (~ 10 µM)	0.5 µl	0.2 µM
• Anti-sense primer (~ 10 µM)	0.5 µl	0.2 µM
• Magnesium sulfate (50 mM)	0.4µl	2m M
• RT Platinum ® Taq Mix	0.5 µl	-
• Autoclaved DEPC treated water	6.6 µl	-

3. It was mixed gently and made sure that all the component were at the bottom of the amplification tube.
4. The mixture was centrifuged briefly.
5. Samples were loaded in the pre-heated (depending on the cDNA step temp.) machine and appropriate amplification program started immediately.
6. The amplification products were analyzed by agarose gel electrophoresis.
7. Observed picture was documented.

Appendix D

Searched internet websites:

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

http://www.iris.irri.org

http://www.undp.org/seed/unso/pub-htm/dryland-population.pdf

http://www.unfpa.org/swp/2001

Appendix E

E1. Chemicals used and their origin

1.	Ammonium per sulfate	-	Sigma, USA
2.	Binder	-	Sigma, USA
3.	Chloroform	-	Mark, Germany
4.	CTAB	-	Sigma, USA
5.	DEPC	-	Sigma, USA
6.	DNA ladders	-	Invitrogen, USA
7.	DNaseI	-	Invitrogen, USA
8.	dNTPs	-	Invitrogen, USA
9.	DTT	-	Sigma, USA
10.	EDTA	-	Sigma, USA
11.	Ethanol	-	Mark, Germany
12.	Formaldehyde	-	Sigma, USA
13.	Glycerol	-	Invitrogen, USA
14.	IPTG	-	Sigma, USA
15.	Isopropanol	-	Mark, Germany
16.	$MnCl_2$	-	Invitrogen, USA
17.	Phenol	-	Mark, Germany
18.	PMSF	-	Sigma, USA
19.	Restriction enzymes and buffers	-	Invitrogen, USA
20.	RNase	-	Sigma, USA
21.	Sigma coat	-	Sigma, USA
22.	Sodium chloride	-	Sigma, USA
23.	Superscript™ One-step RT-PCR with platinum® Taq	-	Invitrogen™ ,USA
24.	TEMED	-	Sigma, USA
25.	Tris. HCl	-	Invitrogen, USA
26.	TRIZOL® Reagent	-	GIBCO BRL, USA
27.	Typing grade agarose	-	GIBCO BRL, USA
28.	Urea	-	Phytotechnology, USA

E2. Instruments used and their origin

1.	Autoclave machine	-	EYELA, TOKYO RIKAKINI CO. LTD., JAPAN (Model # MAC-501)
2.	Bio-documentation system	-	Kodak, USA
3.	Centrifuge machine	-	Eppendorf, Germany (Model # 5415 D)
4.	Electronic balance	-	Sartorius, Germany
5.	Electrophoresis apparatus	-	C.B.S. SCIENTIFIC CO. USA (Model # MGV-202-33)
6.	Electrophoresis apparatus	-	HYBAID, USA
7.	-30°C Freezer	-	SANYO, England
8.	-80°C Freezer	-	Heraeus, England
9.	Horizontal shaker	-	GFL, Germany (Model # 3018)
10.	Ice machine	-	SCOTSMAN, Germany
11.	Incubator shaker	-	New Brunswick Scientific, USA
12.	Laminar air flow cabinet	-	ESCO MICRO PTE LTD., Singapore
13.	Magnetic strirrer	-	SIGMA, USA
14.	Micropipettes	-	Eppendorf, Germany
15.	PCR machine/icycler	-	BIO-RAD, USA
16.	PCR machine/Mastercycler gradient	-	Eppendorf, Germany
17.	P^H meter	-	CONSORT (Model # C831), Germany
18.	Power pac	-	E.C. Apparatus Corporation (Model # EC 3000 P), USA
19.	Power pac	-	BIO RAD (Model # 1000/500), USA
20.	Sequencing gel apparatus	-	ESCO MICRO PTE LTD., Singapore
21.	SORVALL$^{(R)}$ Centrifuge	-	RC 26 PLUS, Germany
22.	Spectrophotometer	-	UV-160A, Shimadzu, Japan
23.	UV-transilluminator	-	Kodak, USA
24.	Vortex machine	-	Gallenkamp Spinmix ^{TM}, England
25.	Water bath shaker	-	Precision Scientific, USA
26.	Water distillation plant	-	Fisons Scientific Apparatus Ltd., England