# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Rice seeds

Six salt tolerance rice cultivars and six salt sensitive rice cultivars were used for the experiment. Six salt tolerant rice cultivars were kindly provided by Pathum Thani Rice Research Center : Gow Ruang 88,Khao Dawk Mali105,Khao Tah Haeng 17,Leuang Pratew 123, Look Daeng Pattani and Pokkali. Among the six salt sensitive cultivars, two were kindly provided by Pathum Thani Rice Research center : IR 28 and Muey Nawng 62 M and four were kindly provided by Phattalung Rice Research Center, which were: Foi Tawng, Leb Nok Pattani, Nahng Pa-yah 132 and Yah Yaw.

## 2.2 Rice genomic DNA extraction

DNA was extracted and purified from leaves according to the method of Saghai *et al.* (1984). One gram of leaf was ground in liquid nitrogen with mortar and pestle extracted with 7.5 ml of preheat 60$^{o}$C CTAB buffer and swirled regularly for 30 min. The mixture was added with 5 ml of chloroform, mixed gently and centrifuged at 3000 rpm for 5 min to separate phases. The aqueous phase was removed, added with 5 ml of cold isopropanol and mixed gently to precipitate the nucleic acids. Crude DNA was collected by centrifugation at 3000 rpm for 5 min. The pellet was washed in 70 % ethanol, dissolved in 0.7 ml of TE, added 20 µl of RNase (10 mg/ml, preboiled) and incubated for 30 min at 37$^{o}$c. The dissolved DNA was extracted twice with 500ul of phenol-chloroform (1:1), added 50 µl of 3 M Na-acetate (pH 5) and ethanol. DNA was recovered by centrifugation and redissolved in 0.3 ml TE.

## 2.3 Enzyme

AmpliTaq DNA polymerase (Perkin-elmer Cetus, Norwalk, Connecticus.)

## 2.4 DNA primers

Oligonucleotide primers used in this research were designed based on RAPD markers previously reported by the Rice Genome Research Project for molecular markers in *japonica* and *indica* rice (Monna *et al* .1994) were purchased from Operon Technologies (Alameda,Calif).

Primer sequences were shown in Table 2.1

Table 2.1 Arbitrary 10-mer primers used in RAPD analysis.

| Primer | Sequence | Primer | Sequence |
|---|---|---|---|
| X1 | CAGGCCCTTC | X2 | CAGCACCCAC |
| X3 | TTCCCCCGCT | X4 | GGACCCAACC |
| X5 | ACCCGGTCAC | X6 | CGTCTGCCCG |
| X7 | TGACCCCTCC | X8 | GCTCCCCCAC |
| X9 | ACGGCCGACC | X10 | GCCCGACGCG |
| C1 | GTCTGACGGT | C2 | CAGCTCAAGT |
| C3 | CGATCGAGGA | C4 | GCAGAGCATC |
| C5 | AAGCAGCAAG | C6 | TCTTCGAGGA |
| C7 | AGCACTTCGG | C8 | CACCGTTCTG |
| C9 | ACTCCGCAGT | C10 | GTCCTCTGAA |

## 2.5 Spectrophotometric measuring of DNA concentration

DNA concentration is estimated by measuring the Absorbance (A) at 260 nm. Absorbance (A) of 1.0 corresponds to 50 μg double-stranded DNA/ml. DNA sample concentration is therefore estimated in μg/ml by $A_{260}$ X dilution factor X 50. An estimation of the purity of sample can be obtained by calculation the ratio of the absorbance at 260 and 280nm. For a pure preparation of DNA, $A_{260}/_{280}$ should be $\geq$1.8 (Kirby, 1992).

## 2.6 Random amplified polymorphic DNA (RAPD) analysis

RAPD-PCR was performed in a reaction volume of 12.5 μl containing: the reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, pH 8.3),100μM each dNTP,0.2 μM oligonucleotide primer,1.5 unit *Taq* DNA polymerase and100 ng of template DNA. Following an initial incubation at 94 $^{o}$C, PCR was carried out for 10 cycles at 94 $^{o}$C for 30 sec, 37 $^{o}$C for 30 sec, and 72 $^{o}$C for 30 sec followed by 25 cycles at 94 $^{o}$C for 30 sec, 40$^{o}$C for 30 sec and 72$^{o}$C for 60 sec. A portion of DNA from the amplification reactions was electrophoresed in 0.5XTBE buffer at 100 V for 60 min in a 1.5% agarose gel containing ethidium bromide, and visualised by UV transillumination (Thomson and Henry, 1993).

## 2.7 Agarose gel electrophoresis

DNA was analyzed by convection gel electrophoresis. Agarose was mixed with Tris-Borate-EDTA (TBE) buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) to make the concentration of 1.6% (W/V) and 0.7%(W/V) for detection of amplification products and quality of extracted genomic DNA respectively. The calculated amount of agarose was dissolved in 1XTBE buffer by heating until complete solubilization. The solution was allowed to cool at room temperature and poured into a chamber set with a comb. After the gel hardened, the comb was carefully withdraw and the seal was removed from the ends of the plateform. Sufficient 1XTBE buffer was added to cover the gel for approximately 0.5 cm. An appropriate amount of RAPD-

amplified DNA samples or extracted genomic DNA was mixed with 1/4 volume of the gel-loading dye (0.25 % bromphenol blue, 0.25 xylene cyanol FF and 15% ficoll) and loaded into the well. The low molecular weight 100 bp DNA ladder and Lambda *Hind* III fragments were used as standard DNA markers. The electrophoresis was carried out in 1XTBE buffer from cathode to anode at 100 volts until the bromphenol blue marker dye migrated almost out of the gel. After finishing, the gel was stained in 2.5 μg/ml ethidium bromide (EtBr) solution for 5 min and destained (to remove excessive EtBr) by submerged in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized as fluorescent bands under UV transilluminator and photographed through a red filter using Kodax tri-X-pan 400 film. The exposure time was usually about 10-15 seconds.

## 2.8 Statistical procedures

The photographs of RAPD patterns were evaluated directly by visual measure with the help of a transparent ruler. Only unambiguous fragments that were 200 bp-2Kbp and bands that could be accurately scored throughout all lanes were chosen to score. Standard DNA markers can be used to assign the size of each RAPD fragment, and were marked for the accurate comparison of fragments among lane, derived from the same or different gels. Each RAPD fragment was estimated as a molecular length and recorded in a binary matrix for each individual as present (1) or absent (0) of a given band. The percentages of polymorphic bands and monomorphic bands were evaluated. The RAPD patterns of individuals were compared within and between the rice cultivars.

### 2.8.1 Similarity index

The index of similarity between individuals was calculated using the formula: $S_{xy} = 2n_{xy}/n_x+n_y$ where $n_{xy}$ is the number of fragments shared by individuals x and y, and $n_x$ and $n_y$ are the number of fragments scored for each individual (Lynch, 1990). Within population similarity (S) is calculated as the average of $S_{xy}$across all possible

comparisons between individuals within a population. Between population similarity, corrected for within population similarity, is;$S_{ij}$=1+$S'_{ij}$-0.5 ($S_i$+$S_j$),where $S_i$ and $S_j$ are the values of S for population i and j, respectively, and $S'_{ij}$ is the average similarity between randomly paired individuals from populations i and j(Lynch, 1990)

2.8.2 Genetic distance

$S'_{ij}$ is also converted to a measure of genetic distance ($D_{ij}$) using the equation :$D_{ij}$ =-ln[$S'_{ij}$ /$\sqrt{(S_iS_j)}$],(Lynch,1991).

2.8.3 Dendrograms

The distance values were subjected to cluster analysis using the unweighted pair-group method, arithmetic average (UPGMA) of Phylip version 3.57c.

2.8.4 Chi-square analysis

A RAPD pattern is referred to as genotype which is generated from RAPD. Chi-square($\chi^2$) test, a Monte Carlo simulation, was also performed on genotypes to ascertain the difference among samples for the number and size of RAPD fragments.

2.9 Salt tolerability of rice cultivars at germination stage of growth

Twelve rice cultivars were tested for salt tolerance at the germination stage under laboratory conditions (Bong *et al.*, 1996). Thirty five seeds of each rice cultivars were germinated in a 10-cm diameter petridish containing 10 ml of varied concentration of NaCl solution and kept at room temperature. The NaCl solutions were varied into 5 levels of salinity which were 0,3,6,8,10 and 12 dS/m (0, 0.18, 0.36, 0.48, 0.60 and 0.72% NaCl). The germination rate was determined after 7 days based on the emergence of the radicle and calculated as follows:

Percent germination (%) under salt stress =[(germination rate in NaCl solution)/ (germination rate in control)] X100.

2.10 Salt tolerability of rice cultivars at vegetative phase of growth

The experiment was conducted under greenhouse conditions.17-day old seedlings of each rice variety were transplanted into a plastic pot which contained 6 liters of mixture of 1:3 clay and sand(v/v) for 3 seedlings per pot. Seedling of each variety were treated with the modified Limpinuntana's nutrient solution (Limpinuntana, 1978) which were adjusted with NaCl for electrical conductivity for 8 dS/m and identical standard Limpinuntana's nutrient solution as control (salt not-treated). Level of salinity in each pot was monitored by measuring electrical conductivity every week and adjusted with NaCl solution to 8 dS/m. Phenology of the rice plants under control and salt stress condition in terms of plant height, tillering as well as leaf damage symptom were weekly recorded through the vegetative phase of growth. Completely Randomized Design with 7 replications were arranged in this experiment.

2.11 Salt tolerance of rice cultivars at reproductive phase

To test the salt tolerance at the reproductive phase, 100 seeds of each cultivar were placed in a pot (15x20 cm) containing soil and water. After 17 days, 3 seedlings of each cultivar were transfered to a pot (22x26 cm) filled with soil and sand. Limpinuntana's nutrient solution was incorporated into the pots. At the end of the vegetative phase, the pots were added with NaCl to reach electrical conductivity of 8 dS/m or Limpinuntana's nutrient solution as control.

2.12 Chemicals

2.12.1 Absolute ethanol (Merck,Germany.)

2.12.2 Agarose gel type 1-A low (Sigma Chemical Co., U.S.A.)

2.12.3 Ammonium acetate (Merck,Germany.)

2.12.4 Boric acid (Merck,Germany.)

2.12.5 Chloroform (Merck,germany.)

2.12.6 Hexadecyltrimethylammonium bromide (CTAB)

2.12.7 100 mM dATP,dCTP,dGTP,dTTP

2.12.8 Ethylene diaminetetraacetic acid disodium salt dihydrate (Fluka Chemika-Bio Chemika,Switzerland.)

2.12.9 Isoamyl alcohol (Merck,Germany.)

2.12.10 25 mM $MgCl_2$ (Perkin-elmer Cetus, Norwalk,Connecticus.)

2.12.11 10XPCR buffer.,10 mM Tris-HCl, pH 8.3, 50 mM KCl (Perkin-elmer Cetus,Norwalk,Connecticus.)

2.12.12 Phenol crystal (Fluka Chemika-Bio Chemika,Switzerland.)

2.12.13 Potassium acetate (Merck, Germany.)

2.12.14 Sodium chloride (Merck,Germany.)

2.12.15 Sodium dodecyl sulfate (Sigma Chemical Co.,U.S.A.)

2.12.16 Sucrose (Sigma Chemical Co.,U.S.A.)

2.12.17 Tris(hydroxy methyl)-amino methane (Fluka Chemical-Bio Chemika, Switzerland.)

2.13 Limpinuntana's nutrient solution (Limpinuntana, 1978)

| | |
|---|---|
| Solution A (30.333 g $KNO_3$ and 47.230 g $Ca(NO_3)_2.4H_2O$ in 1 liter $H_2O$) | 50 ml |
| Solution B (11.502 g $NH_4H_2PO_4$, 12.324g $MgSO_4.7H_2O$ and16.577g NaCl in 1 liter $H_2O$) | 50 ml |
| Solution C (7.887g Fe-Na EDTA in 1 liter $H_2O$) | 50 ml |
| Solution D (0.4323 g $MnCl_2.4H_2O$, 0.342g $H_3BO_3$, 0.0075g $Na_2MoO_4.2H_2O$, 0.0264g $ZnSO_4.7H_2O$ and 0.0117g $CuSO_4.5H_2O$ in 1 liter $H_2O$) | 50 ml |