

CHAPTER IV

DISCUSSION

4.1 Genetic variation

In this research, RAPD was used to detect genetic variation in 10 Thai rice cultivars: Khao Dawk Mali 105, Khao Tah Haeng 17, Leuang Pratew123, Look Daeng Pattani, Gow Ruang 88, Muey Nawng 62 M, Nahng Pa-yah 132, Yah Yaw, Foi Tawng and Leb Nok Pattani, and 2 exotic rice cultivars: Pokkali and IR 28. In the screening experiment, 20 random primers of 10 nucleotides sequence were used to amplify DNA segments from the genomic DNA of 6 rice cultivars. Five primers: X6, X8, X9, X10 and C1 out of 20 primers tested showed polymorphism that can be used as RAPD-DNA fingerprints for Pokkali, Khao Dawk Mali 105, Khao Tah Haeng 17, Leuang Pratew123, Look Daeng Pattani and IR 28. The amplification products were obtained in all 6 cultivars generating a total of 131 amplification products, which ranged in size between 200-1,517 bp (Figure 3.2,3.3 and 3.4). The most significant results from pair-wise similarity coefficient for 6 cultivars using 5 random primers show 2 groups, containing 3 salt tolerant cultivars Leuang Pratew123, Pokkali, and Khao Dawk Mali 105 in the same group. Khao Tah Haeng 17 and Look Daeng Pattani are more closely related to IR 28 than Khao Dawk Mali 105 (Fig.3.5). From RAPD analysis Pokkali is also closer to Leuang Pratew123 than other cultivars. Base on RAPD analysis using 5 random primers all 6 cultivars can be identified from each other easily, so RAPD analysis could be useful for rice cultivar identification. The polymorphism DNA bands can be selected and designed for the construction of the DNA-fingerprints by computerized technique. Using the 1 and 0, which indicate the presence and absence of a specific band, Weng *et al*, 2000 also reported that hybrid rice cultivars in China and their parents were identified by combining RAPD, AFLP and SSLP. RAPD markers can also be converted to sequence tagged sites (STSs) and STS specific primers by placing them onto the available map of RFLP markers established by the Rice Genome Research Project (Monna *et al*, 1994) and Cornell University(Tanksly *et al*, 1992). Among the 5 random

primers, primer 9 has the highest potential to distinguish between salt tolerant and salt sensitive cultivars. Amplification products were therefore generated with primer X9 in 12 cultivars including 5 salt susceptible Thai cultivars. Total of 16 amplification products, which range in size between 200-1517 bp were generated by random primer X9 (Fig.3.6). The most significant results from pair-wise similarity coefficient for 12 cultivars show that all the 10 Thai rice cultivars are closely related comparing to the 2 exotic cultivars: Pokkali and IR 28 (Fig.4.1). Since RAPD analysis is based on random amplification of different templates on genomic DNA and the GC content in the 10 nucleotide may provide variable polymorphism due to variation in annealing temperature with some primers between different genotypes. Analysis of amplification products provides assessments of genetic distances, seed purity, resolution of uncertainties in parentage and can be a precise method for legal protection of improved cultivars (Tancred *et al.*, 1994). PCR markers are also useful for genetic diagnostics and molecular taxonomy studies (William *et al.*, 1993). Only very small quantities of plant material are required for this type of analysis. Variation of this technique have been applied to analyze intra-and inter-specific polymorphism (Kolchinsky *et al.*, 1991). In this research RAPD analysis by 5 random primers can identify individual variety but not able to distinguish geographical distribution, because the 2 group obtained were not separated by regional origin. Ko *et al.*, 1994 used RAPD to study the genetic relationships among 37 rice cultivars by using the polymerase chain reaction (PCR) with 27 arbitrary oligonucleotide primers. There were 144 amplification products, of which 67% were polymorphism and 7 selected primers gave sufficient polymorphism to identify individual cultivar. RAPD polymorphism patterns confirmed that commercial Australian and USA lines and their relatives were very closely related with similarity indices of 88-97%. Three cultivars originating from more distant geographical centers were easily distinguished, producing variety-specific amplification profiles and expressing a lower similarity index of 80% to all other cultivars tested. By comparing 37 rice cultivars analyzed by Ko *et al.*, 1994 and 12 cultivars used in this research, it is obvious that wider genetic variation exists among Thai rice (similarity coefficient in the range of 0.00-0.93), because rice were cultivated in Thailand for more than 7,000 years (Chitrakon, 2001). Yu and Nguyen, 1993 analyzed genetic variation in 13 rice cultivars

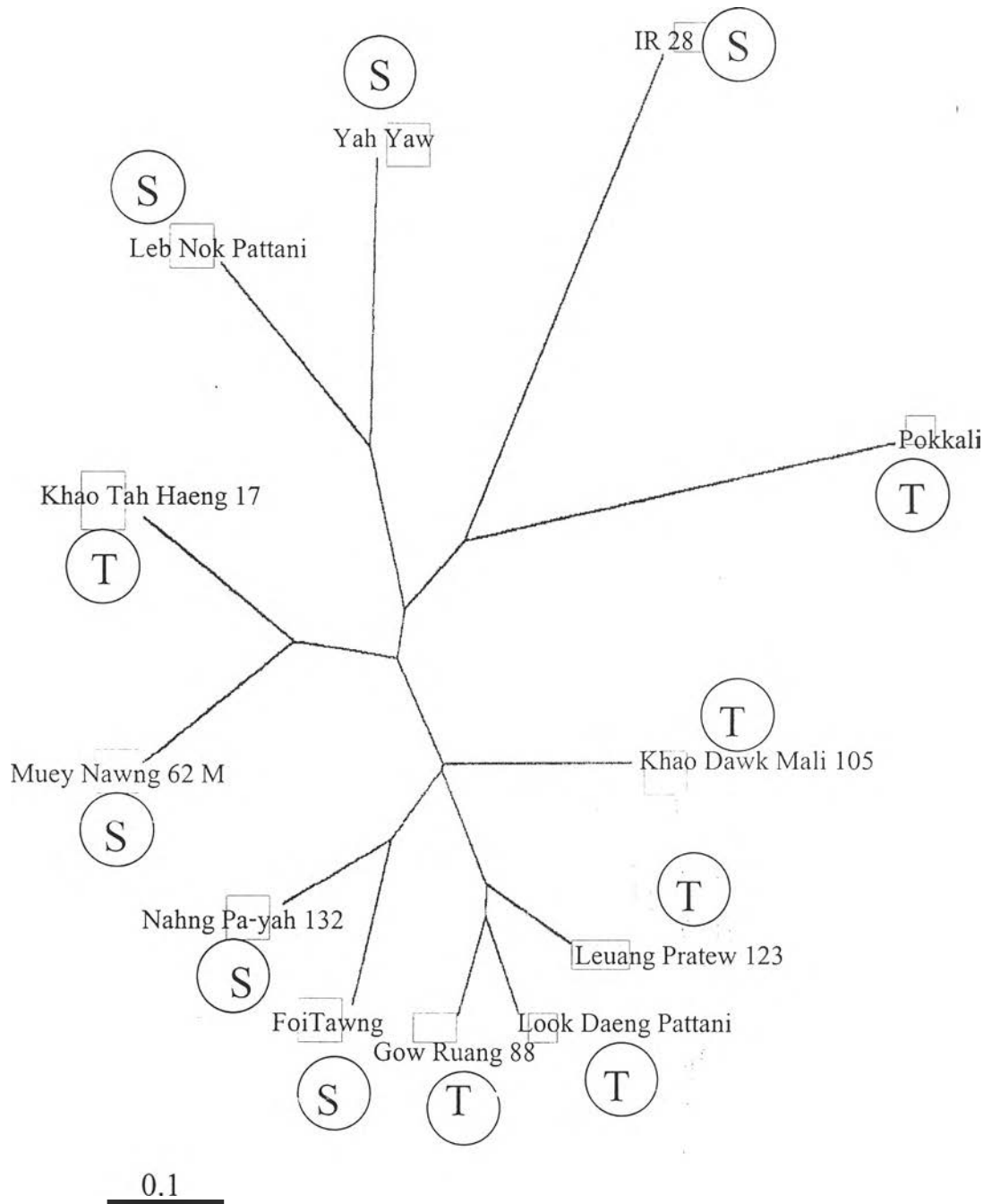


Fig 4.1 Fan-dendrogram showing higher genetic similarity among local rice cultivars comparing to exotic cultivars



in the Philippines, in which 7 cultivars are *japonica* subspecies and 6 cultivars are *indica* subspecies that can be divided into nine upland and four lowland rice cultivars by RAPD method using 42 random primers. They reported 260 PCR products of which 208 PCR products (80%) were polymorphic. All 42 primers used in the experiment were amplified and typically generated one-to-four major bands. Only two primers showed no polymorphisms. In general, a higher level of polymorphism was found between *japonica* and *indica* subspecies while fewer polymorphisms were found between upland and lowland cultivars within the *indica* subspecies. A dendrogram that showed the genetic distances of 13 rice cultivars was constructed based on their DNA polymorphisms in the similarity coefficient range of 0.2-0.8. Noticeably, the genetic variation (similarity coefficient in the range of 0.17-0.93) among 10 local cultivars in Thailand are more diverse than 13 *indica* and *japonica* cultivars collected at IRRI (5 Philippines, 2 India, 2 Ivory Coast, each from Brazil, New Guinea, Nigeria and Japan).

Beside RAPD, progress has been made in the study of genetic index by using simple sequence length polymorphism (SSLP) markers which are effectively assayed by PCR. Wannawichitra, 2001 used SSLP to study the genetic relationships among 48 aromatic, local rice cultivars by using PCR with 64 SSLP markers. Rice could be distinguished into main 3 groups: (1) local Thai rices and the hybrid RD groups, (2) IR groups and (3) exotic groups. A dendrogram showed that the genetic distance of 48 rice cultivars constructed based on their DNA polymorphisms were in the similarity coefficient range of 0.09-0.90. By comparing 48 rice cultivars analyzed by Wannawichitra, 2001 and this research, the results indicated that 12 cultivars used in this research have approximately the same genetic variation as evident by similarity coefficient range of 0.00-0.93, and confirmed very wide genetic variation among Thai local cultivars, and Asian lowland rice of *indica* subspecies.

4.2 Correlation between genetic variation and salt tolerant /sensitive phenological parameters

Among the 4 local cultivars: Gow Ruang 88, Look Daeng Pattani, Leuang Pratew 123 and Khao Dawk Mali 105, which are genetically similar compared by

similarity coefficient equal or higher than 0.80 (Figure 4.1 and Table 3.7), the degree of saline vulnerability observed in the vegetative phase namely; damaged leaf and plant height are classified in the same group by statistical analysis (Table 3.9). So the results from RAPD analysis in these 4 cultivars showed good correlation with these two phenological parameters pertaining to salt tolerance in the vegetative phase. There is no clear correlation between genetic similarity coefficient and phenological index when the genetic similarity coefficients were lower than 0.80 (Table 3.7). In the germination stage and reproductive phase, there was no significant correlation observed between genetic index and phenological indices.

Numerous rice genes are believed to mediate physiological process in response to salt stress. The hormone abscisic acid (ABA) was found to increase in tissues subjected to stress such as salt, drought or desiccation or cold. Mundy and Chua (1988) have reported that an ABA-responsive rice gene, *rab-16*, that is induced by ABA and osmotic stress in vegetative tissues. Four members of rice *rab* gene family were cloned and sequenced by Yamaguchi-Shinozaki *et al.* 1989, to be all transcriptional elements, and DNA-binding proteins with certain motif known as ABA-responsive elements. Claes *et al.* (1990) found eight proteins to be induced in the salt-sensitive rice plant subjected to salt stress. One of them, with the molecular weight 15 kDa were cloned, sequenced and designed for an oligo-nucleotide probe. Using the probe, a cDNA clone, *SaT*, was obtained and already mapped using AFLP markers on rice chromosome 1 (Gregorio 1997). *SaT* mRNA was found to accumulate rapidly in sheaths and roots of seedlings and mature rice plants upon treatment with 1% NaCl, 1% KCl, 20 μ M ABA and 5% polyethyleneglycol, but not observed in the leaf lamina. The organ-specific response of *SaT* is correlated with the pattern of Na^+ accumulation during salt stress. PCR based technology offers great potential to enhance DNA marker assisted selection (MAS), by using two primers pairs based on the sequence tagged site data (STSs) and RAPD markers linked to a specific phenological index.

More investigation is required to understand the underlying genes and processes in saline vulnerability. Different physiological processes may be responsible for salt stress in different developmental stages of rice, starting from seed germination to ripening.

4.3 Genetic variation and various criteria of salt tolerance

There are 8 criteria of salt tolerance used in this research to compare phenological parameters with RAPD analysis starting with percent germination, leaf damage plant height, tillers/hill, shoot/root ratio ((S/R), panicles/plant, fill grains/panicle and 100 grain weight.

4.3.1 Percent germination

In this research various salt levels 3,6,8,10 and 12dS/m were used. Table 3.8 showed that the salt tolerance at the germination stage was not inversely correlated with salt concentration. Noticeably, the results confirmed that Pokkali displayed the highest percent germination at all salinity level ranging from 0-12 dS/m and IR 28 displayed the lowest percent germination comparing with all cultivars. Among 5 local salt tolerant cultivars, Leuang Pratew 123 is the best for salt tolerance and Nahng Pa-yah 132 is the most susceptible cultivar. None of the Thai cultivar is better than Pokkali. The results is similar to that reported by *Bong et al.*, 1996, in which 13 local cultivars in Vietnam were compared with Pokkali and IR 28 using only one salt concentration 1.5% NaCl (25 dS/m). None of the Vietnam rice cultivar is more salt tolerant than Pokkali. However there is no correlation between RAPD markers and percent germination except between Pokkali and IR 28 pair (similarity coefficient =0 and % germination of Pokkali=91.43, IR 28 =0 at 12 dS/m).

4.3.2 Salt tolerance criteria in the vegetative phase

The survival scoring of plants based on visible symptoms in the vegetative phase is a reliable and simple indicator of salinity tolerance and frequently used as a screening criterion (*Yeo et al.*, 1990., *Narayanan and Sree*, 1991). In this study, 3 criteria: damaged leaf, plant height and tillering were evaluated at a salinity level of 8 dS/m comparing to control which contains no salt. Among these 3 criteria leaf damage is considered the best one based on statistical analysis. However any of these 3 criteria, 6 salt sensitive cultivars can be clearly distinguished from 6 salt tolerant cultivars at 3

weeks after treatment. (Figure 3.10, 3.12 and 3.13). At the 6th week, 6 salt sensitive cultivars died, whereas 6 salt tolerant cultivars still survived. By using these 3 criteria, 6 salt sensitive cultivars can be distinguished from 6 salt tolerant cultivars since 3 weeks after salt treatment. According leaf damage Khao Tah Haeng 17 is the most salt tolerance Thai cultivar and Muey Nawng 62 M is the most salt sensitive cultivar.

By comparing the polymorphic DNA between Khao Tah Haeng 17 and Muey Nawng 62 M in Figure 3.6, a faint band at 450 might be a putative marker for distinguishing salt tolerant Khao Tah Haeng 17 from salt sensitive Muey Nawng 62 M. Other salt tolerant cultivars also showed faint bands at this position. This RAPD marker may be enhanced by optimizing annealing temperature in the PCR condition together with the use of double primer RAPD(DP-RAPD), and combination with known RFLP markers in the vicinity of putative salt responsive genes.

Table 4.1 exhibits correlation between RAPD analysis of genetic variation and percent leaf damage the best criterion for salt susceptibility.

Table 4.1 Correlation between RAPD analysis of genetic variation and percent leaf damage in 12 rice

Cultivars Pair	Pair-Wise Similarity coefficient using Primer X9	% Leaf damage* at the 5 th week under salt stress (8 dS/m)	Salt tolerant/salt sensitive
GR/LDP	0.93	15.87 ^{ab} /15.30 ^{ab}	T/T
GR/LPT	0.88	15.87 ^{ab} /13.49 ^{ab}	T/T
LPT/KDML	0.80	13.49 ^{ab} /15.27 ^{ab}	T/T
LPT/LDP	0.80	13.49 ^{ab} /15.30 ^{ab}	T/T
LPT/KTH	0.75	13.49 ^{ab} /10.74 ^a	T/T
FT/NP	0.80	39.37 ^c /48.22 ^{cd}	S/S
YY/NP	0.75	43.33 ^{cd} /48.22 ^{cd}	S/S
FT/LNP	0.75	39.37 ^c /43.37 ^{cd}	S/S
MN/KTH	0.71	49.43 ^d /10.74 ^a	S/T
MN /NP	0.57	49.43 ^d /48.22 ^{cd}	S/S
YY/POK	0.57	43.33 ^d /21.87 ^b	S/T
KDML/POK	0.55	15.27 ^{ab} /21.87 ^b	T/T
IR /LDP	0.36	49.11 ^d /15.30 ^{ab}	S/T
IR /MN	0.33	49.11 ^d /49.43 ^d	S/S

* Means followed by a common letter are not significantly different at 5 % level by DMRT.

POK = Pokkali , KDML = Khoa Dawk Mali 105, KTH = Khao Tah Haeng 17
LPT = Leuang Pratew 123 LDP = Look Daeng Pattani GR = Gow Ruang 88
IR = IR 28 MN = Muey Nawng 62 M NP = Nahng Pa-yah 132
YY = Yah Yaw FT = Foi Tawng LNP = Leb Nok Pattani

From analysis of variance by DMRT, percent leaf damage observed at the 5th week can obviously classify 6 salt tolerant cultivars (10.74-21.87% leaf damage) from 6 salt sensitive cultivars (39.37-49.43% leaf damage). In contrary the pair-wise similarity coefficient from RAPD analysis which is not specific can only show genetic similarity within the group of 5 local salt-tolerant cultivars(0.75-0.93) and within the group of 4 salt-sensitive cultivars(0.75-0.80). When the genetic similarity coefficient was below 0.75, the cultivars pair may be either T/T or T/S.

From archeological evidence and geographical distribution, non-glutinous rice separated from glutinous rice only about 500 years ago, and before the transplanting technology of wetland rice was established in the lowland central plain of Thailand. Khao Tah Haeng 17 is considered the oldest cultivar among all 10 Thai rices. It most likely that Khao Tah Haeng 17 is closely linked with Muey Nawng 62 M more than other cultivars. The results indicates that about 30% genetic distance is enough to explain for different salt tolerant phenology of Khao Tah Haeng 17 with respect to Muey Nawng 62 M.

Other lowland salt tolerant cultivars namely Khao Dawk Mali 105, Leuang Pratew 123 and Gow Ruang 88 originated in the central part of Thailand and adapted to wetland ecosystem. These 3 cultivars are closely associated both in terms of genetic variation and salt tolerance. Salt tolerant lines of Khao Dawk Mali 105 and Leuang Pratew 123 cultivars have been developed through tissue culture (Vajrabhaya *et al.*, 1989) and six salt tolerant lines of RD 23 which showed higher survival rate and accumulated proline and sugar higher than the ancestor line: RD 23 (Sungsuk, 1998). These salt tolerant lines which were somaclonal varied lines obtained from RD 23 should have very high similarity coefficient with the ancestor line : RD 23 but differ in salt tolerance by some genes involved with proline and sugar accumulation.

4.3.3 Salt tolerance criteria in reproductive phase

The 4 criteria used were : ratio of shoot to root dry weight, panicles/plant, fill grains/panicle and 100-grain weight. All these parameters were affected by salt stress to

a certain extent and depend on individual cultivar. There is no clear separation in yield component as to six-salt tolerant / sensitive cultivars.

The results indicated that genetic variation based on RAPD analysis by a single random primer can reflect genetic similarity among 10 local cultivars, and wider genetic distance of Thai rice from Pokkali, the exotic tall cultivar from Sri Lanka, and the semi-dwarf IR28 from the International Rice Research Institute. Within the 10 local cultivars, 4 salt-tolerant cultivars: Gow Ruang 88, Look Daeng Pattani, Leuang Pratew 123 and Khao Dowk Mali 105 are closely associated both by genetic index and phenological parameters in the vegetative phase especially the leaf damage. Khao Tah Haeng 17 which can be ranked the best salt tolerant Thai rice, and Muey Nawng 62 M which is placed as the least salt tolerant cultivar are closely related by this random primer.

The interaction between several salt responsive genes or genotype (G) and environment (E) may lead to the partition coefficient. The effect of geographical distribution can also be observed for the 3 pairs of salt sensitive cultivars: Foi Tawng and Nahng Pa-yah 132, Yah Yaw and Nahng Pa-yah 132, and the pair of Foi Tawng and Leb Nok Pattani.