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Genetic Diversity, Salinity Tolerance and Physiological Responses to NaCl of Six Rice (*Oryza sativa* L.) Cultivars

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Abstract: This study was designed to study genetic diversity and differences in pattern of physiological responses to salt stress among six rice cultivars differing in salt tolerance level. Six cultivars of rice were screened for salinity tolerance at seedling stage based on visual symptoms of salt injury index established at the International Rice Research Institute. The cultivar Pokkali was classified as tolerant, IR29 highly sensitive, three local Thai cultivars; Dang Dawk Kok, Luang Ta Moh and Supanburi 2 moderately tolerant and Khao Dawk Mali 105 sensitive. The genetic relationship between rice cultivars were analysed by using randomly amplified polymorphic DNA. Cluster analysis revealed that the three moderately tolerant Thai cultivars were closely related to one another and together with the tolerant Pokkali formed one group, the sensitive Khao Dawk Mali 105 and the highly sensitive IR29 were closely related to each other and placed in another group. The seedlings had been treated with NaCl at 6 and 12 dS m^{-1} for 7 days. Compared with the non-stressed plants, the magnitude of changes in some physiological parameters were different among different cultivars and were related to the level of salinity tolerance. The weight of shoots and roots and net photosynthesis rate showed high positive correlation with the level of salinity tolerance. The Na^+:K^+ ratio, proline content and electrolyte leakage of shoots were negatively correlated with the level of salinity tolerance. Cluster analysis of physiological responses revealed that salt responsiveness of Pokkali was distinct from all other cultivars. The pattern of responses of Khao Dawk Mali 105 to salinity was more similar to the moderately tolerant group than the highly sensitive IR29.

Key words: Salt tolerance, rice, RAPD, proline, electrolyte leakage

INTRODUCTION

Soil salinity is one of the most important abiotic stress factors adversely affecting crop production. Arid and semiarid lands worldwide suffer from an ever-increasing area of salt-affected land. In northeastern Thailand, 35% of the land area faces varying degrees of salinity problems from the accumulation of NaCl generated by the underground salt dome resulting in low crop productivity, especially rice which is considered to be moderately sensitive to salinity[1]. Growth and yield components of rice are severely affected by salinity[2]. In developing more salt-tolerant plants it is essential to understand what mechanisms make one plant more salt-tolerant than another. In rice, several authors[3-5] have shown that Na-K selectivity of plant roots functions to minimize the entry of Na^+ into plants and maintain effective K^+ uptake together with the mechanism of low salt transport to expanding leaves is very important mechanism directly correlated with salt tolerance. Osmotic adjustment by accumulation and compartmentation of inorganic ions and organic compatible solutes is another well-characterized mechanism relating to salt tolerance[5-7]. The effects of NaCl on chlorophyll content vary depending on the concentration of salt, duration of treatment and age of leaves. Rice plants treated with low salt concentration (<50 mM) for short duration usually have higher chlorophyll content than the non-stressed plants. With the increased duration of treatment or concentration of salts, chlorophyll content decreases, with that of salt-resistant cultivars being less affected than salt-sensitive cultivars[8-10].

The level of salt tolerance has also been related to lipid peroxidation and membrane damage with sensitive cultivars being suffered from enhanced free radical formation and lipid peroxidation leading to loss of membrane integrity, whereas tolerant cultivars were significantly less affected in these respects[11,12]. Several

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rice proteins which accumulated as a result of salinity treatment have been identified such as 15 kDa *salt* protein in leaf sheath[13], 26 and 27 kDa polypeptides in salt-adapted rice cells[14]. Recently three salt-induced proteins have been detected in rice by 2 dimensional polyacrylamide gel electrophoresis and identified using mass spectrometry in combination with bioinformatics database[15]. These proteins; ASR-1 like protein, ascorbate peroxidase and caffeoyl-CoA O-methyl transferase, were markedly up-regulated in tolerant cultivar Pokkali by salt stress but insignificantly changed in sensitive IR29.

Polymerase chain reaction-based DNA amplification has been used to study genetic diversity and relatedness of rice germplasm. Randomly amplified polymorphic DNA (RAPD) has been used to study 134 japonica cultivars[16] and 37 cultivars belonging to 5 different geographical origins[17] and classify and quantify genetic relatedness of rice cultivars based on their origins. Xie *et al.*[18] investigated the genetic diversity of three salinity tolerant and one sensitive rice cultivars and identified four primers which amplified specific fragments in all three tolerant cultivars but not in the susceptible cultivar. These markers if proven to be linked to salt tolerance gene could be potentially useful for salt tolerance breeding study program. This research provides a report on relationship between the level of salt tolerance with genetic diversity based on RAPD markers as well as the pattern of physiological responses to salinity of six rice cultivars representing four levels of salt tolerance.

MATERIALS AND METHODS

Plant materials: Seeds of rice (*Oryza sativa* L. cvs. Pokkali, Dang Dawk Kok, Supanburi 2, Luang Ta Moh, Khao Dawk Mali 105 and IR29) were kindly provided by Pathumthani Rice Research Institute, Thailand. Pokkali (PK) is a tall variety from India, well-known for its tolerance to salinity. IR29 is a modern rice variety from International Rice Research Institute (IRRI), highly susceptible to salinity. Pokkali and IR29 are often included in the experiments to analyse the genetics of salt tolerance[19]. Dang Dawk Kok (DDK), Supanburi 2 (SP2), Luang Ta Moh (LTM) and KDML105 are local Thai varieties not yet well-documented for salinity tolerance. The experiments were performed during August to October 2002, the plants were grown in greenhouse under natural light conditions.

Screening for salinity tolerance: A preliminary test for salt tolerance was carried out based on the system described by Gregorio *et al.*[20]. Seeds were surfaced sterilized with 5% sodium hypochlorite, imbibed for 48 h and sown on plastic grids placed above 4 L black plastic pots containing distilled water. Three replications with 15 seedlings were used. When seedlings were 5 days old, distilled water was replaced with nutrient solution[21]. After seedlings had been grown for 14 days, nutrient solution was replaced with salinized nutrient solution initially at EC = 6 dS m^{-1}. After 3 days the level of salinity was increased to 12 dS m^{-1} and maintained for 7 days. The pH of nutrient solution was maintained between 5.0-5.5 throughout the growth period. Salt stress symptoms were evaluated according to the standard evaluation system used at the International Rice Research Institute[20] with some modifications. A set of non-salinized controlled plants were also grown for comparison.

Physiological studies: Seeds were surface-sterilized, imbibed for 48 h and sown on plastic grids placed above 4 L black plastic pots containing distilled water. When seedlings were 5 days old, distilled water was replaced with nutrient solution[21]. When the plants were 14 days old, the nutrient solutions were replaced by the ones containing NaCl at the salinity level of 0, 6 or 12 dS m^{-1}. After 7 days in salinized solutions, plants were harvested for determination of fresh and dry weight, chlorophyll, proline, malondialdehyde (MDA), Na^+ and K^+ content, osmotic potential and electrolyte leakage (EL). Net photosynthesis rates of the second leaves from the top of the plants were measured between 900-1500 using Portable Photosynthesis system (Li-Cor 6400, USA). Photosynthesis measurement was performed on 5 plants for each treatment. After photosynthesis measurement the leaf was harvested for chlorophyll determination. Chlorophyll was extracted using 80% acetone, the absorbance of chlorophyll extract was recorded at 663 and 645 nm and chlorophyll content was calculated according to Arnon[22]. Free proline content of both shoots and roots was determined using acid-ninhydrin method based on Bates[23]. Lipid peroxidation was determined by measuring the amount of malondialdehyde using thiobarbituric acid method described by Stewart and Bewley[24]. For measurement of Na^+ and K^+ content, shoots and roots were dried at 70°C for 48 h, accurately weighed, digested with perchloric acid and nitric acid (1:2) and the volume of extracts were adjusted to 100 mL. The extracts were diluted 1:100 and Na^+ and K^+ content determined by atomic absorption spectrophotometer (Model GBC 932 AA). For osmotic potentials, plant materials were ground with small tissue grinder in microcentrifuge tubes, centrifuged at 13,000 x g for 15 min and the osmotic concentration of the supernatant was determined in an osmometer (automatic semi-microosmometer A0300 version 0291). The osmotic potential of the extract was

determined according to van't Hoff equation[25]. Electrolyte leakage test was determined by comparing the initial electrical conductivity (EC) of the medium bathing the fresh plant materials at 32°C for 2 h (EC1) with the final EC (EC2) measured after the plant materials had been killed by autoclaving at 121°C for 20 min to release all electrolytes, by using electrolyte leakage formula[11]. All physiological measurements were made in four replicates except the determination of Na^+ and K^+ content (3 replicates). Statistical analysis of physiological data was performed using one-way ANOVA and the difference between the mean values was compared at 5% significant level using Duncan's Multiple Range Test.

RAPD analysis: The genetic diversity among six rice cultivars differing in their salt tolerance was investigated using RAPD. The genomic DNA of 10-day-old seedlings of each cultivar was extracted following the method of Doyle and Doyle[26]. Fifty-nine decamer oligonucleotide primers (Operon Technologies) were used to prime genomic DNA. Each 25 μL PCR reaction contained 150 ng genomic DNA template, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.0 μM oligonucleotide primer, 1x PCR buffer and 1.25 U Taq DNA polymerase (Promega). PCR was initiated by a pre-denaturation step at 94°C for 4 min, then the reaction was subjected to 45 cycles of 94°C for 1 min, 36° for 1 min and 72°C for 2 min with a final extension step at 72°C for 4 min. Amplification products were resolved on 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV transilluminator. PCR products were scored as presence (1) or absence (0) of bands for each of the six cultivars with the 59 primers. Similarity between each pair of cultivars was calculated using Jaccard's similarity coefficient and cluster analysis was performed using the UPGMA method.

RESULTS

Screening for salinity tolerance at seedling stage: The standard evaluating score in rating the visual symptoms of salt toxicity established at IRRI[20] was used with modifications to discriminate the susceptible from the tolerant and moderately tolerant cultivars. Salinization started when seedlings were 14 days old and had 4-5 green healthy leaves. After 3 days in salinized solution at 6 dS m^{-1}, initial signs of salt stress were observed in the oldest leaves, which started to desiccate and roll inward, especially in the highly sensitive cultivar IR 29. When salinity level was increased to 12 dS m^{-1} signs of salt stress also appeared in the moderately tolerant cultivars. Three days after salt treatment at 12 dS m^{-1}, mature leaves of most cultivars started to wilt and those of IR29 have dried up. Two days later, most leaves of IR29 had died, only the youngest leaves of some plants remained green, the oldest leaves of most cultivars have dried but younger leaves remained green. Scoring was performed on day 7 after salinization at 12 dS m^{-1}, a total of 10 days of salinization, when four categories of tolerance can be visually distinguished (Table 1). PK scores 3 (tolerant) because seedlings looked almost normal with only the first oldest leaf wilted and rolled, younger leaves remained green and healthy. Most plants of IR29 have died, only the youngest leaf of some plants remained green and therefore was scored 9 (highly sensitive). DDK, SP2 and LTM exhibited growth retardation, most of the lower leaves rolled, some oldest leaves dried, only the two youngest leaves remained green and elongating and were scored 5 (moderately tolerant). Most leaves of KDML 105 have dried, most plants stopped growing and some plants were dying, therefore it was scored 7 (sensitive). The difference between KDML105 and the three moderately tolerant cultivars was clearly seen on 12th day after salinization, all KDML105 plants have died, some of the moderately tolerant plants have died and the rest still retained green younger leaves.

Effects of salinity on growth and physiology of six rice cultivars: The effects of moderate (6 dS m^{-1}) and high (12 dS m^{-1}) level of salinity on growth and some physiological characteristics of seedlings of six rice cultivars are shown in Fig. 1 and 2. Moderate salt treatment resulted in a slight increase in fresh weight of shoots in PK, small decrease in DDK, SP2 and IR29 and no effect in LTM and KDML105. At 12 dS m^{-1}, shoot fresh weight of PK was not different from that of the control, whereas that of moderately tolerant and sensitive cultivars decreased, with IR29 being the most significantly affected (data not shown). The effects of NaCl on dry weight of shoots followed similar pattern as that of fresh weight. Shoot dry weight of PK treated with moderate and high level of salinity showed 15.51 and 7.02% respectively increase from controlled plants (Fig. 1A). Compared with the controlled plants, IR29 plants treated with salinity level of 12 dS m^{-1} showed 36.36% reduction in shoot dry

Table 1: Salinity tolerance rating of six rice cultivars based on modified standard evaluation score of visual salt injury at seedling stage after 10 days in salinized solution[20]

Cultivar	Observation	Score	Tolerance
PK	Nearly normal growth, only leaf tips or lower leaves whitish and rolled.	3	Tolerant
DDK, SP2, LTM	Growth severely retarded, two basal leaves dried and rolled, 2-3 younger leaves are still elongating.	5	Moderately-tolerant
KDML105	Complete cessation of growth, most leaves dried, some plants dying.	7	Sensitive
IR29	Almost all plants dead or dying.	9	Highly sensitive

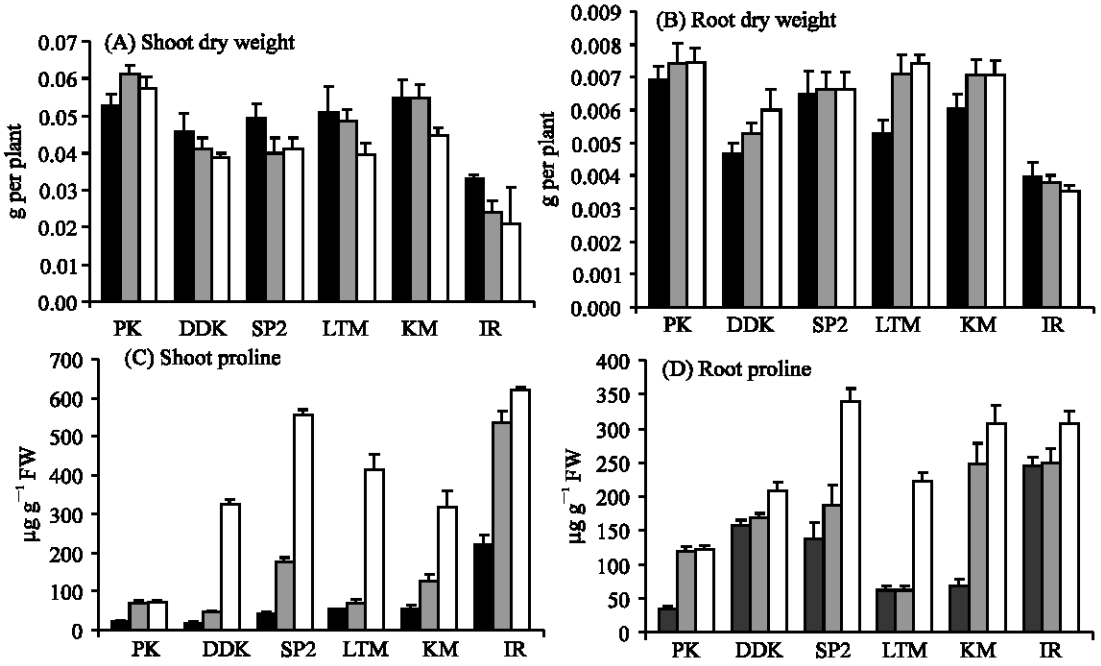

Fig. 1: Effects of NaCl on (A) dry weight of shoots (B) dry weight of roots (C) proline content in shoots (D) proline content in roots of seedlings of six rice cultivars 7 days after addition of NaCl at 0 (■), 6 (▣) and 12 (▫) dS m^{-1}. Values for dry weight and proline are means of 10 and 4 plants respectively. Bars indicate standard errors. (PK = Pokkali, DDK = Dang Dawk Kok, SP2 = Supanburi 2, LTM = Luang Ta Moh, KM = KDML 105, IR = IR29)

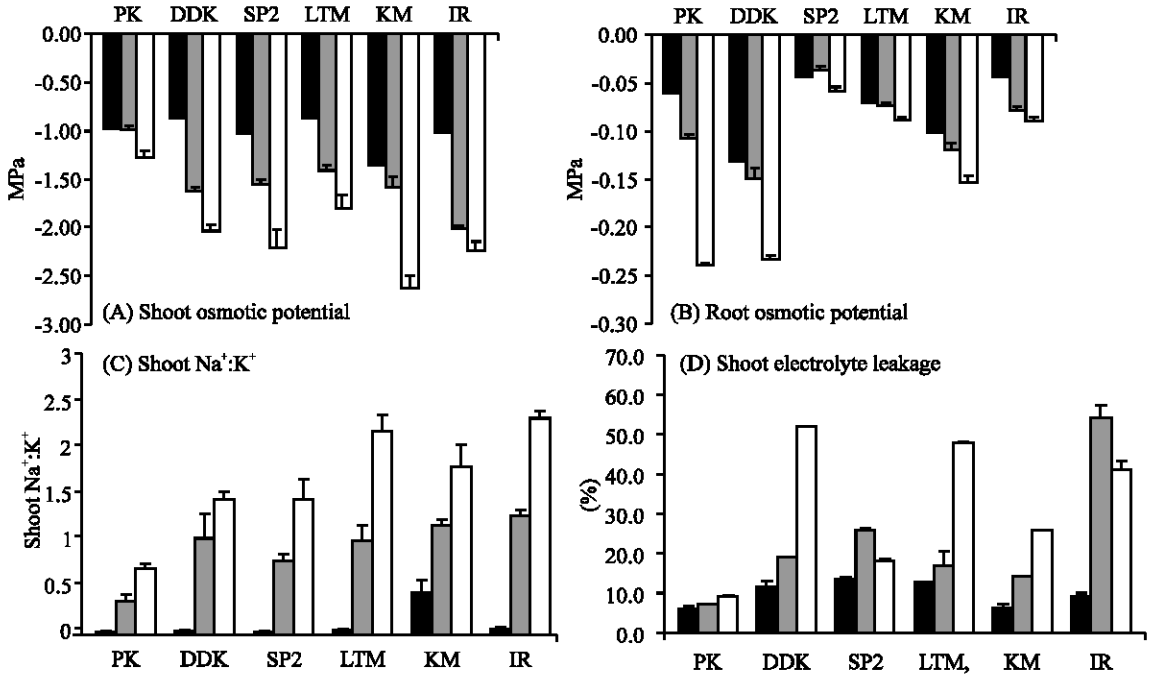

Fig. 2: Effects of NaCl on (A) osmotic potential of shoots (B) osmotic potential of roots (C) Na:K ratio in shoots (D) electrolyte leakage of seedlings of six rice cultivars 7 days after addition of NaCl at 0 (■), 6 (▣) and 12 (▫) dS m^{-1}. Values are means of 4 replicates. Bars indicate standard errors. (PK = Pokkali, DDK = Dang Dawk Kok, SP2 = Supanburi 2, LTM = Luang Ta Moh, KM = KDML 105, IR = IR29)

weight. Among the moderately tolerant cultivars, DDK suffered only 14.66% reduction in shoot dry weight followed by SP2 (16.68%) and LTM (22.45%). The sensitive KDML105 showed 18.02% reduction in dry weight from the controlled plants. The effect of NaCl on root growth was different from that of shoots, fresh weight of roots of NaCl-treated plants of all cultivars increased compared with the non-treated control plants (data not shown). The dry weight of roots of IR29 slightly decreased, whereas those of the other cultivars were either unaffected or slightly increased (Fig. 1B). Depression in shoot growth and stimulation of root growth resulted in lower shoot:Root ratio (on dry weight basis) in NaCl-treated plants of all cultivars except PK which showed a small increase in shoot:root ratio due to higher increase in shoot than root dry weight.

Shoots of tolerant and moderately tolerant cultivars under non-stressed condition accumulated relatively low amount of free proline, unlike the highly sensitive IR29 which contained high free proline content even without salt stress (Fig. 1C). In response to NaCl treatment, rice shoots accumulated higher amount of proline in higher salt concentration. The cultivar IR29 accumulated greatest amount of proline and PK the lowest. Among the moderately tolerant cultivars, SP2 accumulated the highest amount of proline when subjected to salinity at 12 dS m^{-1} followed by LTM and DDK. The pattern of response of KDML105 was more similar to the moderately tolerant group than IR29. Proline content in roots under non-stressed condition was also lowest in PK, highest in IR 29 and the other 4 cultivars displayed values in between (Fig. 1D). Root tissues also responded to NaCl by accumulating higher proline with higher salt concentration. At salinity level of 12 dS m^{-1} tolerant and moderately tolerant cultivars, with the exception of SP2, accumulated less proline than the two sensitive cultivars. The amount of proline in both shoots and roots tends to be negatively correlated to the level of salinity tolerance.

Sap extracted from shoots of all cultivars had similar values of osmotic potential ranging from -0.863 to -1.364 MPa under non-stressed condition (Fig. 2A). The osmotic potential of shoot sap became more negative with increasing salinity reaching -2.625 and -2.244 MPa for KDML105 and IR29 in high-salt treatment, 1.9 and 2.2 times as low as that in the control plants. In moderately tolerant cultivars, osmotic potentials of the shoots were also approximately twice as low compared with the controlled plants. The tolerant cultivar PK, on the other hand, showed only 29% decrease in shoot osmotic potential from the control. Osmotic potentials of root sap (Fig. 2B) under non-stressed condition were more variable ranging from -0.044 MPa to -0.132 MPa. The response of root tissues is somewhat opposite to that of the shoot. PK root osmotic potential under 12 dS m^{-1} salinity was 3.9 times lower than the control, whereas the extent of reduction in other cultivars ranged from 1.24 to 2.02 times lower than the controls. Less tolerant cultivars tend to have lower shoot osmotic potential than more tolerant ones, however, no exact relationship could be drawn between root osmotic potential and salt tolerance.

With salinity treatment the concentration of Na^+ significantly increased in all cultivars. The most sensitive IR29 accumulated the greatest, whereas the most tolerant PK the lowest amount of Na^+. Concentration of K^+, on the other hand, decreases in response to NaCl except in PK which showed a small insignificant increase. It appeared that concentration of Na^+ in shoots negatively, while K^+ positively correlated with the level of salt tolerance. The increase in Na^+ and decrease in K^+ resulted in the increase in Na^+ : K^+ ratio in response to NaCl and the ratio was negatively related to the level of salt tolerance (Fig. 2C).

Membrane integrity was monitored by means of electrolyte leakage (EL) from freshly harvested plant tissues. Under controlled conditions, electrolyte leakage from shoot tissues of different cultivars varied between 5.2% in PK to 13.7% in SP2 (Fig. 2D). With salinity treatment, membranes of most cultivars lost some integrity resulting in significant increase in EL, except for PK in which EL increased only slightly. The relationship between EL and salt tolerance was seen more clearly when comparing EL of the tissues treated with moderate level of salinity (6 dS m^{-1}). Membrane of the sensitive cultivar IR29 tended to be most severely affected by moderate level of salinity as shown by 5.8 times increase in EL from controlled plants, followed by KDML105 showing 2.19 times increase. EL of moderately tolerant and tolerant cultivars was less than twice the EL of the controlled plants. With higher level of salinity (12 dS m^{-1}), however, membranes of moderately tolerant (LTM and DDK) were damaged to the similar degree as those of IR29.

After 7 days of NaCl treatment, lowest leaves at the lower part of the NaCl-treated plants of most cultivars, except PK, have rolled and dried. There are 3-5 green leaves remaining in most cultivars, except IR29 in which only 2-3 green leaves remained. Chlorophyll content and net photosynthesis rate was measured in controlled and 12 dS m^{-1} treatment groups. The photosynthesis rate of the second leaf from the top which is the most actively growing leaf was measured and then the leaf was harvested for chlorophyll determination. Chlorophyll content in most cultivars did not differ from the controlled leaves except SP2 and IR29 in which the chlorophyll content significantly decreased (Fig. 3A). For most cultivars, net photosynthesis rate of the healthy second leaf of the plants treated with NaCl did not differ significantly from that of controlled plants (Fig. 3B). In case of IR29, leaves of both controlled and salt-treated plants exhibited negative net photosynthesis values indicating that the plants are senescing. In the high salt

treatment group, net photosynthesis rates of PK, DDK, LTM, KDML, SP2 and IR29 were 3.234, 2.502, 2.158, 1.775, 1.164 and -0.648 μmol $CO_2 m^{-2} s^{-1}$, respectively.

RAPD polymorphisms of six rice cultivars differing in salt tolerance: The genetic diversity of six rice cultivars was revealed by RAPD using 56 arbitrary decamers. Intra-variety uniformity was tested on five selected primers on 5 individual plants on each cultivar. All primers displayed high intra-variety consistency. Amplification using 56 arbitrary primers produced a total of 544 amplified bands which ranged in size between 120 and 2580 bp. Of these 544 bands, 396 (72.79%) were polymorphic among six rice cultivars. Most primers produced amplified products which showed polymorphisms among different cultivars (Fig. 4). Dendrogram showing genetic relationships between six rice cultivars resulted from UPGMA analysis of 396 polymorphic bands is presented in Fig. 5. Six rice

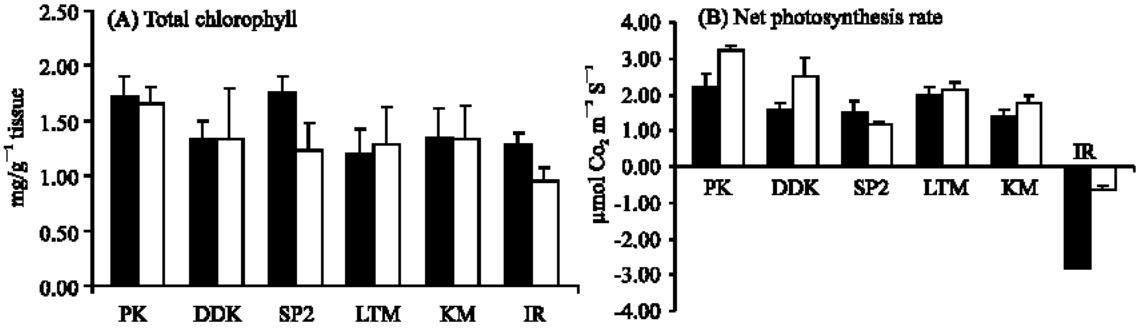

Fig. 3: Effects of NaCl on (A) total chlorophyll and (B) net photosynthesis rate of the second leaf from the top of seedlings on day 7 after addition of NaCl at 0 (▪) and 12 (▫) dS m^{-1}. Values are means of six plants and bars indicate standard errors. PK = Pokkali, DDK = Dang Dawk Kok, SP2 = Supanburi 2, LTM = Luang Ta Moh, KM = Khao Dawk Mali 105, IR = IR 29

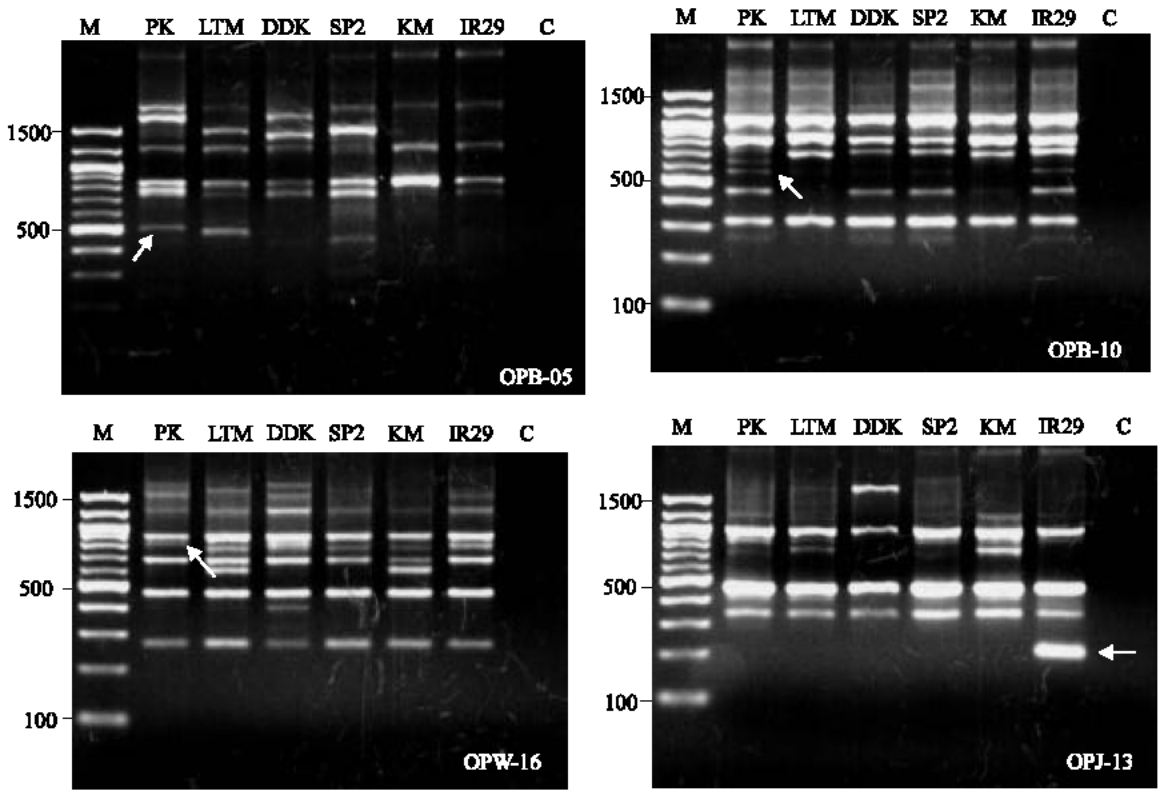

Fig. 4: RAPD profiles of 6 rice cultivars obtained with primers OPB-05, OPB-10, OPW-16 and OPJ-13. Lane M: molecular weight standards in base pairs; Lane C: negative control; PK: Pokkali; LTM: Luang Ta Moh; DDK: Dang Dawk Kok; SP2: Supanburi 2; KM; Khao Dawk Mali 105; IR: IR29.

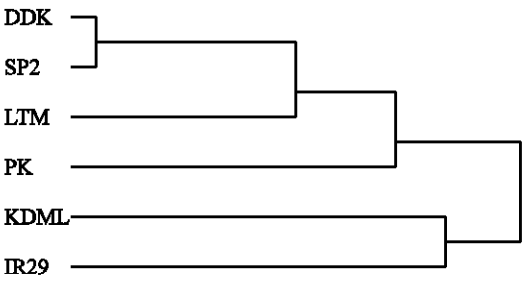

Fig. 5: Dendrogram based on cluster analysis of 6 rice cultivars with 396 PCR amplification fragments produced by 56 arbitrary RAPD primers

cultivars could be divided into two groups, KDML105 and IR 29 were clustered in one group. The second group contained PK, LTM, SP 2 and DDK. Within the second group, DDK and SP2 are closely related to each other and these two cultivars and LTM formed a sub-group, more closely related to one another than to PK.

DISCUSSION

Growth and physiological responses: After 7 days in salinized solutions at 6 and 12 dS m^{-1}, growth of plants of all cultivars, except PK, was retarded leading to the reduction in both fresh and dry weight of shoots. Lower leaves of sensitive cultivars usually rolled and some were desiccated, those of moderately tolerant cultivars started rolling and became slightly chlorotic whereas most leaves of PK appeared almost normal except for a slight chlorosis in the lowest leaves. The distinction between the tolerant PK and other cultivars is clearly evident in changes in shoot dry weight. Shoot dry matter of PK was slightly stimulated by both concentrations of NaCl whereas that of the other cultivars was retarded. The response of root growth to salinity was opposite to that of shoot growth in that fresh and dry weight of roots increased as the concentrations of NaCl increased. In all cultivars except IR29, both fresh and dry weight of roots were higher in salt-treated than the non-stressed control plants.

The parameter which showed the highest correlation with salt tolerance scores was root fresh weight (r = 0.934**), followed by shoot fresh weight (r = 0.840*), shoot dry weight (r = 0.834*) and percentage shoot dry weight compared with the controls (r = 0.824*). Root dry weight (r = 0.791) and percentage of shoot fresh weight compared with the controls (r = 0.789) also showed high, though insignificant correlation (Table 2). Percentage of both root fresh and dry weight compared with the controls showed low correlation with salt tolerance scores. The fact that root fresh weight, shoot fresh weight and shoot dry weight of plants growing in the 12 dS m^{-1} treatment had high correlation with salt tolerance scores was in fact due to the characteristics of each genotype, with PK being the tallest and most robust genotype, IR29 the shortest and Thai cultivars the intermediate type. The best growth parameter which should be used to correlate with the salt tolerance scores is, therefore, the percentage of shoot dry weight of plants growing in the 12 dS m^{-1} treatment compared with the controls. In the two extreme cases, the most tolerant PK (score = 3) showed+8.35% increase in shoot dry weight whereas the most sensitive IR 29 (score = 9) showed highest reduction (-36.36%). Among the three Thai cultivars rated moderately tolerant (score = 5), DDK performed best in terms of reduction in shoot dry weight (-14.66%), followed by SP2 (-16.68%) and LTM (-22.45%). KDML105 which was scored sensitive (score = 7) showed more similar value in shoot dry weight reduction (-18.02%) to the moderately tolerant group than the highly sensitive IR29.

Table 2: Correlation between salinity tolerance level with growth and physiological parameters

Growth / physiological parameter	Correlation
Shoot fresh weight at 12 dS m^{-1}	0.840*
Shoot fresh weight at 12 dS m^{-1} (% of control)	0.789ns
Root fresh weight at 12 dS m^{-1}	0.934**
Root fresh weight at 12 dS m^{-1} (% of control)	0.165ns
Shoot dry weight at 12 dS m^{-1}	0.834*
Shoot dry weight at 12 dS m^{-1} (% of control)	0.824*
Root dry weight at 12 dS m^{-1}	0.791ns
Root dry weight at 12 dS m^{-1} (% of control)	0.514ns
Shoot proline at 12 dS m^{-1}	-0.810ns
Root proline at 12 dS m^{-1}	-0.705ns
Na:K ratio at 12 dS m^{-1}	-0.803ns
Shoot osmotic potential at 12 dS m^{-1}	0.746ns
Root osmotic potential at 12 dS m^{-1}	-0.480ns
Chlorophyll at 12 dS m^{-1} (% control)	0.398ns
Net photosynthesis rate at 12 dS m^{-1}	0.887*
Electrolyte leakage at 6 dS m^{-1}	-0.819*

* correlation is significant at $p<0.05$; ** correlation is significant at $p<0.01$; ns = not significant

The observation that shoot growth was more retarded by salinity than root growth was similar to earlier report[5]. This differential response leads to the reduction in shoot:root ratio (on dry weight basis), ranging between 18.00% (SP2) to 44.48% (LTM) reduction, in moderately tolerant and sensitive cultivars. PK is the only cultivar which maintained similar value of shoot:root ratio under the 12 dS m^{-1}(7.697) compared with that in the non-stressed treatment (7.669). Therefore the ability to maintain high shoot:root ratio could be another useful indicator relating to salt tolerance.

The amount of proline in shoot and root tissues markedly increased in response to NaCl and the extent of accumulation was more pronounced in shoots. As shown in Table 2, the amount of proline in salt-treated tissues was negatively correlated with the level of salt tolerance

(r = -0.810 for shoots and r = -0.705 for roots). Proline has been widely considered to be a compatible solute that accumulates in plants in response to a wide variety of environmental stresses and confers stress tolerance by contributing to osmoregulation and protecting proteins and membranes in conditions of low water potential. Numerous investigations have been performed on a wide range of plant species to show that proline is the major component of the amino acid pools on plant tissue grown under salinity both in laboratory and field experiments. However the potential value of proline accumulation during stress has yet to be fully understood. Overproduction of proline in transgenic tobacco with P5CS (Δ-pyrroline-5-carboxylate synthethase) gene enhanced root biomass and flower development under salinity stress[27]. Transgenic rice transformed with P5CS gene increased biomass production under drought and salinity stress[28]. Free proline markedly increased in 5-day old rice seedlings exposed to salinity and the increase was more pronounced in susceptible than in tolerant cultivars[29]. Liu and Zhu[30] reported that the SOS1 (salt overly sensitive) mutant of *Arabidopsis thaliana* which was more than 20 times more sensitive than wild type to NaCl stress accumulated more proline and expressed P5CS transcripts to higher level than the wild type under salt stress. Negative relationship between proline and salt tolerance, however, have been reported. Lin and Kao[31] investigated the effects of NaCl on changes in proline level in rice roots and found that accumulation of proline promoted root growth inhibition caused by NaCl. Comparing the effects of trehalose and proline on growth and physiology of rice seedlings, Garcia *et al.*[32] found that proline had no effect or, in some cases, exacerbates the effect of NaCl on growth inhibition and chlorophyll loss whereas trehalose offered much better protection. Moreover proline and trehalose displayed an opposite effect on the expression of *salt,* a salt-sensitive marker gene. Proline enhanced, where as trehalose suppressed the expression of *salt* which negatively correlated with Na accumulation. Sivakumar *et al.*[33] reported that proline, at a concentration as low as 100 mM, suppressed *in vitro* activity of Rubisco in seedlings of *Brassica juncea*, *Sesbania sesban* and *Oryza sativa*. The exact mechanisms that proline plays in relation to protection of plants under salt stress needs to be further investigated. In this report, however, the accumulation of proline in salt-treated shoots could be a convenient physiological trait useful for differentiating between the tolerant, moderately tolerant and highly sensitive cultivars

Detrimental effects of NaCl occurred because large quantities of salts are carried through the transpiration stream to the leaves resulting in ultrastructural and metabolic damages which eventually leads to their death. The fundamental mechanism of salinity tolerance in rice is the exclusion or reduced uptake of toxic Na^+ and increased absorption of beneficial K^+ to maintain good Na^+-K^+ balance in the shoot. The data demonstrated the high negative correlation between Na^+:K^+ ratio in the shoots of salt-stressed plants and the level of salinity tolerance (r = -0.803). The most tolerant cultivar PK (score = 3) had the lowest Na^+:K^+ ratio in the 12 dS m^{-1} treatment (0.73) and the most sensitive IR29 (score = 9) the highest (2.37). Four other cultivars exhibited intermediate values. The classification into susceptible, moderately tolerant and tolerant types is clearly related to Na^+:K^+ ratio[20].

Osmotic potential of extracts from shoots and roots decreased in response to increasing NaCl concentration. The reduction in osmotic potential is an essential mechanism to lower the tissue water potential so as to maintain the ability of plants to take up water from outside medium with low water potential. Osmotic potential in shoots tends to have some correlation with the level of salt tolerance (r = 0.746). The most tolerant PK treated with 12 dS m^{-1} salinity exhibited the highest osmotic potential (-1.273 MPa), in the sensitive KDML105 and highly sensitive IR29 the values of shoot osmotic potential dropped to -2.625 and -2.244 MPa, respectively. The lowered osmotic potential was contributed by uptake and compartmentation of inorganic ions, especially Na^+, in the vacuoles and synthesis of non-toxic organic solutes in the cytoplasm[34]. Both accumulation of inorganic ions and synthesis of organic solutes require energy. The energy cost for accumulation of inorganic ions in vacuoles is relatively small in relation to that needed to synthesize organic solutes for osmotic adjustment[5]. Therefore the ability to reduce tissue water potential (osmotic adjustment) by accumulating relatively low amount of both Na^+ and the organic solutes, such as proline, in the tolerant PK is obviously a very important contributing factor to salt tolerance. For other cultivars relatively high concentration of both Na^+ and proline are needed to obtain low shoot osmotic potential values.

The extent of membrane leakiness as a result of NaCl was assessed by measurements of solute leakage from cells. In the absence of salt stress, electrolyte leakage of shoots of controlled seedlings did not vary among cultivars. However, when treated with 6 dS m^{-1} salinity, cultivars differing in salinity tolerance exhibited different degrees of membrane damage. Most significant membrane damage occurred in the most sensitive IR29, where as membrane permeability of PK did not change. KDML105 exhibited similar values of electrolyte leakage to the moderately tolerant cultivars. In the 12 dS m^{-1} salinity treatment, PK still retained very low electrolyte

leakage values. However, electrolyte leakage of the moderately tolerant and sensitive cultivars did not relate well with salinity tolerance. At 12 dS m^{-1} salinity, electrolyte leakage of DDK and LTM was slightly higher than that of IR29. Dionisio-Sese and Tobita[11] and Lutts *et al.*[9] also found that electrolyte leakage increased with increasing NaCl concentration in the medium and that it was higher in salt-sensitive than in salt-resistant rice cultivars. Lutts *et al.*[9] found that alteration in membrane permeability was one of the first symptoms of stress-induced senescence and that electrolyte leakage was strongly correlated with malondialdehyde content, an indicator metabolite of lipid peroxidation reactions. They also suggested that electrolyte leakage was a more reliable and convenient tool for screening rice cultivars for salinity tolerance than chlorophyll fluorescence. In this study significant correlation between salinity tolerance scores and electrolyte leakage at low salinity treatment was observed (r = 0.819*). We also suggest that electrolyte leakage determination may be a useful tool for screening salt tolerance due to its early response, simplicity and low cost.

Net photosynthesis rate of the growing leaf of most cultivars, like the total chlorophyll content, was not significantly affected by NaCl treatment. Salt-treated leaves of SP2 and IR29 which showed significant reduction in chlorophyll content, however, also exhibited a reduction in photosynthesis rate. Net photosynthesis rate of the six cultivars at 12 dS m^{-1} salinity was found to be positively correlated (r= 0.887*) with the level of salinity tolerance (Table 2). The more tolerant tended to have higher net photosynthesis rate than the more sensitive cultivars. Although addition of NaCl up to 150 mM did not elicit a short-term (hours-days) effects on photosynthesis rate[35], salt accumulation in the leaves over a long-term (weeks) reduced photosynthetic efficiency, before leaves die and this in association with senescence, leads to decreasing productivity[36]. Faustino *et al.*[8] also found that, in 3-week-old rice seedlings, photosynthesis rate was affected only at extremely high salt concentrations (150 to 200 mM) and that it was less affected in the salt-tolerant than in the salt-sensitive varieties. In this study, after 7 days in salinity no significant reduction in photosynthesis rate was observed compared with the controlled treatment. This could be due to the fact that the measurement of photosynthesis rate was made on the second leaf from the top which was young actively growing and are less affected by salt than the lower more mature leaves, some of which began to turn yellow. This observation could be accounted for by preferential accumulation of Na^+ in old leaves and its exclusion from young leaves[36]. To be able to correlate CO_2 assimilation rate with biomass production, both photosynthesis rate and total leaf area of the plants have to be taken into account. The observation that both shoot and root dry matter of PK were not affected by salinity could be explained by its highest photosynthetic rate and greatest area of green leaves. Significant reduction in shoot biomass of IR29 could be related to its loss of photosynthetic activity and small number of remaining green leaves. Among the moderately tolerant cultivars, SP2 which exhibited lowest photosynthesis rate also suffered the most reduction in shoot fresh weight (-29.97%), the least increase in root fresh weight (+32.33%) and root dry weight (+1.54%).

Cluster analysis using physiological characters: In an attempt to evaluate the similarity among different rice cultivars in relation to growth and physiological responses to salinity. A cluster analysis was performed using eight physiological characters which gave high correlation with the level of salinity tolerance i.e. root fresh weight at 12 dS m^{-1}, shoot dry weight at 12 dS m^{-1} (% of control), shoot proline, root proline, shoot osmotic potential, photosynthesis rate, Na^+:K^+ ratio at 12 dS m^{-1} and electrolyte leakage at 6 dS m^{-1}. Measurement of association using Jaccard's similarity coefficient and cluster analysis using UPGMA revealed that six cultivars of rice could be divided into 2 groups: Group 1 consists of PK and the other 5 cultivars belong to the second group. The second group could be divided into 2 subgroups: IR 29 in one subgroup and the 4 Thai varieties in the other (Fig. 6). Within the second subgroup, the moderately tolerant DDK, LTM and SP2 displayed physiological responses which are more similar to the sensitive KDML105 than to the tolerant PK. The results showed that PK exhibited highly different characteristics from other cultivars in terms of quantitative physiological parameters in response to NaCl. Important physiological parameters of PK which are distinct from other cultivars included low shoot proline, low shoot Na^+:K^+ ratio, high shoot osmotic potential, low root osmotic potential, high photosynthesis rate and low electrolyte leakage. The highly sensitive IR29, on the other hand, exhibited the values at the opposite extreme for the aforementioned parameters except the shoot osmotic potential. The four Thai cultivars displayed the values for most physiological parameters in between the two extremes. Although being classified as sensitive by scoring using visual salt injury, KDML105 exhibited physiological responses characteristic of the moderately tolerant type.

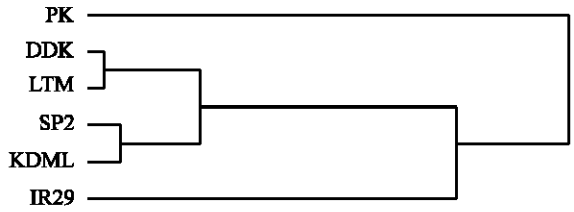

Fig. 6: Dendrogram based on cluster analysis of 6 rice cultivars with 8 physiological parameters responding to salinity stress

Comparison of genetic diversity, salinity tolerance and physiological responses: The study on RAPD polymorphism has divided the six rice cultivars into two groups which coincide with the level of salinity tolerance. The first group contained the two sensitive cultivars, KDML 105 and IR 29. The moderately tolerant (DDK, SP2 and LTM) and the tolerant (PK) cultivars formed the second group. In the present study, 9 primers produced amplified 9 prominent bands which are specific to Pokkali and not present in any other cultivars. Primer OPB-15 produced a band (1515 bp) which is present in PK, DDK, SP2 and LTM but absent from the two sensitive cultivars. Xie *et al.*[18] performed a similar experiment using 100 primers on three salt-tolerant (Pokkali, Nona-Bokra and Bicol) and one salt-sensitive (IR29) and identified four primers which amplified specific fragments that appeared in all the three tolerant cultivars but not in the susceptible IR29. These specific markers might potentially be related or linked to salinity tolerance genes and may be useful in salt tolerance breeding program.

The three Thai moderately tolerant cultivars used in this study i.e. DDK, SP2 and LTM, are closely related to one another in terms of genetic diversity, salinity tolerance rating as well as physiological responses. Based on RAPD polymorphism these three cultivars are genetically more closely related to PK than to KDML105. DDK and LTM are local Thai varieties being grown both for domestic consumption and commercial purposes, mostly in the salt-affected area in the central and eastern Thailand. Compared with SP2, a high-yielding improved cultivar, DDK and LTM are more salt tolerant at flowering stage[37]. It may be useful to incorporate these two cultivars in the breeding program to provide useful genes due to their genetic relatedness to PK and their salt tolerance and adaptability to environmental conditions in Thailand. KDML105 is more closely related to IR29, a check variety for salt sensitivity in relation to RAPD polymorphism and salinity tolerance. In the study involving KDML105, SP2, PK and five other local Thai varieties, KDML105 which scored sensitive (score = 7) at seedling stage also produced the lowest yield in the saline field experiment[38]. PK which scored tolerant (score = 3) at seedling stage gave lower yield than the three moderately tolerant local Thai cultivars (Leuang Anan, Leuang Yai and Khao Med Lek). The field performance of SP2, scored moderately tolerant at seedling stage, was similar to that of KDML105. Tolerance in the field conditions, therefore, does not necessarily conform with the rating at seedling stage. More work on screening germplasm of local Thai rice for salinity tolerance is clearly needed. Local Thai moderately tolerant varieties, in addition to well known tolerant varieties from overseas, should be included in the breeding program to improve salinity tolerance of commercial elite cultivars.

In the present study some physiological parameters were found to be useful indices for salt tolerance. These parameters included low shoot Na^+:K^+ ratio, low shoot proline, high shoot osmotic potential, low root osmotic potential, low electrolyte leakage and high photosynthesis rate. Among these parameters, Na^+:K^+ ratio is the most reliable tools and has been widely used, however the process of determination is time-consuming and expensive. Electrolyte leakage test is suggested to be another appropriate physiological tool for rapid screening of salt tolerance due to its simplicity in performing the test and its early responses.

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