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Characterizing Salt Stress Response in a Rice Variety and Its Salt Tolerant Lines Derived from *In Vitro* Mutagenesis

In Sok Lee[1]*, Dong Sub Kim[1], Si Yong Kang[1], Seung Gon Wi[1], Hua Jin[2,3], Pil-Yong Yun[2] Yong Pyo Lim[2], Young Il Lee[1]

[1]Korea Atomic Energy Research Institute, Daejeon, 305-353, Korea, [2]Department of Horticulture, Chungnam National Univ., Daejeon, 305-764, Korea
[3]Institute of Biological Resources and Environmental Research, Da Lian Nationalties University, 116600, Da Lian, Liao Ning, P. R. China

Abstract

The objectives were to compare the salt tolerance levels in the parental rice cultivar, Dongjinbyeo, and induced mutagenesis derived its lines for plant height, MDA, ATPase, POD, and 2-dimensional protein electrophoresis pattern in NaCl-containing hydroponic nutrient solutions. Rice plants isolated from a population of rice (*Oryza sativa* L. cv. Dongjinbyeo) mutation lines, which were generated in combination with *in vitro* selection and gamma-ray, exhibited salt tolerance. Line No. 18 had the longest plant, whereas NaCl-sensitive line (No. 25) had the shortest plant. The parent, and the sensitive line showed severe damage from salt stress. Tolerant lines (No. 18, 50) had a lower malonaldehyde (MDA) content than the sensitive one (Dongjinbyeo, No. 25) during salt stress. Several proteins showed significant quantitative variation through 2DE; phosphoribulokinase, peroxidase, oxygen evolving enhancer 1 and the H^+-ATPase, which are known to be involved in salt tolerance. The effect of salt on peroxidase and H^+-ATPase activity in the seedlings of two groups with contrasting genotypes of rice was studied. A greater activity was recorded in the tolerant lines as compared to the sensitive ones ($P<0.05$, Duncan's test). The results indicate that salt tolerant lines expressed more salt stress-inducible proteins associated with salt tolerance than the sensitive lines during salt stress.

Key words: ATPase, 2-dimensional electrophoresis, MDA, mutation, POD, salt-tolerance, Summary

* Corresponding author, E-mail: insoklee@hanmail.net
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Introduction

Spontaneous mutations have played an important role in the improvement of varieties (Sanada, 1986), however they have resulted in a low frequency of mutation recovery (Miao et al., 1988), thus encouraging plant breeders to use induced mutagenesis (Pinet-Leblay et al., 1992). Also, when existing germplasm fails to provide the desired genotype, it is necessary to resort to other sources to induce variation. A combination of radiation techniques with *in vitro* culture methods can speed up the breeding programs, by generating new variability (Maluszynski et al., 1995), and has been used by other workers (Ahloowalia, 1990; Chen et al., 2001; Das et al., 2000). Such a technique for crop improvement should enable a greater use of mutated genes, and its cumulation effect may result in providing a more desirable character than the original variety.

Soil salinity is one of the important constraints and a better understanding of the mechanism that enables a plant to tolerate salt stress is necessary for exploiting saline soil. To cope with salt stress, plants respond with physiological and biochemical changes. These changes include retention of water in spite of the high external osmoticum and the maintenance of the photosynthetic activity while stomatal opening is reduced to counter the water stress. An accumulation of low molecular weight compound such as betaine, suger alcohols and proline serves as a mechanism to tolerate the water potential following an increase in salinity. In addition to synthesis of these osmolytic compounds, specific proteins and translatable mRNA are induced and increased by salt stress (Cales et al., 1990, Morabito et al., 1996, Reviron et al., 1992). After exposure to salt stress, a Na^+/H^+ exchange process is activated so that K^+

can be pumped across the cell membrane (Watad et al., 1986) and Na^+ can be pumped into the tonoplasts (Garbarino and DuPont, 1989), which are carried out by the H^+-ATPase (Burgos and Donaira, 1996). Also an increase in the peroxidase activity is a common response to salt tolerance (Sreenivasulu et al., 1999). In several studies, 2-dimensional electrophoresis were used to identify and isolate salt tolerant genes and proteins in addition to the H^+-ATPase and peroxidase under salt stress (Cales et al., 1990, Morabito et al., 1996, Sugihara et al., 2000). Also, Riccardi et al. (1998) found 78 proteins affected by drought.

The present study showed a low rate of lipid peroxidation and a high total peroxidase and H^+-ATPase activity in the tolerant lines compared to the sensitive ones. We also determined the protein sequence, which was specifically increased by salt stress in the tolerant lines.

Materials and Methods

Mutation induction and selection of mutants

Dehusked seeds of cv. 'Dongjinbyeo' were sterilized with 5% sodium hypochlorite, and cultured on a N_6 medium supplemented with 2 mg/L 2,4-D and incubated in the dark at 25℃ ±1 for callus initiation. Callus pieces were irradiated with 0, 30, 50, 70 and 90 Gy γ-ray from a ^{60}Co source. The calli were divided into small pieces (0.5-1mm diameter) and inoculated on a N_6 medium containing 1.5% NaCl after 72 hours. The salt-tolerant calli were maintained at the same concentration for three passages, each of 40 days. For regeneration, selected NaCl tolerant calli were cultured on MS medium supplemented with 0.5mg/L NAA + 2mg/L BAP, but without salt for 30 days. Each of the six hundred regenerated plants (M_1) were assigned numbers, and grown to maturity in NaCl-free soil in a field. Standard crop management practices were followed, which included an application of 11; 7; 8 kg / 975 m^2 of N; P; K.

Three hundred M_2 lines derived from M_1 plants, except those with poor plant type (droopy leaves and weak culm), were harvested and numbered from M_2-1 to 300. The M_2 generation was grown as a plant in rows in a NaCl-free field and 5,000 individual plants showing the normal grain fertility (above 80%) harvested. Each M_3 line was the progeny of a single M_2 line seed bulk and numbered from M_3 -1-1 to M_3 -1-n and from M_3 -300-1 to M_3 -300-n (n was influenced by lines).

Selection of salt tolerant lines

The M_3 lines were screened in trays with 0.75% NaCl with three replications in the green house. Each tray had 20 pots (each of 60 mm × 150 mm × 30 mm), with one pot for each of the 5000 lines. Of the twenty pots per tray one was from the parent, not cultured *in vitro*. The trays were filled with fine soil, commercially used for rice culture in Korea and 80 seeds per line were placed in each pot at a depth of 5 mm. The trays were watered with tap water untill the 3 to 4 leaf-stage. At that stage, excess water was drained, and the trays with rice seedlings were refilled with a solution containing 0.75% (E.C=13 mS) salt and 1 g/L fertilizer. The solution was circulated with an underwater rotator to maintain a uniform salt concentration. The E.C measurements were taken daily. After three weeks of salinization with 13 mS, salinity symptoms were scored according to the Standard Evaluation System (1-3: tolerant, 5: moderate and 7-9: sensitive) developed at the IRRI. To re-estimate the salt tolerance, each thirty seeds of the tolerant and sensitive lines was identified in the first experiment, were placed in glass bottles (5 cm × 7 cm) with a salt-free solution and 0.75% salt treatment in these replicating and cultured for 30 days and 3 weeks, respectively. Plant height was used to assess the salt tolerance or sensitivity of the rice lines for the quantitative measurement of salt tolerance.

Estimation of salt tolerance at the seedling stage and the preparation of the plant material for physiological analysis

To test salt tolerance at the seedling stage, seedlings grown without salt in the second test were cultured on a solution with a 0.75% salt for 8 days. The leaves were prepared for the analyses of malonaldehyde, 2 dimensional electrophoresis, ATPase and peroxidase activity after 72 hours with wilting of the sensitive lines.

Malonaldehyde (MDA)

The levels of the malonaldehyde content in the 3 to 4 leaf stage-seedling grown for 3 days in a 0.75% NaCl solution was determined by the thiobarbituric (TBA) reaction as described by Heath and Packer (1968). One gram of tissue was homogenized in 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 5,000 g for 10 m and 4 ml of 20 % TCA containing 0.5% (w/v) TBA was added to 1 ml of the supernatant. The mixture was heated at 95℃ for 30 min and then quickly cooled on ice. The contents were centrifuged at 5,000 g for 10 m and the absorbance was measured at 532 nm in a spectrophotometer. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. MDA content was expressed as μmol g FW^{-1}.

Two dimensional electrophoresis analysis

Protein for SDS-PAGE was extracted in a modified Laemmli's (1970) buffer containing 65 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 2 mM EDTA. For isoelectrofocusing, the protein was extracted as described by Hurkman and Tanaka (1986). Two-dimensional electrophoresis was done with two replications, which was carried out for the first dimension under isoelectric focusing conditions according to O'Faeerll (1975). In the second dimension we used the discontinuous SDS-PAGE gel system (Laemmil, 1970). Gels were stained according to the method of Damerval et al. (1987).

Protein sequencing and search for similarities in amino acid

N-terminal sequence and the internal amino acid sequences of the leaf protein were obtained from the Korea Basic Science Institute, Seoul Branch. Amino acid sequences were compared with the sequences in the Peptide Sequence Database of the National Center for Biotechnology Information using the BLAST program.

H^+-ATPase activity

Seedlings grown for 3 days in a 0.75% NaCl solution were homogenized. Protein for the H^+-ATPase activity was prepared as described by Leonard and Hotchkiss (1976) with a buffer containing 0.1 M Tris-HCl, pH 7.8. The enzyme activity of the H^+-ATPase activity was tested at 30℃ in a 1 ml buffer containing 30 mM Tris-MES, pH 6.5, 3 mM $MgCl_2$ and 3 mM ATP. After 1h, 25% trichloroacetic acid was added to stop the reaction. Activity was assayed by measuring the amount of phosphatidyl inositol (PI) hydrolyzed from ATP (Fiske and Subbarow 1952).

Peroxidase activity

Seedlings grown for 3 days in a 0.75% NaCl solution were homogenized separately in a 50 mM Tris-HCl, pH 7.5 buffer at 4℃. The homogenate was centrifuged at 5,000 g for 10 min. The pellet was washed with the same extraction buffer and centrifuged in the same way. The protein in the extracts was quantified by the method of Bradford using BSA as the standard. For determining peroxidase activity. the reaction mixture (3 ml) consisted of 0.25% (v/v) guaiacol in 10 mM sodium phosphate buffer, pH 6, containing 10 mM hydrogen peroxide. 50 ul of the crude enzyme extract was added to initiate the reaction that was measured spectrophotometrically at 470 nm min^{-1} g^{-1} FW.

Statistical evaluation

Duncan's test and Statistical Packages for Social Sciences (SPSSX 1983) were used to determine the superiority of the salt tolerant lines over the Dongjinbyeo and salt sensitive lines.

Results

Evaluation of salt tolerance

Repeated cycles of flooding and evaporation leave behind a mixture of salts in the paddy soils, some of which can be more deleterious to plants than others. To mimic the complex nature of this stress in the green house, rice was grown hydroponically in a solution. Salt tolerant and sensitive one, and their parent showed a good germination (more than 90%) in 0.75 % salt and salt free conditions. The plant height of all the lines including the parent was similar in solution without salt. However, the salt tolerant lines (line 18, 50) showed quicker germination in a salt solution. After 3 weeks, the parental variety and sensitive line were about 5 cm in plant height and 10 ±1.3 cm for the salt tolerant lines (Figure 1). To estimate the salt tolerance at the seedling stage, seedlings grown in a salt-free condition were tested in a 0.75% salt concentration. The salt sensitive genotypes began wilting after 3 days. There was a substantial salt tolerant difference between the sensitive and tolerant lines at 8 days (Figure 2).

Malonaldehyde (MDA) content

Salt stress caused an increase in the level of MDA content among the tolerant and sensitive lines. The MDA content of the non-stress treatment of the four genotypes (original pa-

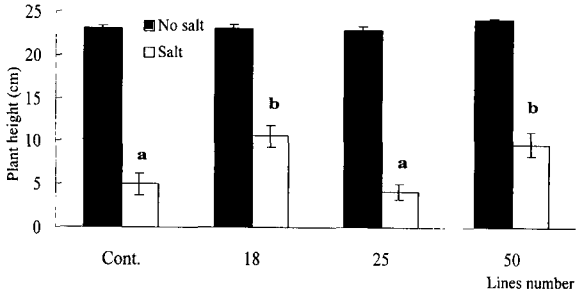

Figure 1. The effect of salt stress on growth of the salt tolerant and salt sensitive lines. The black and white bars indicate the height of plants grown in the solution without salt and with salt (0.75%). The error bars are means±SE with ten replications. The Duncan's test at 1% level.

Figure 2. Salt tolerant and salt sensitive rice lines grown for 3~4 weeks in 0.75% salt solution. Extreme left, Dongjinbyeo; Middle, salt tolerant line No. 18, 50; Extreme Right, Sensitive line, No. 25.

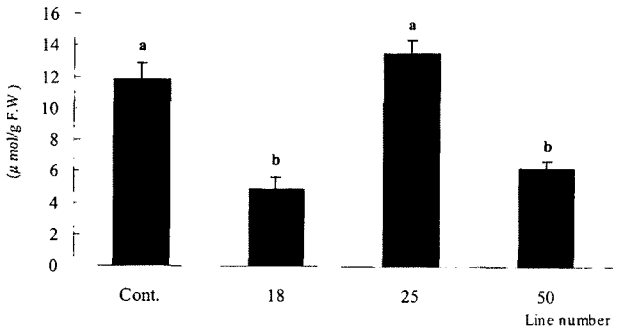

Figure 3. Malonaldehyde content in 3 to 4 leaf stage-seedlings of rice for 3 days under 0.75% salt stress. Cont. (Dongjinbyeo) and Line No. 25: Salt sensitive lines, Line No. 18 and no. 50: Salt tolerant. The error bars are means ± SE with three replications. The Duncan's test at 1% level.

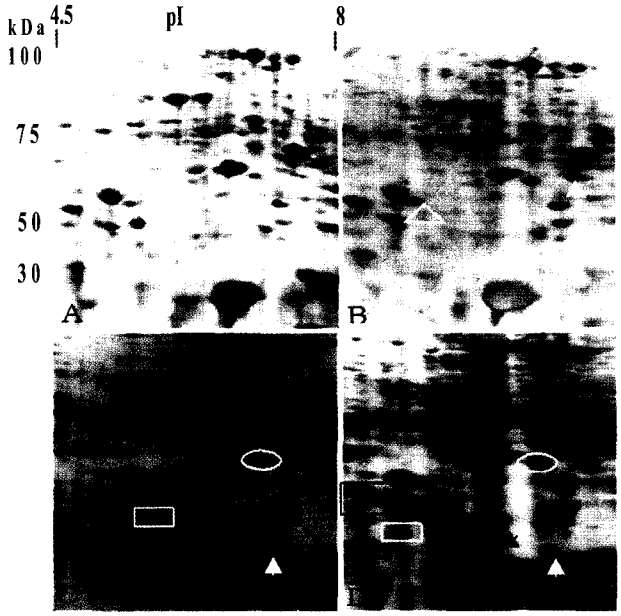

Figure 4. Comparison of a silver-stained 2DE gel of leaf proteins from salt tolerant and sensitive lines subjected to 0.75% salt stress for 3 days. A (Dongjinbyeo), B (No. 18, salt tolerant line): Non-stressed plants. C (Dongjinbyeo), D (No. 18, salt tolerant line): Salt-stressed plants. A, B: Comparison between protein responses of the two genotypes in normal condition revealed differential kinds of genetic variation. Several proteins were specific to one of the two lines (Triangles, Arrows). C. D: White rounds, rectangular and arrows indicate sequenced proteins, which were more induced in salt tolerant one than in the Dongjinbyeo. Black box and arrows mean the higher expressed proteins in the tolerant lines than in the Dongjinbyeo.

rent, No. 18, 25, 50) averaged 1.2 μmol/ g F.W. However, the degree of MDA accumulation under salt stress was more in the two salt sensitive lines (average 12.5 μmol/ g F.W) than in the tolerant lines (average 5.6 μmol/ g F.W), which indicates a higher rate of lipid peroxidation in the sensitive lines due to salt stress (Figure 3).

Selection of protein highly expressed in salt tolerant lines

Proteins were isolated from the leaves harvested from salt-untreated and salt-treated plants, and separated by two-dimensional gel electrophoresis (Figure. 4). After silver staining with $AgNO_3$, we observed the difference the spot intensity between the original parent and salt tolerant plants grown in a salt-free solution. Some proteins of the original parent were not detected in the salt tolerant line. The parental peptide, absent in the leaves compared with the salt tolerant line, is marked in Figure. 4 as a triangle. The protein 2-DE from the salt tolerant lines under salt stress was more abundant than those for the parent. Out of the 328 spots detected, 56 were affected by salt stress; of which, 35 were increased in the salt tolerant line. Among the quantitatively increased proteins, a protein of about 100 kDa with a pI 7, a 60 kDa protein with a pI of 6.8, the 53 kDa, 8 pI protein and the 45 kDa, 7 pI protein were sequenced. Of these, sequencing of 60 kDa protein with a pI of 6.8 was carried out after trypsin digestion and the remaining proteins selected for N-terminal amino acid sequencing (Table 1). Sequence comparisons showed similarities with the proteins previously characterized in rice, wheat and Mangrove with a 70% to 95% identity: phosphoribulokinase, peroxidase, oxygen evolving enhancer 1 and the H^+-ATPase.

Effects of salt stress on H^+-ATPase and Peroxidase activity

Figure 5 indicate that the 0.75% NaCl treatment caused a statistically significant reduction in the total ATPase activity average 74% of the control treatment (average 270 unit/mg protein) in the salt sensitive lines (Dongjinbyeo and No. 25) and 92% and 89% in the salt tolerant ones (No. 18

Table 1. Amino acid sequence similarity of protein induced by salt stress in leaves of rice lines

Genotype	kDa	pI	Amino acid sequence	Protein match	Species	Amino acid identity (%)	References
M-18	90	5	DVDKPVVIGLAADSG GKST	Callus formation[a] (PRK)	Wheat	80	Raines et al. (1989)
M-18	60	6.8	YFDLIAIDPK	Peroxidase[b]	Rice	70	Moons et al. (1996)
M-18	35	5	TRLTATLEIEGPLVVSS DGTIKFEEKDGIDAA	Oxygen evolving enhancer 1[a]	Mangrove	78	Sugihara et al. (2000)
M-18	30	7	ATTVLSRGLASKEIPA VDPLDST	H^+-ATPase[b]	Rice	95	Hiratsuka et al. (1989)

a: N-term: N-terminal sequence, b: Internal sequence, M: Mutant.

and 50). Effects of salt stress on peroxidase activity are presented in Figure 6. Salt stress caused increase in the peroxidase activity in the salt sensitive and in the salt tolerant lines as compared to the peroxidase activity (average 16 unit/mg protein) of the non-stressed treatment of the four genotypes (original parent, Lines No. 18, 25, 50).

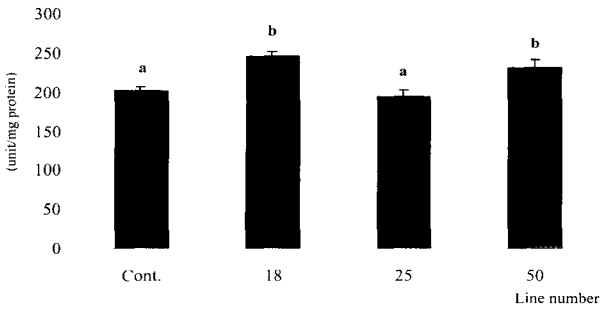

Figure 5. ATPase activity of rice grown for 3 days under 0.75% salt condition. Cont. (Dongjinbyeo) and Line No. 25: Salt sensitive lines, No. 18 and no. 50: Salt tolerant lines. The error bars are means±SE with three replications. The Duncan's test at 5% level.

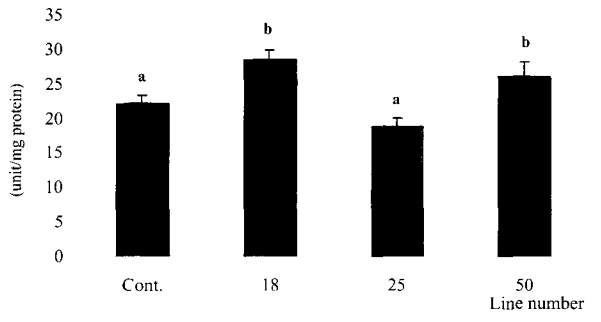

Figure 6. Peroxidase activity of rice grown for 3 days under 0.75% salt condition. Cont. (Dongjinbyeo) and no. 25: Salt sensitive lines, No. 18 and no. 50: Salt tolerant lines. The error bars are means±SE with three replications. The Duncan's test at 5% level.

For the salt sensitive lines, the treatment of 0.75% NaCl increased peroxidase activity from 12.5% (No. 25) to 37.5% (Dongjinbyeo) the non-stressed treatment. However, peroxidase activity of the salt tolerant lines raised from 56% (No. 50) to 75% (No. 18) compared to the non-stressed treatment. There is a statistical difference of 5% in the peroxidase activity IN the salt sensitive and salt tolerant lines ($P<0.05$).

Discussion

Seeds of both the tolerant and sensitive lines were germinated and grown in the salt and salt-free solution. The growth pattern was similar between the genotypes in the solution without salt. However, obvious differences in the salt sensitive lines and tolerant lines were observed in response to the salt stress. This confirms the high effectiveness of mutation in salt tolerance breeding as reported by Neto et al. (2001). Barriga et al. (1990) observed that among the mutants for various characteristics, some had advantages over the control. Based on their results they recommended mutation induction for some characteristics.

The most striking effect of salt stress was manifested by differences in the content of malonaldehyde among the genotypes. We observed protein changes occurring in the rice leaves after salt stress were applied to the plants. Comparison between the protein responses of the two genotypes under normal condition revealed different kinds of genetic variation. These results could be due to silent gene activation, a neutralization of the suppression and chromosome translocations (Lukaszewski et al., 1984; Marais and Marais, 1994). 2DE gel electrophoresis showed that salt stress caused a visible difference in both the salt tolerant and salt sensitive genotypes. Several proteins were specific to one of the two lines. In the comparison of the protein intensity of the two genotypes to salt response, some proteins were found to decrease: this could be due to the repression of the synthesis of some proteins, but also to a different turnover (Riccardi et al., 1998). The effect of salt

stress was accompanied by a genotype effect for some other proteins: although their constitutive level was different, these proteins were similarly increased or decreased by salt in the three genotypes. Finally, the response of the other proteins exhibited a genotype x treatment interaction, i.e. the protein quantity was differentially modified by the stress according to the genotypes. It was found that a number of peptides from the salt tolerant ones under salt stress were richer than those of the parent. A lower number of peptides in the parent than in the salt tolerant ones may be a result of the gene dosage and differences of the gene expression (Aragoncillo et al., 1978).

Microsequencing, the N-terminal amino acid sequence and internal sequencing, was performed to tentatively identify the proteins reproducibly induced with salt stress. Four of these proteins, which increased only in the salt tolerant lines, have been identified and sequences correlated with the salt tolerant genes, which are known to be induced under salt stress (Moons et al., 1997: Sugihara et al., 2000). For further detailed research, analysis of specific enzyme activity was carried out.

The peroxidase is reported to play a key role in salt toerance (Screenivasulu et al. 1999). If the metabolic efficiency and function are to be maintained either in normal or stress conditions, a regulated balance between the oxygen radical production and destruction is required. A constitutively high antioxidant capacity under stress conditions can prevent damage and correlates with resistance to a particular stress. Hence, the mechanisms that reduce oxidative stress are expected to play an important role in imparting tolerance to plants under saline conditions. An increase in the total peroxidase activity under salt stress has been reported (Heath and Packer. 1968; Median et al., 1999). In the present study, a significant elevation in the activity of peroxidase was recorded in the lines during salt condition. Furthermore, the degree of increase in enzyme activity was relatively high in the salt tolerant lines as compared to the sensitive ones. High peroxidase activity in the tolerant lines reflects changed mechanical properties of the cell, which, in turn, could be related to the salt tolerance process (Heath and Packer, 1968). An increase in the total peroxidase activity is a common response with various oxidative stress factors (Gasper et al., 1991). Enhanced production of oxygen free radicals is responsible for stress-dependent peroxidation of membrane lipids (Elstner 1987). Increased peroxidation of the membrane lipids is known to occur during salt stress (Dhindsa et al., 1981). It has been reported that saltstress could modify the membrane structure, and may sti mulate oxygen radical production, which facilitates lipid peroxidation. MDA is one of the decomposition products of the poly-unsaturated fatty acids of biomembranes, and shows greater accumulation under salt condition (Chaudhuri and Choudhuri, 1993). Increase in the total peroxidase activity and low malonaldehyde content in the salt stress tolerant line indicates the involvement of peroxidases for the cell membrane integrity.

In order to avoid salt accumulation in the cytosol, plants have developed various mechanisms involving a secondary transport. A response to the accumulation of toxic ions in the cytosol is their compartmentalization within the vacuole, while another response is their extrusion out of the cell. In each case, Na^+ and K^+/ H^+-ATPase seem to be involved and activation of this process is expected by a vacuolar and plasma membrane proton transporter (Morsomme and Boutry, 2000). The present study found that salt stress caused reductions of the H^+-ATPase activity in both salt-tolerant and sensitive lines. Nevertheless, the salt-sensitive ones had a greater reduction in H^+-ATPase activity than the salt tolerant lines. Niu et al. (1993) were able to detect a greater enhancement of mRNA expression of the H^+-ATPase by salt treatment to a halophyte than that of a glycophyte. Rice is a nonhalophyte plant. Its mechanism for maintaining a higher H^+-ATPase activity under salt stress in the tolerant ones is not yet clear. However, the results suggest that the differential salt tolerances among the genotypes is partially associated with the difference in their ability to maintain a higher H^+-ATPase activity and regulate the salt uptake. Thus, the data in the present study supports this hypothesis. There is evidence, from plant and bacteria systems, that modification of the membrane lipid composition can alter the kinetic properties of an internal protein such as the H^+-ATPase (Kuiper, 1985; Laemmli, 1970). The restructuring of the lipid composition in response to environmental cues implies that lipid metabolism is subject to an environmental influence and ensures the appropriate physical properties and phase behavior to adapt to the environmental conditions (Mansour et al., 1993).

Another important enzyme related to photosynthesis, is phosphoribulokinase (PRK) and the oxygen evolving enhancer protein I. PRK, regulated by light, is involved in the ATP binding and is regulated as a tissue-specific (Raines et al., 1989). The carboxylation reaction is catalyzed by Rubisco and three reactions in the regenerative phase, frustoose-1, 6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase) and phosphoribulokinase (PRK), are involved in the Calvin cycle (Paul et al., 2000). It is known that there is an *in vivo* high relevance for PRK in the dynamic balance of the carbon fixation of the Calvin cycle. We believe that carbon fixation ability of salt tolerant lines is higher than that of salt sensitive ones, although the 2-DE result were in con-

clusive. The 35 kDa protein was an oxygen evolving enhancer protein I (OEEI). Sugihara et al., (2000) demonstrated that oxygen evolving enhancer proteins (OEEs) had three subunits, OEE -I, -2, -3. These are nuclear-encoded chloroplast proteins, and peripherally bound to photosystem II (PS II) on the lumenal side of the thylakoid membrane. Among these, oxygen evolving enhancer I is important to plants for oxygen evolution and PS II stability (Sugihara et al., 200). Two lines (No. 18 and No. 50) showed salt tolerance and we simultaneously identified the OEE-I expression through 2-DE. So, it is believed that OEE-I is associated with the salt tolerant mechanism of rice. Considering the increased amounts of the two enzymes observed with salt stress, activation of the photosynthesis pathway by salt treatment can be suggested. A photosynthesis increase has already been reported in maize and rice subjected to severe water and salt stress, respectively (Riccardi et al., 1998; Tiwari et al., 1997). These enzymes were detected in higher numbers in the salt tolerant lines and can have a physiological advantage. Unfortunately, it is not certain whether the causes of the higher intensity of these peptides, such as POD, H^+-ATPase, OEE 1 and PRK, is due to difference in the number of isoenzymes or increase in isoenzyme activities. These phenomena needs to be subjected to further investigations.

Possible allocations of quantitative trait loci to the induced proteins with salt-stress responsive traits would be consistent with the causal relationship between the proteins and the phenotypic traits (Damerval et al., 1994). The study demonstrates that induced mutation holds to induce salt tolerance and elucidate the molecular mechanism of tolerance.

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