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Isolation of Gamma-Induced Rice Mutants with Increased Tolerance to Salt by Anther Culture

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Abstract

Doubled haploids have long been recognized as a valuable tool in plant breeding since it not only offers the quickest method of advancing heterozygous breeding lines to homozygosity, but also increased the selection efficiency over conventional procedures due to better discrimination between genotypes within any one generation. Salt tolerant mutants were obtained in rice the variety, 'Hawsungbyeo', through *in vitro* mutagenesis of *in vitro* cultured anther-derived calli. Various doses (30, 50, 70 and 90 Gy) of gamma ray were applied to investigate the effect of radiation on callus formation on medium containing 1% NaCl, green plant regeneration, frequency of selected doubled haploid mutants and of the salt tolerant screen. It was demonstrated that the dose of 30 and 50 Gy gamma rays had significant effects on callus formation, regeneration and selection of salt tolerance. No tolerant lines were obtained from non-mutagenized cultures. From gamma ray irradiated cultures, five tolerant lines (M_2 generation) at germination stage and 13 tolerant lines (M_3 generation) at seedling stage were obtained. The frequency of salt tolerant mutants indicates that anther culture applied in connection with gamma rays is an effective way to improve salt tolerance.

Key words: Doubled haploids, gamma ray, mutagenesis, salt tolerance

Introduction

Haploid development by *in vitro* methods includes anther, pollen, ovary culture and chromosome elimination. Among them, anther culture is one of the simplest, most efficient and practical techniques. Doubled haploids (DH) have long been recognized as a valuable tool in plant breeding since it not only offers the quickest method of advancing heterozygous breeding lines to homozygosity, but also increases the selection efficiency over conventional procedures due to better discrimination between genotypes within any one generation (Croughan et al. 1987; Chen et al. 1997).

In addition, anther culture could also be an effective vehicle for producing variation as it allows early expression of recessive genes.

Most of the available genetic variation used in breeding programs has occurred naturally and exists in germplasm collections of new and old cultivars, land races and genotypes. This variation through crosses is recombined to produce new and desirable genotypes. When existing germplasm fails to provide the desired recombinant, it is necessary to resort to other sources to induce variation. Since spontaneous mutations occur with extremely low frequency, mutation induction techniques such as radiation and heavy beam provide tools for the rapid creation and increase in variability in crop species. Combination of radiation techniques with *in vitro* culture methods can speed up breeding programs, from generation of variability, through selection, to multiplication of the new genotypes (Maluszynski et al. 1995; Novak 1991).

Salinity and drought are the two major environmental stresses that limit plant growth and productivity (Boyer 1982). Rice, an important staple food, is consumed by more than half of world's population (IRRI 1990). It is sensitive to salt (Mass and Hoffmann 1977), therefore, development of varieties with increased salt tolerance is urgently required. Success for obtaining abiotic stress mutants by the application of mutation coupled with *in vitro* systems have been attained in some crop

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plants including rice (Ahloowalia 1990; Das et al. 2000; Pathirana et al. 2002; Malmberg et al. 1984). Thus the possibility of screening for abiotic stress in culture makes *in vitro* methods attractive for developing stress tolerant plants. The successful identification of the salt tolerant lines obtained from *in vitro* mutagenesis was carried out at germination and seedling stage on the solution by other authors (Miah et al. 1996; Sathish et al. 1997).

The 2-dimensional electrophoresis (2-DE) technique was used to detect differential protein expression and genetic diversity between original cultivars and mutant. The complex regulatory routes, from post-translational modifications to protein turnover can be studied at the protein level through this technique. The comparative 2-D pattern protein analysis allowed identification of several enzymes that are involved in stress defense (Claes et al. 1990; Moons et al. 1997).

The objectives of the present work were to: (1) produce new salt tolerant lines in rice utilizing *in vitro* mutagenesis of variety cultivated, and (2) identity genetic variation and differential protein expression through 2-DE.

Materials and Methods

Culture media

Mildly desiccated, pre-treated (8 days at 8°C) anthers of rice variety 'Hawsungbyeo' were cultured on N_6 (Chu et al. 1975) basal medium supplemented with 2 mg/L 2,4-D, 60 g/L sucrose and 2 g/L gelrite, pH 5.8 before autoclave. To obtain true haploid calli, diploid tissue such as filament attached at anther was excluded. Healthy calli were selected in 40 days and transferred to fresh medium for propagation. Calli were irradiated with 30, 50, 70 and 90 Gy doses, then divided into about 1 mm in diameter and inoculated on the medium with 1.25 % NaCl, at the rate of 25 calli per 90 mm petri dish in two replications. The survival rate of calli was measured in 40 days. Salt tolerant calli were subcultured for 120 days at intervals of 40 days. After 120 days, the calli were transferred to NaCl-free plant regeneration MS medium (Kinetin(4 mg/L) + NAA(1 mg/L)) to regenerate plants (M_1). Regenerated plants were each assigned a plant line number and grown to maturity in NaCl-free environment.

Screening for salt tolerance in M_2 generation

A salt tolerance screen was done using a split-plot design consisting of 1.0% NaCl treatment for 2 weeks in the green house with three replications. Trays contained 16 pots (30 × 30 × 50 mm per pot), with one pot for each of lines. Of the 16 lines per tray, one was a parent line and this was not subjected to *in vitro* technique. Twenty-five seeds of each line in a replicate were inoculated. After 3 weeks of salinization, tolerant lines were selected by using the relative plant height and root length of seedlings.

Mutant characterization

Each of the tolerant M_2 DH lines was transplanted with a single seedling at a spacing of 30 × 15 cm in NaCl-free field. The morphology of the M_2 lines was carefully studied throughout the growing season. The characters studied included: heading, plant height, chlorophyll deficiencies and leaf shape.

M_3 generation, which was derived from M_2 seeds, showing normal grain fertility (above 80%) was harvested on individual plant basis after 140 days. The chi-square test and Statistical Packages for Social Sciences (SPSSX 1983) were used to compare with the various treatments.

Screening for salt tolerance at seedling stage of M_3 generation

A screen in M_3 generation seeds, derived from M_2, was done using a split-plot design consisting of 1.0% salt treatment. Trays contained 20 pots (60 × 150 × 30 mm per pot), with one pot for each line. Of the twenty lines per tray, one was a parent line and this was not subjected to *in vitro* technique. The trays were filled with fine soil and 80 seeds per line were placed in each pot at a depth of 5mm for germination. The trays were placed on tap water until the plants grew to the 3 ~ 4 leaf stage. At this time excess water was drained, and the trays with rice were refilled with 1.0 % salt solution containing fertilizer of 1 g/L. The solution was circulated by using an underwater rotator to equilibrate the salt concentration. The E.C measurements were taken daily. After 10 days of salinization, tolerant lines were selected over 'Hawsungbyeo'. The two seeds of tolerant lines, which were confirmed as tolerant lines in the first experiment, were replaced in each glass bottle (5 × 7 cm) with 10 replications, cultured until the above-mentioned growth stage and re-estimated salt tolerance on a 1.0% salt solution.

SDS-PAGE and 2-dimensional electrophoresis analysis

Protein for SDS-PAGE was extracted in 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 extraction procedure was done 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,

the discontinuous SDS-PAGE gel system was used in the analysis (Laemmil 1970).

Results

Anther culture, screening of salt tolerant calli and regeneration

The strategy adopted in the study was to isolate salt tolerant mutants to plate callus from a basal medium onto selective medium, and then screen the surviving colonies for salt tolerance. Within 40 days of anther inoculation, callus was obtained on NaCl-free medium (Figure 3A). To understand the effect of NaCl on the growth of calli, they were divided into 1 mm in diameter and cultured on the medium with either 0, 0.5, 0.75, 1.0, 1.25 or 1.5 % NaCl, respectively. The NaCl-LD_{50} value for calli in rice was about 0.75% NaCl and almost all of the calli were dead at 1.5% concentration (Figure 1). Effect of gamma-ray on the mass of calli was tested. The gamma ray-LD_{50} value for calli was 90 Gy, and the mass of calli was significantly affected at more than 120 Gy doses (Figure 2). To select the salt tolerant calli by *in vitro* in connection with radiation, micro-calli were plated onto medium with 1.5% NaCl and irradiated with 0, 30, 50, 70 and 90 Gy dose. Both 30 Gy and 50 Gy at two independent experiments yield higher growing calli than non-irradiation (Figure 3B, C and Table 1).

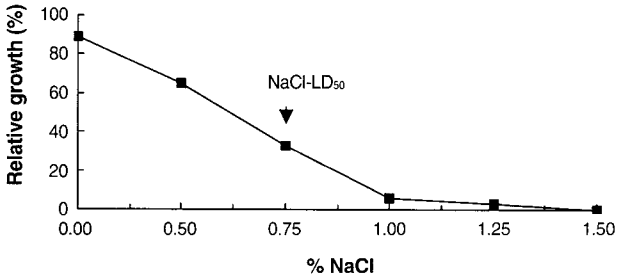

Figure 1. Effects of NaCl on the survival rate of anther-derived callus in 40 days.

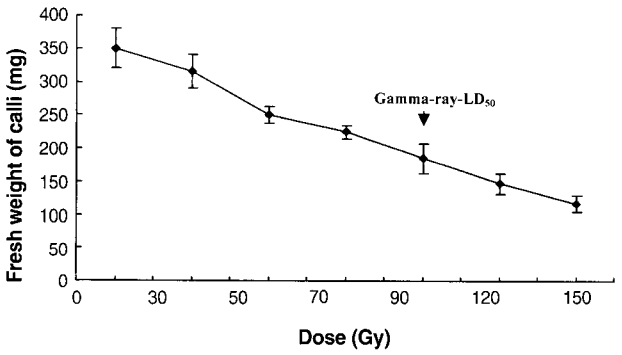

Figure 2. Effects of irradiation on fresh weight of callus derived from Hawsungbyeo anther in 40 days.

The response of tolerant calli to regeneration in media with different hormone combinations is shown in Table 2. Green spot number and plant regeneration number were much higher with the Kinetin (4 mg/L)+NAA (1 mg/L) combination than with the two independent hormones (Table 2). No plant regeneration occurred with Kinetin (1 mg/L)+NAA (1 mg/L) combination. For green plant regeneration, the best dose was 50 Gy, where the number of green plant regenerations was 19, which was more than about four times higher than that of non-irradiated ones (Figure 3D, Table 3). The differentiation of green spots was higher and albino plant formation was lower at all doses of gamma rays than in the non-irradiated control (Table 3). The number of doubled haploids (DH) among green plants is shown in Table 4. The frequencies of fertility of green plant from calli irradiated with 30 and 50 Gy of gamma-ray were 11.2-14.4%, 2.4 times and 3.1 times higher than that of non-irradiated ones, respectively (Table 4).

Protein differences between sensitive and tolerant calli

SDS extractable protein from each two sensitive and tolerant calli was analyzed on SDS-PAGE. Distinct changes in protein patterns were observed between sensitive and tolerant

Table 1. Selection of NaCl tolerant haploid calli irradiated with various radiation doses on the medium with 1.25% NaCl.

Dose (Gy)	Experiment 1			Experiment 2		
	No. of callus inoculated	No. of calli survived	%	No. of callus inoculated	No. of calli survived	%
0	500	27	5.4	500	21	4.2
30	500	48	9.6	500	42	8.4
50	500	40	8.0	500	49	9.8
70	500	18	3.6	500	27	5.4
90	500	9	1.8	500	13	2.6

The number of propagated calli were counted in 40 days

Table 2. The effects of plant growth regulators for regeneration from the tolerant anther calli

Hromone Combination	No. of calli inoculated	No. of green spot	No. of regenerants	No. of albino
Kinetin (1 mg/L) +NAA (1 mg/L)	500	68	0	19
Kinetin (2 mg/L) +NAA (1 mg/L)	500	60	7	14
Kinetin (4 mg/L) +NAA (1 mg/L)	500	78	14	13

Regenerants were counted in 2 months.

calli (Figure 3E). The quantity of each protein as well as total protein was higher in tolerant calli than sensitive calli.

Screening for salt tolerance in M_2 generation

The NaCl-LD_{50} value for rice germination stage in the japonica rice cv. Hawsungbyeo was 0.75% NaCl (Figure 4A). However, 1% NaCl was used as a selective concentration to assure salt tolerance selection in this screening. This concentration extremely inhibited plant shoots as well as roots as a selective concentration. Using a quantitative selection index, such as plant height and root length, 68 M_2 lines were used to screen for salt tolerance.

From the germination to 2 leaf stage screen, none of the M_2 lines obtained from non-irradiated callus grown on medium with 1.0% NaCl was selected, but 5 M_2 lines obtained from irradiated callus grown on medium with 1.0% NaCl exhibited a varying degree of salt tolerance as compared to the original variety. Especially, two lines (M_2-9, -16) were extremely superior to the original variety. The roots of the tolerant lines were more viable than those of original variety on the solution with 1.0% NaCl (Figure 4B). Thus, it was able to obtain salt tolerant lines which were expressing salt tolerance at early growth stage. Like the original variety, the other M_2 lines died early.

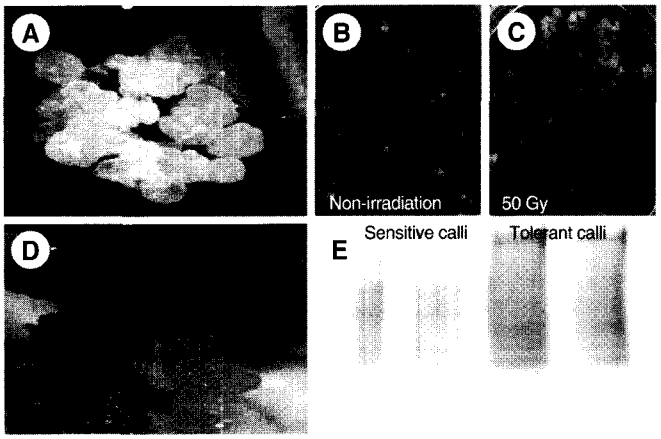

Figure 3. Induction of calli from anther (cv Hawsungbyeo), selection of salt tolerant calli irradiated and plant regeneration. (A) Anther derived calli in N_6 medium after 40 days of culture, (B) Non-irradiated calli on the medium with 1% NaCl, (C) Selection of callus tolerant to 1% NaCl after treatment of gamma-ray (50 Gy), (D) Regeneration of plant from tolerant calli, (E) SDS-PAGE analysis of sensitive and tolerant calli.

Table 3. The number of regenerants from the tolerant anther calli irradiated with various radiation doses.

Dose (Gy)	No. of calli inoculated	No. of green spot	No. of regenerants	No. of albino
0	500	49	5	21
30	500	75	14	17
50	500	65	19	11
70	500	56	8	14
90	500	32	3	9

Tolerant calli were cultured on the medium with 1%NaCl. Regenerants were counted in 2 months.

Mutant characterization

To examine morphological characterization, in addition to salt tolerant lines expressing salt tolerance at early growth stage, a total of 2,720 M_2 DH lines (160 from 0 Gy, 880 from 30 Gy, 1,240 from 50 Gy, 320 from 70 Gy and 120 from 90 Gy) were transplanted to paddy field. Variations for heading date (early and late), plant height, chlorophyll deficient (*albina, xanta, viridies and chlorina*) and leaf shape were observed (Table 5). The total mutation frequency ranged from 34.2% (30 Gy) to 75.8% (90 Gy) in comparison of 11.9% in the non-irradiated control. The most frequent type of morphological mutation observed was during the heading stage.

Screening for salt tolerance at seedling stage of M_3 generation

In this screening study for salt tolerance, about 2,500 M_3

Table 4. The number of doubled haploid (DH) lines in M_2 seeds derived from haploid calli irradiated with gamma ray.

Dose (Gy)	No. of regenerants	No. of DH lines	%
0	86	4	4.6
30	197	22	11.2
50	215	31	14.4
70	220	8	3.6
90	187	3	1.6

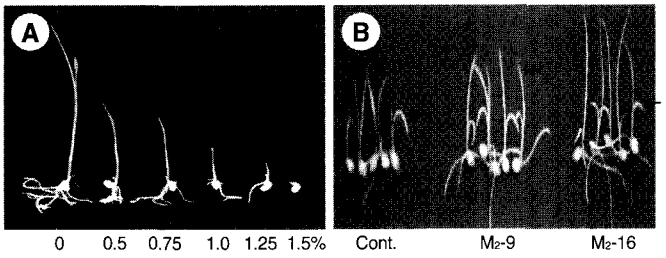

Figure 4. Selection of salt tolerant lines in the solution with 1% NaCl. (A) Effect of plant growth on medium with various NaCl concentrations. The NaCl-LD_{50} value for rice seedling stage in the japonica rice cv Hawsungbyeo was 0.75% NaCl. (B) Difference of growth of Hawsungbyeo (Cont.) and M2 mutants in 14 days on the solution with 1% NaCl. Shoots and roots of mutant were more increased than those of Hawsungbyeo. Diamonds are coleoptile, arrows are foliage leaf.

lines with an average 80% of fertile grain, from non-salt tolerant lines as well as salt tolerant M_2 lines previously selected were used. The salt tolerance mechanism was different according to the growth stages of germination and seedling. Based upon the seedling screen, none of the M_3 lines was obtained from non-irradiated callus grown on medium with 1.0% NaCl, but 13 M_3 lines obtained from irradiated callus grown on medium with 1.0% NaCl exhibited a varying degree of salt tolerance as compared to the original variety. Generally, rice seedlings were damaged on saline solution. External symptoms exhibited by plants included leaf chlorosis and wilting. Most leaves in the original variety turned yellow in ten days after culture in NaCl-connecting medium. However, the leaf damage in lines M_3-9-5 and -16-2, which were confirmed as salt tolerant lines at early growth stage of M_2 generation, was significantly less than that observed in the original variety. The ranking for salt tolerance of the different phenotypes is shown in (Figure 5A). It is known that these lines were salt tolerant beyond the germination stage. Like the original variety, the other M_3 lines died soon. In comparison of M_3-9-5 and M_3-16-2, the leaf damage in M_3-16-2 appeared less than in other lines. The salt tolerance of those lines was retested under the same condition with the same results (Figure 5B). From the findings of Figure 5, these lines (M_3-9-5 and M_3-16-2) are uniform to the salt stress at M_3 generation. Therefore, it is our thought that these lines are expressing the salt tolerance from germination to seedling stage. It is also believed that salt tolerance in M_2 generation bred true in M_3 generation.

Figure 5. (A) M_3 trials testing for salt tolerant screen at seedling stage (3-4 leaf) on the solution with 1% NaCl for 10 days. The original variety and tolerant genotype were diagonally placed and cultured and on the same tray. (B) Re-evaluation of Hawsungbyeo and mutant at above-menationed condition. M_3-16 indicates M_3-16-2. The damaged leaves of mutant were less than those of Hawsungbyeo.

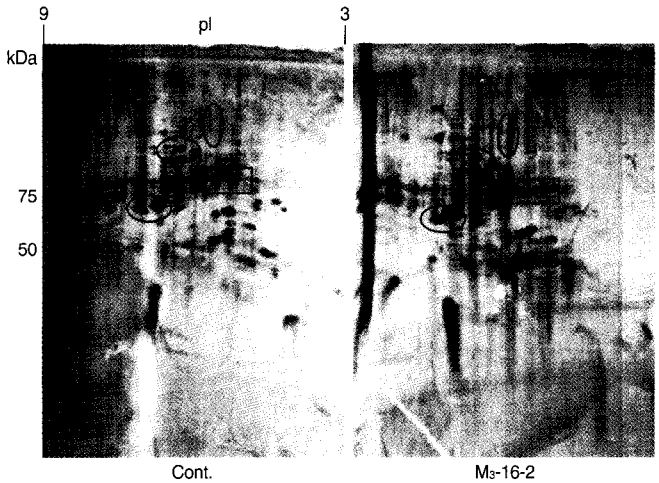

Figure 6. Effects of salt treatment on protein profile separated by two-dimensional gel electrophoresis. Proteins were extracted from leaves grown for 3 days on the solution with 1% NaCl. Protein marked by circle were more increased at tolerant line (16-2) and protein marked by rectangle was more induced at Hawsungbyeo. Arrow indicates that novel spot appeared in tolerant line.

Two-dimensional electrophoresis analysis

The 3-4 leaf staged plants of original variety and tolerant line M_3-9-5 and M_3-16-2 were supplied with 1% NaCl solution for 3 days. No visible change was observed until 48 hours after the treatment. The color of the lower leaves of 'Hawsungbyeo' turned from green into yellowish-green before it occurred in the tolerant lines after exposure to the saline solution for 72 hours. At this time the higher leaves of each genotype (M_3-9-5 and M_3-16-2) were harvested with ten plants, and the proteins were extracted. To verify gene expression in seedlings exposed to 1% NaCl for 3 days, the *in vitro* translation products were analyzed by two-dimensional electrophoresis (Figure 6). Among the expressed polypeptide spots, a new one about 52 kDa with a pI of 5.5 was observed in the tolerant line (Figure 6 →). The level of 100 kDa (pI 5), 90 kDa (pI 6.3), 85 kDa (pI 6) and 65kDa (pI 6.8) protein increased (Figure 6 ○) in relative amount at tolerant line, and two proteins, 77 kDa with a pI of 5.4 and 75 kDa with a pI of 5, decreased in tolerant line (Figure 6 □). Thus, significant changes seem to occur at this seedling stage.

Discussion

The present study showed that salt tolerance in calli and plant stage can be generated by irradiation. Mutagenesis of callus tissue may prove to be more efficient in producing useful mutants (Bhagwat and Duncan 1998). Cellular macromolecular components, membranes, enzyme activity, specific protein and DNA are markedly affected by ionizing radiation (Casarett 1986). Since the aim of this study was to focus on stable heritable variations rather than epigenetic variations on salt stress response, the combined radiation with an *in vitro* culture method was examined to select salt-tolerant lines. It is important to know that NaCl and gamma ray-LD_{50} are effective in selecting salt tolerance with irradiation. The first step of the

present study for irradiation was to choose the proper dose of gamma-ray. High doses will give a high frequency of mutation but few regenerated plants. Although the LD_{50} is generally used, some authors prefer to apply lower doses (LD_{20} and LD_{30}) since mutants are also induced with levels of mutagenic treatment that are not highly toxic for the tissues (Colijn et al. 1979). Therefore, 30, 50, 70 and 90 Gy under LD_{50} were selected as treatment doses. The findings summarized on Table 1 and Table 3 reveal that a range of 30 and 50 Gy radiation was effective for the selection of salt-tolerant calli and for regeneration. Similar results were observed by other authors (Chen et al. 2001; Lu et al. 1999; Zapata and Aldamita 1986), and gamma radiation, 20-30 Gy, was reported to stimulate growth and regeneration in *Atropa belladonna* and *Lavandala angustifolia* callus (Onisei et al. 1992). It was believed that minimal or proper stress on the callus by irradiation may not induce irreversible genotypic changes but can stimulate callus formation and plant regeneration. It was known that a dose of 30 and 50 Gy was stable for the generation of DH lines (Table 4). In the present experiment, wide genotypic differences were identified among the variants (Table 5).

It was not possible and also impractical to evaluate all of the regenerates plant progeny lines for salt tolerance up to maturity. Changes in salt tolerance at different growth stages were observed in rice (Pearson and Bernstein 1959; Lutts et al. 1995). Pearson and Ayers (1960) described the seedling stage in rice as the most salt-sensitive stage. Therefore, in this study, lines were screened for salt tolerance at different growth stages such as germination (Figure 4) and seedling stages (Figure 5). It was noticed that variations between and within lines for tolerance to salt stress and some lines at M_2 and M_3 generation showed higher salt tolerance than 'Hawsungbyeo'. It responded very uniformly to the imposed stress of 1% NaCl at M_3 generation. The technique of anther culture applied in connection with induced mutations was an effective way to increase mutation frequency, enhance selection efficiency and improve rice cultivars. The selected mutant could be utilized as salt tolerant varieties and/or as a source of salt tolerance in the hybridization breeding program.

Table 5. The frequency of mutants in M_2 generation.

Dose (Gy)	Plant Lines	Mutated character				
		Heading Date (%)	Plant Height (%)	Chlorophyll Deficient (%)	Leaf Shape (%)	Total (%)
0	160	2 (1.25)	1 (0.6)	2 (1.3)	14 (8.8)	19 (11.9)
30	880	150 (17.1)	34 (3.9)	94 (10.7)	23 (2.6)	301 (34.2)[b]
50	1240	267 (21.5)	184 (14.8)	120 (10.0)	30 (2.4)	601 (48.5)[a]
70	320	41 (12.8)	67 (21.0)	54 (17.0)	64 (23)	226 (70.6)[a]
90	120	21 (17.5)	9 (7.5)	37 (31.0)	24 (20)	91 (75.8)[a]
Total (%)	2720	481 (17.7)	295 (10.8)	307 (11.2)	155 (5.7)	1298 (47.7)[a]

[a, b]Significantly different within column at $P < 0.01$ and $P < 0.05$ using chi-square test, respectively.

The results of this study demonstrated that some proteins were enhanced at tolerant calli (Figure 3E) and plants (Figure 6) after salt stress. A correlation between increasing levels of salt tolerance and the level of some polypeptides in tolerant calli and plants suggests that altered expression of the genes for those proteins may be functionally involved in the ability of the cells to survive and grow in salt containing medium and solution. Particularly, it was detected that several proteins increased or newly induced by 2-DE analysis in tolerant line. It was believed that some proteins are involved in salt tolerance among these proteins. Therefore, the studies on the functions of protein are yet to be cleared.

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