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Physiological characterization of gamma-ray induced salt tolerant rice mutants

J.Y. Song[1,2*], D.S. Kim[1], M.-C. Lee[2], K.J. Lee[1], J.-B. Kim[1], S.H. Kim[1], B.-K. Ha[1], S.J. Yun[3] and S.-Y. Kang[1]

[1]Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, 1266 Sinjeong, Jeongeup 580-185, Rep. of Korea
[2]National Agrobiodiversity Center, National Academy of Agricultural Science, RDA, 88-20, Seodun-Dong, Suwon, Gyunggi-do 441-707, Rep. of Korea
[3]Department of College of Crop Agriculture & Life Science Agriculture & Life Sciences, Chonbuk University, Deokjin, Jeonju 561-756, Rep. of Korea

***First two authors equally contributed: J. Y. Song and D. S. Kim**

***Corresponding author: bioplant@kaeri.re.kr**

Abstract

High salinity in soil is one of the major abiotic stresses leading to the reduction of rice yield. In this study, two salt-tolerant rice mutants induced by gamma-irradiation were selected for *in vivo* and *vitro* screening techniques, and their physiological characteristics were evaluated. Using a salt stress screening based on the Standard Evaluation System, 2 candidate lines for salt tolerance (ST-87 and ST-301) were selected out of 1,500 M_6 mutant lines. The two ST-lines were further characterized through a physiological analysis, which examined factors such as the electrolyte leakage (EL), malondialdehyde (MDA), antioxidant, chlorophyll, total amino acid, and Na^+/K^+ contents. Compared with the WT, the ST-lines demonstrated lower levels then the activity of the enzymatic catalase in the contents of EL and MDA and the higher peroxidase under the salt condition. The chlorophyll and total carotenoid contents decreased more severely in the WT-lines than in those of the ST-lines. The total amino acid contents in the seedling leaves of the ST-lines were 2.2 and 1.4-times higher than in the WT. In the ratio of K^+/Na^+ of the ST and WT-lines, the ST-87 (2.7 fold) was significantly higher than that of the WT (1.4 fold). The selected rice mutant lines in our study will be used to increase the salt tolerance in the rice breeding program. Also, the screening methods used in this study will help to develop an excellent variety of rice with a tolerance to salt.

Introduction

When exposed to salt stress, plants usually generate a wide range of reactions, including cellular dehydration with concomitant osmotic changes. Prolonged exposure to salt stress triggers other consequences from several physiological responses, such as ion imbalance, mineral nutrition and water deficiencies, or a reduction in photosynthetic efficiency (Tsai et al., 2004). High-salt concentrations can also cause an ion imbalance and hyper-osmotic stress (Rhoades and Loveday, 1990). Plants have evolved physiological, biochemical, and metabolic mechanisms to increase their chance of survival against various environmental stresses (Ingram and Bartels, 1996; Pastori and Foyer, 2002). There are several strategies to cope with salt stress in plants: (1) the exclusion of salt ions from the cytoplasm into extracellular space, (2) the control of ion uptake by the roots and transport into the leaves, (3) toxic ion compartmentalization into the vacuole, and (4) the changes in membrane structure and antioxidative enzyme activity (Parida and Das, 2005). In addition, many plants accumulate proline in response to osmotic stress caused by salinity. An important staple food like rice is consumed by more than half of the world's population. However, salinity is one of the major abiotic stresses leading to the reduction of the rice yield (Mass and Hoffmann, 1977). Therefore the development of varieties with an increased salt tolerance is an important way to meet the growing food demand. Usually, salt tolerant plants are developed through *in vitro* and *vivo* screening techniques. The following parameters can be used to evaluate salt tolerance in rice: the seed germination, shoot length and root growth, fresh weight, electrolyte leakage (EL), malondealdehyde (MDA), chlorophyll contents, antioxidant enzyme activity, ion balance including sodium (Na) and potassium (K), and the total amino acid contents. Environmental stress induces cell membrane damage. Moreover, the reactive oxygen species (ROS) generated by salt stress can damage cellular structures, membrane lipids, and metabolism (Bartels and Sunkar, 2005). Therefore many studies have pointed to the elimination of ROS in plant cells under environmental stresses. Salinity can also cause a progressive loss of chlorophyll content, which leads to a corresponding reduction in light absorption by the plant leaves (Šiler et al., 2007). Plants develop defense systems against salt stress through the induction of antioxidant enzymes (Dionisio-Sese and Tobita, 1998). Plants have evolved to develop an antioxidant enzyme activity, which is a stress defense mechanism that reduces the harmful effects of free radicals during salt stress conditions. Numerous studies have indicated that the activity of antioxidant enzymes is correlated

with a plant's tolerance to salt, drought, and low-temperature stresses. In the present study, we described the selection of salt-tolerant lines via *in vivo* and *in vitro* screening techniques and tested the salt-tolerant lines in a saline field.

Results

Determination of optimum salt concentration

To determine the optimum concentration for the selection of NaCl-tolerant lines, Donganbyeo seeds were initially cultured for 40 days in various media, each containing a different NaCl concentration (0 to 2.5%). The shoot length, root length, number of roots, and fresh weight of the plantlets all decreased with an increase in the NaCl concentration. Because Donganbyeo did not grow in more than 1.5% NaCl, the optimum NaCl concentration for the selection of the salt-tolerant (ST) plants was determined to be 1.0% (171 mM) NaCl (Fig 1). The results of salt concentration based on the physiological responses to salt stress can be applied toward a screening of salt tolerant mutants.

Screening and selection of rice mutant lines under salt stress

To screen salt-tolerant mutants at the seedling stage, a total of 1,500 mutant lines (M_6) were used and then applied with a 171 mM NaCl salt solution. Among the 1,500 mutant lines, 56 mutant lines (those that scored less than 3 based on the Standard Evaluation System (SES)) were selected as putative salt tolerance lines. We then further investigated the germination frequency and survival rate of the 56 selected mutant lines. 12 mutant lines out of the 56 lines exhibited a survival rate 10% to 100% higher than the WT (data not shown). The exposure to salt stress resulted in a reduction in plant growth, the death of older leaves or whole plants, and fewer numbers of tillers in most of the 12 selected mutant lines, as well as in the WT. Based on a growth comparison among the 12 mutant lines, the two putative ST-lines (ST-87 and ST-301) were selected (Fig 2). To estimate whether a tolerance to salinity was improved in the ST-lines during the differential stage, we planted WT and ST-lines in soil trays without salt stress for 3 weeks, and then we irrigated them with 171 mM NaCl salt solutions. We demonstrated that salt stress significantly affected the growth of both the ST-lines and WT plants. After a salt level treatment in soil, the WT plant gradually wilted and did not show shoot elongation for 3 days. In contrast, ST-87 and ST-301 continued to grow for 7 days (Fig 3). The symptoms of major damage caused by salt stress, such as the wilting and yellowing of old leaves, and the death of older leaves or whole plants, were more moderate and delayed in the 2 ST-lines as compared with the WT symptoms.

Seed germination of salt-tolerant lines in salt solution

The germination rate and biomass production under salt conditions were used as indicators for salt tolerance (Zhang et al., 1995). To evaluate the differential responses of the 2 ST-lines to salt stress during germination, we analyzed the germination rate under 171 mM NaCl concentrations. The germination rate in the 2 ST-lines was affected differently by a treatment with a ½ MS medium that contained a 171 mM NaCl medium (Fig 4). The germination rates of the 2 ST-lines were higher than WT under the MS medium that contained the 171 mM NaCl. The ST-lines and WT plants germinated slowly, with a delay of approximately 3 or 5 days before the emergence of shoots (Fig 4A). At 7 days after the treatment, the germination rate of ST-87 and ST-301 was nearly 80-83%, whereas the germination rate of WT was 62% under the 171 mM NaCl treatment (Fig 4B). These results show that the 2 ST-lines were tolerant to high salt stress during the germination stage.

Test of salt tolerant mutant lines in a saline field

Rice is very salt-sensitive during the seedling stage. However, the salt tolerance in the early growth stages does not always correlate with salt tolerance at maturity. Therefore, the 2 selected ST-lines were screened for salt tolerance at different growth stages on reclaimed saline land. The characteristics of the 2 ST-lines were estimated in a saline field. To evaluate the agronomic characteristics of the 2 ST-lines, the WT plants were employed as controls. The plant height, panicle length, number of tillers per plant, number of grains per spike, grain sterility, 1000-grain weight at maturity for the 2 ST-lines, and the WT plant genotype in saline- and non-saline fields are given in Table 1. High salinity reduced the height of the plants. The average height of the WT, ST-87, and ST-301 was 95.9, 80.1, and 82.9 cm in a non-saline field and 53.5, 50.5, and 48.3 cm in a saline field, respectively. The plant height of the WT in the salt conditions was reduced by approximately 45.2% compared to the height of the WT in the non-saline field, while ST-87 and ST-301 showed a 37% and 41.8% reduction in plant height, respectively. During salt stress, the WT plant showed a significant reduction in the number of grains when compared to the 2 ST-lines. The number of grains per spike of the WT, ST-87, and ST-301 were 81.4, 100.0, and 96.0, in the saline field, respectively. The WT, ST-87, and ST-301 had 26.0, 25.6, and 24.6 g per 1,000 seeds in the non-saline field and 21.3, 23.1, and 23.7 g in the saline field, respectively. The 1,000 brown grain weight of the WT under salt conditions was reduced by approximately 18% when compared to the WT in the non-saline field, while ST-87 and ST-301 showed 9.7% and 3.6% reductions, respectively (Table 1). The reductions of the 1,000 brown grain weight in 2 ST-lines was significantly lower than that in the WT at $P < 0.01$.

Measurement of electrolyte leakage, chlorophyll and total carotenoid contents

Electrolyte leakage (EL) was considered to be an indicator of stress-derived cell damage (Allen et al., 1997). The ELs of the 2 ST-lines and the WT plants were evaluated in order to study the degree of membrane injury caused by salt stress. The WT and 2 ST-lines showed almost no change in electrolyte leakage under non-salt stress, while the EL was much higher in the WT than in the ST-87 and ST-301 after a 7-day salinity treatment. Compared to the WT, the EL was observed to be less than 50% for the two tolerant mutants under the salt treatment (Fig 5A).
A decrease in the degree of the chlorophyll and total carotenoid content is a common phenomenon under salt stress conditions. The chlorophyll a, b, and a + b contents in the WT plant were not only gradually decreased during the initial 7 days after the salt treatment, but were also significantly diminished more than 7 days after the salt treatment. However, the salt-tolerant lines did not show any notable change during the period (Fig 5B). The total carotenoid contents decreased more in the WT plant than in the 2 tolerant mutants during the entire process after the salt treatment (Fig 5C). Decreased chlorophyll and total carotenoid contents after the salt treatment in the WT were

Table 1. Agronomic characteristics of the WT and salt-tolerant lines in saline and non-saline fields. Each value is the mean ± SE of three independent experiments.

Plants	Test field	Culm Length (cm)	Panicle Length (cm)	No. of tiller	No. of seeds per spike	Seed fertility rate (%)	1,000 brown grain weight (g)
WT	NSF†	95.9±1.7a	19.8±0.6ab	13.2±0.8	110.3±2.6ab	90	26.0±0.6a
	SF‡	53.5±0.5c	17.4±0.4c	13.3±0.2	81.4±7.3d	89.7	21.3±0.1d
ST-87	NSF	80.1±0.4b	20.9±0.1a	14.2±0.9	108.3±2.6abc	92.3	25.6±0.2ab
	SF	50.5±0.2cd	18.9±0.3bc	13.7±0.6	100±5.4bc	96	23.1±0.3c
ST-301	NSF	82.9±0.6b	20.9±0.4a	15±1.1	116±3.7a	90.5	24.6±0.2ab
	SF	48.3±1.2d	18.6±0.2bc	15.3±1.1	96±3.6c	94.2	23.7±0.2c

†NSF, Non-saline field. ‡SF, Saline field. Note: Means with the same letters are not significantly different at $p<0.01$ as determined by Duncan's multiple test. -: Not significantly different at $p<0.05$

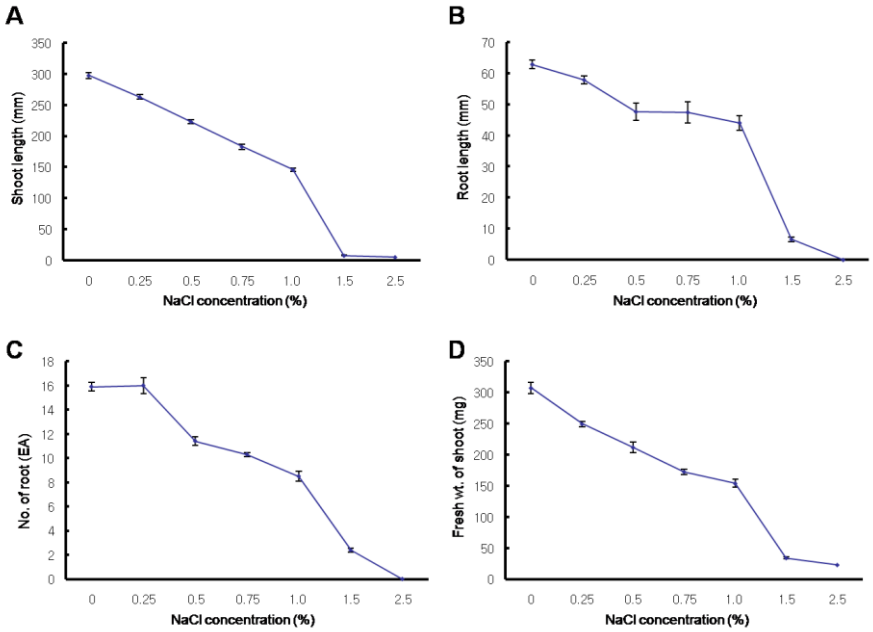

Fig 1. Growth of Dongan (WT) for 40 days in different media, each containing various NaCl concentrations: (A) shoot lengths; (B) root length; (C) number of roots; (D) fresh weight of seedlings.

observed from 7 days after the treatment. The degradation of the chlorophyll and total carotenoid contents is a normal status under abiotic stress conditions (Sultana et al., 1999). The chlorophyll pigment in rice is very sensitive to salt stress (Bahaji et al., 2002), especially in salt susceptible varieties (Asch et al., 2000). In this study, the chlorophyll and total carotenoid contents decreased more in the WT plant than in the two tolerant mutants.

Measurement of lipid peroxidation

The lipid peroxidation contents in the shoots of the two ST-lines and the WT (measured as the content of the MDA) are recorded in Fig 6. Salt stress caused an increase in the MDA contents among the tolerant and sensitive lines. The MDA contents showed almost no difference (5.4 nmol g^{-1} F.W.) in the 2 ST-lines and WT under normal growth conditions while the mutant lines gradually increased during the earlier days until 7 days after the salt treatment. After 7 days, the difference in the MDA contents between the mutant lines and the WT became apparent. However, the WT plant (23.6 nmol g^{-1} F.W.) was enhanced greater than ST-495 (19.0 nmol g^{-1} F.W.) and ST-532 (15.2 nmol g^{-1} F.W.) (Fig 6). Luna et al. (2000) mentioned that the MDA contents are higher in sensitive groups than in tolerant groups. The accumulation of MDA in seedlings was increased with prolonged salinity stress in all plants, including the ST-lines and WT, but showed a greater change in the WT plant than in the tolerant lines.

Antioxidant enzyme activities

Salt stress induces the production of active oxygen species, which are the product of an altered metabolism in chloroplasts and mitochondria during stress. The plant response to salt stress includes an elevated level of antioxidant enzyme activity, which because of salt stress, reflects the presence of oxidative stress (Gossett et al., 1994; Hernandez et al., 1995). To evaluate the contribution of the inductive responses of the antioxidant enzymes in the tolerance to salt stress, we measured the activities of the enzymes in the salt tolerant mutant lines and

Table 2. Effects of salinity on sodium (Na^+) and potassium (K^+) contents and the K^+/Na^+ ratio in the leaves of the WT and ST-lines after a 7-day treatment with 171 mM NaCl.

Plants	0 mM NaCl			171 mM NaCl		
	ppm		K/Na ratio	ppm		K/Na ratio
	Na	K		Na	K	
WT	21.7	708.3	32.7	625.1	870.0	1.4
ST-87	22.6	651.3	28.9	269.2	719.4	2.7
ST-301	32.3	888.4	27.5	495.5	886.0	1.8

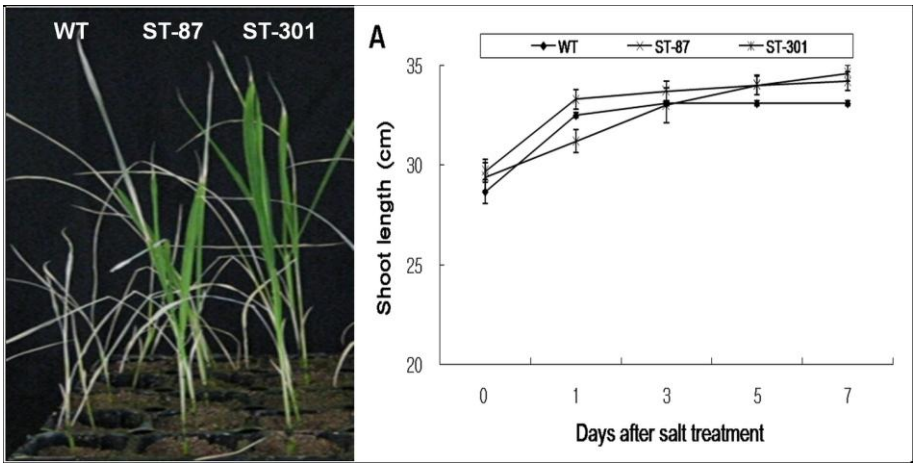

Fig 2. Growth comparison of the WT and 2 selected mutants. The seeds from the WT and 2 mutants were germinated and grown in soil trays (without salt) for 21 days, and then irrigated with 171 mM NaCl solutions for 7 days.

the WT plants. Before the salt stress treatment, there were no remarkable differences between the WT and the two ST-lines in CAT activity levels. However, after the treatment, the results showed that the CAT activity of the WT was decreased by approximately 23.8%, while the ST-87 increased 15.1% and the ST-301 showed no difference at 7 days (Fig 7A). The differences in the activity changes between the WT and 2 ST-lines were also shown in the POD activity. The POD activities of the WT plant had no change in the initial days up to 5 days after the salt treatment, but they decreased approximately 18.4% after 7 days. Compared with the 18.4% decrease of the WT, the POD activities of the ST-87 and ST-301 increased approximately 36.5% and 7.1% at 7 days after the salt treatment, respectively (Fig 7B). Consequently, the two mutant lines showed an increase in catalase (CAT) and peroxidase (POD) activity when compared to the WT at different points in time during the 171 mM salt treatment (Fig 7).

Determination of sodium and potassium ion contents

We measured the Na^+ and K^+ contents in the leaves of the salt tolerant line and wild-type plants under normal and salt-stress conditions. The ratios of Na^+ and K^+ are known to play an important role in the cytosol of plant cells under NaCl stress (Shi et al., 2002; Zhang et al., 2008). Under non-salt stress conditions, the Na^+ contents remained similar in both the WT and salt-tolerant lines. However, after 7 days of the salt treatment, the Na^+ contents were much higher in the leaves of the WT plant (28.8 fold) than in the ST-87 (11.9 fold) and ST-301 (15.3 fold). The K^+ content increased slightly in both the tolerant line and the wild-type plants, but no significant difference was observed in the WT and ST-87 plants (Table 2). The genotypes differed in their K^+/ Na^+ ratio. The ratio of K^+/ Na^+ in the ST-87 (2.7) and ST-301 (1.8) lines were much higher than in the wild-type (1.4) (Table 2).

Determination of total amino acid contents

The complete amino acids were determined in the seedling leaves of the two salt-tolerant lines and the wild-type plant before and after salt stress. Under normal conditions, the complete amino acid contents were higher in the WT plant when compared with the two ST lines. In detail, the contents of the ST-87 and ST-301 were observed to be approximately 31.5 and 39.8% lower than that of the WT. The amount of each amino acid was also higher in the wild-type plant. However, in the presence of salt stress, the total amino acid contents of the WT were significantly decreased by approximately 70.5% as compared with the WT plant in the absence of salt stress, while the contents of the ST-87 and ST-301 were slightly decreased by approximately 4% and 29.7%, respectively. Consequently, the complete amino acid contents in the seedling leaves of the ST-87 and ST-301 were 2.2 and 1.4 times higher than the wild-type after 7 days of the 171 mM NaCl treatment (Table 3).

Discussion

The present study was performed in order to select salt-tolerant mutant lines through an *in vivo* and *in vitro* screening and to investigate the physiological responses of the selected salt mutant lines under salt stress. The number of induced mutants is an important resource for the improvement of specific agronomic traits. In this study, 1,500 M_6 mutant lines were used for an initial screening under a 171 mM NaCl salt condition during the seedling stage. Based on the germination rate, survival rate, and seedling growth, we selected 12 salt tolerant

Table 3. Amino acid profiles in the leaves of the WT and ST-lines after a 7-day treatment with 171 mM NaCl.

Amino acid	0 mM NaCl			171 mM NaCl		
	WT	ST-87 (Ratio[a])	ST-301 (Ratio)	WT	ST-87 (Ratio)	ST-301 (Ratio)
Asp[b]	19.8	11.5(0.6)	14.1 (0.7)	2.5	9.0(3.6)	6.9(2.8)
Glu[c]	38.1	25.0(0.7)	25.9 (0.7)	9.1	22.2(2.4)	16.5(1.8)
Ser	17.6	12.3(0.7)	11.2 (0.6)	6.1	12.8(2.1)	9.2(1.5)
Gly	29.6	21.0(0.7)	16.7 (0.6)	6.5	18.0(2.8)	9.6(1.5)
His	12.3	8.5(0.7)	7.3 (0.6)	3.3	7.9(2.4)	5.0(1.5)
Arg	12.8	9.9(0.8)	7.8 (0.6)	4.2	9.6(2.3)	5.7(1.4)
Thr	26.7	20.0(0.7)	15.2 (0.6)	6.7	17.8(2.7)	9.9(1.5)
Ala	24.9	16.5(0.7)	15.0 (0.6)	8.8	16.7(1.9)	11.3(1.3)
Pro	37.0	26.3(0.7)	18.6 (0.5)	7.1	21.2(3.0)	12.0(1.7)
Tyr	4.6	4.2(0.9)	2.4 (0.5)	1.1	3.1(2.7)	1.4(1.3)
Val	23.8	16.6(0.7)	14.3 (0.6)	9.1	17.6(1.9)	11.7(1.3)
Met	3.4	2.5(0.7)	2.1 (0.6)	1.2	2.5(2.1)	1.6(1.3)
Cys	0.3	0.1(0.2)	0.2 (0.7)	0.1	0.1(2.1)	0.1(1.2)
Ile	14.6	9.1(0.6)	8.9 (0.6)	6.2	11.1(1.8)	7.6(1.2)
Leu	24.7	14.5(0.6)	15.1 (0.6)	10.8	18.4(1.7)	12.9(1.2)
Phe	23.8	17.1(0.7)	12.9 (0.5)	7.7	17.2(2.2)	9.6(1.2)
Trp	0.5	0.3(0.5)	0.3 (0.6)	0.3	0.4(1.5)	0.3(1.2)
Lys	3.7	2.4(0.6)	3.2 (0.9)	2.9	3.3(1.1)	2.9(1.0)
Total	318.1	217.8(0.7)	191.3(0.6)	93.6	208.9(2.2)	134.4(1.4)

Numbers given are ug/mg dry weight. [a] Ratio of the mean values of ST-lines to WT. [b] Aspartate + asparagine.
[c] Glutamate + glutamine.

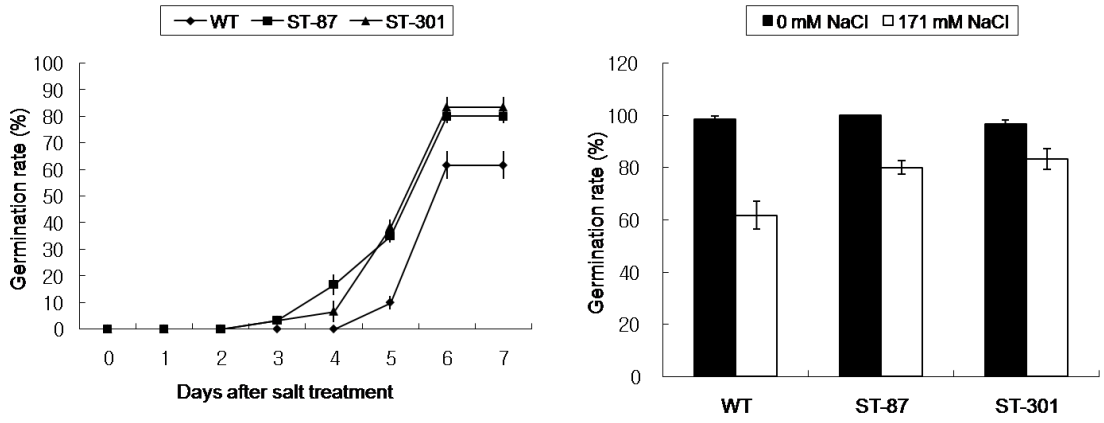

Fig 3. Seed germination of the WT and 2 ST-lines under a 171 mM NaCl treatment. The seeds were germinated for 6 days under a half-strength MS medium containing 171 mM NaCl (A). The germination rate of the WT and 2 ST-lines under a half-strength MS medium containing 171 mM NaCl after 6 days (B).

mutant lines under salt stress. Rice plants are highly sensitive to salinity during the early seedling stage (Pearson and Bernstein, 1959; Flowers and Yeo, 1981). However, in terms of the final yield components, the salt tolerance during the seedling growth stages does not always correlate with the salt tolerance during the maturity stages. Therefore, we carried out a screening of genotypes for salt tolerance in a saline field during the life-cycle of rice plants. We selected two salt tolerant lines, ST-87 and ST-301. The agronomic characters of the two mutant lines were much better than those of the original variety (WT) under a saline field. Electrolyte leakage (EL) in the cell membranes was considered to be an indicator of stress-derived cell injury (Allen et al., 1997). The degree of EL was low in the two ST-lines when compared to the WT plant under the salt treatment. In the larger plants, the cell membrane stability was used as a standard for tolerant and sensitive plants, and it was correlated with the abiotic stress tolerance (Blum and Ebercon, 1981). In the membranes of the ST-lines, a low EL might have less effect on either the membrane permeability or the maintenance of membrane integrity under salt stress. The chlorophyll and total carotenoid contents decreased more in a WT plant than in the two tolerant mutants. The ST-lines did not show any notable change during this period. The degradation of chlorophyll and total carotenoid contents is a normal occurrence during abiotic stress conditions (Sultana et al, 1999). Chlorophyll degradation in salt-stressed rice plants is positively related to the net photosynthetic rate (P_n) reduction or CO_2 assimilation, as well as the stomatal conductance and transpiration rate (Sultana et al, 1999). In addition, the chlorophyll and P_n reduction percentages in salt tolerant rice plants are lower than those in

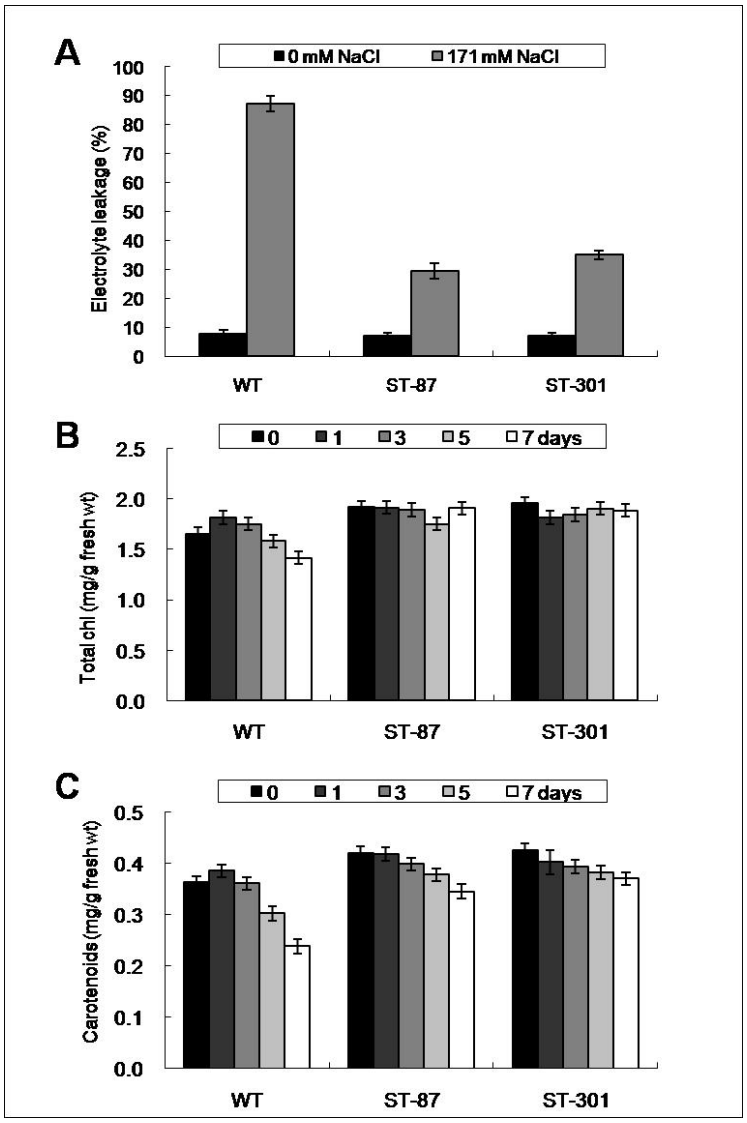

Fig 4. The electrolyte leakage analysis of the wild type and 2 salt tolerant mutant lines under a 171 mM NaCl after 7 days (A). Total chlorophyll (B) and carotenoid content (C) of the wild type and 2 salt-tolerant lines in a 171 mM salt solution for 7 days.

salt sensitive rice (Cha-um et al., 2007) plants. The physiological changes in salt-stressed rice plants directly impair their overall growth (Khan and Panda, 2008). High lipid peroxidation is a symptom of membrane damage under salt-stress conditions (Katsuhara et al., 2005). The accumulation of MDA in seedlings increased with prolonged salinity stress in all of the plants, including the ST-lines and WT, but it showed a greater change in the WT plant than in the tolerant lines. The low values of the MDA and EL contents are important signs of the oxidative-damage-limiting capacity under salinity, as demonstrated in various prior studies (Liang et al., 2003; Ruiz et al., 2005). The MDA is an end product of lipid peroxidation in the biomembranes, and the MDA content usually reflects the level of lipid peroxidation, while it also indirectly reflects the extent of membrane injury (Wang et al., 2009). Our study showed that the 2 ST-lines had significantly lower MDA contents than the WT lines. Exposure to salt stress induces a reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). An ROS causes negative oxidative stress effects, such as lipid peroxidation, membrane damage, and plant senescence in cellular structures (Foyer and Noctor, 2005). Plants have developed various strategies to cope with environmental stresses. The antioxidant system, a type of plant defense system, is activated for the scavenging of ROS through complex reactions (Wang et al., 2009). A superoxide radical is converted by superoxide dismutase (SOD) to H_2O_2 and the detoxification of H_2O_2 by catalase (CAT) and various peroxidases (POD). In our study, we obtained an increase in the CAT and POD activities in the shoot of the two ST-lines for different periods of time under salt stress; however, those activities decreased in the WT plant. An increase in the activities of the antioxidant enzymes are correlated to the salt tolerance of many plants, as has been reported in previous studies (Gossett et al., 1994; Wang et al., 2009). The accumulation of amino acids in salt-stressed plants equalizes the osmotic potential of the cytoplasm, thus maintaining the cellular function and structure (Dubey and Rani, 1989; Seki et al., 2007). Livia et al. (2007) reported that common salt stress induces an increase in the free amino acid concentration, while severe salt stress results in cellular damage which prevents the accumulation of free amino acids. In the present study, it is likely that the WT plant did not accumulate amino acids through severe salt stress. However, the ST-lines maintained their amino acid contents. The contribution of amino acid accumulation toward improved salt tolerance was demonstrated by the comparison of nine rice genotypes with different salt tolerances, because the tolerant genotypes had a higher total soluble amino acid content than the sensitive genotypes following salt stress (Dubey and Rani, 1989). In combination with gamma-ray induced mutations, implementing an *In vitro* culture in rice plants is an effective way to improve salt-tolerance. In this study, we introduced the systemic procedures for the selection of salt tolerant rice plant mutants, and 2 promising mutant lines, ST-87 and ST-301, were finally selected.

Materials and methods

Plant materials

The high quality seeds of *Oryza sativa* L. cv. 'Dongan' that demonstrated high yield traits, along with good lodging and disease resistance (Shin et al 1997), were irradiated with gamma-rays that were generated using a ^{60}Co gamma-irradiator (150 TBq of capacity; ACEL, Ontario, Canada) at the Korea Atomic Energy Research Institute (KAERI). The irradiated seeds and the controls were sown at the Breeding Research Farm at the KAERI in 2003. The M_1 plants were harvested individually and carried forward to the M_2 generation. The genetically fixed mutant lines (M_6 generation) with excellent agricultural characteristics were selected in 2004. To select the salt tolerant rice, 1,500 mutant lines were used in this study.

Selection of optimum salt concentration in vitro

In order to determine the concentration of the 50% lethal dose (LD_{50}) under the salt concentration, the hulled seeds of cv. Dongan were sterilized with 5% sodium hypochlorite and cultured in a ½MS agar medium containing 0, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.5% NaCl concentrations for 40 days. The plants were grown in a controlled growth room at 27±1°C and 45±5% relative humidity under a 16 h d^{-1} photoperiod of 1500 lux light intensity.

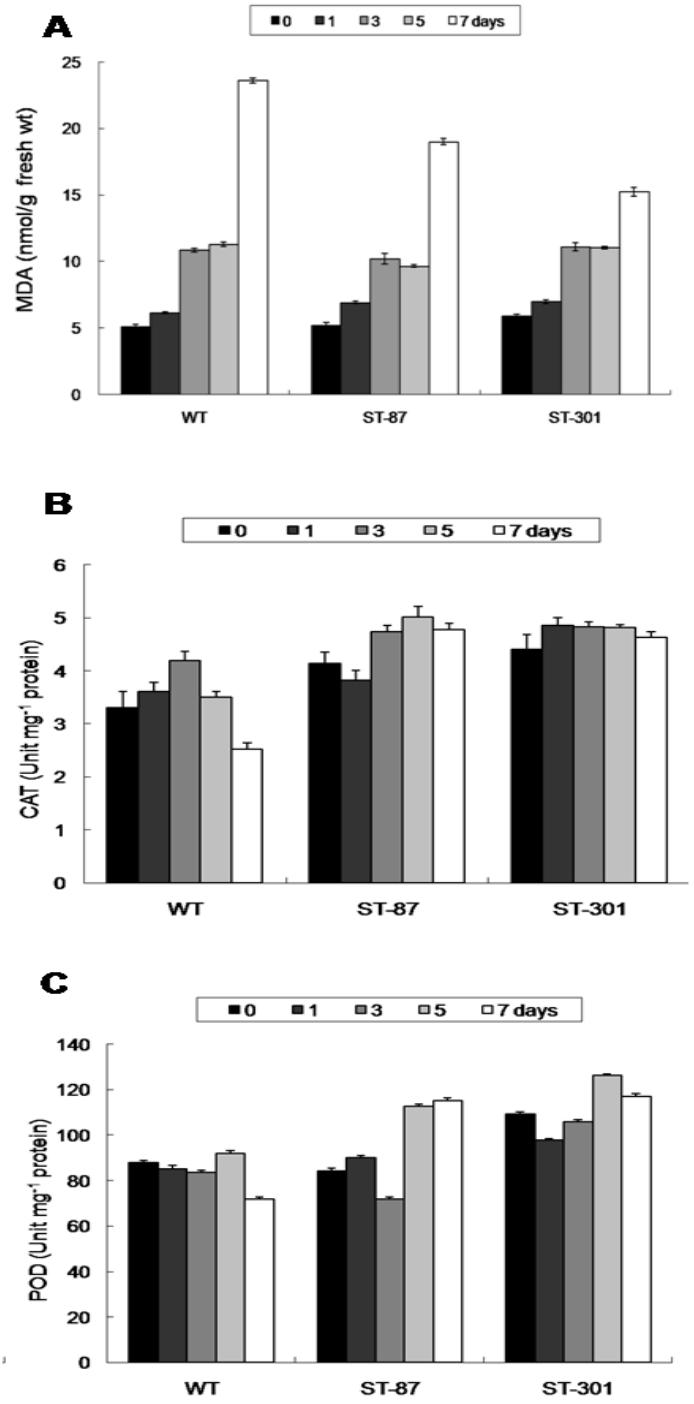

Fig 5. Changes in the Malondealdhyde (MDA) content and antioxidant enzyme activity of the wild type and 2 salt-tolerant lines in a 171 mM salt solution for 7 days. (A) MDA, content; (B) Catalase avtivity; (C) peroxidase activity.

Selection of salt tolerant rice mutants

To test the salt tolerance at the seedling stage, the seeds of cv. Dongan (WT) and the 1,500 mutant lines (M_6 generation) derived from cv. Dongan were planted in trays with three replications and with 15 seeds per pot for each of the mutant lines. After the WT and mutant line seeds were germinated under normal condition, the seedlings were treated with a 171 mM NaCl salt solution for 7 days in a glass house. To select the salt tolerant mutant lines, the germination frequency and survival rate of the mutant lines and the WT were investigated. To evaluate the 56 selected mutant lines at the differential stage, they were grown in soil trays (without salt) for 30 days and then irrigated with a 171 mM NaCl formulated solution for 25 days. A germination test was conducted using two mutant lines that were germinated in a ½MS media containing 171 mM NaCl with three replications (n = 3), and 15 seeds per line. The percentage of germination was obtained by counting the number of germinated seeds for 7 days. Salinity symptoms were scored according to the Standard Evaluation System (1 - 3: tolerance, 5: moderate and 7 - 9: sensitive) developed at IRRI (IRRI, 1996).

Testing salt-tolerant rice mutants lines in a saline field

The selected mutant lines at the seedlings stage were tested each year in a saline field at the Gyehwado Substation of the Honam Agricultural Research Institute. The salinity level of the saline field was approximately 0.8% (136 mM NaCl, E.C = approximately 13 dS/m). After the mutant lines (ST-87 and ST-301) grew seedlings, the mutant lines were transplanted into normal and saline fields in three replicates 30 × 15 apart. The original variety was used as the control, and every mutant line was transplanted. The seeds were harvested on an individual plant basis.

Determination of electrolyte leakage

To determine the electrolyte leakage (EL), fresh leaf (0.1 g) sample cuttings of 5 mm segments were placed in test tubes containing 10 mL of distilled deionized water and then incubated in the water bath at 32°C. After 2 hours, the initial electrical conductivity of the medium (EC_1) was measured using an electrical conductivity meter (TOA Electronics Ltd., Kobe, Japan). The samples were autoclaved afterwards at 121°C for 20 min to completely kill the tissues and release all of the electrolytes. The samples were then cooled to RT, and the final electrical conductivity (EC_2) was measured. The rate of electrolyte leakage (EL) was calculated based on the following formula: $EL = EC_1/EC_2 \times 100$.

Measurement of lipid peroxidation

Lipid peroxidation was determined in terms of the malondialdehyde (MDA) contents through a trichloroacetic acid (TCA) reaction, as described by Dhindsa and Matowe (1981) and Ding et al. (2008).

Chlorophyll extraction and measurement

A fresh leaf sample (0.5 g) was ground in 5 ml of 100% acetone. The sample was centrifuged at 10,000 g and 4°C for 10 min. One milliliter of the supernatant fraction was removed and re-centrifuged for 5 min at 10,000 rpm. For the pigment determination, the supernatant was diluted with 100% acetone. The absorbance of the mixture was measured at 470, 644.8, and 661.6 nm with a spectrophotometer (UVIKON 923, Bio-Tek Instruments, Winooski, Vermont, USA). The chlorophyll content was calculated using the extinction coefficients reported by Lichtenthaler (1987).

Determination of antioxidant enzyme activities

The frozen-leaf samples (0.5 g) were ground into a fine powder with a chilled mortar and pestle and then placed in liquid nitrogen. The powder from each sample was homogenized in a 50 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was used directly for the enzyme assays. The total protein concentration was measured according to the Bradford method (Bradford, 1976). According to the method described by Aebi (1974; 1984), the catlase (CAT) activity measurement was based on a decrease in absorbance at 240 nm for 1 min due to the degradation of H_2O_2. The reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.0) and 10 mM of H_2O_2. One unit of CAT is defined as the amount necessary to decompose 10 mM of H_2O_2 per minute at 25°C. The peroxidase (POD) activity was determined using a modification of the method described by Putter (1974) and Kwak et al. (1995), which used pyrogallol as a substrate. The reaction mixture contained a 0.1 M potassium phosphate buffer (pH 6.0), 8 mM of H_2O_2, and 15 mM pyrogallol. One unit of POD activity was defined as the amount of enzyme necessary to obtain H_2O_2 from pyrogallol in 20 s at 420 nm.

Determination of the cation concentrations

The cations (Na^+ and K^+) were analyzed using a Dionex ICS3000 ion chromatograph (IC, Dionex, CA, USA). Separation was achieved using an IonPac Analytical CS12A cation column (4 × 250 mm, Dionex, CA, USA) with 20 mM MSA (methansulfonic acid). The samples were injected at a rate of 1 mL min^{-1} at the National Instrumentation Center for Environmental Management (NICEM)

Total amino acid analysis

A total amino acid analysis of the seedlings was measured using the Pico-Tag method (Waters) (Tarr, 1986).

Statistical data analysis

All of the collected data were subjected to an analysis of variance and a means separated using Duncan's Multiple Range Test at a 5% or 1% probability level.

References

Aebi H (1974) Catalase. In: Bergmeyer HU (Ed) Methods of Enzymatic Analysis, Academic Press, New York

Aebi H (1984) Catalase in vitro. Method Enzymol 105:121-126

Allen RD, Webb RP, Schake SA (1997) Use of transgenic plants to study antioxidant defenses. Free Rad Biol Med 23:473-479

Asch F, Dingkuhn M, Dorffling K (2000) Salinity increase CO_2 assimilation but reduces growth infield-grown, irrigated rice. Plant Soil 218:1-10

Bahaji A, Mateu I, Sanz A, Cornejo MJ (2002) Common and distinctive responses of rice seedlings to saline- and osmotically-generated stress. Plant Growth Regul 38:83-94

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24:23-58

Blum A, Ebercon A (1981) Cell membrane stability as measure of drought and heat tolerance in wheat. Crop Sci 21:43-47

Bradford M (1976) A rapid and sensitive method for the quantitation of microgramquantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248-254

Cha-um S, Supaibulwatana K, Kirdmanee C (2007) Glycinebetaine accumulation, physiological characterizations, and growth efficiency in salt tolerant and salt sensitive lines of indica rice (*Oryza sativa* L. spp. *indica*) response to salt stress. J Agron Crop Sci 193:157-166

Dhindsa RS, Matowe W (1981) Drought tolerance in two mosses correlated with enzymatic defense against lipid peroxidation. J Exp Bot 32:79-91

Ding YC, Chand CR, Luo W, Wu YS, Ren XL, Wang P, Xu GH (2008) High potassium aggravates the oxidative stress induced by magnesium deficiency in rice leaves. Pedosphere 18:316-327

Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. Plant Sci 135:1-9

Dubey RS, Rani M (1989) Influences of NaCl salinity on growth and metabolic status of protein and amino acids in rice seedlings. J Agron Crop Sci 162:97-106

Flowers TJ, Yeo AR (1981) Variability in the resistance of sodium chloride salinity within rice (*Oryza sativa* L.) varieties. New Phytol 88:363-373

Foyer CH, Noctor G (2005) Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ 28:1056-1071

Gossett DR, Eddie PM, Cran LM (1994) Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci 34:706-714

Hernandez JA, Olmos E, Corpas FJ, Sevilla PF, Del Rio LA (1995) Salt-induced stress in chloroplast of pea plants. Plant Sci 105:151-167

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47:377-403

IRRI (1996) Standard evaluation system for rice. 4th ed. Manila: international Rice Reasearch Institute. P. 52

Katsuhara M, Otsuka T, Ezaki B (2005) Salt stress-induced lipid peroxidation is reduced by glutathione S-transferase, but this reduction of lipid peroxides is not enough for a recovery of root growth in Arabidopsis. Plant Sci 169:369-373

Khan MH, Panda SK (2008) Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. Acta Physiol Plant 30:81-89

Kwak SS, Kim SK, Lee MS, Jung KH, Park IH, Liu JR (1995) Acidic peroxidases from suspension-cultures of sweet potato. Phytochemistry 39:981-984

Lee BH, Zhu JK (2010) Phenotypic analysis of Arabidopsis mutants: electrolyte leakage after freezing stress. CSH Protoc 1: doi:10.1101/pdb.prot4970

Liang Y, Chen Q, Liu Q, Zhang W, Ding R (2003) Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare* L.). J Plant Physiol 160:1157-1164

Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Method Enzymol 148:350-382

Livia SS, G´abor K, Zolt´an S, G´abor G (2007) Deletions of chromosome 5A affect free amino acid and polyamine levels in wheat subjected to salt stress. Environ Exp Bot 60:193-201

Luna C, Seffino LG, Arias C, Taleisnik E (2000) Oxidative stress indicators as selection tools for salt tolerance in *Chloris gayana*. Plant Breed 119:341-345

Mass EV, Hoffmann GJ (1977) Crop salt tolerance-current assessment. J Irr Drain Div ASCE 103:115-134

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Safe 60:324-349

Pastori GM, Foyer CH (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of ‘‘redox’’ and abscisic acid-mediated controls, Plant Physiol 129:460-468

Pearson GA, Bernstein L (1959) Salinity effects at several growth stages of rice. Agron J 51:654-657

Premachandra GS, Saneoka H, Fujita K, Ogat S (1992) Leaf water relations, osmotic adjustment, cell membrane stability, epicuticular wax load and growth as affected by increasing water deficits in sorghum. J Exp Bot 43:1569-1576

Putter J, Peroxidase. In: Bergmeyer HU (Ed) Methods of Enzymatic Analysis, Academic Press New York

Rhoades J, Loveday, J (1990) Salinity in irrigated agriculture. Amer Soc Civ Eng Irrigation Agric Crop 30:1089-1142

Ruiz JM, Blasco B, Rivero RM, Romero L (2005) Nicotine-free and salt tolerant tobacco plants obtained by grafting to salinity-resistant rootstocks of tomato. Physiol Plant 124:465-475

Seki M, Umezawa T, Urano K, Shinozaki K (2007) Regulatory metabolic networks in drought stress responses. Curr Opin Plant Biol 10:296-302

Shi H, Quintero FJ, Pardo JM, Zhu JK (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. Plant Cell 14:465-477

Shin M-S, Ko J-K, Kim B-K, Lee K-S, Lee J-K, Ha K-Y. Yang B-K, Shin H-T, Lee S-Y, Kim C-H, Cho S-Y. (1997) A new hight yielding and good quality rice cultivar adaptable to direct seedling “Donganbyeo”. Korean J Breed Sci 29:495

Šiler B, Mišiæ D, Filipoviæ B, Poroviæ Z, Cvetiæ T, Mijoviæ A (2007) Effect of salinity on in vitro growth and Photosynthesis of common cemtuaty (*Centaurium erythraea* Rafn.). Arch Biol Sci 59:129-134

Sullivan CY, Ross WM (1979) Selection for drought and heat resistance in grain sorghum. In: Hussel H, Staples R (Eds) Stress Physiology in Crop Plants, John Willy and Sons, New York

Sultana N, Ikeda T, Itoh R (1999) Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. Environ Exp Bot 42:211-220

Tarr GE (1986) Manual Edman Sequencing System. In: Shively JE (Ed), Methods of Protein Microcharacterization (pp. 155–194). Humana Press, Clifton, NJ

Tsai YC, Hong CY, Liu LF, Kao CH (2004) Relative importance of Na^+ and Cl^- in NaCl induced antioxidant system in roots of rice seedling. Physiol Plant 122:86-94

Wang WB, Kim YH, Lee HS, Kim KY, Deng XP, Kwak SS (2009) Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. Plant Physiol Biochem 47:570-577

Zhang GH, Su Q, An LJ, Wu S (2008) Characterization and expression of a vacuolar Na/H antiporter gene from the monocot halophyte *Aeluropus littoralis*. Plant Physiol Biochem 46:117-126

Zhang GY, Guo Y, Chen SL, Chens SY (1995) RFLP tagging of a salt tolerance gene in rice. Plant Sci 110:227-234