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Selection and Characterizations of Gamma Radiation-Induced Submergence Tolerant Line in Rice

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Abstract

The combination of a radiation technique with an *in vitro* culture system was appiled to develop submergence tolerant rice. The 3,000 M_3 lines with an average 80 percent of fertile grain were utilized for the selection of submergence tolerance. Salt tolerant lines were selected based on high plant height, root length and root number after submergence in plastic pots. Of the lines tested, the tolerant line (403-6) showed a dramatic difference in morphological traits under submergence compared to its original variety (Dongjinbyeo). It was suggested that genetic variations between the original variety and M_3-403-6 did exist. The levels of α-amylase and alcohol dehydrogenase activities were significantly increased in the mutant line compared to its original variety. The mutant with greater tolerance showed less electrolyte leakage indicating a greater membrane integrity and better survival. Also, this line was much more resistant to a salt stress of 1.25% than the original variety. The proline level of the line was significantly (P<0.01) higher than that of the original variety. The relationships between the inhibition of growth caused by stress and the physiological changes in the plant cell were discussed.

Key words: Irradiation, submergence tolerance, α-amylase, alcohol dehydrogenase, electric conductivity, proline

Introduction

Seedling vigor is a plant's ability to emerge rapidly from soil or water (Heydecker 1960). Also, root vigor is important for direct -seed cultivation of rice. To develop rice varieties with vigorous seedling and root, it is necessary to introduce/generate genetic factors related to the traits. Conventional plant breeding is being employed to develop varieties resistant to stresses, but the progress is time-consuming. There is a great need to exploit all genetic variabilities that can be used in breeding for stressful environments. When existing germplasm fails to provide the desired recombinant, 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, from generation of variability, through selection, to multiplication of the new genotypes (Maluszynski et al. 1995; Novak 1991).

The vigor of seedling and root is difficult to measure in the field because of the large environmental effects. Therefore, greenhouse and laboratory screening procedures have been used to measure seedling and root vigor in rice. A greenhouse technique, slantboard tests and laboratory screening based on shoot length were utilized to estimate submergence tolerance, respectively (Adair 1968; Jones and Peterson 1976; Redona and Mackill 1996; Miura et al. 2002). There is a need to develop lines with enhanced seedling and root vigor to improve rice stand establishment and for use in genetics, molecular and physiological studies.

A subsequent submergence is the cause of severe hypoxia or anoxia during germination and the early growth stage. The submergence tolerance was associated with the supply of sugar maintained by α-amylase, the generation of ATP through ethanolic fermentation produced by alcohol dehydrogenase (Hwang et al. 1999; Drew 1997), and the looseing of electrolytes and ions, which are index of the plant cell death (Pfister-Sieber and Braendle 1994). Also, salt causes the most serious problem with plant growth and productivity in agriculture. The salt tolerance is involved in proline accumulation in plant cells

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(Greenway and Munns 1980), which serve as a protective agent for cytoplasmatic enzymes (Paleg et al. 1984), and a reservoir of nitrogen and carbon sources for post-stress growth (Fukutaku and Yamada 1984).

The objectives of the present work were to produce and assess a new submergence tolerant line in rice by utilizing *in vitro* mutagenesis of variety cultivated; Determine the relationships between the factors related to the tolerant mechanism and submergence tolerance; And investigate cross protection for salt.

Materials and Methods

The development of plant materials

After sterilizing dehusked seeds of 'Dongjinbyeo' with 5% sodium hypochlorite, the seeds were cultured on a N_6 medium with 2 mg/L 2,4-D and incubated in the dark at 25±1°C for callus induction. Callus was exposed to different strength of r-ray (0, 30, 50, 70 and 90 Gy) from a ^{60}Co source in a gamma room of the KAERI (Daejeon, Korea) for 24 hours. The calli divided into small pieces (0.5-1.0 mm diameter) were inoculated on the N_6 medium within 72 hours. The surviving calli were maintained for 3 passages, one passage was 40 days. For regeneration, the surviving calli were transferred on to the MS medium (0.5 mg/L NAA + 2 mg/L BAP) for 30 days. Regenerates (M_1) were assigned a plant line number and grown to maturity in a field. Two hundred M_2 lines derived from M_1 plants, except for the expressed poor plant type (droopy leaves and weak culm), were harvested on an individual plant basis.

Screening for submergence tolerance at seedling stage of M_3 generation

The 3,000 M_3 lines, derived from M_2, with an average 80 percent of fertile grain were used in the experiment. A screen of the M_3 generation was done in a pot with distilled water with a 50 mm depth for 10 days at 25°C for three replications. Screening was completed at 10 days to avoid the overgrowth of seedling. Trays contained 16 pots (20×50×30 mm; 1×w×h), with one pot for each line. Of the sixteen lines per tray, one was a parent line and this was not subjected to the *in vitro* technique. The trays were filled with 30 seeds per line. The trays were kept until the plants grew to the 3- leaf stage. At this time, tolerant lines were selected based on plant height, root length and root number. The thirty seeds of the tolerant lines, which were confirmed as tolerant lines in the first experiment, were retested in the above-mentioned conditions.

Enzyme activity, electric conductivity value and proline content

Twenty whole caryopses were germinated to 5 mm in length for α-amylase, five rice seedlings, which were grown till the 3-leaf stage, for alcohol dehydrogenase and five rice seedlings for electric conductivity value were ground for one determination. The analysis was carried out with three replications. Total protein was measured using the Bradford (1976) method. *Alpha*-enzyme activity was assayed at pH 4.5 (Terashima et al. 1995) using soluble potato starch as the substrate (Kumagai et al. 1990). The increase in reduced sugar equivalents was determined using the 3,5-dinitrosalicylic acid assay method. Using maltose as a standard, one unit of α-amylase activity was defined as the amount of enzyme needed to produce 1μmol of maltose per min. Alcohol dehydrogenase activity was measured spectrophotometrically by monitoring the oxidation of NADH as described by Kato-Noguchi (2000). Electric conductivity value was measured using a conductivity meter by stirring distilled water with the chopped rice seedling of 5 mm in length. Proline was extracted and its concentration was determined by the method of Bates et al. (1973). Proline level was expressed as μg/g fresh weigth.

Cross protection for salt

To identify cross protection for salt, the submergence tolerant lines were raised on a solution containing 1.25% NaCl in tray with 16 pot (20×50×30 mm; 1×w×h) for 14 days. After 14 days of salinization, salt tolerant lines were selected from the original variety, 'Dongjinbyeo'. The fifteen seeds of the tolerant lines, which were previously confirmed as tolerant lines, were replaced in a petridish (9 cm in diameter) with a 1.25% salt solution, and cultured in the above-mentioned growth conditions. Then, the tolerant lines were transferred to a pot with NaCl-free water for recovery and cultured for 7 days. A grain was considered alive if it continued to grow and produced normal roots and shoots.

Performance evaluation

The t-test value at 1% and 0.1% level of significance was used to determine the superiority of the tested lines from the original variety, 'Dongjinbyeo'.

Results

Genetic variation for vigor traits

The original variety and mutant seeds were exposed to 10 days submergence stress. All rice seedlings survived, but the elongation of the rice seedlings was inhibited by the anoxic stress. In the 3,000 M_3 lines, there were 64 submergence tolerant lines. Of these lines, the M_3-403-6 line was superior to the original variety and the other lines (Figure 1). From Table 1, it can be seen that the M_3-403-6 line and the original variety had a significant difference (P<0.01 and 0.001) in plant height, root length and root number during the submergence period. It was confirmed that genetic variation between the original variety and M_3-403-6 did exist.

Enzyme activity and electrolyte leakage

During the germination of cereal seeds, there is a rapid breakdown of starch in the endorsperm (Dennis and Blakeley 2000). The most studied of the starch-hydrolyzing enzymes is α-amylase, which is synthesized in the aleurone layer surrounding the starchy endorsperm. Note that ethanolic fermentation regenerates NAD^+, which is required for glycolysis. *Alpha*-amylase and alcohol dehydrogenase activity were measured to identify submergence sensitivity and tolerance. *Alpha*-amylase activity was measured untill the length of coleoptile reached 5 mm in length.

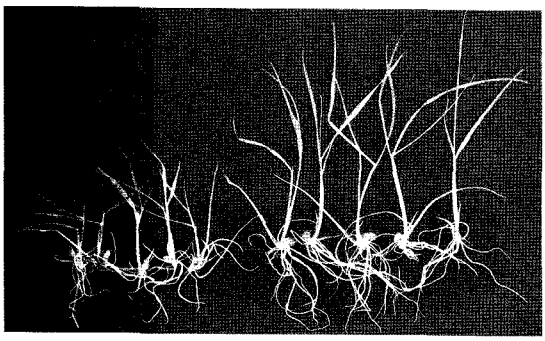

Figure 1. M_3 trials testing for submergence tolerant screen on the distilled water for 10 days. The original variety and tolerant genotype were placed on the same pot. The shoot height and root length of tolerant line are higher than those of Dongjinbyeo.

Table 1. Performance of submergence tolerant M_3 lines cultured in pot with distilled water of the 5 cm in dept for 14 days

Entries	Shoot height	Root length	No. of roots
Dongjinbyeo	3.4±0.18	4.1±0.15	8.0±0.3
M_3-403-6	8.2±0.19[a]	6.0±0.17[b]	11.8±0.5[b]

[a, b]Significant at 0.1% and 1.0% level. Average of 5 repeats.

The α-amylase activity of original variety and tolerant line was similar in the coleoptile grown in the aerobic condition about at 3.7 unit/mg protein. But the α-amylase of the M_3-403-6 seeds in submergence condition showed about 1.55-fold higher activity than the original variety (Figure 2). In submergence tolerant species, ethanol production was enhanced through the fermentation pathway. The end enzyme of the pathway was alcohol dehydrogenase (ADH). The ADH activity of the original variety and the tolerant line was similar in the shoots grown in the aerobic condition at about 6.1 μmol/f.w. (g)/min. The tolerant line showed about a 1.4 fold increase in ADH activity level compared with the original variety when grown in submergence (Figure 3). Rapid growth of plants rapidly was inhibited

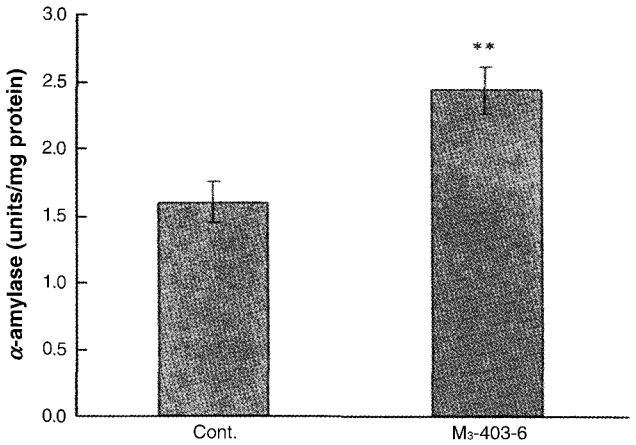

Figure 2. *Alpha*-amylase activity in rice seeds. Rice seeds were imbibed and incubated under submergence conditions. Seed caryopses were homogenized and assayed in triplicate for α-amylase. Average of 3 repeats. Error bars represent the standard error of the mean. ** indicates significant at 1.0% level.

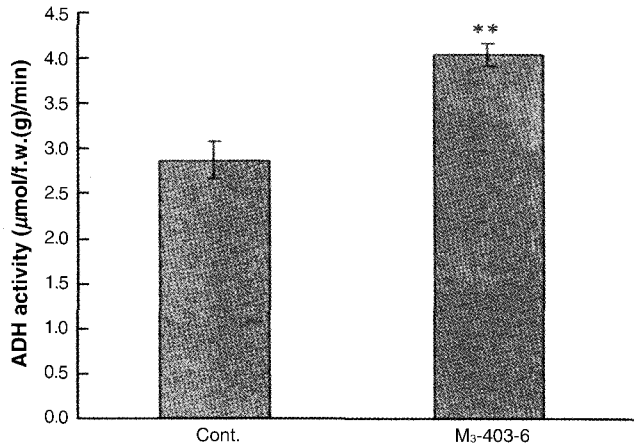

Figure 3. Alcohol dehydrogenase activity in rice seedlings. Rice seeds were imbibed and incubated under submergence conditions. Seedlings were homogenized and assayed in triplicate for alcohol dehydrogenase. Average of 3 repeats. Error bars represent the standard error of the mean. ** indicates significant at 1.0% level.

because of the cell acidifies of the cytoplasm. The electric conductivity value was indexed so that it will make an estimate of the damage to plant cells.

The original variety showed about a 1.7-fold increase in electric conductivity value compared to its tolerant line (Figure 4). In the non-stressed treatment, the electric conductivity value was too small to detect.

Cross protection for salt

To identity cross protection for salt, the 64 submergence tolerant lines were cultured on a solution with 1.25% salt for 14 days, then transferred to a salt-free solution for 7 days. Of the 64 lines, the submergence tolerant line with salt tolerance was M_3-403-6. The line showed salt tolerance on a solution with 1.25% salt, but there was clearly a difference when transferred to a salt-free solution (Figure 5B). The original variety, cultured on a solution with 1.25% salt, did not survive after the second culture on a salt-free solution (Figure 5B). The plant height, root length and root number of the tolerant line were statistically significant at 1% and 0.1% level compared with its original variety (Table 2).

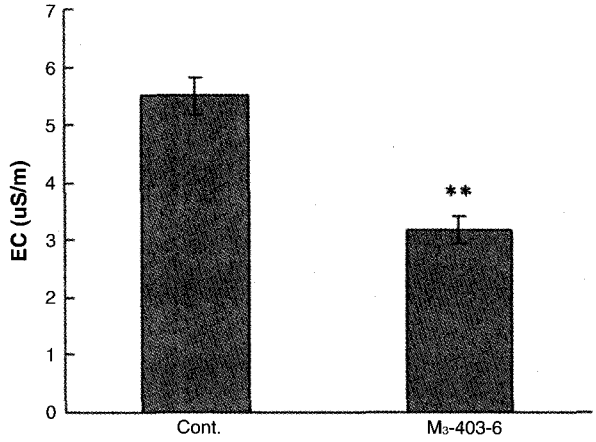

Figure 4. Leakage on metabolites of seedlings. Rice seeds were imbibed and incubated under submergence conditions. Seedlings were chapped at a interval of 10 mm and assayed in triplicate for electrolytes and ions. Average of 3 repeats. Error bars represent the standard error of the mean. ** indicates significant at 1.0% level.

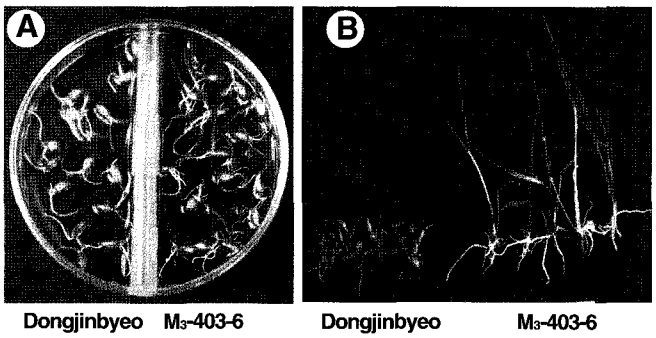

Figure 5. M_3 trials testing for salt tolerant screen on the solution with 1.25% salt for 14 days (A) and performed the second culture on salt-free solution (B). The original variety and tolerant genotype were placed on the same pot. The shoot height and root length of tolerant line are more than those of Dongjinbyeo.

Proline content

Proline is believed to protect plant tissues against salt by acting as an N-stotage compound, osmo-solute and hydrophobic protectant for enzymes and cellular structures. The proline levels in shoots are shown in Figure 6. The proline level of the original variety and tolerant line was similar in shoots grown in a NaCl-free condition 16 ug/f.w. (g). However, the M_3-403-6 line accumulated more proline than the original variety. In the tolerant line the proline content increased ~ 1.52-fold compared to the control (Figure 6).

Discussion

In this study, the comparative effects of submergence on shoot and root were studied with the aim of developing submergence tolerant lines. It was able to induce mutants by the

Table 2. Characterization of submergence tolerant M3 lines respond to salt

Entries	Shoot height	Root length	No. of roots
Dongjinbyeo	1.0±0.05	2.2±0.4	2.0±0.1
M_3-403-6	3.9±0.17[a]	6.0±0.2[b]	3.2±0.1[b]

[a, b]Significant at 0.1% and 1.0% level. Average of 5 repeats.

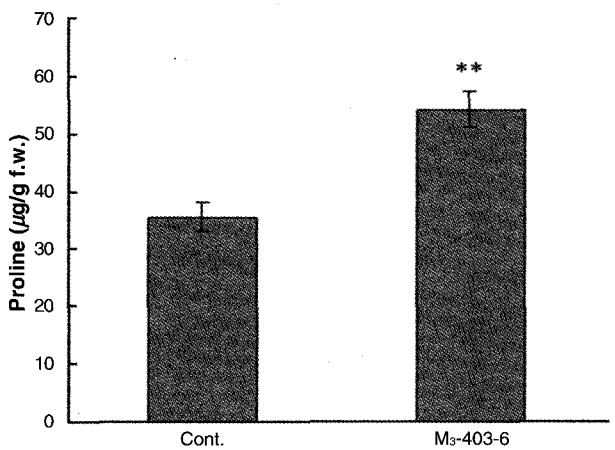

Figure 6. Comparison of proline content in rice shoot. Rice seeds were imbibed and incubated under 1.25% salt concentration. Seedlings were homogenized and assayed in triplicate for proline contents. Average of 3 repeats. Error bars represent the standard error of the mean. ** indicates significant at 1.0% level.

combination of irradiation and *in vitro* culture selection and selected submergence tolerant lines, a near isogenic line of Dongjinbyeo, by using a simple method in trays filled with distilled water. Among them, the vigor of seedling and root of M_3-403-6 was far superior to those of the Dongjinbyeo. It is believed that the length of shoot and root was good indicators of seedling vigor. And this confirms the effectiveness of irradiation and the simple method with a tray for developing and evaluating submergence tolerant line. Substantial genetic variations that can be exploited to improve rice cultivars for submergence tolerance were identified in this study. The similar genetic variations for submergence tolerance were confirmed by using test tube and slantboard tests of rice (Miura et al. 2002; Redona and Mackill 1996). And it indicates that some mutagenesis affecting better growth ability against submergence stress occur by the irradiation of plants.

Besides shoot and root length, there are some factors affecting submergence tolerance, such as germinability (Sasaki 1974), the resistance to oxygen deficiency (Sasahara and Ikarashi 1989), α-amylase activity (Hwang et al. 1999), alcohol dehydrogenase activity (Kato-Nobuchi 2002) and electrolyte leakage (Buwalda et al. 1988). The interactions among these factors seem to affect submergence tolerance. Therefore, the separation of the mixed effects is necessary to elucidate the effect of each factor. Near isogenic lines like mutation for a target factor would be useful to identify the effect of each factor.

Maintaining the supply of sugar is especially important because of the low energetic efficiency of fermentation. One of the features that clearly distinguishes plants that are tolerant vs. sensitive to anoxia is the ability of anoxia-tolerant species to break down starch to maintain their sugar pools under anoxic conditions (Kennedy et al. 1980). Mobilizing the starchy endorsperm of seeds into sugars requires the concerted action of a battery of hydrolytic enzymes, including α-amylase, β-amylase and α-glucosidase. Of these, the regulation of α-amylase has been studied because of its key role in the hydrolysis of native starch granules (Sun and Henson 1991). The α-amylase activity and growth of rice seedlings under anoxia correlates with the increased activity of the α-amylase in the embryo are illustrated (Figure 3, Table 1, Figure 4). This could help to determine whether the differences in α-amylase activity contribute to the differential tolerance under submergence.

When oxygen is limited, which is formed under submergence, fermentation was carried out. These reactions are finally catalyzed by alcohol dehydrogenase. The tolerance for submergence is associated with a greater concentration of ATP and with sustained levels of glycolysis and ethanol fermentation (Drew 1997). The increase in the tolerance was paralleled by an increase in alcohol dehydrogenase (Andrews et al. 1994). From Figure 5, the increase of alcohol dehydrogenase activity in the mutant indicates that anaerobic glycolysis may be more active in the mutant than in the original variety, since alcohol dehydrogenase is the main end enzyme of anaerobic carbohydrate catabolism in plants (Drew 1997). The shoot increase was much greater in the M_3-403-6 than in the original variety (Figure 3, Table 1). This shoot elongation was found to closely correlate with the activity of ethanolic fermentation in the shoots of rice (Setter et al. 1994; 1997). The ethanolic fermentation pathway allows the continuation of glycolysis owing to pyruvate consumption and the recycling of NAD^+ when the oxygen supply is limited and glycolysis replaces the Krebs cycle as the main source of ATP (Kennedy et al. 1992; Ricard et al. 1994). It was confirmed that the growth rates of these tissues correlate closely with their energy charge and enzyme activity.

Leakage of ions as a consequence of oxygen deprivation has been described previously for wheat (Chirkova et al. 1991) and potato (Blom 1999). It occurs when the cells suffer from a lack of energy and the ATP-active ion carrier-proteins cannot function. As a consequence, the membrane becomes depolarized and leaky and the cells loose their electrolytes and ions (Pfister-Sieber and Braendle 1994; Buwalda et al. 1998). Therefore, plants with enough energy (ATP) available during submergence or with a low energy requirement can survive better (Guglielminatti et al. 1995). The electric conductivity value of the M_3-403-6 line was much more decreased than that of the Donjinbyeo ($P<0.01$).

According to the findings of the experiment for salt, the M_3-403-6 line was resistant to salt when salt stress occurred 7 days after placing and a clear difference was observed after the second culture on a salt-free solution, and there was no recovery of the original variety (Figure 7, Table 2). It was known that the M_3-403-6 line has tolerant trait for salt and submergence. The golden promise variety has occurred mutation for yield, plant height (semi-dwarf) and salt tolerance (Forster 2001). Proline accumulation in plant cells exposed to salt or water stress is widespread phenomenon (La Rosa et al. 1991; Lutts et al. 1999). It has been speculated that it can serve as an osmotic regulator (Pollard and Wyn Jones 1979), a protector of enzyme denaturation (Paleg et al. 1984), a reservoir of nitrogen and carbon sources (Fukutaku and Yamada 1984). Proline contents in the M_3-403-6 line treated by 1.25% salt increased significantly compared to the original variety ($P<0.01\%$). It is confirmed that the differences in the accumulation patterns of proline in Dongjinbyeo and its mutation line indicate the involvement of different proline mechanisms related to salt tolerance. The submergence tolerant plant line with salt tolerance developed here has the potential to elucidate the molecular mechanism involved in tolerance as well as in rice breeding for stress tolerance.

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