Review Paper

Selection of Radiation-induced Salt Tolerant Rice Mutants by *in vitro* Mutagenesis

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INTRODUCTION

Radiation induced mutagenesis has been studied extensively for the identification and isolation of a plant gene and in plant breeding programs. Rapid progress in plant tissue culture methods is attributed to the availability of many mutants that can be generated for specific biochemical events that are difficult or impossible to use with intact plants (Carlson 1970; Larkin and Scowcroft 1981; Schaeffer and Sharpe 1983; Kim *et al.* 2004). This may be achieved by co-culturing plant tissues with pathogens or their toxins and by manipulating a medium composition, e.g. selection for a resistance to salinity, drought and amino acid analogs. Although, it is possible to select mutants with a salt tolerance by a somaclonal variation, mutant frequencies are often lower in plant systems due to inefficient recovery techniques, as well as the presence of multiple gene copies located on different chromosomes, and by cell-to-cell interactions in even the most ideal suspension culture systems (Schaffer and Sharpe 1983). Therefore, *in vitro* mutation induction techniques provide tools for a rapid creation and increase of the selection frequencies of cell types for which there is no obvious counterpart in field environments (Maluszynski *et al.* 1995). Such techniques enable a greater use of mutated genes for a crop improvement, the cumulative effect of which may result in a more desirable character than the original variety. Rice, an important staple food, is consumed by more than half of the world's population. It is sensitive to a salt stress; therefore, development of varieties with an increased salt tolerance is required. The possibility of a screening for a salt stress in a culture makes *in vitro* methods attractive for developing stress tolerant plants (Lee *et al.* 2003). In the present protocol, we described the selection of salt tolerant cell lines via *in vitro* mutagenesis, regeneration, a consecutive selection through each generation (M_2-M_3), and a screening of salt tolerant lines in a saline field. Fig. 1 shows a flow chart of the procedures for the selection of the salt tolerant lines.

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PROTOCOL

Section 1: *In vitro* selection of salt tolerant rice mutant cell lines

Embryogenic callus induction

Calli were initiated from embryos of hulled seeds of a japonica rice cultivar 'Dongjinbyeo', a high quality and high yielding cultivar with a lodging and disease resistance. Seeds were sterilized in 10% sodium hypochlorite with 2 ~ 3 drops of Tween 40 for 30 min. The embryos were placed on a callus induction medium that was a N_6 basal medium supplemented with 2 mg l^{-1} 2,4-D 30 g l^{-1} sucrose and 0.4% (w/v) phytagel in sterile Petri dishes, and incubated in the dark at 25 ± 1°C for a callus initiation (Table 1). The medium was adjusted to pH 5.8 and autoclaved for 20 min at 1.1 kg cm^{-2} pressure at 121°C. Induced calli were once more sub-cultured for proliferation with 4 weeks intervals.

Decision of optimum NaCl concentration and irradiation dose for salt tolerant calli

It is very important to decide the optimal salt concentration

for a selection of salt tolerant calli. The calli were divided into small pieces (0.5 ~ 1 mm diameter) and inoculated on to a N_6 medium containing various concentration (0, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5%) of NaCl, and the survival rate and fresh weight of the surviving calli for 40 days were investigated. The callus pieces were irradiated with 0, 30, 50, 70, 90, and 120 Gy gamma-ray from a ^{60}Co source, and the fresh weight and survival rate of calli for 40 days were also investigated (Fig. 2A). In each experiment, there were three replicates for each treatment.

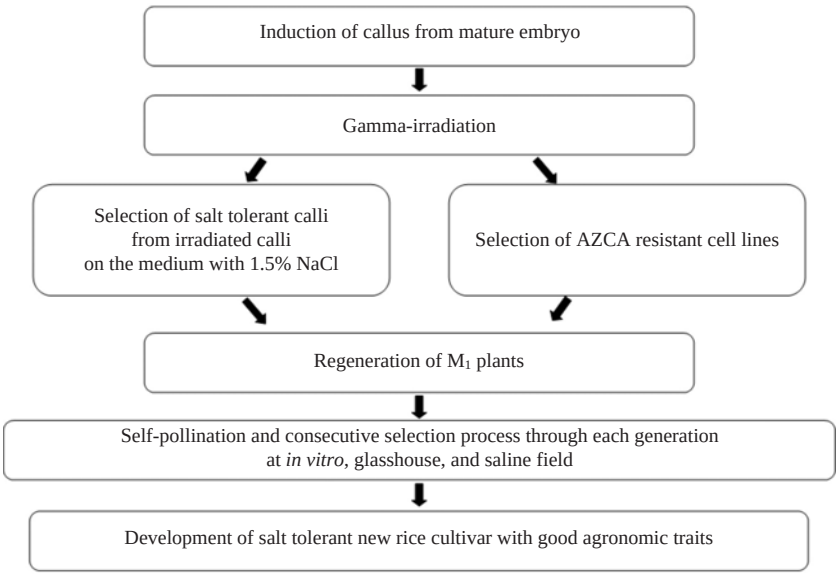

Fig. 1. Flow chart of procedures for the selection of salt tolerant lines.

Table 1. Composition of callus induction and regeneration media for *in vitro* selection of salt tolerant cell lines

Medium type	Components
Callus induction	N_6 basal, 30 g l^{-1} sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, pH 5.7 ~ 5.8, 4 g l^{-1} phytagel
Callus selection	Same as callus induction medium with 1.0% NaCl
Regeneration	MS basal, 30 g l^{-1} sucrose, 0.5 mg l^{-1} Naphthaleneacetic, 2 mg l^{-1} Benzylaminopurine, pH 5.7 ~ 5.8, 4 g l^{-1} phytagel
Plant selection	MS basal, 30 g l^{-1} sucrose, 1.0% NaCl, pH 5.7 ~ 5.8, 4 g l^{-1} phytagel
Root formation	Same as plant selection medium, but no NaCl

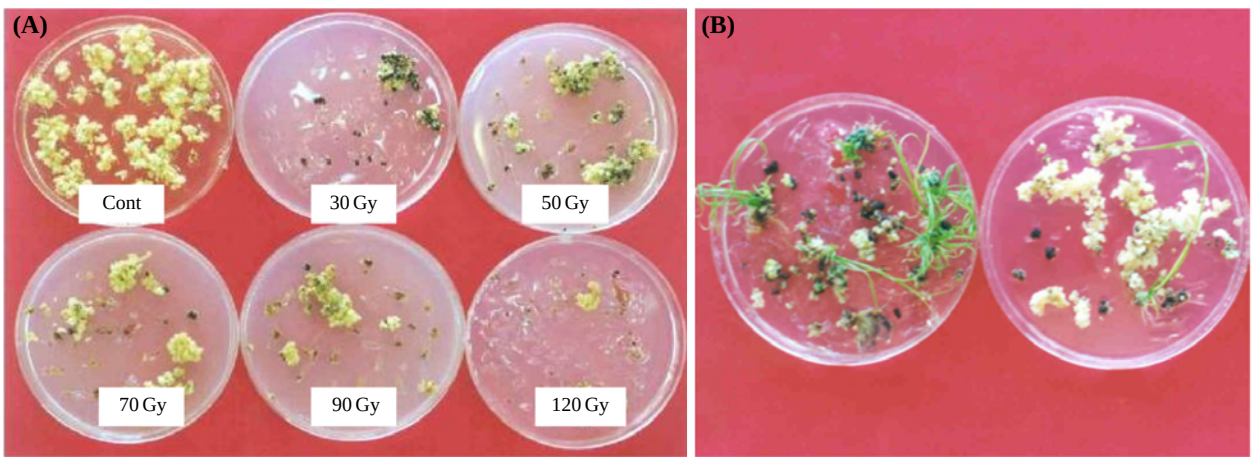

Fig. 2. Comparison of growth calli irradiated with various radiation does on the medium with 1.5% NaCl. Control plate contains non-mutagenised callus on the medium without salt (A). Regeneration from salt tolerant calli on the medium (B).

Selection for NaCl tolerance

The propagated calli were divided into small pieces (0.5～1 mm diameter) and inoculated on a N_6 medium supplemented with 1.5% NaCl and 70 Gy, which were the optimum concentration of NaCl and irradiation dose for the salt tolerant calli, respectively (Table 1). The selected salt-tolerant calli were maintained at the same concentration.

Regeneration

The selected NaCl tolerant calli were transferred and cultured for a regeneration on a MS medium supplemented with 0.5 mg l^{-1} NAA and 2 mg l^{-1} BAP, but without salt for 30 days (Table 1, Fig. 2B). Plantlets were cultured at 10 cm on a half-MS medium followed by an acclimation at room temperature. Some of the regenerates were transferred to a medium with 0.75% NaCl to confirm a transmission of a tolerance from the callus to the regenerated plants. Each of the regenerated plants (M_1) were assigned numbers, and grown to maturity in a NaCl-free soil in a field. Standard crop management practices were followed, which included an application of 11; 7; 8; kg/10a of N; P; K. Harvested lines were sterilized with a disinfectant for the seed.

Section 2: Selection of salt tolerant rice using high proline accumulating rice mutants induced by *in vitro* mutagenesis

When plant cells are exposed to stresses, such as drought, salinity and cold, a proline accumulation is a widespread phenomenon. Under stress conditions proline increase plays a key role for an osmotic adjustment in a large number of plant species (Delauney and Verma 1993). Mutants resistant to proline analogs are thought to be a useful material for studying the proline biosynthesis and saline, drought, and cold resistance in higher plants (Miflin *et al.* 1983). Azetidine-2-carboxylic acid (AZCA), a proline analog, is a natural product found in some species of *Liliaceae*, which inhibited an irreversible cell growth by competition with proline for incorporation into protein (Cella *et al.* 1982; Hyun *et al.* 2003). This incorporation presumably leads to an altered protein conformation and function, and accounts for the cytotoxic effects of AZCA (Lodato *et al.* 1984; Song *et al.* 2007). In many cases, the mechanism of a resistance to amino acid analogs has been shown to be due to the insensitivity to a feedback inhibition (Widholm 1974). Mutants with an altered feedback mechanism express more resistant to a feedback inhibition and this results in a greatly elevated free proline accumulation. The decrease of uptake inhibitors by a change in cell membrane permeability and an inactivation of inhibitors have also been reported as mechanisms of an amino acid analog resistance.

Embryogenic callus induction

Dehusked seeds cv. 'Donganbyeo' were sterilized in 70% ethanol for 30 sec followed by an addition of 5% sodium hypochlorite with 2～3 drops of tween-20 for 20 min. The embryos of seeds were rinsed 3～4 times with sterile distilled water and placed on a N_6 medium supplemented with 2 mg l^{-1} 2,4-D 30 g l^{-1} sucrose and 0.4% (w/v) phytagel in sterile Petri dishes, and incubated in the dark at 25±1°C for a callus initiation. The medium was adjusted to pH 5.8 and autoclaved for 20 min at a 1.1 Kg cm^{-2} pressure at 121°C. Induced calli were sub-cultured once more for a proliferation.

Decision of on the optimum AZCA concentration

Induced callus was divided into 30 pieces (about 1～2 mm) with a sterile razor and transferred to the N_6 basal medium containing 1.5, 2, 3, and 4 mM AZC concentrations. Thirty callus pieces were plated per each concentration with 10 replications and the data on the survival rate and fresh weight were obtained at 40 days after culture. Based on the survival rate and fresh weight, the optimum AZCA concentration was assessed as 3 or 4 mM.

Gamma ray treatment and selection of AZCA resistant cell lines

Effect of gamma rays on the growth of the callus was investigated after 40 days of irradiation. Callus was irradiated with 0, 30, 50, 70, 90, and 120 Gy of gamma rays. Selection of AZCA resistant cell lines was conducted on a selection medium containing 3 or 4 mM AZCA.

Regeneration of AZCA Resistant Cell lines

AZCA resistant callus was transferred to the MS medium containing 0.2 mg l^{-1} indoleacetic acid (IAA), 3 mg l^{-1} kinetin, and 30 g l^{-1} sucrose solidified with 0.5% (W/V) phytagel. The medium was adjusted to pH 5.8 and autoclaved for 20 min at 121°C. Regenerants were obtained at 30 days after being transferred to the regeneration medium. The plantlets were grown further in a bottle containing a half strength MS medium without hormone. Regenerants from the resistant callus were grown in a solution containing a half strength MS basal and 2 mM AZCA to determinate their resistance.

The shoots of the regenerants were removed except for 5 cm in length and the roots were removed thoroughly for the 2～3 leaf stage seedlings for a comparison of their revival ability. AZCA resistant regenerants were selected after 2 weeks.

Section 1 & 2 (continued)

Selection of the optimum salt concentration at a plant level

It is very important to determine the salt concentration which can cause 50% lethal (LD_{50}). Hulled seeds were sterilized with 5% sodium hypochlorite, and cultured on a half strength MS agar medium containing 0, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.5% NaCl concentrations for 40 days. During this period, the highest efficiency of a plant culture was achieved when the plants are grown in a controlled growth room temperature at 27±1°C 16 h and 8 h of light and dark conditions, respectively, for the determined optimum salt concentration.

M_2 generation and screening of the M_3 seedlings

Three hundred M_2 lines from M_1 plants, except those with a 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 harvested as M_3 generation (5,000 lines) showing normal grain fertility (above 80%) on an individual plant basis. Each M_3 lines 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). A salinity tolerance of the M_3 lines was screened *in vitro* and in a glasshouse with trays for 0.75% NaCl (Fig. 3A & B). In the screening in the glasshouse, each tray had 20 pots (60 × 150 × 30 mm), with one from the parent, not cultured *in vitro*. The trays were filled with fine soil, which was commercially made for a rice culture, and 80 seeds per line were placed in each pot at a depth of 5 mm. The trays were watered with tap water until the 3 to 4 leaf stages. At that stage, excess water was drained, and the trays with rice seedlings were refilled with solution containing 0.75% (E.C=13 mS) salt and 1 g l^{-1} 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 a 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 IRRI. To re-estimate the salt tolerance, each thirty seeds of the tolerant and sensitive lines were placed in glass bottles (5 × 7 cm) with a salt-free solution and 0.75% salt treatment 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 a quantitative measurement of their salt tolerance.

Selection of salt tolerant lines in a saline field

M_3 or M_4 lines selected at the seedlings stage were tested in a saline field near the sea coast (Fig. 3C). The salinity level of the saline field was cal. 10～14 mS. Heterogeneity of the soil salinity in the field was an inherent problem for evaluating their performance. However, this problem was minimized with plot sizes of 2.4 × 4.5 m^2. From each line, 30 plants were transplanted in two replicates 30 × 15 apart in a saline and normal field. The original variety was used as the control, and transplanted once every 8 lines. Standard crop management practices including an application of 20-8-8 kg/10a of NPK were applied. Seed was harvested on an individual plant basis.

Performance evaluation at maturity

All lines were tested in a field trial in five replications, and evaluated at maturity (Fig. 4). Plant height, panicle length, tiller number, survival rate, spikelets per plant and grain weight were recorded. Analysis of the variance and means

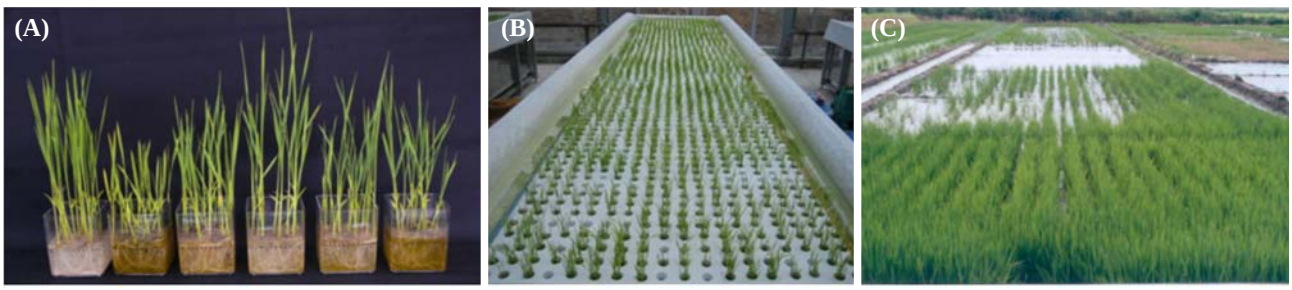

Fig. 3. Screening of selected salt tolerant rice mutant lines in *in vitro* (A), glasshouse (B), and saline field (C).

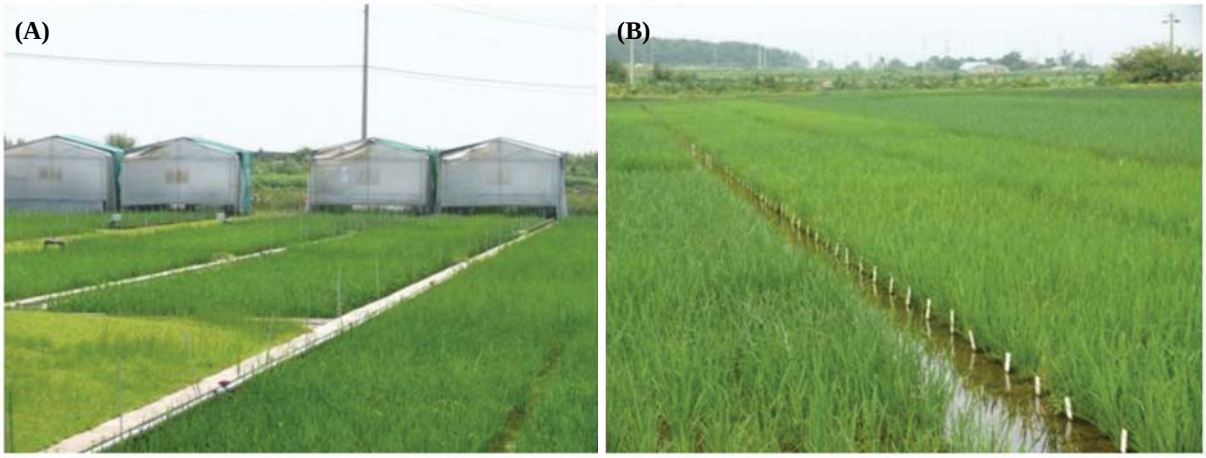

Fig. 4. Comparison of agronomic characteristics of M_3 lines cultured in the 0.8% saline field at the Gyehwado Substation of the Honam Agricultural Research Institute, the Rural Development Administration (A) and non-saline field (B).

were compared with the data from the saline and non-saline fields. The t-test value at 5 and 1% level of significance was used to determine the superiority of the tested lines over the control.

Efficiency and applications

Variations in the progenies of the *in vitro* mutagenized plant materials have already been reported and they probably originated from the genetic instability created during a plant tissue culture in combination with induced mutations (Maluszynski *et al.* 1995). *In vitro* culture in rice in combination with gamma-ray induced mutations is an effective way to improve the salt-tolerance in rice. The mutation and selection frequency were greatly increased, and the selected mutants were stable. Such mutant lines could be utilized as a source of salt-tolerant a germplasm. New rice cultivar "Wonhaebyeo" with a salt tolerance was developed by the above mentioned *in vitro* mutagenesis with 70 Gy gamma-rays on 2007 in the KAERI. This rice mutant will be released for cultivation at reclaimed land and used as a control plot for genetic research about salt tolerance.

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