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Establishment of salt stress tolerant rice plants through step up NaCl treatment *in vitro*

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Abstract

Establishment of salt tolerant rice plants was examined by single step or step up NaCl treatments of shoot bud clumps *in vitro*, and variation among *in vitro* salt tolerant plants were examined by rapid amplified polymorphic DNA (RAPD). Shoot bud clumps were necrotic, stubbed or dead when subjected to single step treatment with 1.5 or 2.0 % NaCl. Conversely all the clumps could grow vigorously when subjected to step up salt treatment with 0.5, 1.0, 1.5 and 2.0 % NaCl at 3 week intervals and 2 % NaCl tolerant plants were established. RAPD revealed shoot bud clumps with and without different NaCl treatments, seedlings from field and grown *in vitro*, and regenerants from callus were genetically close to one another. Conversely, callus cultures were genetically isolated. Growth under different salt stress conditions was not correlated with the genetic variation, suggesting that 2.0 % NaCl tolerant plants might not result from genetic mutation but were due to adaptation of plants by step up NaCl treatment *in vitro*.

Additional key words: adaptation, multiple shoot buds, *Oryza sativa*, salt tolerance, step up salt selection.

Introduction

Establishment of tolerant plants *in vitro* against some environmental stress such as salinity has been attempted *in vitro*, by combination of salt treatment of callus, cultured cells and transgenic cells *in vitro*, and subsequent plant regeneration (Nabors *et al.* 1980, Vajrabhaya *et al.* 1989, Hasegawa *et al.* 1997). However, regenerants often did not keep environmental stress tolerance *in vitro* and/or in field except transgenic regenerants (Smith and McComb 1983, Chandler and Vasil 1984, Oono and Sano 1986). Therefore, how to establish stress tolerant plants *in vitro* is the first hardship to establish stress tolerant plants in the field.

Theoretically, salt tolerant individual plants might also be established by the combination of mutagen treatment and subsequent salt stress selection of a large number of seedlings, shoots, buds or shoot bud clumps. We have been trying salt stress selection of shoot bud clumps with or without chemical mutagen treatment to establish salt tolerant plants *in vitro* (Arai *et al.* 1993). Preliminary experiments revealed that no mutagen treatment was required to establish 2.0 % NaCl tolerant plants *in vitro* by NaCl stress selection of pea (*Pisum sativum* L.) shoot bud clumps under high benzyl-aminopurine (BAP) concentration *in vitro* (Arai *et al.* 1993). Although BAP might induce mutation (Chatterjee and Gupta 1997), mutation during *in vitro* culture was not examined.

In this paper, the establishment of salt tolerant plants of rice by step up salt treatment of single-seed derived shoot bud clumps *in vitro* and DNA variation in salt tolerant plants are examined.

Materials and methods

Plants and culture conditions: Multiple shoot bud clumps were induced from a single seed of rice (*Oryza sativa* L. cv. Nipponbare) according to the procedures previously reported (Hisajima 1982). Sterilized seeds were cultured on 40 cm^3 of modified Murashige and Skoog's (MMS) medium, which consists of the basal

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medium, containing 50 μM BAP, 3 g dm^{-3} of sucrose and 0.8 g dm^{-3} of agar. Except otherwise stated, cultures were kept at temperature of 27 °C under 16-h photoperiod [cool-white fluorescent tubes, irradiance of 35 μmol(photon) m^{-2} s^{-1})]. The multiple shoot buds from a given seedling were cut into several small shoot bud clumps consisting of 8 - 10 shoots about 5 cm in length and some buds, and the clumps were subcultured in MMS medium containing 50 μM BAP and 1 μM indolbutyric acid (IBA) at 14-d intervals. Multiple shoot bud clumps derived from one seed, which showed similar growth characteristics on MMS medium without plant growth regulator were employed throughout the salt treatment.

For DNA analysis, plants regenerated from callus according to Nakano and Maeda (1974), and rice seedlings grown in a greenhouse in the usual manner and grown in MMS medium without plant growth regulator were used.

Salt treatment: Two types of salt stress treatments were designed, namely the single step and step up salt treatments. In the single step salt treatment, shoot bud clumps were cultured in MMS medium containing 0, 0.5, 1.0, 1.5 or 2.0 % NaCl for 3-week intervals. In the step up salt treatment, shoot bud clumps were cultured in MMS medium containing 0.5 % NaCl in the first 3 weeks and then in 1.0, 1.5 and 2.0 % NaCl medium for 3-week intervals, successively. Materials were cultured for 12 weeks in total and the results were recorded.

DNA extraction and RAPD: Total genomic DNA was extracted from the leaves of 5 plants using a modified cetyltrimethylammonium bromide method (Murray and Thompson 1980). RNA in crude DNA preparation was eliminated by the digestion with DNase-free RNase (*Nippon Gene Co.*, Tokyo, Japan). The DNA concentration was adjusted to 25 μg cm^{-3} before use.

A rapid amplified polymorphic DNA (RAPD) procedure is as follows. Five arbitrary primers, AP-1 (5'-AGCCAGCGAA-3'), AP-4 (5'-CAAACGTCGG-3'), AP-5 (5'-GTTGCGATCC-3'), AP-9(5'-CTGGCTACAC-3') and AP-10 (5'-CCGTCGCATA-3'), which gave clear and reproducible results, were selected from 30 primers in preliminary experiments. Reaction mixtures contained 60 nmol each of dATP, dCTP, dGTP and dTTP, 45 pmol primer, 60 ng genomic DNA, Δ*Tth* buffer (*Toyobo Co.*, Tokyo, Japan) and 0.75 units of Δ*Tth* DNA polymerase (*Toyobo Co.*) in a total volume of 0.03 cm^{-3}. Amplification reaction was carried out in a Quarter Bath DNA thermal cycler (*Inotech AG.*, Dottikon, Switzerland) under the following conditions: 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min for 35 cycles. After the last cycle, the reaction mixture was incubated at 72 °C for 8 min. It was confirmed to produce reproducible results in the above condition by preliminary experiments. Electrophoresis was carried out using 5 % polyacrylamide gel in Tris-borate-EDTA buffer, pH 8.0, 100 V, followed by staining with ethidium bromide and visualizing under UV radiation.

Data analysis: DNA amplification profiles of 5 shoot bud clumps from 20 shoot bud clumps in respective treatments were compared. Amplified DNA fragments were scored as present (1) or absent (0). The data for all the five primers were used to estimate genetic distances on the basis of the number of shared amplification products (Nei and Li 1979). Using the Neighbor-joining method (Saitou and Nei 1987), a dendrogram based on genetic distance was generated. A dendrogram was drawn by *PHYLIP* (Phylogeny Inference Package) Version 3.5c (Joseph Felsenstein, University of Washington 1986-1995).

Results

Single step salt treatment: When small shoot bud clumps were cultured in MMS containing 0, 0.5 or 1.0 % NaCl, it seemed that the clumps were green and grew normally. In three treatments, the numbers of shoots longer than 1cm in length from shoot bud clumps were almost the same (Table 1). The NaCl treatments resulted in an increase in the root number from shoot bud clumps grown in medium (Table 1). However, the effect decreased with increase in NaCl concentration. The average leaf length decreased with an increase in medium NaCl concentration.

In 1.5 % NaCl medium, about 35 % of shoot bud clumps died within two weeks. In addition, parts of the remaining individual clumps were necrotic.

In 2.0 % NaCl medium, some clumps were necrotic but produced shoot buds even if clumps turned partially brown during the first week in culture (Fig. 1). About 50 % of clumps cultivated were necrotic in 2 weeks and all clumps died when the cultivation was prolonged beyond four weeks.

Step up salt treatment: All the shoot bud clumps grew vigorously when the clumps were cultured in medium containing NaCl increased from 0.5 to 2.0 % (Fig. 1, Table 1) at 3 week intervals. All the shoot bud clumps examined grew and were green. No necrotic part was observed in any shoot bud clumps. Average shoot number and root number of the clumps with salt treatment were greater than those of control clumps (Table 1). Average leaf length of the clumps was the same as that of the control clumps. The clumps grew as vigorously as control clumps.

Table 1. Effect of single and step up NaCl treatment on growth of rice plants. Clumps were cultured in respective NaCl media at 3 week intervals and for 12 weeks in total. The results were recorded after 12 weeks in culture. The same letter in each indicator except the survival rate indicates that there is no significant difference at $P \leq 0.05$.

Treatments	Shoot number over 1 cm	Shoot number under 1 cm	Root number	Leaf length [cm]	Survival rate [%]
Single step salt treatment at 0 % NaCl	7.2a	0.6a	16.9a	39.4a	100a
Single step salt treatment at 0.5 % NaCl	9.9a	0.5a	30.0b	39.0a	100a
Single step salt treatment at 1.0 % NaCl	9.2a	0.6a	23.6c	35.6ab	100a
Single step salt treatment at 1.5 % NaCl	12.9b	5.8b	21.6c	5.2c	65a
Single step salt treatment at 2.0 % NaCl	0.0c	0.0c	0.0d	0.6d	0b
Step up salt treatment up to 2.0 % NaCl	11.5b	3.3b	29.2b	36.0ab	100a

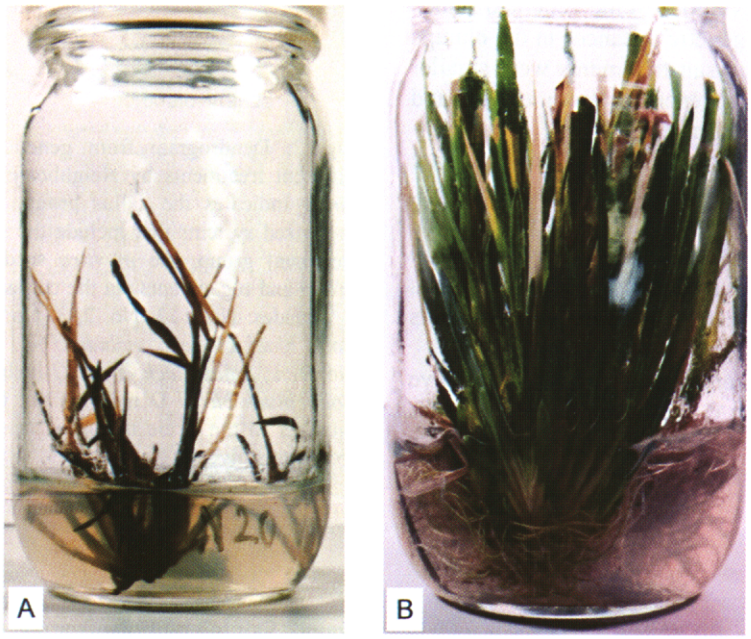

Fig. 1. Effect of single and step up salt treatments on growth of rice plant *in vitro*. Culture conditions are the same as described in Table 1. *A* - single step salt treatment at 2.0 % NaCl; *B* - step up salt treatment at 2.0 % NaCl.

Table 2. Effect of NaCl treatment on average genetic distance of group.

Treatments		Average genetic distance of respective groups	Standard deviation
1. Single step salt treatment at 0 % NaCl	Cultured with 0 % NaCl for 12 weeks	0.0059	0.0041
2. Single step salt treatment at 0.5 % NaCl	Cultured with 0.5 % NaCl for 12 weeks	0.0043	0.0037
3. Single step salt treatment at 1.0 % NaCl	Cultured with 1.0 % NaCl for 12 weeks	0.0014	0.0013
4. Single step salt treatment at 1.5 % NaCl	Cultured with 1.5 % NaCl for 12 weeks	0.0051	0.0040
5. Step up salt treatment up to 2.0 % NaCl	Subcultured in turn of 0.5, 1.0, 1.5, and 2.0 % NaCl in every 3 weeks	0.0051	0.0040
6. Seedlings in field	Germinated and grown in field for 2 months	0.0021	0.0018
7. *In vitro* seedlings	Germinated and grown *in vitro* for 2 months	0.0043	0.0037
8. Callus	Induced from rice grain by 2.4-D and grown for 7 months	0.0279	0.0087
9. Regenerants from callus	Regenerated from 4-month-old rice callus	0.0101	0.0060

Variation of genomic DNA in salt tolerant plants determined by RAPD analysis: All the 5 primers used in this study amplified different DNA fragments. Thirty-six DNA fragments from 146 fragments (24.6 %) showed polymorphism. The number of polymorphic DNA fragments detected in callus and regenerants from callus were 22 (15.0 %) and 14 (9.6 %), respectively.

The average genetic distances in seedlings grown in field and *in vitro* were 0.0021 and 0.0043, respectively, and the standard deviations of those were 0.0018 and 0.0043, respectively. A rice plant is a self-line. However, all the seedlings are not expected to be genetically the same. The present result is not against the idea (Table 2).

Generally speaking, genetic mutation occurred in callus cells. The average genetic distances of callus were 0.0279 and the standard deviations of the genetic distance were 0.0087. The average genetic distances in the clumps with single step and step up salt treatments were smaller than those in callus and regenerants from callus, and close to that from seedlings (Table 2).

In the dendrogram (Fig. 2), callus cultures are separated from organized systems such as seedlings, shoot bud clumps and regenerants. Each material consisting of seedlings from the field, seedlings grown *in vitro* or shoot bud clumps with different NaCl treatments did not form a corresponding group, but individual plants are intermixed in the dendrogram.

In the organized systems, the maximum relative genetic distance was about 0.0037 and the average was 0.0048 (Fig. 2). A lot of the individual explants are intermixed and mainly present in the solid circle (Fig. 2). It suggests that callus, a disorganized system, has more DNA variation than that of all of seedlings and shoot buds in the organized systems.

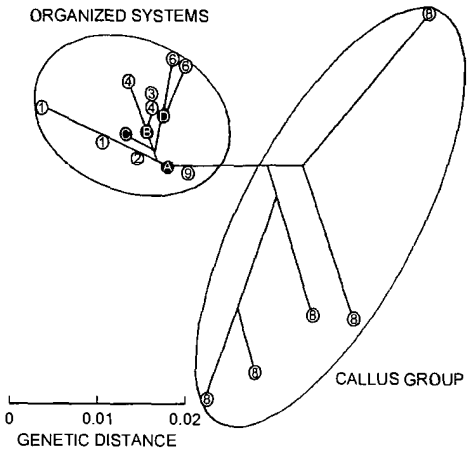

Fig. 2. Dendrogram from genetic distances of plants in different treatments by Neighbor-joining method. The *right circle* indicates the callus group. The *left circle* indicates organized systems that include the single step or step up salt treatment group, the *in vitro* seedling group, the seedling group and regenerants via the callus group. *Solid black circle A* includes: No. 1, 3, No. 2, 3, No. 5, 2, No. 7, 4, No. 9, 1; *Solid black circle B includes*: No. 2, 1, No. 3, 4, No. 4, 3, No. 9, 1; *Solid black circle C* includes: No. 5, 3, No. 9, 1, *Solid black circle D* includes:No. 6, 3, No. 7, 1, No. 9, 1. Number in the circle indicates the corresponding number of treatments given in Table 2.

Discussion

Single step treatment with 1.0 % NaCl has been widely used to establish salt stress tolerant cells *in vitro* (Vajrabhaya *et al.*1989, McCoy 1987, Cano *et al.* 1996).

In the present research, shoot bud clumps were selected by step up NaCl treatment and 100 % of shoot bud clumps could grow vigorously in high stress condition, 2.0 % NaCl medium (Fig. 1, Table 1). The present step up salt treatment procedure using shoot bud clumps is an alternative procedure to establish salt tolerant rice plants *in vitro* which is different from that using cell or callus cultures (Oono and Sano 1986). Whether the present *in vitro* salt tolerant plants have salt tolerance in the field is attempted in the following paper (Miki *et al.* 2001).

In preliminary experiments with pea, during the step up salt treatment up to 2.0 % NaCl, all the old shoots in the clumps were necrotic and new shoots developed (Arai *et al.* 1993). Therefore, simple adaptation could not explain the phenomenon. However, in the present experiment, shoot buds in every rice clumps grew during the step up salt treatment up to 2.0 % NaCl. In addition, any DNA variation responded to salt tolerance is not found. Salt tolerant plants are genetically close to original plant, namely seedlings grown in the field and *in vitro*, and non-selected shoot bud clumps (Table 2). It may suggest salt-tolerance does not correlate with genetic variation and the present salt tolerance of rice plants does not result from any mutation. These results also led us hypothesize that the present salt tolerant plants were due to the adaptation of plants to saline environment by step up salt treatment.

If the present establishment of salt tolerant plants by step up salt stress treatment is a kind of adaptation, the procedure will be applicable to establish salt tolerant plants in the field.

It was reported that BAP induced genome mutation, chromosome mutation and plasma mutation in plants (Chatterjee and Gupta 1997). The present multiple shoot bud clumps were induced by culturing a single seed in high BAP concentration and amplified in high BAP concentration. Although morphologically no aberrant rice plant was reported among plants amplified from one rice seed by the present procedure (Hisajima 1986, Nishikawa and Hirohara 1989), possible small mutations can not be

excluded in the present experimental system. RAPD analysis is useful and effective to detect somaclonal variants (Brown *et al.* 1993, Munthali *et al.*1996, Al-Zahim *et al.* 1999). RAPD and statistical analysis revealed that some variation occurred among the present shoot bud clumps, namely the so-called clone plants (Fig. 2, Table 2). Genetic uniformity in cloned plants is the next question.

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