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Establishment of stable NaCl-resistant rice plant lines from anther culture: distribution pattern of K^+/Na^+ in callus and plant cells

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Abstract Soil salinity markedly suppresses the growth of rice (*Oryza sativa* L.). We established rice anther culture to select for rice callus lines adapted to NaCl stress and regenerated plant progenies resistant to a NaCl stress of E.C. 16–18 mS. When exposed to NaCl, NaCl-adapted rice calli lost K^+ and accumulated little Na^+. Conversely, plant cells lost relatively little K^+ and accumulated Na^+. It is plausible that, NaCl-resistant mechanisms are different at callus and plant level. The stable NaCl-resistant lines produced have potential use in elucidating the molecular mechanisms behind NaCl resistance in rice and in rice breeding.

Key words Anther culture · NaCl-resistance · K^+/Na^+ analysis · *Oryza sativa* L. · Rice

Introduction

Rice, *Oryza sativa* L., which is consumed by more than half of the world's population (IRRI 1990), is sensitive to salt (Maas and Hoffmann 1977). Despite this, more than 150 million acres of rice-growing land are saline (Jenning et al. 1979). The threshold for NaCl tolerance in rice is E.C. 4 mS; beyond this growth and yield are compromised. The genetics of salt tolerance in rice is as poorly understood as its physiology and biochemistry, and rice breeding for NaCl tolerance has produced only limited results. Yeo and Flowers (1986), have suggested a "pyramiding" approach to overcome the hurdles in breeding for salt-resistant rice plants.

Tissue culture has been shown by one of us (Nabors et al. 1980) to be a means to select for salt-resistant plants. Since then, rice somatic cell cultures have been attempted to produce salt-tolerant rice plants (Oono and Sakaguchi 1978; Rains et al. 1980; Kishor and Reddy 1985; Zapata and Abrigo 1988; Subhashini and Reddy 1989; Vajrabhaya et al. 1989). In many cases, the results have been disappointing. An early report described progenies of rice plant regenerants obtained from NaCl-tolerant somatic callus lines that were able to tolerate NaCl stress for only a short duration over generations (Vajrabhaya et al. 1989). Unless the progeny of rice plant regenerants can mature in NaCl-stressed environment, the in vitro selection approach contributes little to NaCl-resistance breeding of rice.

During salt stress, the cellular K^+ concentration has been implicated in cellular osmotic adjustment and growth capacity (Ben-Hayyim et al. 1987). Many halophytes and partial salt-excluding glycophytes that involve osmotic adjustments for negating salt stress are known to retain high levels of internal K^+ despite the uptake of Na^+. The response is reversed in salt-accumulating glycophytes that do not involve osmotic adjustments (Ben-Hayyim et al. 1985). The status of K^+ and Na^+ in cells of NaCl-selected rice callus and in their plant progenies have not been characterized so far.

The two objectives of the study presented here were to: (1) isolate progenies of NaCl-resistant rice plant regenerants obtained from in vitro NaCl-selected rice anther-derived callus; (2) analyse the cellular K^+/Na^+ levels in cells of rice anther-derived callus and plant regenerant progenies in order to understand how the cells combat environmental Na toxicity.

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Materials and methods

Handling of rice anther-derived callus and plant regenerants

Mildly dessicated, pre-treated anthers of rice cv 'Yamahoushi' were cultured as described by Sathish et al. (1995) on 1% agar-solidified B5 medium (Gamborg et al. 1968) supplemented with either 0 m*M*, 85 m*M* or 130 m*M* NaCl. Callus from each anther was maintained separately in conjunction with our NaCl selection strategy (Fig. 1). Except for the first transfer, which was done after 42 days, healthy embryogenic calli (e-calli) were selected at intervals of 28 days and transferred to fresh medium at the rate of 55 ± 2.5 mg per vial. Growth was measured as the increase in the mass of total callus and e-callus at the end of the culture period over that inoculated. After 126 days, the calli were transferred to NaCl-free plant regeneration medium to regenerate plants (R_1). Regenerated plants were each assigned a plant line number and grown to maturity in NaCl-free environment as described previously (Sathish et al. 1995).

Screening of R_2 rice lines for NaCl resistance

A NaCl resistance screen was done using a split-split plot design consisting of two treatments (NaCl free and 100 m*M* NaCl supplemented), two blocks per treatment and three replications, Trays (blocks) contained 18 plastic pots ($75 \times 75 \times 50$ mm; $l \times w \times h$), with three pots for each of six lines. Of the six lines one was a control – a "parent" line that was not subjected to in vitro technique. The pots were filled with water-saturated artificial soil and two seeds were placed in each pot at a depth of 10 mm for germination. The trays were kept moist with de-ionized water for 14 days. At this time the seedlings were thinned to have 1 healthy seedling per pot. Excess water was drained, and the trays were refilled with 4 l of nutrient solution (Yoshida 1973). The NaCl stress was applied from day 21, with one of the treatments receiving 4 l of 100 m*M* NaCl-supplemented nutrient solution (NSS). The other treatment received 4 l of NaCl-free nutrient solution (NFS). The E.C. and pH measurements were taken once every 3 days, after which 1 l of the appropriate solution was added to each tray. After 63 days, the E.C. in NaCl-supplemented tanks was maintained around 18 mS by adding required quantities of either NFS or NSS and were filled to the brim with de-ionized water. The plants were grown at $30^\circ \pm 2^\circ$C and $65\% \pm 5\%$ relative humidity until the plants were harvested. The plants were selfed and seeds harvested 30 days after pollination on an individual plant basis. Grain yield per line in the NaCl-stressed medium was noted.

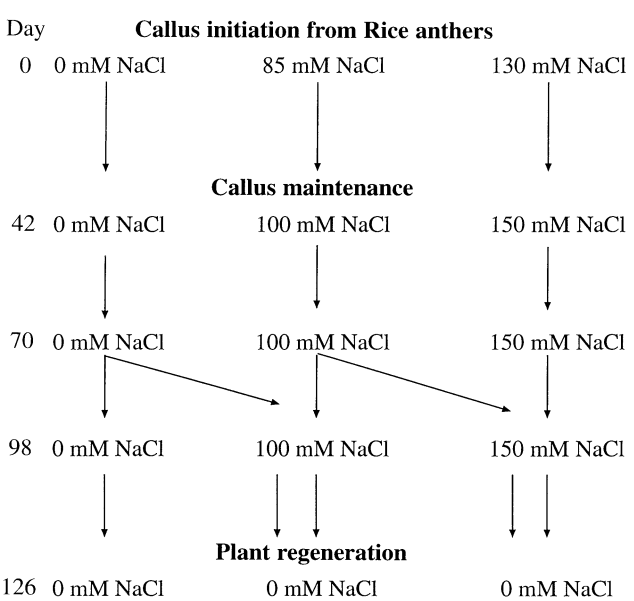

Fig. 1 A diagrammatic summary of the anther-culture NaCl-selection strategy

Analysis of soil samples

Soil samples from randomly chosen pots were collected after harvesting the plants and cleaned free of root materials. The soil samples were dried for 48 h at 90°C and ground into fine powder. A soil slurry was prepared by mixing 5 g of soil in de-ionized water. The soil slurry was equilibrated overnight, then diluted with some more water and the soil extract filtered under vacuum using Whatman filter paper no. 1. The soil extract was used to measure pH, E.C., Na^+ and K^+ as described earlier (Havlin and Soltanpour 1980).

X-ray microanalysis and light microscopy of callus samples

Identical dual samples of healthy e-callus (approx., 3 mm^3) were removed from the upper surface of e-calli (i.e. not in direct contact with the medium) growing on NFM and NSM on day 102 (4 days after transfer to fresh medium) for X-ray microanalysis and light microscopy. The e-calli samples for X-ray microanalysis were glued onto individual aluminium discs using silicone glue. The discs were immersed in liquid freon for 1 s, blotted using a filter paper and then immersed in liquid nitrogen slush. Each disc was placed on a sample holder and then transferred to a cold (-170°C) pre-chamber to fracture the e-callus mass with a pre-cooled (-170°C) knife. Samples were observed at 20 kV without any anti-charge coating. From each treatment two samples were analysed and each sample was analysed at three different locations selected randomly within the fractured surface. The operating parameters for the X-ray microanalyses were as follows: scanning electron microscope, Philips® model number 505; accelerating voltage, 20 kV (tungsten filament); aperture, 0.14–7.5 μm; raster area, 100 μm^2 at 1000-fold magnification; point analysis, 100 s each; detector system, KEVEX® Super 8000™ microanalysis system (Kevex Int Corp, Calif. USA). The X-ray peak height data for selected minerals were quantified using the Quantex III™ software program (Kevex Int Corp).

For light microscopy, callus were fixed overnight in 3% glutaraldehyde prepared in 0.1 *M* phosphate buffer (pH 7.4), dehydrated in an ethanol series to 100% and embedded in Spurr's low-viscosity embedding medium (Polysciences, Warrington, Pa.). Sections were cut at 5 μm with glass knives on a rotary microtome, mounted on slides and stained with 1% toluidine blue.

Plant tissue chemical analysis

The root and shoot samples from NFS and NSS were collected and the roots rinsed in de-ionized water. Shoot samples were collected separately as dead shoots (basal mature shoots) and live shoots (distal young shoots) from NaCl-resistant plants growing in NSS. The samples were dried for 24 h at 95°C and then homogenized using a tissue homogenizer. Homogenized tissue weighing 0.25 g was digested in 4 ml of concentrated HNO_3. Na^+ and K^+ were quantified using Inductive Coupled Plasma-Atomic Emission Spectroscopy as described earlier (Havlin and Soltanpour 1980).

Results

Rice anther culture for NaCl-adapted callus selection and plant regeneration

Within 42 days from inoculation, we obtained shiny-white nodular e-callus from 17.5%, 6.1% and 0.5% of the 1200 anthers plated on NaCl-free medium (NFM) and 85 m*M* NaCl- and 130 m*M* NaCl-supplemented medium (NSM), respectively. NaCl-selected calli, total and e-callus, grew more slowly on NSM than control calli on NFM (Fig. 2). The proportion of e-callus in total callus, however, was better in callus growing on NSM. The growth of NaCl-selected calli declined after 70 days when grown on 150 m*M* NSM. From nine NaCl-selected anther-derived callus lines, 30 rice plants (R_1) were regenerated (Table 1). All of the plant regenerants were grown to maturity under NaCl-free conditions and selfed seeds harvested.

Isolation of NaCl-resistant rice plant lines

The NaCl-LD_{50} value for rice seedling stage in the *japonica* rice cv 'Yamahoushi' was 85 m*M*; E.C. 8–10 mS (data not presented). Using a combination of quantitative and qualitative selection indices, we screened for NaCl resistance using 6 and 30 NFM- and NSM-derived R_1 progeny (R_2) seedlings, respectively (data not presented). None of the R_2 seedlings obtained from callus grown exclusively on NFM and very few R_2 seedlings obtained from callus initiated and grown exclusively on NSM had a higher NaCl resistance than the control R_2 seedlings obtained from a callus line that

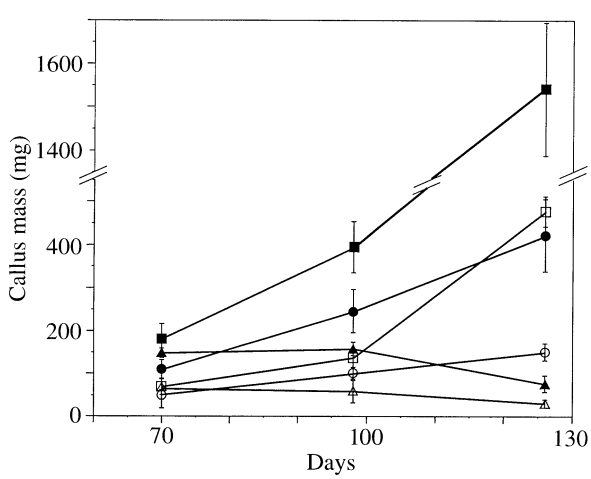

Fig. 2 Growth measurements of rice anther-derived callus. Growth of total callus on: ■ NaCl-free medium, ● 100 m*M* NaCl-supplemented medium, ▲ 150 m*M* NaCl-supplemented medium. Growth of e-callus on: □ NaCl-free medium, ○ 100 m*M* NaCl-supplemented medium, △ 150 m*M* NaCl-supplemented medium. Standard deviation is indicated by *bars*

Table 1 Plants regenerated from rice anther-derived callus

NaCl level[a] × days	Number of callus lines used to regenerate plants	Number of plant lines regnerated
0 × 126	1[b]	6
0 × 98; 100 × 28	6	14
85 × 42; 100 × 84	1	10
85 × 42; 100 × 56; 150 × 28	1	6
130 × 42; 150 × 84	1[c]	0

[a] NaCl (m*M*) supplemented in the medium
[b] Only 1 of the many callus lines was selected from this treatment for plant regeneration
[c] Sufficient e-callus was not present in this treatment after 126 days for regenerating plants

was initiated and grown on NFM for 98 days and then screened on 100 m*M* NSM for 28 days were found to have a higher NaCl resistance than the control.

Based upon the seedling screen, a selection of 9 R_2 lines exhibiting varying degrees of NaCl resistance and a control were grown to maturity in NFS and NSS (Fig. 3A–D). The E.C. of the NSS in the growth tank and that of the soil was greater than 15 mS, while in NFS it was less than 4 mS (Fig. 3A, B). All lines tested in NFS, including the control, matured approximately at the same time. NaCl-resistant rice plants grown in NSS matured 14 days later than their siblings grown in NFS (Fig. 3C). Of the R_2 lines tested, plants from four lines matured and set seeds in NSS (Table 2). The control succumbed to NaCl stress when the E.C. of the solution in the growth tank reached 13 mS. Like the control, other R_2 lines died soon after reaching the tillering stage when grown in NSS. Of the four R_2 lines that set seed, three were the progenies of plants regenerated from a single callus line. Yet, there were considerable variations among the lines in their ability to resist NaCl. Line number 73 proved to be the best line based on the percentage of surviving plants, seed weight and grain yield (Table 2; Fig. 3D). A R_3 (progenies of R_2) seedling screen involving line number 73 showed no loss in its ability to resist NaCl stress (data not presented).

Distribution pattern of K^+/Na^+ in rice callus and plant cells grown in NaCl environments

Due to the limited availability of e-calli, the technique of X-ray microanalysis was used to measure the levels of K^+ and Na^+. The X-ray spectra collected had similar Bremsstrahlung. The peaks for different ions, within the fractured face of callus from a given treatment, were very similar when analysed at different random locations. As the level of NaCl in the medium increased, the K^+ level in the e-callus decreased dramatically. There was, however, a negligible increase in the levels of detectable Na^+ (Fig. 4A–C). The starch levels in

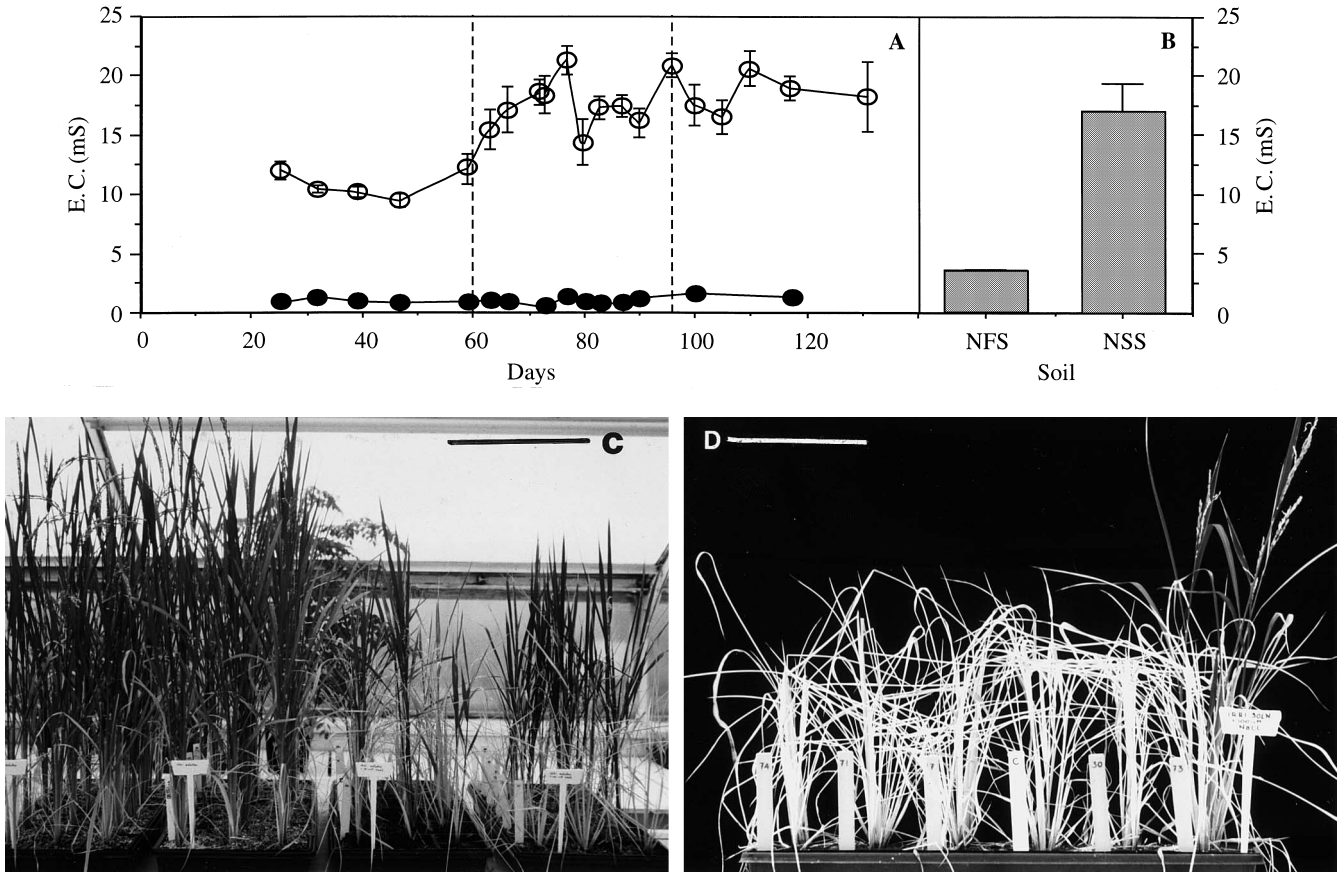

Fig. 3A–D R_2 maturity trials testing for NaCl resistance in the rice plant progenies obtained from selected anther-cultured, NaCl-selected callus. **A** E.C. of the NaCl-free nutrient solution (●) and NaCl-supplemented nutrient solution (○) during the seedling (*first column*) tillering (*second column*) and maturity (*third column*) stage. **B** E.C. of the soils used in R_2 maturity trials. **C** Early maturity in the plants growing in NaCl-free solution (*left* 2 trays); late maturity in plants growing in NaCl-supplemented solution (*right* 2 trays). **D** The most NaCl-resistant rice line maturing in NaCl-supplemnted nutrient solution. Standard deviation is indicated by *bars*. *bar*: 0.25 m

the cells growing on NSM dropped, suggesting some sort of energy cost that probably facilitated Na^+ efflux (Fig. 4D–F).

Unlike the situation, in the cells of anther callus, plant cells challenged with NaCl accumulated ten fold more Na^+, and the K^+ level decreased only by half (Fig. 5A, B). The roots of live plants growing in NFS and NSS exhibited higher levels of K^+ and Na^+ than their respective live shoot counterparts. In plants that

Table 2 Maturity of rice lines growing on NaCl-supplemented solution

Plant; callus line numbers	In vitro history[a]	Plants maturing (%)	Total seed yield per line[b]	
			Number	Weight (mg)
Control	–	0	0	0
48; 4	0 × 126	0	0	0
54; 4	0 × 126	0	0	0
46; 8	0 × 98; 100 × 28	0	0	0
71; 25	0 × 98; 100 × 28	22.22	21	134.1
73; 25	0 × 98; 100 × 28	33.33	30	855.9
74; 25	0 × 98; 100 × 28	22.22	8	143.1
17; 10	85 × 42; 100 × 84	0	0	0
30; 10	85 × 42; 100 × 84	11.11	1	8.1
29; 10	85 × 42; 100 × 56; 150 × 28	0	0	0

[a] NaCl (m*M*) NaCl supplemented in the medium × days
[b] Data from 9 plants from each R_2 line and 18 control plants

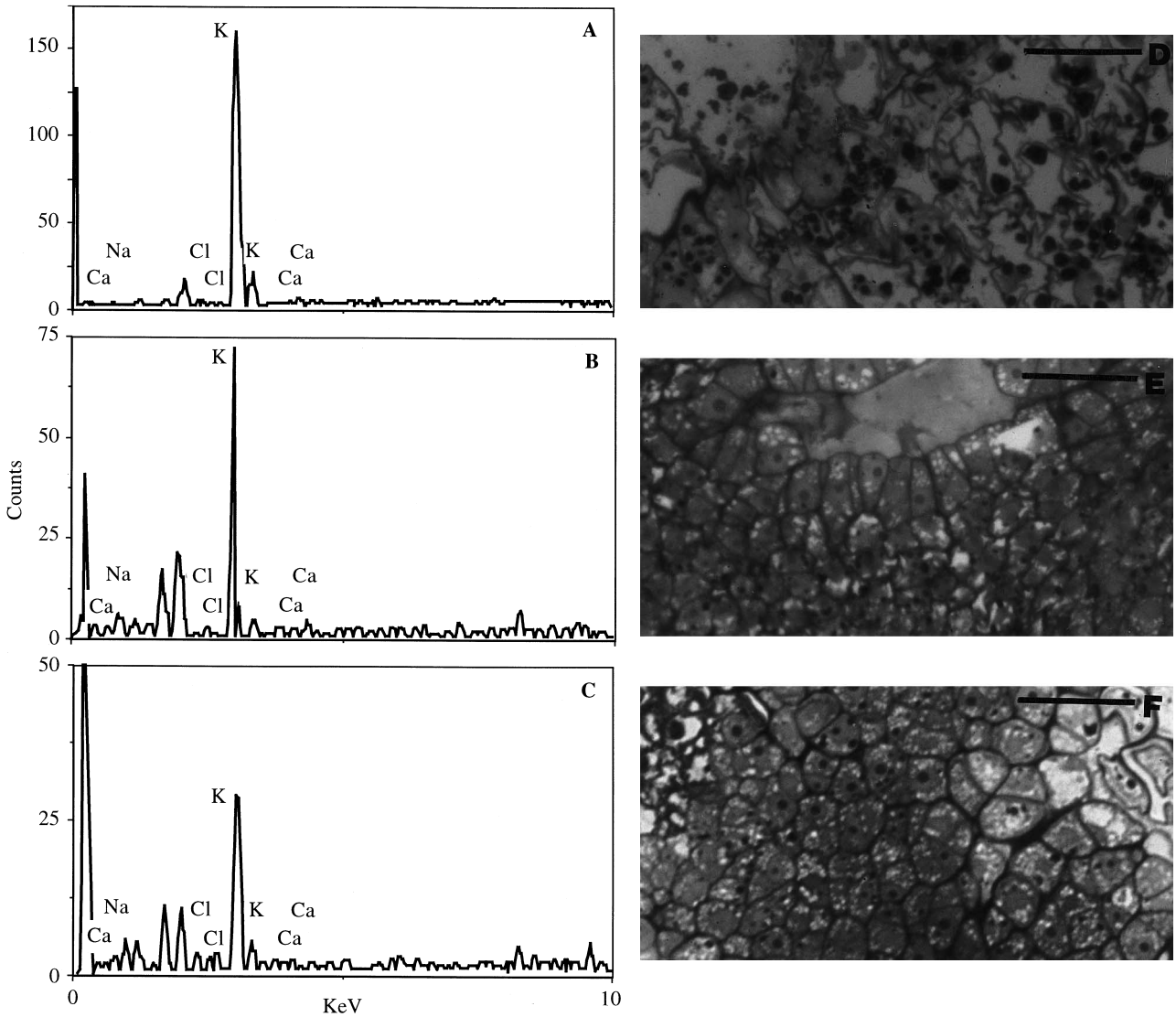

Fig. 4A–F X-ray microanalysis and ultrastructural analysis of e-calli grown on medium supplemented with different levels of NaCl. X-ray microanalysis for sodium, potassium, calcium and chloride ions of e-calli grown on **A** 0 m*M* NaCl, **B** 100 m*M* NaCl, **C** 150 m*M* NaCl. Ultrastructural analysis revealing levels of starch in e-callus cells growing on **D** 0 m*M* NaCl, **E** 100 m*M* NaCl, **F** 150 m*M* NaCl. *Bar*: 50 μm

succumbed to NaCl stress, shoots had relatively higher levels of both K^+ and Na^+. In NaCl-resistant rice plants, dead basal mature leaves contained higher Na^+ levels than their root and shoot counterparts, suggesting the sequestration of Na^+ to older leaves.

Discussion

We believe this to be the first report demonstrating the production of stable NaCl-resistant rice plants by anther culture. Unlike previous investigators (Wong et al. 1983; Chantprem et al. 1984; KrishnaRaj and Sree Rangasamy 1993), we succeeded in regenerating fertile rice plants from NaCl-adapted, anther-derived callus mainly because of our conscientious e-callus selection procedure. We refrained from stressing the regenerant rice plants with NaCl in order to improve the chance of seed set. Besides, we were interested in the stable heritable variations rather than epigenetic variations towards NaCl stress.

It was not possible to evaluate all of the regenerant plant progeny lines for NaCl resistance upto maturity due to a limited availability of greenhouse space. Pearson and Ayers (1960) described the seedling stage in rice as the most NaCl-sensitive stage. So, using a seedling screen and different selection parameters we classified the lines based on their ability to tolerate NaCl stress. We noticed variations between and within lines for resistance to NaCl stress. Variations in progenies of

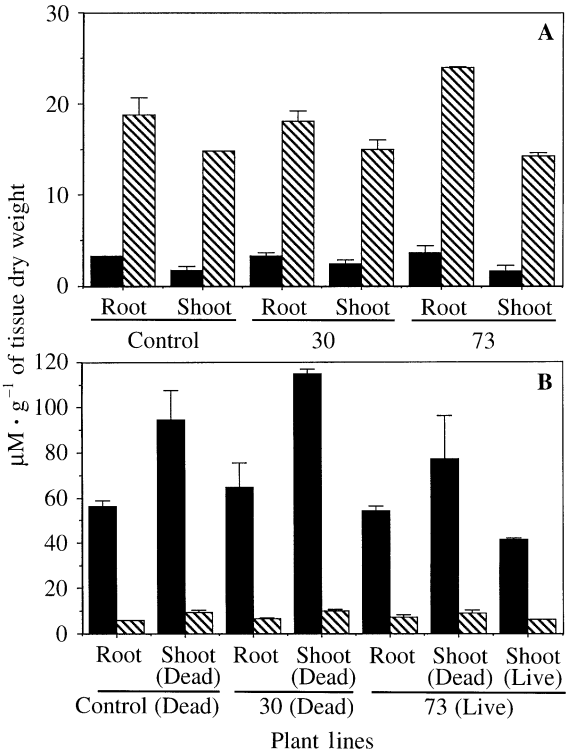

Fig. 5A, B Levels of K^+ and Na^+ in the cells of rice plants. K^+ (striped columns) and Na^+ (*black columns*) levels in roots and shoots of rice plants growing in **A** NaCl-free nutrient solution (NFS) and **B** in NaCl-supplemented nutrient solution (NSS). Standard deviation is indicated by *bars*

tissue-cultured plant materials have been widely documented and probably stem from the genetic instability created during plant tissue cultures (Phillips et al. 1994).

While the K^+/Na^+ value in both callus and plant cells decreased when exposed to NaCl stress, the way by which this was accomplished was different. In callus cells grown in NSM the cellular K^+ level decreased considerably with only a negligible increase in Na^+ level. A high level of Na^+ has been shown to inhibit K^+ uptake mediated by a low-affinity system (Rains and Epstein 1967). It is plausible that the low cytoplasmic Na^+ level, might be the result of an efficient Na^+ efflux mechanism. This, however, remains to be determined. Such a Na^+ efflux mechanism has been described in the charophyte, *Chara longifolia* (Kiegle and Bisson 1996). Unlike in callus cells, plant cells contained increased levels of Na^+ with relatively little change in the K^+ level.

Also, in plant cells the distribution of Na^+ was blocked at different levels. The roots are the primary barrier in preventing excess Na^+ from reaching the shoots. In shoots, the older shoots proximal to the roots and soil accumulate more Na^+ than the distal young shoots. When the roots fail to prevent the uptake of Na^+ the plant dies due to excessive accumulation of Na^+ in the shoot, provided there is no partitioning of the offending ions into mature shoot tissues. This two-tier of defence against Na^+ toxicity in rice plants perhaps helps the distal younger shoot to survive and bear seeds. Current information on Na^+ efflux and K^+ uptake in glycophytic plant systems is not sufficient to discuss seriously about how they function. It is probably best approached on a genotype basis.

The differences in the accumulation patterns of Na^+ and K^+ in the callus and plant cells indicate the involvement of different NaCl-resistant mechanisms in the undifferentiated and differentiated cells. Recently, a gene similar to the *S*-phase-specific cyc07 gene of *Catharanthus roseus* was shown to be more highly expressed in NaCl-stressed rice callus cells than in seedling cells (Kidou et al. 1994). Also, expressions of certain genes in NaCl-stressed rice plants have been shown to be organ-specific (Claes et al. 1990). Considering the differences involved in NaCl resistance at the callus and plant level, it is not surprising why all plant lines regenerated by us from NaCl-selected anther callus lines were not NaCl-resistant. The NaCl-resistant plant lines developed here hold the potential to elucidate the molecular mechanism involved in rice NaCl resistance as well as in rice breeding for NaCl resistance.

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