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Phenotypic characterization of the progenies of rice plants derived from cryopreserved calli

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Abstract The progenies of rice plants (*Oryza sativa* L.) differentiated from calli that had been cryopreserved and from control (non-cryopreserved) calli were used to study the influence of selection pressure during cryopreservation. The phenotypic evaluation of these progenies was based mainly on the response of seedlings and calli to freezing stress and on the characterization of protoplast and cell populations by flow cytometric analyses. The patterns of response to freezing stress, as well as the variations in some morphological and physiological cell parameters, were unrelated to the origin (cryopreserved or control calli) of the parental plants.

Key words Cryopreservation · Flow cytometry · Freezing stress · *Oryza sativa* L. · Rice

Introduction

Cryopreservation of plant cells has long used to maintain the stability of selected clones with respect to specific physiological or biochemical traits such as morphogenic ability or secondary-product synthesis (Bajaj 1995; Kartha 1987; Withers 1985, 1987). In rize (*Oryza sativa* L.), cryopreservation has allowed the storage of transgenic lines (Meijer et al. 1991), transformation-competent calli (Cornejo et al. 1995) and embryogenic cell suspensions (Huang et al. 1995; Lynch et al. 1994), while retaining their competencies.

Studies on the recovery of cryopreserved cells of two gramineous species, rice (Sala et al. 1986) and Guinea grass (*Panicum maximum*) (Gnanapragasam and Vasil 1992), have shown that, under optimal cryopreservation conditions, structural and physiological damage is repaired within a short period. The ability to survive cryopreservation may be influenced by the cell type used, its physiological condition, as well as by the procedure used. Thus, cryopreservation experiments with various rice tissues, as well as with calli or cell suspensions (for reviews see Bajaj 1995, Bajaj and Sala 1991), have shown wide variation in both extension of the post-freezing lag phase and in cell survival. The depletion of viable cells related to cryodamage may select either specific cell types, epigenetic variants or genotypes. Although cryoselection by direct immersion in liquid nitrogen has been used with wheat calli to regenerate freezing-tolerant plants (Kendall et al. 1990), such a trait would be undesirable were it to occur following cryopreservation of important clones under suboptimal conditions.

In previous research (Cornejo et al. 1995, cryopreservation of rice calli did not affect their competence for plant regeneration or genetic transformation. However, post-freezing lag phases of 4–8 weeks and the fact that cell division took place in localized areas of the calli suggested that some selection might have taken place. Progenies of plants regenerated from these calli and from calli of the same lines that had not been cryopreserved have provided us with a useful tool to evaluate the effects of selection pressure during cryopreservation. As freezing-stress resistance might have been involved in the putative selection, we developed and used in vitro screening techniques to test the response to freezing stress in seedling and callus cells.

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Materials and methods

Calli induced from mature embryos of rice (*Oryza sativa* L., cv. Taipei 309) were used in previous cryopreservation experiments (Cornejo et al. 1995) to obtain callus lines from individual calli that had been cryopreserved (Cryo-1 callus lines) and from calli that had not been cryopreserved (Control-1 callus lines). Fertile plants were obtained from these callus lines (Cryo-1 and Control-1 plants). Seeds of Cryo-1 and Control-1 plants were used to provide the seedlings and calli used in this research, and will be designated hereafter as Cryo-2 or Control-2 seedling and callus lines. The response of these lines to periods of freezing stress at –14°C (seedlings) and –20°C (calli) was characterized as described below.

Freezing stress procedure using seedlings

Cryo-2 and Control-2 seedlings of 0.5–2.5 cm in height and similar age were used to test the response to freezing. Seedlings with this size range were uniformly distributed for the freezing treatments. For this purpose, seeds were germinated in Petri dishes and transferred to Magenta boxes (five seedlings/box) containing 100 ml of solidified basal medium (its composition is described in the following section). Seedlings were kept at 27°C under a 16-h photoperiod before and after the freezing treatment. Two types of treatments were tested. Direct freezing at –14°C for periods ranging from 40 to 120 min and freezing at the same temperature for 40 min but with pre- and post-acclimation. For pre-acclimation, seedlings were maintained for 15 h at 14°C then for 4 h at 4°C. For post-acclimation, seedlings were maintained for 4 h at 4°C followed by 15 h at 14°C. Post-freezing seedling growth was monitored over a period of 7 days. Those seedlings that did not continued to show growth 7 days after freezing were considered dead.

To study leaf structure and alterations related to freezing, leaf fragments from 2-week-old seedlings that had or had not been exposed to –14°C for 80 min were infiltrated with Tissue-Tek embedding medium and sectioned with a Cryotome 620 M (Anglia Scientific). The 14-μm cross-sections were then stained with safranine and fast green. All sections shown were obtained from the central area of leaves when seedlings were about 2 weeks old.

Freezing-stress procedure using calli

Cryo-2 and Control-2 calli were induced and maintained on a basal medium containing to 10 μM of 2,4-dichlorophenoxyacetic acid (2,4-D). The basal medium was composed of MS salts (Murashige and Skoog 1962), R-2 organic compounds (Ohira et al. 1973), 3% (wt/vol) sucrose and 0.7% agarose, pH 5.7. Calli were kept in darkness at 27°C. The nine callus lines used in these experiments were established from individual seeds of two Cryo-1 plants and one Control-1 plant (three callus lines from three seeds of each plant). To test the freezing response, callus samples (24 samples/callus line) were placed in wells of Corning plates (24 wells with an individual diameter of 16 mm) containing 1.5-ml aliquots of solidified culture medium. This was composed of the basal medium with 10 μM 2,4-D and the pH indicator chlorophenol red at 50 mg/l (Kramer et al. 1993). Samples were maintained at –20°C for 2 h and then incubated in darkness at 27°C. Color changes in the culture medium (from red at pH 6 to yellow at pH 4.8), indicating active cell growth, were monitored over a period of 7 days. Resistant calli were then transferred to fresh culture medium and allowed to regrow for 14 days prior to a second freezing. A similar procedure was followed for a third freezing.

Microscopic observations and flow cytometric analyses

Protoplasts were prepared from Cryo-2 and Control-2 callus lines as described elsewhere (Cornejo et al. 1993) and resuspended in a buffer solution composed of 5 mM MES, 5 mM $CaCl_2$ and 0.55 M mannitol, pH 5.7. Protoplasts were counted with a Fuchs-Rosenthal chamber and their viability determined with methylene blue. The number of elongatd cells that remained undigested in protoplast preparations from several callus lines was also recorded. Prior to flow cytometric analyses, samples of each protoplast batch were stained separately with fluorescein diacetate (FDA) and rhodamine B. FDA was diluted with buffer from a stock solution of 5 mg/ml acetone and rhodamine B was directly dissolved in the buffer solution, to a final concentration of 5 μg/ml for each dye.

All flow cytometric analyses were performed with an EPICS Elite Cell Sorter (Coulter Electronics, Hialeah, Fla.) using a 15-mW air-cooled argon-ion laser turned at 488 nm. The protoplast buffer solution was used as sheath fluid. To avoid protoplast damage, suspensions were analyzed through a 200-μm nozzle EPICS Profile flow chamber at low sample pressure and without agitation. Measurements of forward angle light scatter (proportional to cell size), right angle light scatter (proportional to intracellular complexity) and fluorochrome fluorescence were registered in logarithmic amplification. Green fluorescence emission of rhodamine B or FDA was collected through a 525-nm band-pass filter. Data were acquired in the form of list mode files which were analyzed off-line using the EPICS Elite Workstation software. Data were displayed as frequency histograms of fluorescence distribution or as scattergram dot plots correlating fluorescence distribution and cell size, estimated by forward angle light scatter. Each measurement included at least 10,000 cells.

Results and discussion

Freezing stress response in seedlings

Table 1 shows the effect of the duration of freezing at –14°C on the subsequent growth of five seedling lines. The term seedling line is used to designate seedlings obtained from seeds of the same plant. We tested one line of Control-2 seedlings and three lines of Cryo-2 seedlings. Two of the latter were from seeds of two Cryo-1 plants regenerated from the same Cryo-1 callus line (Cryo-2 B_1 and B_2) while the third line (Cryo-2 A) was from another Cryo-1 callus line obtained in a different cryopreservation experiment. We also tested a line of seedlings from seeds of similar age but obtained from a greenhouse-grown plant (Control GH). A total of 500 seedlings of similar size and age were used in this experiment (20 seedlings per seedling line per freezing period). Data are expressed as differences between seedling growth for a given number of days after the freezing treatment and growth prior to freezing.

The survival of seedlings at –14°C decreased with the length of freezing exposure from a range of 75–50% in plants treated for 40 min to 25% to no survival in plants treated for 120 min. As expected, the reduction in growth observed in the surviving seedlings was related to the length of exposure to the freezing treatment in the five lines of seedlings tested (Table 1).

Some differences in growth rate between seedlings lines were found in both unexposed seedlings and in seedlings exposed to freezing for only 40 min. Thus, the growth rate, while slight, was significantly higher in Control-2 seedlings unexposed to freezing as well as in both Control-2 and Cryo-2 B_2 seedlings following exposure to freezing for 40 min. Response to more extended freezing periods (60 and 80 min) was more uniform in all seedling lines,

Table 1 Effect of the duration of freezing stress (–14°C) on the post-freezing growth of five seedling lines. A total of 500 seedlings were used (20 seedlings per seedling line per freezing period). From two-way ANOVA and Tukey's test ($P=0.01$) performed within each freezing period, growth response of seedling lines followed by the same letter is not significantly different. Post-freezing growth of each seedling line after 2, 4, and 7 days was significantly different

Freezing period (min)	Seedling line		Post-freezing seedling growth (cm)			Survival after 7 days (%)
			2 days	4 days	7 days	
0	Cryo-2 A	a	2.46	6.44	13.24	100
	Cryo-2 B_1	a	1.92	3.08	13.40	100
	Cryo-2 B_2	ab	2.34	7.66	15.40	100
	Control-2	b	2.98	10.88	19.14	100
	Control GH	a	2.48	6.44	14.22	100
40	Cryo-2 A	ab	0.96	2.85	10.97	60
	Cryo-2 B_1	a	0.81	2.30	8.42	50
	Cryo-2 B_2	b	1.52	4.47	11.70	75
	Control-2	b	0.91	4.22	11.60	60
	Control GH	ab	0.96	3.71	10.79	50
60	Cryo-2 A	a	0.70	2.56	7.77	50
	Cryo-2 B_1	a	1.37	3.28	8.70	85
	Cryo-2 B_2	a	0.86	2.71	7.84	75
	Control-2	a	0.55	3.13	8.24	30
	Control GH	a	0.72	4.47	10.27	75
80	Cryo-2 A	a	0.40	2.36	9.56	25
	Cryo-2 B_1	a	0.66	1.95	7.96	40
	Cryo-2 B_2	a	0.87	2.79	8.18	45
	Control-2	a	0.62	3.02	10.44	55
	Control GH	a	0.88	2.58	9.88	25
120	Cryo-2 A	a	0.52	2.30	7.12	25
	Cryo-2 B_1	b	0.00	0.35	3.15	20
	Cryo-2 B_2	–	–	–	–	0
	Control-2	c	0.82	5.70	11.7	20
	Control GH	–	–	–	–	0

Table 2 Effect of a freezing treatment of 40 min at –14°C with pre- and post-acclimation on the survival and growth of seedlings after 7 days. Within each column, values followed by the same letter are not significantly different using one-way ANOVA and Tukey's test

Seedling line	Post-freezing seedling growth* (cm)	Percent survival** (number of seedlings growing/total number of seedlings)
Cryo-2 A	11.51±0.71 ab	68.42 (26/38) ab
Cryo-2 B_1	9.90±0.66 a	80.77 (21/26) b
Control-2	13.25±1.20 b	41.93 (13/31) a

* $P=0.05$: ** $P=0.01$

Table 3 Percentage of cali surviving one, two, and three freezings for 2 h at –20°C, with recovery periods of 3 weeks between each freezing treatment. The number of surviving calli/total number of calli is also indicated.) From two-way ANOVA and Tukey's test ($P=0.01$) survival between the first and second freezings was not significantly different, while values of the third freezing are significantly lower. The patterns of survival among the nine callus lines to the three freezings were not significantly different

Callus line	Percentage of calli surviving freezing		
	First freezing	Second freezing	Third freezing
Cryo-2 A L-1	29.2 (7/24)	41.6 (5/12)	25.0 (3/12)
Cryo-2 A L-2	45.8 (11/24)	41.6 (5/12)	16.7 (2/12)
Cryo-2 A L-3	50.0 (12/24)	33.3 (4/12)	25.0 (3/12)
Cryo-2 B_1 L-1	45.8 (11/24)	50.0 (6/12)	25.0 (3/12)
Cryo-2 B_1 L-2	29.2 (7/24)	50.0 (6/12)	25.0 (3/12)
Cryo-2 B_1 L-3	29.2 (7/24)	33.3 (4/12)	16.7 (2/12)
Control-2 L-1	33.3 (8/24)	41.6 (5/12)	16.7 (2/12)
Control-2 L-2	33.3 (8/24)	41.6 (5/12)	16.7 (2/12)
Control-2 L-3	41.7 (10/24)	41.6 (5/12)	30.0 (3/10)

However, under the longest freezing stress (120 min), differences between seedling lines were more pronounced. Thus, two lines of seedlings, Cryo-2 B_2 and Control GH, did not survive this freezing stress, while the remaining three groups (Control-2, Cryo-2 B_1, and Cryo-2 A) showed significant differences in growth during the post-freezing period.

This pattern of response indicates that under the most severe freezing stress (120 min), differences in post-freezing growth of the resistant seedlings become significant, while after moderate freezing periods (60 and 80 min) the growth of resistant seedlings is more uniform than that of unfrozen seedlings or seedlings exposed to freezing stress for a short period (40 min).

We also tested the effect of pre- and post-acclimation to freezing exposure (Table 2). Under the conditions of acclimation used, differences in post-freezing growth between the three lines tested were comparable to those observed in seedlings exposed directly to freezing for the same period (40 min, Table 1). But, in this case (Table 2), the ability to survive the freezing treatment was inversely related to the growth of the surviving seedlings.

In addition to the reduction in seedling growth, freezing stress also caused leaf rolling, a reduction in their width compared to that of unfrozen seedlings of the same age and line, as well as some structural changes. Figure 1 shows cross-sections of leaves from Cryo-2 A and Control GH seedlings unexposed to freezing stress and from seedlings of the same two lines exposed to –14°C for 80 min, with a recovery period of 7 days. Although Cryo-2 A and Control GH seedlings unexposed to freezing were of similar age and size, leaves of Cryo-2 A (Fig. 1A, C) were wider and had more developed sclerenchyma than those from Control GH (Fig. 1B, D). None of the leaves from unexposed seedlings of either line had noticeably bulliform cells, although some of these cells were noticed near the tip of the leaves (sections not shown). Seven days after exposure to freezing stress, leaves of both lines were 30–35% narrower than those from unexposed seedlings of the same age and showed abundant, very distinct bulliform cells (Fig. 1E, F).

The role of bulliform cells in the control of folding and opening of leaves has been questioned, as in some instances, leaf rolling occurs while bulliform cells are turgid. In our case, the common and most evident structural change in all leaves subjected to freezing stress was the presence of bulliform cells. However, the physiological implication of this observation is unclear.

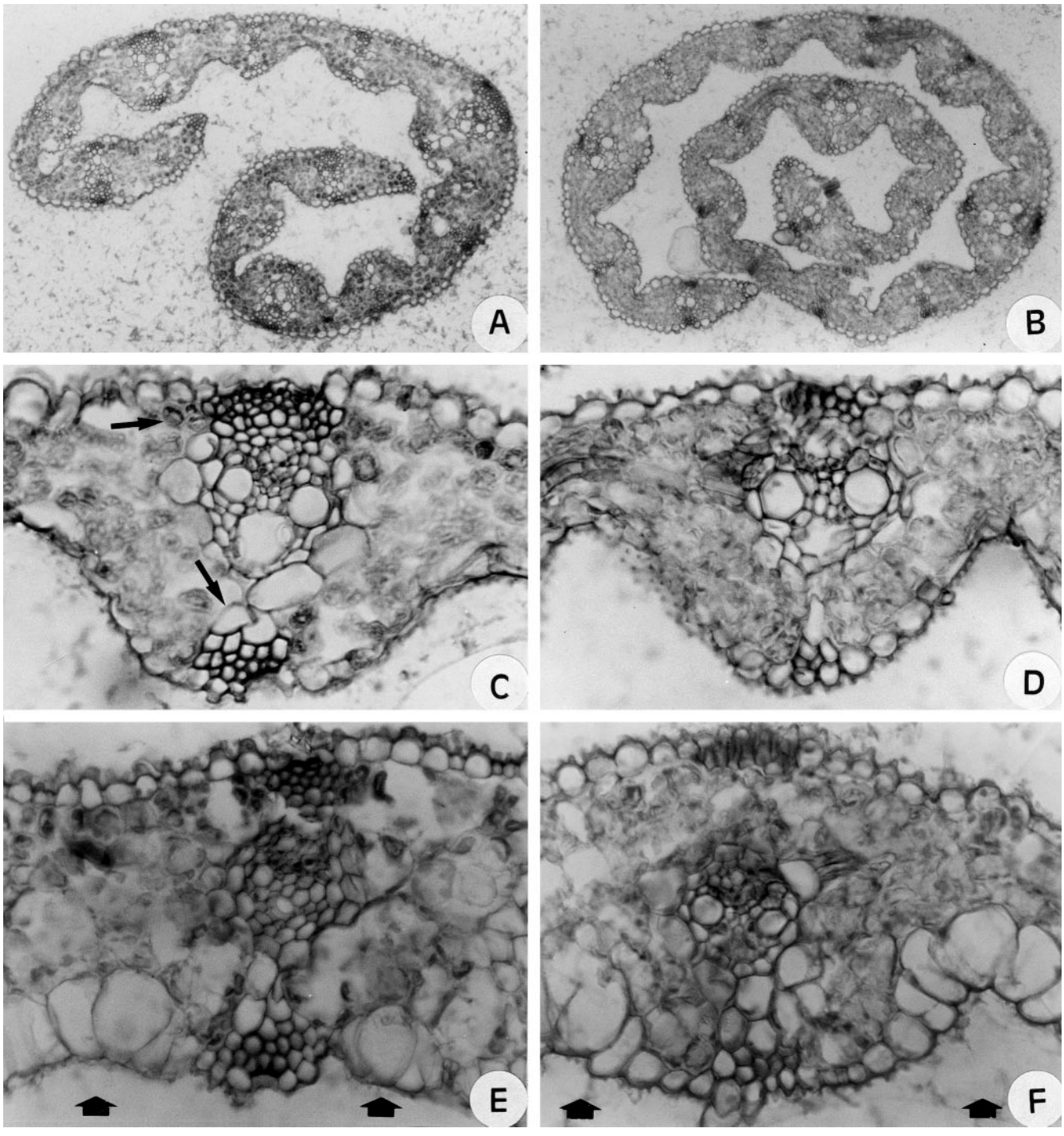

Fig. 1A–E Cross-sections of leaves from Cryo-2 and Control-2 seedlings. **A–D** Seedlings unexposed to freezing stress. **A** Cryo-2 A. **B** Control GH. **C** Cryo-2 A (*arrows* sclerenchyma cells). **D** Control GH. **E, F** Seedlings exposed to –14°C for 80 min (*arrows* bulliform cells). **E** Cryo-2 A. **F** Control GH. Magnifications **A, B** ×100; **C–F** ×400

Freezing stress response in calli

The pH indicator chlorophenol red proved to be reliable for detecting cells that survived freezing stress. This indicator has been previously used with rice calli to detect putative stable transformants containing the *bar* gene, that were later confirmed by phosphinothricin acetyl transferase activity and Southern analysis (Cornejo et al. 1995). Preliminary experiments showed that 2 days after placing calli, unexposed to freezing, in the incubation medium, the medium color turned from red to yellow. On the other hand, all calli that initially showed resistance to freezing stress based on pH-related changes in the culture medium were able to continue normal growth.

Based on the response to a severe freezing stress (–14°C for 120 min) of the seedling lines (Table 1), we selected

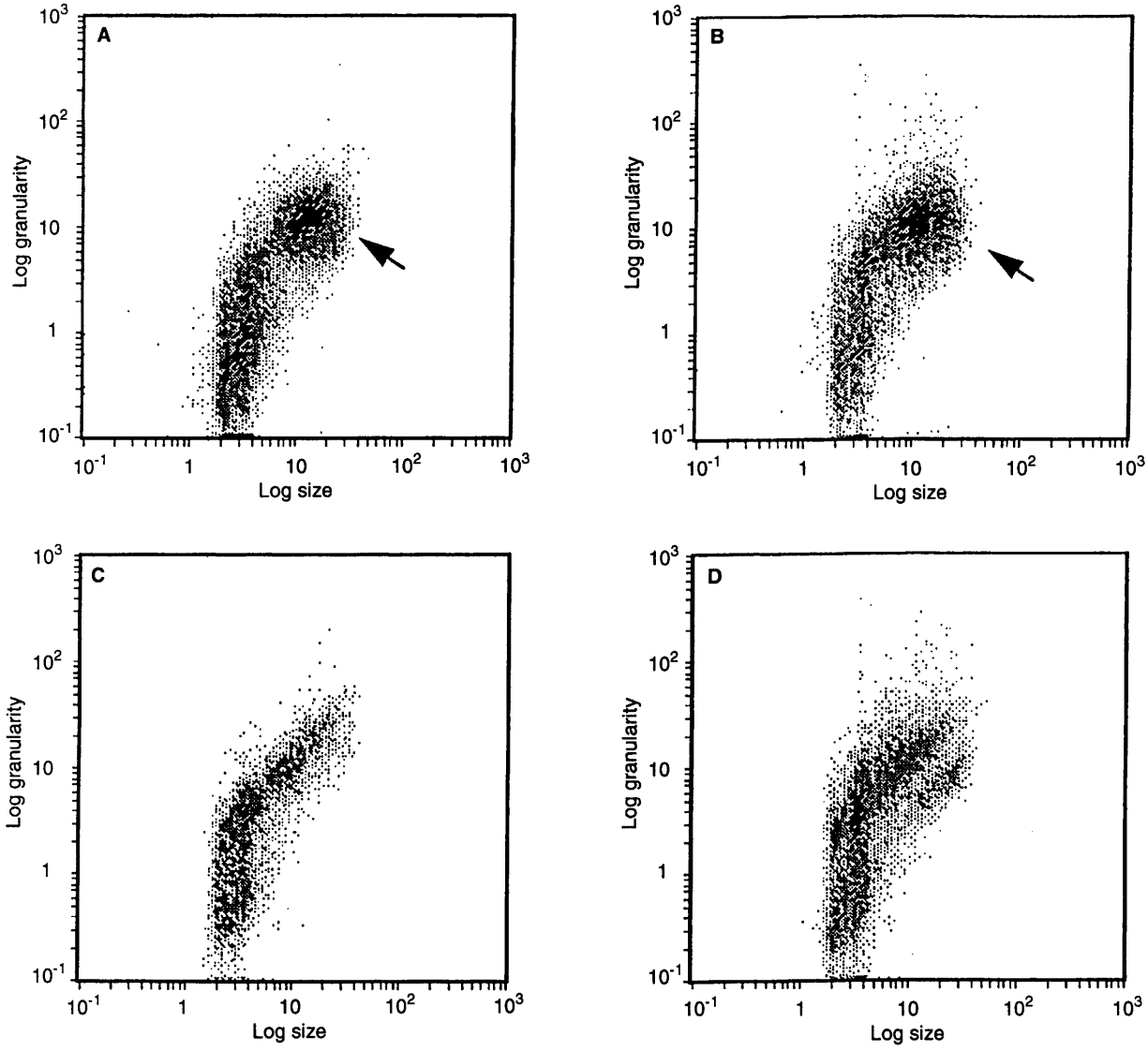

Fig. 2 A–D Morphological analysis by flow cytometry of protoplast suspensions obtained from Cryo-2 and Control-2 callus lines. Dot plots show representative patterns of forward-angle (log size) versus right-angle (log granularity) light scatter of protoplast suspensions. Cryo-2 A L-1 and L-2 suspensions, which have substantial numbers of undigested cells, show an increased content of larger cells with a higher degree of cytoplasmic complexity (*arrows*). **A** Cryo-2 A L-1 cells. **B** Cryo-2 A L-2 cells. **C** Cryo-2 A L-3 cells. **D** Control-2 L-1 cells

seeds of three different plants (Cryo-1 A Cryo-1 B_1, and Control-1) to establish the nine callus lines used in these experiments (three callus lines from three seeds of each plant). Table 3 shows the effect of three freezings (–20°C for 2 h), separated by recovery periods of 3 weeks, on the survival of the callus lines. No significant differences in the pattern of response to either one, two, or three freezings were detected between the nine callus lines tested. Following the first freezing, survival decreased to an average of 37.7%. A second exposure of the surviving calli increased the resistance in most lines, although not to significant levels. The third freezing significantly decreased survival in all lines to an average of 21.9%.

Characterization of protoplast and cell populations from Cryo-2 and Control-2 callus lines

Protoplasts prepared from the Cryo-2 and Control-2 callus lines, used to study the response to freezing stress, were characterized by microscope observations and subsequent flow cytometric analyses.

Protoplast yield and viability as well as the number of elongated cells that remained undigested were monitored for each protoplast preparation (Table 4). The protoplast yield varied in the different lines from approximately 1 to 13×10^6 per gram fresh weight. Protoplast viability was determined with methylene blue which is taken up by all cells, but only those with sufficient reducing power are able to produce the leuco form of the dye. The percentage of living protoplasts was similarly high (90–95%) in all protoplast preparations. Some lines showed a high number of elongated cells (Cryo-2 A L-1, Cryo-2 A L-2, and Cryo-2 B_1 L-2), while the remaining cell lines showed few or no elongated cells. The abundance of cells appeared to be unrelated to the response to freezing stress of the different

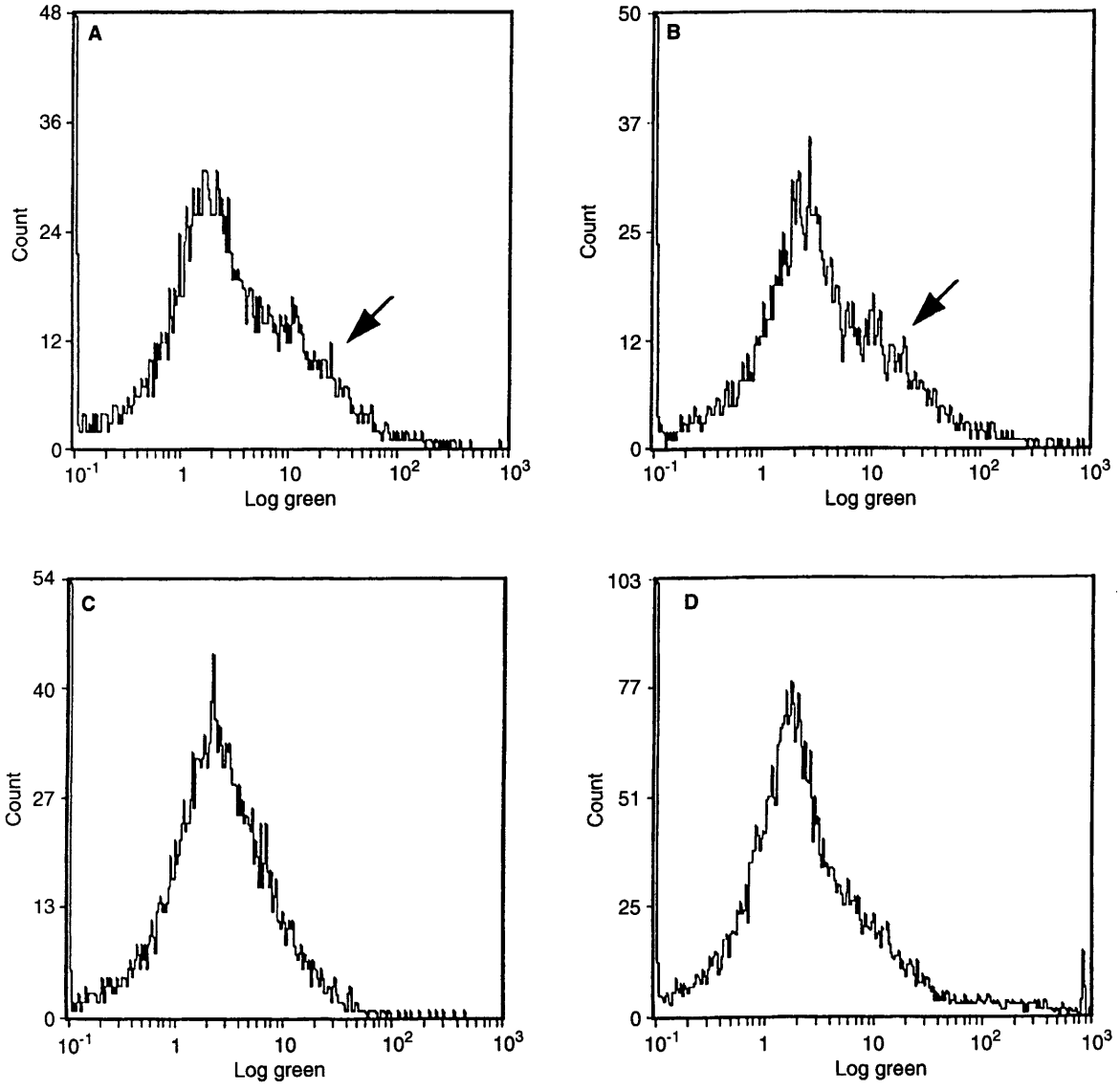

Fig. 3A–D Analysis of cell viability by flow cytometry of protoplast suspensions obtained from Cryo-2 and Control-2 callus lines. Histograms show representative distribution of intracellular FDA content (log green) in protoplast suspensions. While all suspensions analyzed were essentially composed of viable cells (FDA positive), the wider fluorescence distribution in Cryo-2 A L-1 and L-2 suspensions, which have substantial numbers of elongated cells, suggests the presence of a subpopulation with increased cytoplasmic accumulation of FDA (*arrows*). Cryo-2 A L-3 and Control-2 L-1 suspensions are more homogeneous for FDA fluorescence. **A** Cryo-2 A L-1 cells. **B** Cryo-2 A L-2 cells. **C** Cryo-2 A L-3 cells. **D** Control-2 L-1 cells

Table 4 Protoplasts and elongated cells obtained from the nine callus lines that were also used to study the response to freezing stress

Callus line	Number of protoplasts per gram fresh weight	Living protoplasts (%)	Elongated cells (%)
Cryo-2 A L-1	1.68×10^6	94.7	44.1
Cryo-2 A L-2	2.50×10^6	90.0	53.8
Cryo-2 A L-3	9.46×10^6	90.9	0.0
Cryo-2 B_1 L-1	9.99×10^6	91.2	0.0
Cryo-2 B_1 L-2	6.01×10^6	90.0	14.3
Cryo-2 B_1 L-3	13.40×10^6	90.2	2.2
Control-2 L-1	8.30×10^6	91.1	0.0
Control-2 L-2	10.40×10^6	92.8	0.0
Control-2 L-3	5.07×10^6	90.7	8.5

callus lines and was consistent in the same lines in three additional protolast preparations (data not shown).

We also characterized the suspensions by flow cytometry in order to correlate microscopical observations with structural and metabolic features which could help to reveal functional differences in protoplasts originated from Cryo-2 and Control-2 callus lines. While flow cytometry is extensively used in clinical and basic studies in human and animal cells, its applications in plant research is much less frequent, and there are still few functional studies in protoplasts (Galbraith 1994).

Cell size and intracellular complexity (due to cytoplasmic organelles and vesicles) were estimated by the scattering of laser light at a small angle along the laser axis

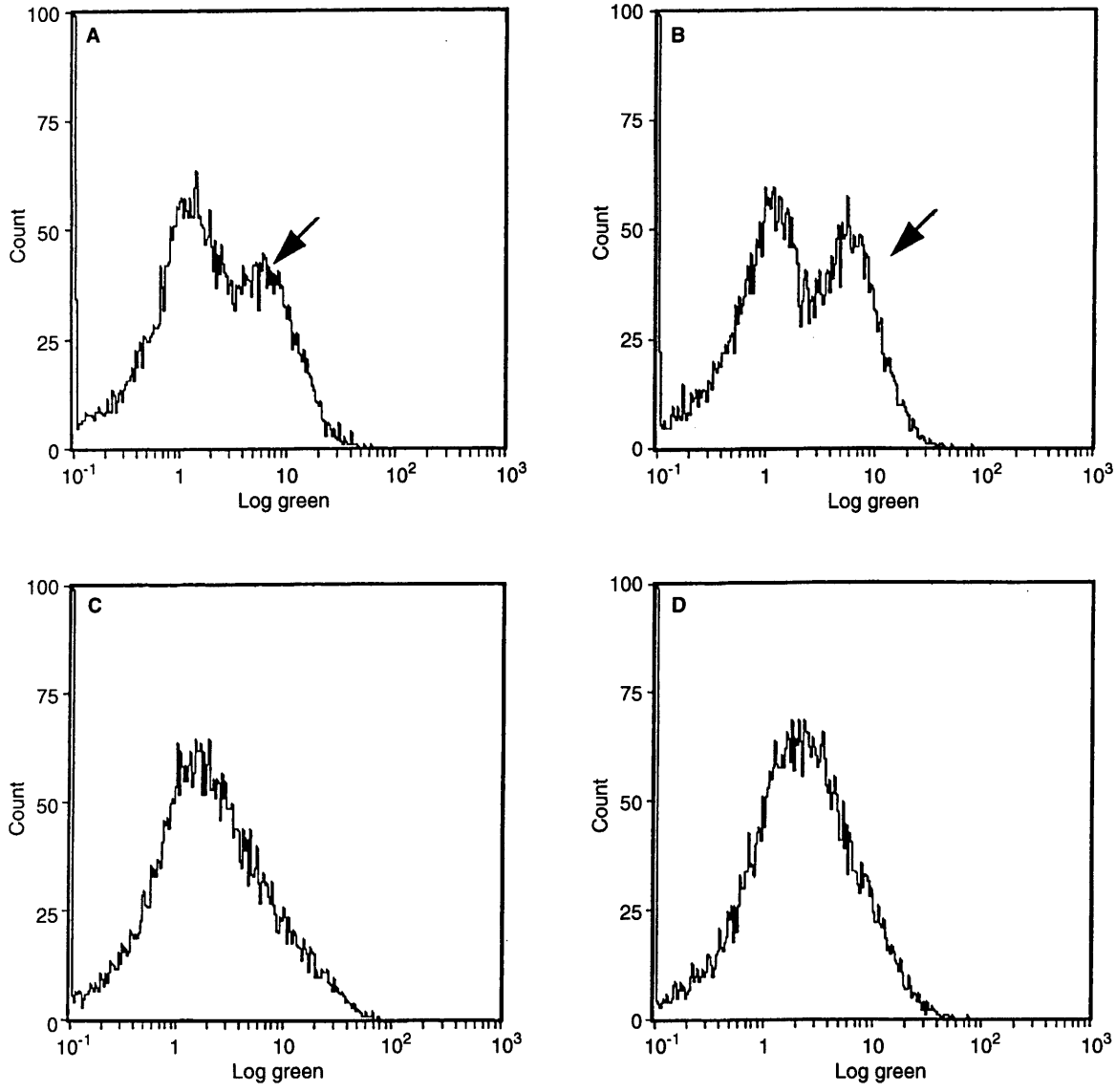

Fig. 4A–D Analysis of lipids by flow cytometry of protoplast suspensions obtained from Cryo-2 and Control-2 callus lines. Histograms show representative distribution of intracellular rhodamine B content (log green) in protoplast suspensions. Rhodamine B fluorescence distribution in Cryo-2 A L-1 and L-2 protoplasts, which are the suspensions with substantial numbers of elongated cells, show a cell subpopulation of higher intracellular lipid content (*arrows*). Cryo-2 A L-3 and Control L-1 suspensions show a single population for rhodamine B fluorescence. **A** Cryo-2 A L-1 cells. **B** Cryo-2 A L-2 cells. **C** Cryo-2 A L-3 cells. **D** Control-2 L-1 cells

(proportional to cell size; Kerker 1983) and at right angles to the laser beam (indicative of intracellular complexity; Dubelaar et al. 1987). As seen in Fig. 2, protoplast suspensions from Cryo-2 and Control-2 lines showed similar patterns of light scattering, with two types of cell subpopulations, one of lower forward and right-angle scatter and one of higher forward and right-angle scatter. However, the suspensions were different in their relative proportions of each subpopulation. Thus, protoplasts from Cryo-2 A L-1 and L-2, which were the suspensions with the highest number of undigested cells (Table 4), were rich in high-scatter cells, consistent with larger cell size and granularity. In contrast, Cryo-2 A L-3 and Control-2 L-3, which were both devoid of elongated cells, comprised mostly cells with low forward and right-angle scatter, i.e., were composed of smaller, less complex protoplasts. These data show that flow cytometry analysis based upon scatter properties, i.e., without the need of fluorescent staining, might be useful for screening and quality control of protoplast preparations.

To assess the viability of protoplast suspensions, we performed flow cytometric assay with FDA. FDA is a nonpolar molecule that upon entering the cells releases fluorescein, a polar and fluorescent molecule, into the cytoplasm due to esterases activity (Shapiro 1995). Therefore, viability determinations with either FDA or methylene blue are based on protoplast metabolic activity. As shown in Fig. 3, all cell preparations analyzed were positive for FDA, and thus comprised almost only living cells, a result consistent with the high viability scored by optical micros-

copy using methylene blue. Analysis of the fluorescence distribution in the FDA-stained suspensions indicated that the two lines previously shown to contain a high number of elongated cells and a high-scatter subpopulation, exhibited a more heterogeneous distribution than the lines that did not contained elongated cells. The heterogeneity was suggested by the presence of a shoulder due to an increased number of cells with a higher FDA content.

A further degree of cellular heterogeneity within the Cryo-2 A L-1 and L-2 lines could be observed when the suspensions were stained with rhodamine B, a basic fluorescent marker for charged lipids in animal cells (Von Steyern et al. 1996) and plant protoplasts (Arnalte et al. 1991). In this case (Fig. 4), Cryo-2 L-1 and L-2 exhibited two subpopulations of rhodamine-B-stained cells: one of lower fluorescence with a mean fluorescence intensity similar to that of Cryo-2 L-3 and Control-2 L-3 lines, and one distinct subpopulation of higher rhodamine B content with a mean fluorescence intensity which was about tenfold that of the first subpopulation. This finding is consistent with the presence of cells of greater size and intracellular complexity demonstrated by morphological analysis, by flow cytometry, and microscopical examination of the suspensions.

The morphological and structural studies of seedlings and callus cells, as well as their response to freezing stress, have shown some expected variability between lines from individual seeds. However, these differences were unrelated to the origin of the parental plants (cryopreserved or control calli), and freezing stress resistance was not transmitted to the progenies. Therefore, we conclude that selection pressure during cryopreservation did not affect the phenotypic characteristics of the progenies evaluated in this research.

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