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Cryopreserved callus: a source of protoplasts for rice transformation

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ABSTRACT

We cryopreserved whole rice calli (*Oryza sativa* L cv Taipei 309) to investigate the ability of the surviving cells to regenerate plants and yield protoplasts competent for genetic transformation. Four out of six callus lines cryopreserved after four months in culture contained small sectors able to continue cell division and subsequently regenerate fertile plants. Both cryopreservation efficiency and regeneration ability decreased when using eight month old cultures. High yields of protoplasts were obtained from different cryopreserved callus lines. Protoplasts were transfected with chimeric genes consisting of the maize ubiquitin 1 promoter, first exon and first intron fused to the coding region of either the GUS or BAR marker genes. Levels of transient gene expression from both marker genes were similar to those previously obtained using protoplasts derived from callus that had not been frozen. Stable transformants were selected by their resistance to Bialaphos and could be identified with the pH indicator chlorophenol red. Southern blot analysis confirmed the integration of the BAR gene into the rice genome. Therefore, cryopreservation does not affect the ability of rice cells to integrate and express foreign genes.

Abbreviations: BA: 6-benzylaminopurine; BAR: Bialaphos-resistance; CaMV: cauliflower mosaic virus; CPS: cryoprotectant solution; CR: chlorophenol red; 2,4-D: 2,4-dichlorophenoxyacetic acid; DMSO: dimethyl sulfoxide; FW: fresh weight; GUS: β-glucuronidase; IOD: interoptical density; MS: Murashige and Skoog; MU: methyl umbelliferone; NAA: naphthaleneacetic acid; PAT: phosphinothricin acetyl transferase; PEG: polyethylene glycol; TTC: 2,3,5, triphenyltetrazolium chloride; UBI: maize ubiquitin 1 promoter, first exon and first intron.

INTRODUCTION

Interest in the cryopreservation of rice tissues has increased with the development of biotechnology. The ability to recover plants from frozen tissue became important with the progressive need to maintain the genetic stability of selected clones obtained by induced mutagenesis or somaclonal variation (Bajaj and Sala, 1991). At present, cryopreservation also offers the possibility of storing transgenic rice lines (Meijer et al. 1991).

The production of transgenic rice plants, either by direct DNA uptake into protoplasts or by particle bombardment of embryogenic calli, generally requires cell lines with competence for both plant regeneration and genetic transformation (for a review see Cao et al. 1991). Such lines have limited lifespans. Therefore, the ability to cryopreserve lines of proven competence would allow the use of the same lines for transformation experiments performed over extended periods. This would make such experiments more reproducible and bypass the need for the continuous establishment and testing of new cell lines.

Previous reports on cryopreservation of undifferentiated rice cells have focused mainly on establishing the freezing and thawing conditions that allowed subsequent growth (Cella et al. 1982, Kuriyama et al. 1989, Meijer et al. 1991, Sala et al. 1979, Ulrich et al. 1984). In these investigations, the cryopreserved cultures were either cell suspensions or friable calli that were dispersed prior to freezing. The competence of cryopreserved rice cells for genetic transformation has not been studied to date.

We previously demonstrated that protoplasts prepared directly from calli are useful for the production of transgenic plants (Cornejo et al. 1993). We now test whether the same type of calli used in that research could be cryopreserved without having to establish cell suspensions. Our results show that the callus cells surviving the freezing and thawing procedures are competent for plant regeneration and prove to be a reliable source of protoplasts for genetic transformation experiments.

MATERIALS AND METHODS

Cryopreservation of rice callus. Callus lines were in use from mature embryos of rice (*Oryza sativa* L, cvs Taipei 309 and Calmochi 101) as described previously (Cornejo et al. 1993). Calli were induced and maintained on an MS medium composed of MS salts (Murashige and Skoog 1962),

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R-2 vitamins (Ohira et al. 1973), 3% (w/v) sucrose and 0.7 % w/v agarose, pH = 7 and supplemented with 10μM 2,4-dichlorophenoxyacetic acid (2,4-D).

To cryopreserve callus lines that had been in culture for 4 to 8 months, calli (100 to 150 mg of fresh weight) with a compact and nodular appearance were used. Individual calli were transferred to sterile cryovials containing an ice-cold cryoprotectant solution (CPS) at half strength and left on ice for 15 min. This solution was replaced with full strength CPS and the vials were left on ice for an additional 15 min before transfer to a programmed freezer (RTE-110, Neslab Instruments Inc., Portsmouth NH*), previously cooled to ca. 4ºC. Samples were cooled at an approximate rate of 1 ºC/min to -25 ºC, maintained at this temperature for 30 min and stored in liquid nitrogen for at least one week.

For thawing, vials were placed in a water bath at 30 ºC until the solution melted. Then, vials were placed on ice and the CPS removed by gradual dilution with ice-cold culture medium lacking ammonium ions (Kuriyama et al. 1989). Cryopreserved calli as well as control calli that had not been frozen were plated on solidified media (MS with 10 μM 2,4-D or N6). N6 medium contains N6 salts (Chu et al. 1975), 2 % (w/v) sucrose, 100 mg/l casein hydrolysate, 2.9 g/l L-proline, 2 mg/l glycine, 1 mg/l thiamine HCl, 0.5 mg/l each of nicotinic acid and pyridoxine, and 5 μM 2,4-D, pH = 5.8. Cultures were incubated in darkness at 27ºC and transferred to fresh medium every three weeks.

Immediately after thawing, the decrease in viability of the cryostored calli with respect to controls that had not been frozen was tested by measuring cellular respiration, based on the reduction of 2,3,5,-triphenyltetrazolium chloride (TTC). The TTC test was performed essentially as described by Towill and Mazur (1975), using samples of approximately 50 mg fresh weight (FW) and loss of color intensity was quantified by recording the absorbance at 530 nm (Steponkus and Lanphear 1967). Each value was expressed as percent of the absorbance / g FW with respect to the absorbance / g FW of the control, with the latter set to 100 %.

* The use of a brand name by the USDA implies no approval of the product to the exclusion of others that also may be suitable.

Plant regeneration from cryopreserved rice calli. One to two months after thawing, the small callus portions showing cell regrowth were separated from the remaining (non-dividing) callus cells and subcultured individually for three to six more weeks to establish callus lines.

Twelve cryopreserved callus lines (cv Taipei 309) were transferred to regeneration media and incubated under a 16 h photoperiod at 27ºC. For each line, the following two media were used: MS with 0.8 μM 6-benzylaminopurine (BA) and MS with 2.7 μM naphthaleneacetic acid (NAA) and 11.6 μM kinetin. Plantlets were moved to pots and kept under mist sprayers in the greenhouse until flowering.

Isolation, transformation and culture of protoplasts from cryopreserved callus lines. Protoplasts were prepared from several cryopreserved callus lines, 4 to 8 months after thawing. Protoplast isolation, transformation by the PEG method, and subsequent culture were performed as described previously (Cornejo et al. 1993).

The following plasmids were used for transformation: Plasmids UBI:GUS and UBI:BAR consist of the maize *Ubi 1* promoter and 5' untranslated sequence, including the first intron (Christensen et al. 1992) fused to the coding region of either the GUS (β-glucuronidase, Jefferson et al. 1987) or BAR (Bialaphos-resistance, Thompson et al. 1987) marker genes followed by the 3' untranslated region and polyadenylation signal of the nopaline synthase gene (Bevan et al. 1983). UBI:GUS was provided by Alan Christensen and Peter Quail (Plant Gene Expression Center, UC Berkeley-USDA, Albany, CA) and UBI:BAR by Douglas Gurian-Sherman (Western Regional Research Center, USDA-ARS, Albany, CA). BAR is the sequence from the *bar* gene of *Streptomyces hygroscopicus* (Thompson et al. 1987) that encodes phosphinothricin acetyl transferase (PAT), which inactivates phosphinothricin. For a comparison of promoter activity in transient assays, we used CaMV 35*S* promoter /*Adh 1* intron 1:GUS, our designation for plasmid BP1 that was constructed by Michael Fromm (Monsanto Corp., St. Louis, MO).

To select for stable transformants, two weeks after protoplast transformation, colonies were transferred to N6 medium with 0.5 mg/l of Bialaphos (Meiji Seika Kaisha Ltd., Tokyo, Japan), a herbicide that contains phosphinothricin as active ingredient. The resistance to Bialaphos was also tested with the pH indicator chlorophenol red (CR, Sigma) (Kramer et al. 1993). Control and Bialaphos-resistant calli were placed individually on 1 ml aliquots of solidified N6 medium with 0.5 mg/l Bialaphos and 50 mg/l CR, both added from filter-sterilized stock solutions. Color changes in the culture medium were monitored over a period of three days.

Enzyme and DNA analyses. Preparation of protein extracts from protoplasts and calli as well as enzyme assays are described elsewhere (Cornejo et al. 1993). Basically, fluorimetric GUS assays were performed according to Jefferson et al. (1987) and PAT activities were determined as described by Spencer et al. (1990). The incorporation of ^{14}C into the acetylated phophinothricin products was quantified by computing the integrated optical density (IOD) of the bands with whole band analysis software (Bio Image, Ann Arbor, MI).

Genomic DNA was isolated from rice calli as described by Gordon-Kamm et al. (1990), except that samples were of approximately 300 mg FW. Undigested and *Eco* RI-digested DNAs were size-fractionated by electrophoresis, blotted onto a nylon filter and hybridized to a probe consisting of the coding region of the BAR gene as described in Cornejo et al. (1993).

RESULTS AND DISCUSSION

Cryopreservation of rice calli and plant differentiation

To establish the cryopreservation conditions for morphogenic rice calli (cvs Taipei 309 and Calmochi 101), we tested several CPS in the freeze-thaw regimen described in Materials and Methods. Based on post-freezing viability determinations (data not shown), a solution composed of 2 M sucrose, 1 M glycerol, 1 M DMSO and 0.03 M proline was selected. A similar CPS except with 0.09 M proline, was used by Meijer et al. (1991) for the cryopreservation of rice cell suspensions. The CPS containing 1.3 M DMSO and 1 M glucose, used by Kuriyama et al. (1989) was also effective in supporting cell regrowth, although the post-freezing survival rates were approximately half those obtained with the selected CPS. In both cases, removal of CPS at 0 ºC was more effective (data not shown) than removal at room temperature (Finkle and Ulrich 1982) or no removal at all (Gnanapragasam and Vasil 1992).

Cell division was noticeable 4 to 8 weeks after thawing and took place within small portions of the calli located at or close to the surface. The dividing cells appeared yellowish, while the rest of the callus remained white (Fig. 1A). Similar results were obtained with both cultivars. The low proportion of dividing cells suggests that some selection might have taken place in

favor of either specific cell types, epigenetic variants or genotypes that were more adapted to withstand freezing and thawing. In this respect, Kendall et al. (1990) reported that cryoselected callus of wheat had higher tolerance to subsequent immersion in liquid nitrogen and plants recovered from these cryoselected calli also showed higher tolerance to freeze injury than control plants.

Table 1 shows the initial viability and subsequent cell division and plant regeneration from cryopreserved Taipei 309 calli prepared from either 4- or 8- month old cultures (cv. Taipei 309). These culture ages were selected because 8-month old calli that had not been frozen exhibit reduced morphogenic potential relative to the highly regenerative 4-month old calli, but protoplast yields are higher (M.J. Cornejo, unpublished observations). Callus lines frozen after 4 months in culture (lines 7 to 12) retained the ability to grow better than those frozen after 8 months (lines 1 to 6). Their initial survival rates, as measured by their respiration ability, were also higher: Values ranged from 2.7 to 22.1 and from 1.1 to 12.5 for 4- and 8-month old lines, respectively. However, we did not find a direct correlation between initial viability values of individual calli and further cell divisions, especially in the older calli. Therefore, the survival data give a rough estimate of the post-freezing viability but do not predict accurately whether the samples will continue growing.

Table 1. Effect of cryopreservation of 4-and 8-month old rice calli (cv Taipei 309) on their initial viability and subsequent growth and plant regeneration.

Callus line [a]	Viability [b] (% control callus)	Callus growth	No. of calli regenerating plants/ No. of calli plated [c]	
			BA	NAA+kinetin
1	1.4	+	0/7	0/7
2	6.5	-	-	-
3	1.1	+	1/7	0/7
4	12.5	-	-	-
5	1.6	-	-	-
6	1.8	-	-	-
7	22.1	+	2/14	10/14
8	10.4	+	3/14	ND
9	5	-	-	-
10	2.7	+	0/7	2/7
11	7.8	+	1/14	0/14
12	4.4	-	-	-

[a] Callus lines 1 to 6 and 7 to 12 were 8- and 4-month - old, respectively.

[b] Callus viability was determined by the TTC test and expressed as described in Methods.

[c] MS medium was supplemented with either 0.8 µM BA or 2.7 µM NAA and 11.6 µM kinetin.
ND = Not determined.

The cryopreserved Taipei 309 calli retained their competence for plant regeneration. Meijer et al. (1991) reported that protoplasts derived from cryopreserved rice suspensions are also competent for plant regeneration. Some cryopreserved calli differentiated into plants after transfer from the maintenance media (MS with 10 µM 2,4-D or N6) to MS media supplemented with either BA or NAA and kinetin (Table 1 and Fig. 1B). The percentage of plant regeneration was higher in cryopreserved calli derived from younger callus cultures (Table 1). All 20 plantlets that were transferred to soil set seeds (Fig. 1 C). Seed set was slightly reduced when compared to plants originated from seeds, but similar to plants regenerated from calli that had not been frozen.

These experiments demonstrate that cryopreservation of morphogenic rice cells can be achieved directly from calli and does not require the establishment of cell suspensions or the use of clumps from very friable calli.

Transient gene expression in protoplasts from cryopreserved calli The procedures for protoplast isolation and transformation previously established for rice calli (Cornejo et al. 1993) were also effective when using cryopreserved calli. At the time of protoplast preparation, their appearance was similar to that of calli that had not been frozen, i.e., compact and nodular. The average yield of protoplasts obtained in 6 experiments (4 with cv Taipei 309 and 2 with cv Calmochi 101) was 8 x 10^6 protoplasts/g FW, approximately 4-fold higher than the typical yield obtained from rice calli of the same total age that had not been frozen (M.J. Cornejo and D. Luth, unpublished observations). The basis for the increased protoplast yield is not understood.

Protoplasts from both cultivars could be transfected, as indicated by their transient gene expression levels approximately 40 h after the introduction of DNA. For example, protoplasts (cv Calmochi 101) transfected with UBI:GUS had a GUS specific activity of 0.6959 pmol methyl umbelliferone (MU)/min/µg of protein, 80-fold higher than the background activity from the controls. Table 2 shows the specific GUS activities of a batch of protoplasts (cv Taipei 309) separated into three aliquots and transfected under identical conditions with CaMV 35*S*:GUS, UBI:GUS or no plasmid DNA.

Table 2. Transient GUS expression driven by the CaMV 35*S* and maize ubiquitin promoters in protoplasts prepared from cryopreserved calli (cv Taipei 309).

Promoter/ intron combination	GUS specific activity [a] (pmol MU/min/ µg protein)	GUS activity relative to control (no DNA)
Ubiquitin/ ubiquitin intron 1	1.7284	82.70
CaMV 35*S*/ ADH intron 1	0.1716	8.21
Control (no DNA)	0.0209	1

[a] Data are the average of two separate experiments.

As occurred with protoplasts derived from rice calli that had not been frozen (Cornejo et al. 1993), UBI directed approximately 10-fold higher expression levels than CaMV 35*S*. The high levels of transient GUS expression driven by this ubiquitin promoter as compared to levels from other promoters have been reported for maize protoplasts (Christensen et al. 1992) and for several other cereal/grass cell suspensions and immature embryos (Taylor et al. 1993).

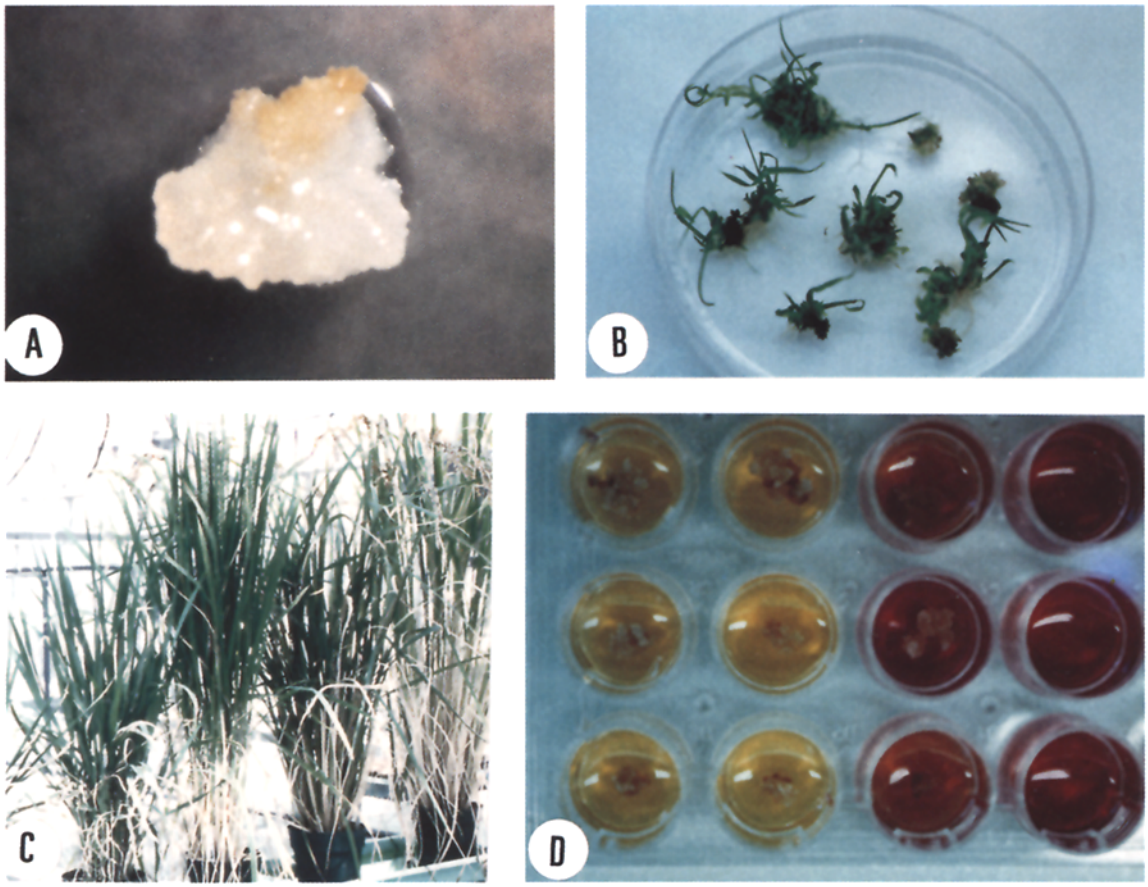

Fig. 1. Plant regeneration from cryopreserved rice calli (cv Taipei 309) and detection of stable UBI:BAR transformants. A) Cryopreserved callus showing yellowish tissue that retained viability upon storage and reinitiated growth. B) Plant regeneration from a cryopreserved callus line. C) Flowering rice plants obtained from cryopreserved callus cultures. D) Rapid detection of stable UBI:BAR transformants. Two Bialaphos-resistant callus lines and one control (untransformed) line were placed (3 samples/line) on solidified selection medium containing 50 mg/l of chlorophenol red. After 2 days, the medium below tranformed calli (columns 1 and 2) has turned yellow while that below control calli remains red (column 3). Three wells containing only medium are also shown (column 4).

Protoplasts derived from cryopreserved calli were also transfected with UBI:BAR. Transient expression of the BAR gene (Fig. 2) was measured by scanning the area of the autoradiogram corresponding to the acetylated product (lane P, arrow). In the extract from protoplasts that had not been transfected (lane NTP), the acetylated product was not detectable.

These results show that protoplasts prepared from several cryopreserved callus lines of two rice cultivars are competent for transformation and transient expression of both a screenable (GUS) and selectable (BAR) marker gene. The levels of GUS expression varied as a function of the promoter used (UBI or CaMV 35*S*), as previously reported for protoplasts prepared from callus lines that had not been frozen (Cornejo et al. 1993).

Selection of stable transformants and DNA analyses

The maize ubiquitin promoter has been used to drive the expression of the GUS and/or BAR genes in transgenic rice obtained by transformation of protoplasts (Cornejo et al. 1993, Toki et al. 1992) and in transgenic wheat obtained by particle bombardment (Weeks et al. 1993). Therefore, we tested whether stable transformants could be obtained from protoplasts derived from cryopreserved calli and transfected with UBI:BAR.

The protoplasts used in these experiments were prepared from a callus line that was cryopreserved after 8 months in culture (Table 1, #3) and propagated for 4 more months prior to protoplast isolation and transformation. Protoplast-derived calli were selected under a somewhat low dosage (0.5 mg/l) of Bialaphos. The resistance to Bialaphos of putative transformants was further tested with the pH indicator chlorophenol red (CR) (Fig 1D). This assay was first used by Kramer et al. (1993) for the selection of protoplast-derived maize colonies containing the BAR gene. Controls and Bialaphos-resistant calli were placed on selection medium that also contained CR. After incubation for only 1 1/2 to 2 days, the media carrying putative transformants turned yellow (pH=4.8) or yellow orange while the media with control calli remained red (pH=6). The color change was indicative of the decrease in pH that occurs in media supporting actively growing cells.

Fig. 2 includes the PAT assays of two of the callus lines (C-1 and C-2) selected by their resistance to Bialaphos. The levels of PAT activity, as determined by the IOD values of the bands corresponding to the acetylated products, are 7.42 and 4.56. No activity was detected in the control (lane NTC). These levels of PAT activity are in the same range that levels found in stable BAR transformants obtained from protoplasts prepared

from callus that had not been frozen and transformed with the same plasmid (Cornejo et al. 1993).

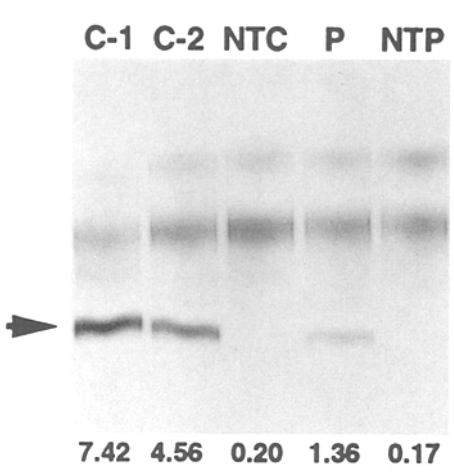

Fig. 2. Autoradiogram showing PAT activity in transformed protoplasts isolated from cryopreserved calli and two transgenic callus lines derived from these protoplasts. Arrow indicates the migration position of the acetylated form of phosphinothricin. Lanes NTC and NTP contain extracts from nontransformed calli and protoplasts, respectively. Lanes C-1 and C-2 contain extracts from two transgenic callus lines and lane P contains extracts from transformed protoplasts. Numbers under each lane indicate the IODs of the bands corresponding to the acetylated product and the background IODs in the controls.

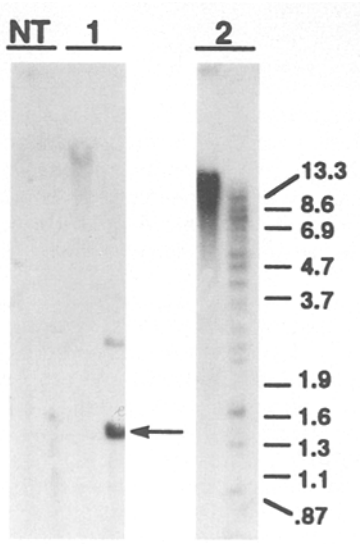

Fig. 3. Southern blot analysis of DNA from control and transgenic calli obtained by transformation of protoplasts prepared from cryopreserved calli. DNA was hybridized to a probe specific for the BAR coding region. Lanes labelled NT contain DNA from nontransformed calli and lanes labelled 1 and 2 contain DNA from two stable transformants selected by resistance to Bialaphos. For each numbered pair of lanes undigested DNA is to the left and DNA digested with *Eco* RI is to the right. The arrow indicates the predicted position of the vector DNA digested with *Eco* RI. The sizes of molecular weight standards are indicated in Kbp to the right.

Analysis of DNA from two callus lines exhibiting PAT activity confirmed the stable integration of the BAR gene into the rice genome. Fig. 3 shows the Southern blot of undigested and *Eco* RI-digested DNA hybridized to the coding region of the UBI:BAR plasmid. When digested with *Eco* RI, the transformant in lane 1 shows a prominent band of about 1.4 Kbp, which is the size expected for an intact BAR coding region (Fig. 3, arrow). In addition, several larger DNA fragments hybridized to the probe. A second transformant (Lane 2) shows multiple bands homologous to the BAR probe, although it does not appear to have the expected 1.4 Kbp band. This indicates the incoming plasmid was rearranged. In both cases, undigested DNAs contain a high molecular weight band at the position of chromosomal DNA that hybridizes to the BAR probe, suggesting that the DNA has been integrated into the genome.

We did not obtain plants from these transgenic calli. This might be related to the fact that the cryopreserved callus line used to prepare protoplasts was approximately one year old (including the period before and after cryopreservation). Since we have shown that following cryopreservation, calli are able to regenerate into plants, it appears that cryopreservation *per se* does not affect the morphogenic potential of rice cells.

In summary, the ability of protoplasts prepared from calli to stably integrate and express foreign genes is reliably reproduced following cryopreservation. Although, further research is needed to optimize the cryopreservation conditions in order to make the procedure fully useful for the routine production of transgenic rice plants.

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