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Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.)

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ABSTRACT

Protoplasts isolated from cultured rice cells of an A-58 cytoplasmic male sterile line (A-58 MS)(Oryza sativa L.) were used to investigate the regeneration of rice plants. A cultured cell line (T_3) of A-58 MS with a high growth rate and dense cytoplasm was selected. About 10% of the protoplasts prepared from this established cell line plated in RY-2 (a new medium) formed colonies. The calli formed shoots and roots in the regeneration medium and developed into whole plants.

Protoplasts also were prepared from suspension cultures of 25 other varieties of rice using the same methods. The protoplasts isolated from two of the 25 varieties, Fujiminori and Toyotama, had high rates of cell division in RY-2 medium. Only protoplast-derived calli from Fujiminori, produced whole plants in the regeneration medium.

ABBREVIATIONS

LS : Linsmaier and Skoog (1965)
2,4-D : 2,4-dichlorophenoxyacetic acid
BA : 6-benzyladenine
MES : 2-(N-Morpholino)ethanesulfonic acid, monohydrate

INTRODUCTION

Pioneering work in methods to prepare protoplasts from monocotyledonous plants has developed and established techniques which produce callus from various cereals including maize (Potrykus et al 1979, Chourey & Zurawski 1981), pearl millet (Vasil & Vasil 1979), sorghum (Brar et al 1980), and rice (Deka and Sen 1976, Cai et al 1978, Wakasa 1984). Although many investigators have attempted to regenerate plantlets from cereal protoplasts, in only a few cases, e.g. sugar cane (Srinivasan & Vasil 1985) and pearl millet (Vasil & Vasil 1980), has success been reported. Our interest in the culture of rice protoplasts and the regeneration of plants from these protoplasts is based on the objective of forming hybrids by cell fusion with a cytoplasmic male sterile cell line.

Formation of callus from protoplasts of rice has been reported by Deka and Sen (1976), Cai et al (1978) and Wakasa (1984). Although their reports have added to the information available on formation from rice protoplasts, they did not succeed in regenerating plants from the calli they derived from rice protoplasts.

According to several reports on the regeneration of plants from rice calli (Nishi et al 1968, Guha-Mukherjee 1973, Wakasa 1981, Tamura 1981), the genotype or variety of the original plants is critical for the success of plant regeneration. Therefore, we considered it important in our experiments on plant regeneration from rice protoplasts to screen for promising varieties from the large number in existence.

The regeneration of plants from calli of a number of varieties of rice has been reported (Wernicke et al 1981, Genovesi and Magill 1982, Lai and Liu 1982, Heyser et al 1983, Fukui1983, Yamada and Loh 1984). The varieties of rice used in those regeneration experiments also may be suitable for plant regeneration from rice protoplasts. We here report the selection of suitable rice varieties for plant regeneration, and the results of our experiments on the regeneration of plants from rice protoplasts.

MATERIALS AND METHODS

Callus formation : Seeds of the Oryza sativa A-58 cytoplasmic male sterile line (A-58 MS) were a gift from professor T. Kinoshita, Faculty of Agriculture, Hokkaido University. The husked seeds were sterilized using 70% (v/v) ethanol for 30 sec then 1% (v/v) sodium hypochlorite for 40 min, followed by 3 washings with sterilized distilled water.

LS basal medium supplemented with 20 μM 2,4-D, 3% (w/v) sucrose and 1% (w/v) agar adjusted to pH 5.6 was used for callus formation. A-58 MS seeds were placed on the medium and incubated in the dark at 25°C. After 40 days, 1 g F.W. of each primary callus was transferred to 300-ml flasks containing 70 ml of LS liquid medium and 20 μM 2,4-D, and the callus cultured on a gyratory shaker at 100 rpm in the dark, with subculturing every 3 days.

Callus from the other 25 varieties of rice were produced by the same methods.

Selection of the cell line for protoplast culture from A-58 MS: The T_3 cell line used for protoplast isolation and culture was selected by the following methods from suspension cultures of 30 cell lines of A-58 MS because of its fast growth and dense cytoplasm:

1) Every three days the total fresh weight of the cells was determined under aseptic conditions.

2) At the time of selection, the cells chosen were viewed by microscopy in order to select those cell lines with large numbers of clusters composed of 10-15 cells. In addition, those clusters in which cells were round and rich in cytoplasm were selected.

3) To maintain the selected clusters, the medium containing floating cells was decanted and the clusters transferred to fresh medium.

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Protoplast isolation: One gram (F.W.) of a 3-day-old suspension of cultured cells was added to 20 ml of filter-sterilized enzyme solution that consisted of 2% Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co.,Ltd.), 2% Macerozyme R-10 (Yakult Pharmaceutical Industry Co., Ltd.), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd.), 1% Driselase (Kyowa Hakko Kogyo Co., Ltd.), 0.8 % calf serum(Nakarai Chem. Co., Ltd.), 80 mM $CaCl_2 \cdot 2H_2O$, 0.125 mM $MgCl_2$ and 0.5 mM MES, in hormone-free LS medium containing 0.3 M glucose. The pH of the solution was adjusted to 5.6 before it was filter sterilized.

After ca. 9h of incubation in the dark without shaking, protoplasts were separated from the undigested cells by filtration through a 40 µm nylon filter then centrifugated at 250 x g for 4 min. The protoplasts were collected and washed 3 times with washing medium (0.8 % calf serum (Nakarai Chem. Co., Ltd.), 80 mM $CaCl_2 \cdot 2H_2O$, 0.125 mM $MgCl_2$ and 0.5 mM MES, in hormone-free LS medium containing 0.3 M glucose, pH 5.6), then sieved again through a 40 µM nylon filter. All protoplasts passing through the filter were collected. Yields of protoplasts were calculated with a hemocytometer before plating.

Protoplast culture: The A-58 MS and 25 varieties of rice protoplasts were cultured in modified medium in which the inorganic elements were the same as in LS medium and the vitamins and organic elements the same as in Kao's meduim. Modifications were made by checking the effects on protoplast division concentrations of ammonium and ferric ions and of the addition of calf serum.

The isolated protoplasts at a final plating density of 2.5×10^5/ml were cultured in 60 x 15 mm plastic petri-dishes containing 2 ml of newly prepared liquid medium. These dishes were sealed with Parafilm M and kept at 25°C in the dark without shaking.

Plant regeneration: After 40-50 days of culture in liquid RY-2 medium (Table 1), 0.5 g (F.W.) of the protoplast-derived callus formed was transferred to 100-ml flasks containing 15 ml of liquid N_6 medium (Chu et al 1975) (supplemented with 8% sucrose, but without hormones), and subcultured on a gyratory shaker at 100 rpm in the dark for 17 days. The cell aggregates that formed were transplanted to a regeneration agar medium (LS medium supplemented with 8% (w/v) sucrose, 0.8% (w/v) agar and 4×10^{-6}M BA, pH 5.6). These cultures for plant regeneration were maintained at 25°C under continuous fluorescent light of 3500 lux. The extent of plant regeneration was assessed visually. All the plants that regenerated were potted in vermiculite and placed in a transparent plastic cabinet to acclimatize them, after which they were transferred to a greenhouse.

Table 1. Composition of RY-2 medium for rice protoplasts.

a) Mineral Salt (mg/l)			
$(NH_4)_2SO_4$	67	KI	0.83
KNO_3	1900	H_3BO_3	6.20
KH_2PO_4	170	$ZnSO_4 \cdot 4H_2O$	8.60
$CaCl_2 \cdot 2H_2O$	440	$Na_2MoO_4 \cdot 2H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	370	$CuSO_4 \cdot 5H_2O$	0.025
$MnSO_4 \cdot 4H_2O$	22.3	$CoCl_2 \cdot 6H_2O$	0.025
EDTA-Na-Fe Salt	19.25		
b) Organic acid (mg/l)(adjusted to pH 5.5 with NH_4OH)			
Sodium pyruvate	5	Malic acid	10
Citric acid	10	Fumaric acid	10
c) Vitamins (mg/l)			
Nicotinamide	1	Biotin	0.005
Pyridoxine HCl	1	Choline chloride	0.5
Thiamine HCl	10	Riboflavin	0.1
D Calcium pantothenate	0.5	Ascorbic acid	1
Folic acid	0.2	Vitamin A	0.005
p-Aminobenzoic acid	0.01	Vitamin D_3	0.005
		Vitamin B_{12}	0.005
d) Inositol (mg/l)	100		
e) Hormone ($x10^{-5}$M)			
2,4-D	2		
f) MES (mM)	1		
g) Calf serum (Nakarai Chem. Co.) (ml)	8		
h) Glucose (M)	0.5		
i) pH	5.6 (NaOH)Filter Sterilized.		

a): Linsmaier-Skoog medium (1965) with modifications.
b,c,d): According to Kao (1977).

RESULTS

Selection of the cell line for the source of protoplasts: A cell line, T_3, selected from suspension-cultured A-58 MS cells after 10 months had a high growth rate and opaque, slightly yellow cytoplasms with small vacuoles. These T_3 cells were almost round. In contrast, the unselected A-58 MS cells had various shapes and colorless, transparent cytoplasms with large vacuoles.

Protoplasts were isolated from suspension cells of both the T_3 and original A-58 MS cell lines. The protoplast yield from T_3 was 6 times that from the original A-58 MS (Table 2). After purification, by two filtrations through a 40 um filter, we confirmed by microscopy that only protoplasts were present.

Improvement of the LS medium for protoplast culture: Effects of various factors on T_3 protoplast division were examined. Sugars, sugar alcohols and the concentrations of NH_4^+ and Fe^{3+} were very important factors for the culture of rice protoplasts. No protoplasts survived after 4 weeks in media in which the osmotic pressures of 0.2, 0.3, 0.4, 0.5, and 0.6 M had been adjusted with mannitol or sorbitol. Only at the osmotic pressures 0.4 and 0.5 M produced by glucose did protoplasts divide well. The rate of colony formation was 9.6% at 0.5 M osmotic pressure but decreased to 4% at 0.4 M (with calf serum, see below). At the 0.3 M osmotic pressure produced by sucrose, colonies formed with only 0.4% efficiency.

Ammonium ion was an important factor in obtaining a high rate of protoplast division. The original LS medium (1965) contains 1650 mg/l of NH_4^+ ion, but at this concentration, no colonies formed. We therefore determined the optimum concentration of NH_4^+ for protoplast division by trial and error and found that a concentration of 67 mg/l of NH_4^+ gave the best colony formation. Ferric ion also was an important factor for rice protoplast culture. But at the concentration of 38 mg/l used in standard LS medium (1965), no colony formation took place. At the Fe^{3+} concentration of 19 mg/l, marked colony formation occurred. Another important improvement was made by the addition of calf serum to the culture medium. Without calf serum, colony formation at osmotic pressures of 0.4 and 0.5 M glucose were 2% and 5%. An addition of 8% calf serum to 0.4 and 0.5 M glucose increased colony formation to 4% and 9.6%.

Eventually, a suitable medium (RY-2) for T_3 protoplast culture was obtained, the composition of which is listed in Table 1. Approximately 10% of the plated protoplasts produced cell colonies in this medium after 4 weeks of culture.

Isolation and culture of protoplasts from 25

Table 2. Properties of the T_3 cell line selected from A-58 MS cells.

	T_3	Original A-58 MS
Growth rate (% increase in fresh weight in 3 d)	103	37
Yield of protoplasts (10^6/g fresh weight)	8.1	1.3
Cytoplasm	dense	clear
Vacuole	small	large

varieties of rice: Protoplasts were prepared from suspension cultures of 25 varieties of rice by the methods used to get A-58 MS protoplasts (Fig 1-A), but no cell selection was done. Suspension-cultured cells of Fujiminori and Toyotama, however, grew very rapidly and had dense cytoplasms like the A-58 MS (T_3) cell line, and even though there was no special selection of these cells, significantly different yields were obtained (Fig 2). RY-2 medium, which was suitable for the growth of protoplasts isolated from A-58 MS (T_3) cells, was used to culture these protoplasts. Only two varieties, Fujiminori and Toyotama, showed a high frequency of protoplast division and colony formation (respectively 8.4% and 5.6% of the plated protoplasts formed colonies,)(Fig 1-B,C,D). Norin-1, Norin-22 and Akage had low rates of cell division, and no colonies formed. None of the other varieties showed protoplast division or colony formation (Table 3).

Regeneration of plants from A-58 MS (T_3) and Fujiminori cells: After colonies that had formed from protoplasts were cultured in N_6 liquid medium for 17 days, the cells (Fujiminori, Toyotama and A-58 MS (T_3)) were placed in regeneration medium. After 30 days of subculture, green spots appeared on some cells in the Fujminori and A-58 MS (T_3) calli (Shimada & Yamada 1979). No spots were present on cells in Toyotama callus. Some green spots grew, eventually forming shoots. After 40 days, approximately 1-cm long coleoptiles and first leaves emerged from these shoots (Fig 3-A). After 50 days, second and third leaves had developed and the rooted plantlets grew to about 5 cm (Fig 3-B). After 60 days, the leaves of the regenerated plants had reached the tops of the flasks and roots had elongated and expanded throughout the agar. These plants were potted and transferred to a greenhouse where they continued to grow (Fig. 3-C). One rice plant was regenerated from the protoplast-derived A-58 MS (T_3) cells and eight from the Fujiminori cells.

DISCUSSION

Rice plants were successfully regenerated from protoplasts of A-58 MS (T_3) cells and Fujiminori cells. This is the first reported case of plant regeneration from protoplasts of rice (O. sativa L.). Our success is due to the fact that (1) a cell line with a good growth rate and dense cytoplasm was selected, (2) that RY-2, a medium suitable for protoplast culture, was developed and (3) that a variety of rice genotypes were used.

The cell line (T_3) selected from A-58 MS cells showed a very good growth rate and its cells had opaque, yellow cytoplasms with small vacuoles and a high frequency of protoplast division. The Fujiminori cells, which we did not put through the selection procedure, had characteristics similar to the selected T_3 cells. Under microscopy these characteristics are useful markers for the selection of cells capable of regenerating plantlets. By selecting a cell line that has a dense cytoplasm, one may obtain a line that can differentiate plants.

Of the other 25 varieties, about 10 (including Fujiminori and Toyotama) showed high, or and relatively high, cell growth rates. But as stated, only protoplasts of Toyotama and Fujiminori divided well; therefore, the selection of a cell line that has a good growth rate is necessary, but not sufficient, condition for the regeneration of rice plantlets.

Protoplast division was strongly affected by the culture medium used. By improving standard LS medium we developed RY-2 medium which is effective for protoplast division. As shown in Table 2, calf serum was added and glucose used as the osmoticum, the amounts of ammonium and ferric ions decreased. These changes together with the other modifications listed

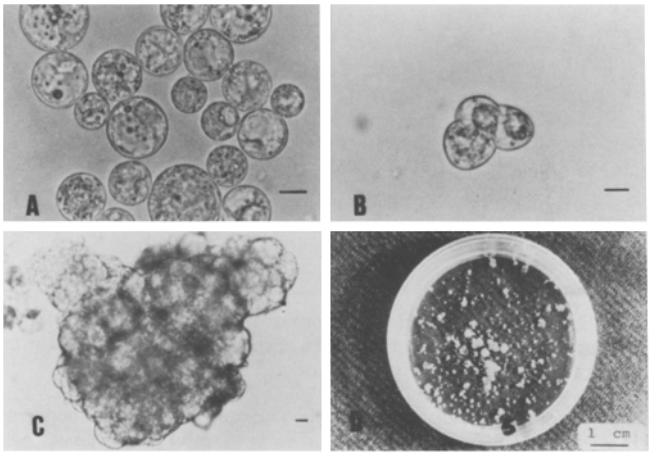

Fig 1. Protoplasts, cell division and colony formation by Fujiminori. (A) Isolated protoplasts from suspension-cultured cells of Fujiminori. (B) Cell division after 12 days of culture. (C) Cell clusters formed after 25 days of culture. (D) Colonies derived from Fujiminori protoplasts after 30 days. Bar: 10 μm.

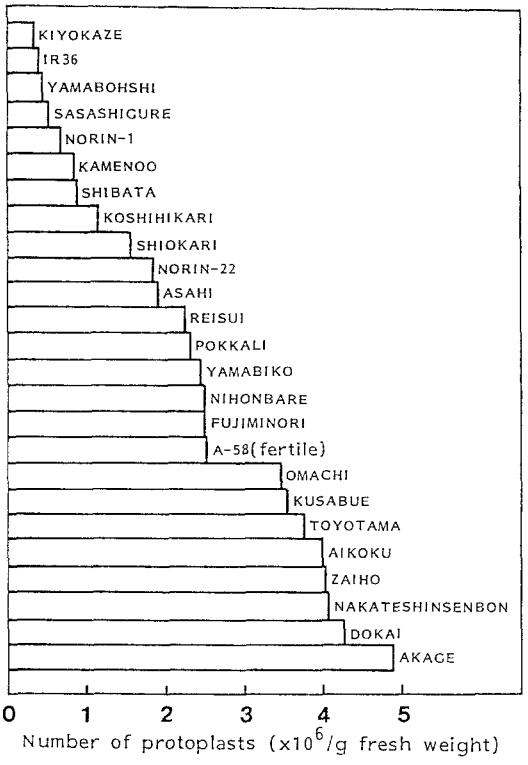

Fig. 2. Yields of protoplasts isolated from cultured cells of 25 rice varieties.

Table 3. Rice varieties forming colonies or showing cell division of protoplasts.

Colony formation	A-58MS (T_3)	(9.6%)[1]
	Fujiminori	(8.4%)[1]
	Toyotama	(5.6%)[1]
Cell division [a]	Norin-1	(0.01%)[2]
	Norin-22	(0.01%)[2]
	Akage	(0.01%)[2]

1) (No. of coloniesformed)/(No. of inoculated protoplasts) x 100 2) (No. of divided protoplasts)/(No. of inoculated protoplasts) x 100 3) No cell division took place in the unlisted varieties.

Fig. 3. Rice plant regeneration from Fujiminori protoplasts. (A) Shoot and root regeneration from the protoplast-derived cells. (B) Developing rice plants regenerated from the protoplasts. (C) Regenerated plants growing in a greenhouse.

were needed to obtain a high frequency of protoplast division in A-58 MS (T_3), Fujiminori and Toyotama cells. We were able to regenerate nine plants from the protoplast-derived cells of two of these three varieties, (8 plants from Fujiminori and one from A-58 MS (T_3)).

A critical point is whether there were cells mixed with our protoplasts. It is almost impossible to confirm whether all the colonies originated from single protoplasts. But, we isolated both the T_3 and Fujiminori protoplasts from suspension-cultured cells then filtered them twice through a 40 μm nylon filter to purify them as much as possible. The Fujiminori protoplast preparation purified in this way contained about 8 single cells per 1 x 10^5 protoplasts. We plated samples 5 x 10^5 protoplasts in petri dishes, and the average colony formation was about 10%. Even assuming that all single cells present in the dish formed colonies, at most there would be only 40 single-cell derived colonies among the 5 x 10^4 colonies in the dish; the ratio of the former to the latter being less than 0.1%.

In our regeneration experiments with Fujiminori protoplast, 396 clones from 5 x 10^4 colonies were used. Four clones regenerated rice plants; a regeneration ratio of about 1%. Therefore, the possibility that all the regenerated plants were derived from contaminating single cells is statistically negligible.

The problem addressed in this study is how to increase the plant regeneration ratio from rice protoplasts. Although differentiation of shoots and regeneration of plantlets from callus has been achieved with several varieties of rice, the calli of many other varieties could not initiate shoots (Kawata 1968, Daroyan & Smetamin 1979, Bhattachary & Sen 1980) or the ability to initiate shoots was lost after protracted subculturing (Nakano et al 1975, Daroyan & Smetamin 1979, Inoue & Maeda 1980). The calli (Fujiminori and A-58 MS) we used to isolate protoplasts had been cultured for more than two years. Therefore, the regeneration totipotency should have been lower than that of the original callus.

It may be possible to increase the plant regeneration ratio from protoplasts by using newly induced calli. Improvement of the regeneration ratio has been reported by Heyser and his associates (1983) who found that about 20% of their root-derived embryogenic callus produced shoots after 50 weeks of culture, whereas only 6% of root-derived non-embryogenic callus did so. The regeneration of pearl millet plants also has been reported from embryogenic callus (Vasil & Vasil 1980).

We also found a few embryogenic calli during protoplast culture in N_6 medium. If the regeneration of rice plants from protoplasts requires the formation of embryogenic cells, we must find a way to increase the embryogenic calli of rice in order to increase rice plant regeneration. Several possibilities that might operate in a higher regeneration frequency of plants from protoplasts of rice are currently being investigated in our laboratory.

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