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Enrichment of heterokaryocytes between mesophyll and epidermis protoplasts by density gradient centrifugation after electric fusion

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Summary. Mesophyll protoplasts from *Nicotiana glauca* were fused with epidermal protoplasts from *N. langsdorffii* by an electric pulse. After the fusion products were centrifuged on stepwise density gradient centrifugation using Percoll and sea water, somatic hybrids were observed at 70%–80% in the fraction recovered from the intermediate specific gravity fraction between epidermis and mesophyll protoplasts. From offsprings of these somatic hybrids, teratomatous plants were regenerated. Since the difference of specific gravity between mesophyll and epidermis protoplasts is inherent, this procedure can be essentially applied to obtain somatic hybrids between any combination of plants. The significance of this study is discussed in relation to obtaining somatic hybrids between plant materials without any appropriate genetic markers.

Key words: Density gradient centrifugation – Electric fusion – Epidermis protoplasts – Heterokaryocyte enrichment – Mesophyll protoplasts – *Nicotiana*

Introduction

Examples of somatic hybrids by protoplast fusion are increasing, and hybrids between sexually incompatible combinations have been reported in many cases (Harms 1983). Since utilized fusion methods cannot induce fusion between specific combinations of protoplasts, fusion products retain homologous as well as heterologous combinations of cells. Thus, the selection of heterokaryocytes from fusion products is a very important step in somatic hybridization. The only reported instances of selection methods are the complementation between genetically defective mutants (Melchers and Labib 1974; Glimelius et al. 1978) and the use of specific characteristics appearing in hybrids such as hybrid vigor (Schieder 1978; Nagao 1978). However, generally applicable selection procedures have not been established. Although the endowsement of different genetic defects on plant cells (such as nitrate reductase deficiency) might be utilized, it takes a long time to obtain such mutants. To place plant cells on artificial conditions for longer periods induces other undesirable characteristics such as losing regeneration ability, chromosome aberration, and other mutations. Thus, it is worthwhile to search for more generally applicable selection procedures to select heterokaryocytes from fusion products.

In this context, the use of mesophyll and epidermis protoplasts of leaves looks promising. Since the difference in specific gravity of mesophyll and epidermis cells is inherent, the fusion products between these two cells have an intermediate specific gravity. Thus, heterokaryocytes could simply be separated from the fusion products by density gradient centrifugation. Although this principle was tried after fusion with polyethylene glycol (PEG) by Harms and Potrykus (1978), heterokaryocytes were only observed at the frequency of ca. 30%.

Recent progress in protoplast fusion by electric pulses has produced many successful instances on the regeneration of plants from fusion products (Kohn et al. 1985; Bates and Hasenkampf 1985; Morikawa et al. 1986). A characteristic of this technique is that fusion can be induced instantaneously, which is in sharp contrast to other chemical treatments. The combination of electric fusion of mesophyll and epidermis protoplasts with density gradient centrifugation produces an ef-

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ficient procedure for separating heterokaryocytes, as longer treatment of protoplasts encountered in the fusion induced by chemicals which could affect the change of specific gravity of protoplasts, could be avoided.

Materials and methods

Preparation of protoplasts

Protoplasts were prepared from leaves of *Nicotiana glauca* and *N. langsdorffii* grown in a greenhouse. The leaves of these plants (whose lower epidermis were peeled off) were treated with an enzyme solution consisting of 0.05% Cellulase YC (Seishin Pharmaceutical Ltd., Nihonbashi-Koamicho, Tokyo), 0.05% Driselase (Kyowa Hakko Kogyo Ltd., Otemachi, Tokyo) and 0.02% Macerozyme R10 (Yakult Honsha Ltd., Higashi-Shinbashi, Tokyo) dissolved in To medium according to Chupeau et al. (1978). Incubation was carried out at 23 °C for 12–15 h with rotary shaking at 10 rpm in the dark.

The mesophyll protoplasts were purified from protoplast fraction of *N. glauca* after overnight enzyme treatment. First, protoplasts were centrifuged in 0.6 M sucrose using Babcock bottles (Shepard and Totten 1975) at 180×g for 5 min. Floated protoplasts were diluted with 7 times volumes of sea water (870 mOs) and collected by centrifugation at 120×g for 1 min. Precipitates were again centrifuged at 180×g on stepwise sea water-Percoll density gradient composed of 30%, 25%, 20% and 15% Percoll from the bottom. Mesophyll protoplasts were recovered from the interphase between 25%–20% Percoll layers with Pasteur pipets. The yield of mesophyll protoplasts was $1–5\times10^6$ from 1 g of leaves.

Epidermis protoplasts from *N. langsdorffii* were purified according to the same procedure as that for *N. glauca,* except that centrifugation in Babcock bottles was carried out in 0.6 M mannitol and stepwise sea water-Percoll gradient consisting of 20%, 15%, 10% and 0% of Percoll from the bottom. Epidermis protoplasts were recovered from the interphase between 15%–10% Percoll gradient. The yield of epidermis protoplasts from this source was $2–8\times10^5$ from 1 g of leaves. Although epidermis protoplasts were also isolated from *N. glauca,* their yield was ten times lower than that from *N. langsdorffii.* Thus, the epidermis protoplasts from *N. langsdorffii* were used throughout this study.

Fusion of protoplasts

Fusion was carried out using Electro Cell Manipulator (BTX Inc., San Diego, U.S.A.) equipped with 1 ml fusion chamber according to Zimmermann and Scheurich (1981). Protoplasts from both sources (2×10^5) were suspended in 0.6 M sucrose and dielectrophoresis was carried out using 1 MHz high frequency waves at 160 V/cm for 60 s. After adhered protoplasts were further compressed with 240 V/cm for 4 s, fusion impulse was given at 780 V/cm for 50 μs.

Selection of fusion products by stepwise sea water-Percoll density gradient centrifugation

After fusion treatment, protoplasts were recovered from the floated fraction by centrifugation at 150×g for 3 min and loaded on stepwise sea water-Percoll gradient of 25%, 20%, 15% and 10% of Percoll from the bottom. After centrifugation at 150×g for 5 min, enriched fusion products were recovered from the interphase between 20%–15% Percoll layer with Pasteur pipets. After adding four volumes of sea water to the recovered fractions, the fusion products were precipitated by centrifugation at 120×g for 1 min. The reason for using sea water to prepare density gradient was that with other conventional osmotica such as mannitol, sorbitol or sucrose, the protoplasts from epidermis with lower specific gravity could not be precipitated.

Culture of heterokaryocytes

Heterokaryocytes enriched fractions were cultured in a modified 8p medium (Kao and Michayluk 1975) in which 0.6 M mannitol and 1% sucrose were replaced with 0.38 M glucose. Fusion products were suspended at a density of 5×10^4/ml, and one drop was placed in the center of plastic Petri dish of 3 cm in diameter and cultured under continuous light of 3,000 lux at 25°–28 °C. After 19 days small colonies consisting of more than ten cells each were transferred to Murashige and Skoog (1962) (MS) medium without plant growth regulators. Obtained hormone autotrophic calluses were further subcultured on the MS basal medium solidified with 0.6% agar.

Staining of epidermis protoplasts with Carboxyfluorescein

To identify heterokaryocytes after density gradient centrifugation, epidermis protoplasts were stained with a fluorescent dye, 5(6)-Carboxyfluorescein (10 mg/l) dissolved in the enzyme solution described above according to Kanchanapoom et al. (1985). After fusion only heterokaryocytes should fluoresce red color of abundant chloroplasts from mesophyll protoplasts and yellow green color of Carboxyfluorescein from epidermis protoplasts under an epifluorescence microscope.

Results

Specific gravity of mesophyll and epidermis protoplasts

First we examined the distribution of specific gravity of mesophyll and epidermis protoplasts. As shown in Fig. 1, the specific gravity of mesophyll protoplasts of *N.*

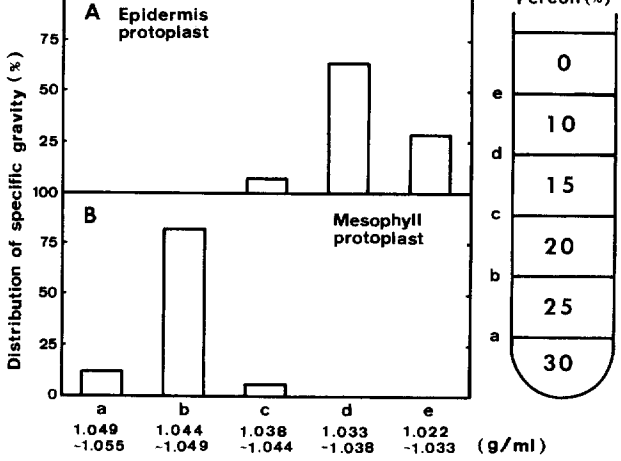

Fig. 1A, B. Distribution of the specific gravity of protoplasts from *N. glauca* and *N. langsdorffii.* Upper histogram (**A**) shows the distribution of specific gravity of epidermis protoplasts from *N. langsdorffii.* Lower histogram (**B**) shows the distribution of specific gravity of mesophyll protoplasts from *N. glauca.* Specific gravities of 0%, 10%, 15%, 20%, 25% and 30% of Percoll dissolved in sea water are 1.022 g/ml, 1.033 g/ml, 1.038 g/ml, 1.044 g/ml, 1.049 g/ml and 1.055 g/ml, respectively

glauca was in the range of 1.038–1.055 g/ml, while that of epidermis protoplasts of *N. langsdorffii* was in the range of 1.022–1.044 g/ml. Such a distribution pattern was reproduced in repeated preparations, although there was little fluctuation, if any. For fusion experiments, ranges between 1.044–1.049 g/ml and 1.033–1.038 g/ml were chosen for mesophyll and epidermis protoplasts, respectively, as the frequency of heterokaryocytes was significantly higher in this combination than in that by fusion between simple mixtures of mesophyll and epidermis protoplasts.

Protoplast fusion

The mesophyll and epidermis protoplasts were mixed in 0.6 M sucrose solution, and fusion was carried out. Under this condition both protoplasts floated on the osmoticum. After 5 min, fusion percentage was determined under a microscope, as the fusion products could be easily identified from the appearance of cells. The fusion frequency was 5%–10%, in which heterokaryocytes were one-half.

However, when fusion was carried out using 0.6 M mannitol as an osmoticum, only less than 0.05% of fusion percentage was observed. This difference occurred because in the 0.6 M mannitol solution mesophyll protoplasts tend to sink and epidermis protoplasts tend to float. Thus, these protoplasts had few occasions to associate.

Enrichment of the heterokaryocytes

Protoplasts after fusion treatment were loaded on stepwise sea water–Percoll density gradient, and after centrifugation the fusion products were recovered from the interphase between the specific gravity of 1.038–1.044 g/ml as described in "Materials and methods". As the fusion products became completely spherical after this treatment and mixing of both cytoplasms proceeded, the fusion products could not be easily identified under a microscope. Thus, the heterokaryocytes were identified under an epifluorescence microscope. Only the fusion products between mesophyll and epidermis protoplasts showed red fluorescence originating from abundant chloroplasts in mesophyll cells, and yellow-green fluorescence from epidermis which had been stained with Carboxyfluorescein. As shown in Table 1, frequency of heterokaryocytes in the enriched fractions was calculated to be 70%–83%, and the factor of enrichment was 35–50 times that of the initial value. Although in this study adjustment of osmotic pressure in density gradient was not considered, preparation of density gradient retaining the equality of osmotic values using sea water and sucrose (Harms and Potrykus 1978) showed essentially the same results as described in this study (data not shown).

Table 1. Frequency of the heterokaryocytes after electric fusion

	Heterokaryocytes (%)[a] ± SEM
Experiment 1	2.4 ± 0.6
Experiment 2	1.9 ± 0.5

[a] Frequency of the heterokaryocytes was calculated according to the following equation:

$$\text{Heterokaryocytes (\%)} = \frac{\text{Heterokaryocytes}}{\text{Total no. of protoplasts}} \times 100$$

Table 2. Frequency of the heterokaryocytes after the enrichment by sea water-Percoll density gradient centrifugation

	Heterokaryocytes (%)[a] ± SEM
Experiment 1	73.9 ± 9.2
Experiment 2	83.2 ± 3.8
Experiment 3	70.8 ± 4.4

[a] Frequency of the heterokaryocytes were calculated according to the following equation:

$$\text{Heterokaryocytes (\%)} = \frac{\text{Heterokaryocytes}}{\text{Total no. of protoplasts}} \times 100$$

Heterokaryocytes were identified under an epifluorescence microscope as described in "Materials and methods"

Culture of the heterokaryocytes enriched fractions

Prepared fusion products were cultured in the modified 8p medium. After three days, when cell division was observed, dilution of cells was carried out with the supplementation of the fresh medium. After ten days of culture, cells were diluted with an equal volume of hormone free MS medium. After 19 days small colonies consisting of 10–20 cells were embedded in the hormone free MS medium solidified with 0.6% agar. After two months, 58% of the heterokaryocytes formed colonies in the hormone free medium. When small colonies 2–3 mm in diameter were further transferred to the hormone free medium solidified with 1% agar, they differentiated many malformed shoots, 10% of which formed roots. Four percent of these offsprings did not show any tendency toward differentiation. The chromosome number of regenerated plants was examined in five randomly chosen materials, in which two plants showed 42 corresponding to an amphiploid of *N. glauca* and *N. langsdorffii*, and three other plants showed aneuploidy between 28–38.

Discussion

It has been shown in this study that heterokaryocytes were enriched to become 70%–80% in the fusion prod-

ucts by stepwise density gradient centrifugation, after mesophyll and epidermis protoplasts were fused by electric fusion. This procedure can be essentially applied to any combination of protoplasts and can be utilized as a general means to obtain somatic hybrids, when protoplasts are separated from mesophyll and epidermis from various plant species. It is especially useful for plant protoplasts which do not have any appropriate genetic markers.

Although the same principle was tried by Harms and Potrykus (1978), their results were not necessarily successful, as they could enrich heterokaryocytes to be 30% in the fusion products after the treatment with PEG. One of the improved points in this study is the combination of the enrichment of heterokaryocytes by density gradient centrifugation with electric fusion. According to this procedure, the time between fusion and density gradient centrifugation after treatment with electric fusion is very short. Thus, it is probable that thepossible change of specific gravity of the fusion products during the longer incubation could be avoided.

Another important point is the use of protoplasts whose specific gravity is located in a limited range. Specific gravity of 1.044–1.048 g/ml of mesophyll protoplasts and 1.032–1.038 g/ml of epidermis protoplasts were used. When we used non-selected mesophyll and epidermis protoplasts of wider specific gravity range of protoplasts than those used in this study, the frequency of the heterokaryocytes in the enriched fraction was significantly lower than that reported here. The condition utilized seems to be optimal for the present combination of materials, but when this procedure is applied to other plant species, it is necessary to examine the range of specific gravity of respective materials in advance. It should be noted that heterokaryocytes after density gradient centrifugation are composed mostly of 1-1 fusion products, which is probably due to the elimination of other combinations of protoplasts during density gradient centrifugation.

However, frequency of heterokaryocytes in the initial fusion products was ca. 2–5%, and improvement of the increase of this frequency has not been tried. The results from the small scale experiments by Kohn et al. (1985) and by Hibi et al. (1986) showed that fusion frequency can be increased by improvement of the fusion chamber. Thus, improvement of the fusion chambers in combination with the procedures to select heterokaryocytes from the fusion products described will produce an efficient procedure to obtain heterokaryocytes from fusion products.

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