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The Isolation and Fusion of Pea and Barley Mesophyll Protoplasts

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완두와 보리의 葉肉細胞 原形質體 分離 및 融合

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ABSTRACT

The optimal conditions for the protoplast isolation from the leaves of pea (*Pisum sativum* L. cv. Sparkle) and barley (*Hordeum vulgare* L. cv. Baecdong) were determined in order to achieve a somatic hybridization between two species.

It was revealed that the use of 0.5 M sorbitol as an osmoticum was appropriate for pea. The yield of intact protoplasts was the highest (40%) when pea leaves were incubated in the enzyme solution for 4 hours.

In case of barley, the optimal concentrations of cellulase, pectinase and mannitol as the enzyme solution were 2%, 1% and 0.35 M, respectively. And the yield of barley protoplasts was the highest (87%) when leaves were incubated in this enzyme solution for 3.5 hours.

A fusion of protoplasts from pea and barley was induced by PEG treatment enriched with calcium salts within 60 minutes.

INTRODUCTION

Enzymatic method suitable for preparing large numbers of protoplasts from tissues of higher plants was first described by Cocking (1960). Thereafter, isolated protoplasts have been used in a very wide range of experiments extending from investigations of the purely physical properties of the plasma membrane (Ruesink, 1973) to studies on wall synthesis (Grout, 1975), uptake of organelles and micro-organisms (Potrykus, 1975; Davey and Power, 1975) and hybridization (Nickell and Torrey, 1969; Carlson *et al.*, 1972; Melchers *et al.*, 1978).

Especially because the plant cell has a totipotency, it is possible to obtain a hybrid plant by protoplast fusion. Fusion of somatic cells of higher plants requires the aggregation of isolated protoplasts as a preparative step. Several methods to obtain protoplast aggregation have been described (Power *et al.*, 1970; Withers, 1973; Hartmann *et al.*, 1973; Keller and Melchers, 1973; Kao and Michayluk, 1974). The high frequency of interspecific protoplast fusion as a result of incubation with polyethylene glycol indicated that aggregation by this method was extremely efficient (Constabel and Kao, 1974).

The fact that fusion products from such widely divergent species as barley and soybean (Kao *et al.*, 1974) or barley and carrot (Dudits *et al.*, 1976) can be obtained and cultured for a time suggests that, at the initial stage of fusion, there may be no incompatibility barrier.

The purpose of this experiment is to obtain an interspecific somatic hybrid between barley and

pea by using PEG as a fusion inducer. The optimal conditions for the protoplast isolation from the leaves of both species were also determined.

MATERIALS AND METHODS

Protoplast Isolation Pea (*Pisum sativum* L. cv. Sparkle) and barley (*Hordeum vulgare* L. cv. Baecdong) grown in an environmental growth chamber at 24~28°C under a fluorescent white tube illumination (2500~3500 lux) with a relative humidity of 50~70% and a photoperiod of 16 hours were used as a source of leaf material.

Fully expanded pea leaves grown for 20~30 days were used to isolate protoplasts. Excised pea leaves were surface sterilized with 70% ethanol for 1.5 min and then washed thoroughly in sterile distilled water for several times. With the help of forceps the lower epidermis was peeled from excised leaves aseptically and floated with exposed surface downwards on a solution of 0.5 M sorbitol for an hour in a 9 cm diameter petri dish.

After preplasmolysis sorbitol solution was removed from below the leaves and replaced with a filter(0.45 μm pore size membrane) sterilized enzyme mixture. The enzyme mixture was composed of 0.6% cellulase (Sigma) and 0.4% pectinase (Sigma) in sorbitol solution, pH 5.8. Approximately 0.8g of material was placed in 10 m*l* of enzyme mixture and shaken at 25°C on a reciprocal shaker (30 rev/min) in the dark.

Undigested leaf tissues were removed through 60 μm pore size nylon filter after enzyme reaction.

The filtrate was centrifuged at 80*g* for 5 minutes, and the supernatant was removed with a pasteur pipette. The pellet was resuspended with pea protoplast culture medium (Constabel *et al.*, 1973) for 3~4 times and incubated in a 5 cm diameter plastic petri dish.

The number of protoplasts was estimated by microscopic protoplast counts using a Levy hemacytometer.

In case of barley, the first leaf of 6~9 days old plants (8~10 cm long) was used as a source of leaf material. Excised leaves were surface sterilized with 1% sodium hypochlorite solution for 20 minutes and washed 4 times with sterile distilled water for 30 minutes. With a razor blade and pincers the lower epidermis was peeled from excised leaves aseptically and the stripped leaves were then cut into pieces of approximately 1~2 cm^2.

After that the processes were similar with those of pea leaves. 0.8g of material was placed in 5 m*l* of enzyme mixture composed of cellulase (Sigma), pectinase (Sigma), 0.01 M potassium citrate and mannitol, pH 5.8.

Protoplast Fusion Freshly prepared protoplasts from pea and barley leaves were mixed together, and 1.2 m*l* of polyethylene glycol (PEG) solution was poured slowly into 0.4 m*l* of this protoplast suspension. The composition of PEG solution is as follows; 500 g/*l* PEG 4000, 0.1 M glucose, 10.5 mM $CaCl_2$ and 0.7 mM KH_2PO_4.

After an hour, addition of 1.6 m*l* Constabel's culture medium was followed by addition of 3.2 m*l* culture medium after 30 minutes. The mixture was then centrifuged at 80*g* for 5 minutes. Sedimented pellet was incubated in Constabel's culture medium.

The mixing of protoplasts from etiolated barley leaves with protoplasts from green pea leaves was made for ready identification of the interspecific aggregates.

RESULTS AND DISCUSSION

Protoplast Isolation from Pea Leaves The results of the preliminary experiments showed that the sorbitol solution was a suitable osmoticum for protoplast isolation from pea leaves. To determine the optimal concentration of the osmoticum and the optimal incubation period in enzyme solution, various concentrations of sorbitol solution were used, and the yield of protoplasts was investigated at 1 hr interval. The quality of the isolated protoplasts was estimated by a microscopic examination of the protoplast structure.

The morphologically intact protoplasts had a regular spherical shape with a size of about 30 μm. The chloroplasts were regularly arranged around a central vacuole, adjoining the protoplast membrane (Fig. 1). When the sorbitol concentration was above or below the optimal, the protoplast shape

Table 1. Determination of the stabilizing sorbitol concentrations when isolating pea mesophyll protoplasts

Concentration of sorbitol (M)	Number of isolated protoplasts(%)		
	Intact	Partially damaged	Damaged or destroyed
0.3	21	75	4
0.4	39	54	7
0.5	40	50	10
0.6	36	32	32
0.7	16	26	56

was irregular. The arrangement of the chloroplasts was in disorder. They often gathered at one side and even protruded below the optimal concentration. Many of the protoplasts (over 50%) bursted above the optimal.

An increase or decrease from the optimal concentration caused destruction of the protoplasts, and the preparations turned into suspensions of chloroplasts and other cell organelles.

The data shown in Table 1 summarize the results of experiments for the determination of stabilizing sorbitol concentrations. It was revealed that 0.5 M sorbitol was the best osmoticum for pea mesophyll protoplasts. When the enzymatic treatment was carried out in the presence of 0.5 M sorbitol, the percentage of intact protoplasts was the highest (40%).

And the amount of intact protoplasts was the highest when pea leaves were incubated in enzyme solution for 4 hours in the presence of 0.5 M sorbitol solution (Table 2).

When enzyme treatment period was over the optimal, the number of intact protoplasts was reduced and many of the protoplasts bursted. Thus, 3~4 hours incubation in enzyme solution was thought to be appropriate to produce intact protoplasts.

Table 2. Effect of the incubation period of pea leaves in enzyme solution on protoplast isolation

Incubation period (hr)	Amount of intact protoplasts isolated from 1 g of plant tissue
1	2.0×10^{3}
2	3.7×10^{5}
3	1.1×10^{6}
4	1.6×10^{6}
5	1.3×10^{5}

Protoplast Isolation from Barley Leaves The age of the plants used for preparing protoplast suspensions of barley mesophyll was of great importance. It was impossible to obtain the protoplast suspension when plants were more than 20 days old, and it was also very difficult to peel the lower epidermis from the plants.

To determine the optimal concentration of enzyme solution the protoplasts were isolated using different concentrations of cellulase and pectinase in 0.35 M mannitol.

The yield of intact protoplasts was good when the concentrations of cellulase and pectinase in solution were 2% and 1%, respectively (Table 3).

Table 3. Effect of enzyme concentration on protoplast isolation from barley mesophyll

Concentration of enzymes (%)		Amount of intact protoplasts from 1 g of plant tissue	Number of damaged or destroyed protoplasts (%)
Pectinase	Cellulase		
0	2	4.4×10^{4}	—
1	1	3.8×10^{5}	31
1	2	6.0×10^{5}	31
1	3	5.3×10^{5}	41
2	2	8.9×10^{5}	52

The ratio of the destroyed protoplasts increased when more concentrated enzyme solution was used. Schaskolskaya *et al.*(1973) reported that the treatment of stripped barley leaves with pectinase was not successful in the maceration of barley mesophyll tissue. In this experiment, however, treatment with cellulase only resulted in a conspicuous decrease of the protoplast yield.

To determine the effect of concentration of osmoticum the mannitol concentration was varied from 0.3 M to 0.6 M. The yield of intact protoplasts was very high (over 70%) when 0.35 M to 0.5 M mannitol solution was used (Table 4). The number of intact protoplasts produced in these concentrations

Table 4. Determination of the stabilizing mannitol concentrations when isolating barley mesophyll protoplasts

Concentration of mannitol (M)	Number of isolated protoplasts (%)		Amount of intact protoplasts from 1 g of plant tissue
	Intact	Damaged or destroyed	
0.30	27	73	0.9×10^5
0.35	87	13	5.6×10^5
0.50	72	28	3.4×10^5
0.60	19	81	1.2×10^5

of mannitol was greater by more than two times than the number in the other concentrations.

These results accord well with those reported by Schaskolskaya (1973) and Hughes *et al.* (1978), in whose experiments the optimal concentration of mannitol solution was 0.34 M and 0.45 M, respectively.

Table 5. Effect of incubation period of barley leaves in enzyme solution on protoplast isolation

Incubation period (hr)	Amount of intact protoplasts isolated from 1 g of plant tissue
1.0	0.3×10^5
2.0	1.9×10^5
2.5	2.9×10^5
3.0	4.4×10^5
3.5	5.6×10^5
4.0	4.4×10^5
5.0	2.0×10^5

The yield of isolated intact protoplasts was the highest at 3.5 hours of incubation and decreased after 4 hours (Table 5). According to Schenk and Hildebrandt (1969) it was thought that this result was due to the presence of a considerable amount of low molecular weight materials in crude enzyme preparations, which caused a considerable increase in the osmotic potential of the isolation medium and also accounted for some of toxicity during long incubation period.

The morphologically intact barley mesophyll protoplasts had a regular spherical shape like pea mesophyll protoplasts. The size of most barley mesophyll protoplast was 20±5μm. Chloroplasts were also regularly arranged at the periphery of the protoplast (Fig. 2).

Protoplast Fusion The PEG solution was very effective to induce the aggregation and fusion of protoplasts from pea and barley mesophyll cells. The strong adhesion of the protoplasts brought about by PEG probably represents the first step of cell fusion (Fig. 3). It is likely that small cytoplasmic bridges are formed at this stage (Withers and Cocking, 1972). Most of the fusion occurred during the dilution of PEG and not during the period of aggregation. Fig. 4 shows that a fusion between pea and barley was occurred during the dilution of PEG. Cytoplasm of a protoplast then moved into another protoplast leading to a global shape, and finally a heterokaryocyte was formed (Fig. 5).

Agglutination by PEG may involve attraction of protoplasts by electrostatic forces (Kao and Michayluk, 1974). The slightly polar PEG and the plasmalemma surface preferentially exhibit negative charges. Bivalent cations are assumed to link PEG molecules with protoplasts. Thus enrichment of PEG solution with Ca salts enhances the agglutination.

A mixing of protoplasts which arise from etiolated leaves of one species with protoplasts from green leaves of another allows the interspecific aggregates to be readily identified. Fig. 6 shows that protoplasts from etiolated barley leaves (with light-colored, non-well developed plastids) and illuminated pea leaves (with green, well developed chloroplasts) were fused leading to an interspecific fusion product. The problems of division and differentiation of this fusion product leading to a mature plant still remain to be solved hereafter.

摘 要

완두(*Pisum sativum* L. cv. Sparkle)와 보리(*Hordeum vulgare* L. cv. Baecdong) 사이의 種間 體細胞雜種을 얻기 위하여 양 植物體의 잎으로부터 葉肉細胞原形質體를 分離하는 最適條件을 구한 후 種間의 原形質體 融合을 시도하였다.

原形質體 分離에 있어서 완두의 경우 0.5 M sorbitol을 osmoticum으로 使用하는 것이 가장 適切하였으며 이 酵素溶液에서 4時間 동안 處理하였을 때 原形質體의 수득율이 40%로 가장 높았다.

한편 보리에서는 1% pectinase, 2% cellulase, 0.35 M mannitol의 酵素溶液이 最適이었으며 이 溶液에서 3.5時間 處理하였을 때 原形質體의 수득율이 87%로 가장 높았다.

分離된 완두와 보리의 原形質體는 Ca鹽을 包含한 PEG溶液에 의해 60分間 處理하여 完全한 融合體를 얻었다.

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EXPLANATION OF THE PLATE

Fig. 1. A typical intact protoplast isolated from pea mesophyll incubated in the enzyme solution (0.4% pectinase, 0.5% cellulase and 0.5 M sorbitol) for 4 hrs. (× 1,000)

Fig. 2. Protoplasts isolated from barley mesophyll incubated in the enzyme solution (1% pectinase, 2% cellulase and 0.35 M mannitol) for 3.5 hrs. (× 900)

Fig. 3. A protoplast aggregation between pea and barley mesophyll by PEG (500 g/*l* PEG 4000, 0.1 M glucose, 10.5 mM $CaCl_2$ and 0.7 mM KH_2PO_4) treatment. Two barley protoplasts (b) and four pea protoplasts are aggregated. (× 1,000)

Fig. 4. A fusion of a dense green pea mesophyll protoplast (p) and a protoplast from light green barley mesophyll (b) during a dilution of PEG with Constabel's culture medium.(× 1,200)

Fig. 5. A heterokaryocyte formed by a fusion between pea and barley mesophyll protoplasts. Pea chloroplasts are gathered at the surface with which two species are in contact. (× 1,200)

Fig. 6. A fusion product between pea and barley mesophyll protoplasts. Cytoplasm of two species is about to intermix. (× 1,000)

PLATE