Efficient Production of Somatic Hybrids between *Raphanus sativus* and *Brassica oleracea*

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An efficient procedure of somatic hybridization between radish (*Raphanus sativus*) and cauliflower (*Brassica oleracea*) was developed by assessing the techniques at the steps from protoplast isolation to plant regeneration. Hypocotyl protoplasts of radish and cotyledon protoplasts of cauliflower were fused by treatment with polyethylene glycol solution containing dimethylsulfoxide. Cauliflower protoplasts having high plant regeneration ability were inactivated by iodoacetamide before fusion treatment. In eight fusion trials, 2965 calli were obtained, and after transfer to a shoot regeneration medium, 163 calli formed shoots. Of the 111 plants obtained, 62 had leaves of intermediate type between the two parents. The other 49 plants had similar leaves to cauliflower. Analysis of rDNA and acid phosphatase isozyme demonstrated the hybrid nature of the plants having leaves of intermediate type. Some of somatic hybrids had high pollen fertility and set seeds, indicating the possibility of utilizing these plants for breeding materials.

KEY WORDS: *Brassica oleracea*, *Raphanus sativus*, cell fusion, somatic hybrid, polyethylene glycol, iodoacetamide.

Introduction

In the breeding of *Brassica* crops, interspecific gene transfer has been successfully carried out (NAMAI *et al.* 1980). Soft rot resistance of *Brassica oleracea* was introduced into Chinese cabbage (SHIMIZU *et al.* 1962), and early maturing of *Brassica napus* was incorporated from *Brassica campestris* (SHIGA 1970). However, there are few examples of intergeneric gene transfer being used successfully for plant breeding in *Cruciferae*, though genetic studies have been intensively carried out in intergeneric hybrids between *Brassica oleracea* and *Raphanus sativus*, *Raphanobrassica*, and their backcross progenies (KANEKO *et al.* 1991).

Both *Brassica oleracea* and *Raphanus sativus* are liable to infestation by *Fusarium oxysporum*, but the disease caused by this fungus is not so serious in the production of cabbage, cauliflower and broccoli as it is for radish. On the other hand, clubroot caused by *Plasmodiophora brassicae* is a serious disease of cabbage, cauliflower and broccoli, but some radish lines are highly resistant to this disease (ASHIZAWA *et al.* 1980). Production of intergeneric hybrids and back crossing with one of the parents are necessary to introduce these traits from one species into another species.

Protoplast fusion and the following cell culture make it possible to produce somatic hybrids between distantly related species. In *Brassica*, many somatic hybrids were obtained by use

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of various techniques of fusion treatment, selection methods and culture procedures (SCHENK and RÖBBELEN 1982, SUNDERBERG and GLIMELIUS 1986, TAGUCHI and KAMEYA 1986, TERADA *et al.* 1987, NISHIO *et al.* 1987c, PRIMARD *et al.* 1988, YAMAGISHI *et al.* 1989, SJÖDIN and GLIMELIUS 1989). Intergeneric hybrids were also produced by somatic hybridization (GLEBA and HOFFMANN 1980, PELLETIER *et al.* 1983, TORIYAMA *et al.* 1987, KLIMASZEWSKA and KELLER 1988, FAHLESON *et al.* 1988, CHATTERJEE *et al.* 1988, SAKAI and IMAMURA 1990).

In this report, we describe a series of procedures which provide a method for the efficient production of somatic hybrids between *Brassica oleracea* and *Raphanus sativus*. More than 60 hybrid plants were obtained in the trials lasting less than 4 months. The hybrids had various chromosome numbers, and some of them were fertile and set seeds.

Materials and Methods

Materials: Seeds of Cauliflower (*Brassica oleracea* L. ssp. *botrytis*, cv. 'Bridal' Sakata Seed Co.) were sterilized in a solution of sodium hypochlorite (active chlorine 1%) for 15 min by gentle agitation, and rinsed three times with sterilized distilled water. The seeds were placed on an agar medium containing 0.3% Hyponex (Hyponex Co.) and 2% sucrose. Cotyledons which appeared 5 to 7 days after sowing the seeds were used for protoplast isolation. For the radish material (*Raphanus sativus* L.), seeds of cv. 'Fukuyo 40 days' (Nakahara Seed Co.) were sown on the agar medium in the same manner as cauliflower. Protoplasts were isolated from hypocotyls 4 to 5 days after sowing the seeds.

Protoplast isolation: Cotyledon protoplasts of cauliflower were isolated according to the method of NISHIO *et al.* (1987a). Cotyledons were sliced into 1 mm strips in a modified MS medium containing half-strength MS salts and vitamins (MURASHIGE and SKOOG 1962) except for 200 mg/l NH_4NO_3 , and 0.4 M mannitol. The sliced cotyledons were incubated in enzyme solution containing 0.1% Pectolyase Y-23 (Seishin Pharmaceutical Co.), 0.5% Cellulase Onozuka RS (Yakult Honsha Co.), 0.4 M mannitol and modified MS for 90 min at 25°C on a rotary shaker (60 rpm). The protoplasts were filtered through a nylon sieve (pore size: 50 μm), and collected by centrifugation at $100 \times g$ for 2 to 3 min.

Hypocotyl protoplasts of radish were prepared by the method of NISHIO *et al.* (1987b). The hypocotyl tissue was cut longitudinally into two pieces, placed in enzyme solution containing 0.06% Macerozyme R10 (Yakult Honsha Co.), 0.3% Meicelase (Meiji Seika Kaisha Ltd.), 0.4 M Mannitol, 1% sucrose and modified MS, and kept at 25°C in the dark without shaking. After treatment for 16 hours, the protoplasts were liberated by gentle shaking, filtered through a nylon sieve, and washed twice with W5 solution (MENCZEL and WOLFE 1984).

Fusion treatment: Cauliflower protoplasts were inactivated by 10 mM iodoacetamide for 10 min at room temperature, then washed twice with 0.4 M mannitol containing CPW salts (ZAPATA and SINK 1981), and suspended in the washing solution at a density of $2 \times 10^6/\text{ml}$. Radish protoplasts were also suspended in the same solution at 4×10^6 , and mixed with an equal volume of the suspension of cauliflower protoplasts. Fusion treatment was carried out according to the method of KAO *et al.* (1974) with a little modification. Four drops (ca. 150 μl) of the protoplasts mixture were placed in a plastic petri dish (6 cm diameter), and

left for 30 min to precipitate the protoplasts. Four drops of 30% polyethylene glycol solution containing dimethylsulfoxide (DMSO) were added to the area surrounding the protoplast suspension, and left for 2 min. Four drops of high pH and high Ca solution (KELLER and MELCHERS 1973) were added, and again left for 2 min. After addition of 1 ml W5 solution, the liquid was removed with a Pasteur pipet. The protoplasts were washed 5 times with W5 solution, and twice with culture medium. Finally, 2 ml of culture medium (NISHIO *et al.* 1987a) containing modified MS, 1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), 1 mg/l naphthaleneacetic acid (NAA), 0.5 mg/l benzylaminopurine (BA), 0.4 M mannitol, 1% sucrose, and 500 mg/l casamino acid, were poured into the petri dish, and cultured at 23°C under dim light.

Plant regeneration: Equal volumes of the culture medium containing less mannitol was added to each petri dish every 7 to 10 days. The culture for callus formation and plant regeneration was performed according to NISHIO *et al.* (1987a). After 3 weeks of initial culture, the colony suspension was spread on a callus culture medium solidified with 0.2% gellan gum, containing salts and vitamins of MS, 0.5 mg/l 2,4-D, 5 mg/l kinetin and 3% sucrose. Further 2 weeks later, calli having ca. 1 mm diameter were transferred to a shoot regeneration medium containing salts and vitamins of MS, 1 mg/l BA, 0.03 mg/l gibberellic acid (GA₃), 3% sucrose, and 0.2% gellan gum, and cultured under fluorescent light (3,000 lx) at 16 hours photoperiods. After shoot formation, the calli were transferred to 1/2 MS agar medium for rooting.

Analysis of DNA: Total DNA was prepared from 0.5 g young leaf following MURRAY and THOMPSON (1980), and digested with several restriction enzymes. Ribosomal DNA was analyzed by the method of HONDA and HIRAI (1990). The DNA fragments were separated by agarose gel electrophoresis, and transferred to a nylon membrane (Hybond N, Amersham). Rice rDNA pRR217, was labeled with digoxigenin according to the manufacture's manual (Boelinger Mannheim), and used as a probe. Hybridized DNA was detected by binding of anti-Dig alkaline phosphatase conjugate and color reaction with NBT and X-phosphate.

Isozyme analysis: Young leaf was homogenized with extraction solution (0.3 M Tris-HCl, 0.1 M NaCl, 0.2 M ascorbic acid and 2% TritonX-100) using a mortar and a pestle. The homogenate was centrifuged for 1 min. at 14,000 × g, and the resulting supernatant was used as a sample for electrophoretic analysis. The electrophoresis was carried out using slab acrylamide gel with the buffer system by DAVIS (1964). Isozymes of leucin aminopeptidase (LAP), 6-phospho-gluconate dehydrogenase (6PGDH), asparagin amino transferase (GOT), malate dehydrogenase (MDH) and acid phosphatase (AP) were detected by the method of VALLEJOS (1983).

Chromosome number: Chromosome number was counted using root tip cells by the method of NISHIBAYASHI (1990). After pre-treatment with 0.02 M 8-oxyquinoline for 4 hours, root tips were fixed with acetic acid-ethanol (1:3), and treated with enzyme solution (4% Cellulase Onozuka RS. 1% Pectolyase Y-23, 75 mM KCl, 7.5 mM Na₂EDTA, pH 4.0). Root tip cells were spread and attached to slide glass with one drop of acetic acid-methanol (1:3). The chromosomes were stained using 2% aceto-orcein.

Pollen fertility: Pollen grains stained by aceto-carmin were counted as fertile pollen.

Results

1. Effect of DMSO on protoplast fusion

Cauliflower protoplasts were prepared from cotyledons, and radish protoplasts were from hypocotyls. Therefore, hybrid cells were easily distinguished from both parent cells by observing chloroplasts and cytoplasmic strands for several hours after fusion treatment (Fig. 1a). Some hypocotyl protoplasts floated in mannitol solution, and did not fuse with cotyledon protoplasts. To increase hybrid cell formation, hypocotyl protoplasts were mixed with cotyledon protoplasts in a ratio of 2:1, and the protoplast mixture was left in the petri dish

Fig. 1. Production of somatic hybrids by protoplast fusion between radish and cauliflower.

- a: Protoplasts fused by polyethylene glycol treatment
- b: Calli obtained by cell culture after protoplast fusion
- c: Shoot formation on a hybrid callus
- d: A hybrid plant cultured in a pot

for 30 min before PEG treatment.

Fusion frequency was investigated after treatment with polyethylene glycol solution containing 0, 5 and 10% DMSO. The percentage of hybrid cells in the total protoplasts was 0.5 with 0% DMSO, 10 with 5% DMSO, and 15 with 10% DMSO. Ten percent DMSO gave the highest fusion frequency, but most of protoplasts treated with this solution died within a few days. Five percent DMSO did not damage protoplasts, and gave a twenty fold increase of hybrid cells in comparison with 0% DMSO.

2. Plant regeneration from hybrid cells

Cell division was observed two days after culture, and colonies with about 200 μm in diameter were obtained in 2 weeks of culture. A fusion experiment using a protoplast mixture of equal number of cauliflower and radish protoplasts gave no colony, but the mixture of cauliflower protoplasts with $2 \times 10^6/\text{ml}$ and radish protoplasts with $4 \times 10^6/\text{ml}$ gave many colonies. Many calli were formed after plating the colony suspension on callus culture medium (Fig. 1b). After transfer of 2965 calli obtained from 8 fusion experiments to a shoot regeneration medium, 163 calli formed shoots (Fig. 1c). These calli were transferred to a half-strength MS medium without growth regulators, and 111 out of them grew into plantlets with roots (Fig. 1d). Some of them were planted in pots.

3. Characterization of regenerated plants

1) *Morphology*: Leaves of the regenerated plants and their parents are shown in Fig. 2. The leaf of cauliflower is round and bluish green in color. The young shoot shows anthocyanin pigmentation. Radish has a long, lobed leaf. The color is light green. Of the 111 regenerated plants, 62 were of an intermediate type, 49 were very similar to cauliflower, and none was radish-like plant. The leaf of the intermediate type is a little roundish and has lobes. The shoot tips did not have anthocyanin pigmentation. Some intermediate types had curled and shrunk leaves. The leaf color of intermediate types segregated into bluish-green and light green.

Flowers of the intermediate type were white. The plants with cauliflower type leaves were not distinguished morphologically from cauliflower, producing curds and blooming yellow flowers.

2) *Analysis of DNA*: Ribosomal DNAs of the parents were compared after digestion with 8 restriction enzymes. *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III and *Sal*I gave species specific restriction fragments. *Bgl*II and *Hind*III were mainly used for the analysis of the regenerated plants because of the distinctive patterns of both parents (Fig. 3). Of the 92 plants analyzed, 50 plants had both the bands of cauliflower and radish, and 42 plants had only the bands of cauliflower. There was no plant having only the radish bands. The leaves of the plants having both bands were of the intermediate type. The plants with only the

Fig. 2. Leaves of a somatic hybrid and the parents
a: cauliflower b: somatic hybrid c: radish

Fig. 3. Analysis of rDNA of somatic hybrids and the parents rDNA of cauliflower (1), radish (2) and regenerated plants (3~5: plants with intermediate type leaves, 6~8: plants with cauliflower type leaves) from fused protoplasts were detected by hybridization with labeled rice rDNA after digestion of plant DNA with BglII, electrophoresis and Southern transfer.

Fig. 4. Acid phosphatase isozymes of somatic hybrids and the parents
1&9: cauliflower, 2&10: radish, 3~8: somatic hybrids

bands of both cauliflower and radish, and was used for analysis of the regenerated plants. Radish had a specific band near the front of the sample, while cauliflower had another band a short distance from the front. Of 29 plants tested, 2 plants having cauliflower type leaves showed the same pattern as cauliflower, and 27 having intermediate type leaves showed an additional band besides both specific bands of cauliflower and radish (Fig. 4). This additional band is considered to be a hetero-dimer of the specific isozymes of both parents. The presence of the hetero-dimer is the evidence which shows that these plants are not kimeric plants but hybrids.

4) *Chromosome number*: The chromosome numbers of ten plants with intermediate leaf shape, which were judged to be somatic hybrids by the analysis of rDNA and acid phosphatase isozyme, varied from 34 to 66. Three plants had 36 chromosomes, and two plants had 54 chromosomes. The other five plants showed aneuploidy (Fig. 5, Table 1).

5) *Pollen fertility*: The pollen fertilities of nine plants with intermediate leaf shape were from 0 to 65 percent. The two plants with 0% fertility had short stamens and few sterile pollen

cauliflower bands had leaves of the cauliflower type. There was no exception in the relation between leaf shape and DNA type.

3) *Isozyme analysis*: Intraspecific variation in radish was found in the isozyme pattern of LAP. Clear bands of 6-PGDH were not detected. In the isozyme of GOT and MDH, radish had an additional band to the bands of cauliflower, and the cauliflower specific band was not detected. Only acid phosphatase gave specific

Fig. 5. Chromosomes of cells of a root tip in a somatic hybrid
a: $2n=36$, b: $2n=54$, c: $2n=34$

Table 1. Characteristics of the somatic hybrids raised through protoplast fusion between *R. sativus* and *B. oleracea*.

Hybrid no.	Leaf shape	Leaf color	Petal color	rDNA ^{a)}	Isozyme ^{b)}	2n	Pollen fertility
2	Intermediate	Bluish green	White	H	H	36	37.5
8	Intermediate	Green	White	H	H	36	0.6
9	Intermediate	Bluish green	White	H	H	34	3.2
10	Intermediate	Bluish green	White	H	H	52	6.4
35	Intermediate	Green	White	H	H	64	0
40	Intermediate	Bluish green	White	H	H	54	65.1
41	Intermediate	Bluish green	White	H	H	36	32.5
45	Intermediate	Green	White	H	H	54	42.2
51	Intermediate	Bluish green	White	H	H	66	0
61	Intermediate	Bluish green	White	H	H	52	—
<i>R. sativus</i>		Green	White	R	R	18	
<i>B. oleracea</i>		Bluish green	Yellow	B	B	18	

a) R: *R. sativus* type, B: *B. oleracea* type, H: hybrid type

b) Acid phosphatase isozyme

R: *R. sativus* type, B: *B. oleracea* type, H: hybrid type

grains, while the other plants had normal stamens. Two plants with 54 chromosomes had relatively high pollen fertility, 65 and 42%. Two plants with 36 chromosomes had intermediate pollen fertility. The other 5 plants were almost sterile. The plants having high pollen fertility set seeds by open pollination. Viable seeds were also obtained by a cross between radish and the hybrid plants.

Discussion

One of the problems frequently encountered in tissue and cell culture work is the difficulty of reproducing reported results. In particular, somatic hybridization requires great skill in the techniques of culture procedure, that is, protoplast isolation, cell fusion, protoplast culture, selection of hybrid cells, and plant regeneration. Therefore, it is difficult to use somatic hybridization as a breeding tool, although it has great potential in introducing nuclear genes from distantly related species to crops. Another important use of somatic hybridization is recombination of cytoplasmic genes. Since there is no established technique of recombining DNA from mitochondria or chloroplast, somatic hybridization is the only possible method for the improvement of chondriome or plastome. We have developed a reliable and efficient technique for somatic hybridization between *Brassica oleracea* and *Raphanus sativus* by assessing and combining procedures for isolation and culture of protoplasts, fusion treatments and selection methods reported in the literature up to now.

The isolation and culture procedure of protoplasts in *Brassica oleracea* has already been established, and cotyledon protoplasts of cauliflower cv. 'Bridal' regenerated plants efficiently by this procedure (NISHIO *et al.* 1987a). Radish protoplasts did not regenerate plants, but formed calli efficiently. Hypocotyl protoplasts of radish were used as a fusion partner of cotyledon protoplasts from cauliflower, because hybrid cells can be distinguished easily from the parent cells. Besides, cotyledon and hypocotyl were good sources of protoplasts having constant viability, which provides highly reproducible results.

In our experience, fused cells between protoplasts of hypocotyl and cotyledon were obtained with low frequency by electric cell fusion. This may be due to the difference of sensitivity of protoplasts to electric pulse, and partly due to the differences in density between them. PEG treatment gave higher percentage of hybrid cells. Precipitating protoplasts on the surface of a petri dish was necessary to increase fusion efficiency. In this work, DMSO increased the formation of hybrid cells, as reported by MENCZEL and WOLFE (1984) and TERADA *et al.* (1987). Addition of 5% DMSO to PEG solution gave the best result without damage to protoplasts.

Iodoacetamide was used for inactivation of cauliflower protoplasts. As our culture procedure did not give plant regeneration from radish protoplasts, plants obtained in this experiment were expected to be derived from hybrid cells. More than half of the plants, 62 plants out of 111 regenerated plants, showed a hybrid nature. This selection method was successfully used for the production of somatic hybrids between *Brassica oleracea* and *Brassica campestris* (TERADA *et al.* 1987, NISHIO *et al.* 1987c). Selection of somatic hybrids by iodoacetamide treatment and regeneration ability can be widely used for the combination of *Brassica oleracea* and related species.

The chromosome number of somatic hybrids varied from 34 to 66. Three plants with 36 chromosomes and 2 plants with 54 chromosomes were considered to be tetraploids and hexaploids respectively. The other five plants were aneuploids, and thought to be derived from the elimination of chromosomes from polyploids. These aneuploids had low pollen fertility, less than 7%. One plant with 36 chromosomes also had low pollen fertility, 0.6%.

High ploidy level of somatic hybrids indicates a high proportion of multiple fusion. Somatic hybrids obtained from fusion events of three or more protoplasts have been reported in many papers (GLEBA and HOFFMANN 1978, SUNDBERG and GLIMELIUS 1986, TERADA *et al.* 1987, FAHLESON *et al.* 1988, CHATTERJEE *et al.* 1988).

The high percentage of aneuploids suggests instability of chromosomes in hybrid cells. Regenerated plants having the same leaf shape and rDNA as cauliflower might have been generated by elimination of radish chromosomes from the hybrid cells. KAMEYA *et al.* (1989) obtained 2 plants having a nucleus of cabbage and chloroplasts of radish by protoplast fusion. Analysis of mitochondrial DNA of our cauliflower type plants is now in progress, and showing the presence of mtDNA bands specific to radish (NISHIO *et al.* unpub.).

Most of intergeneric somatic hybrids were sterile, and therefore unsuitable for use as breeding materials (MELCHERS *et al.* 1978, GLEBA and HOFFMANN 1980). Some of the somatic hybrids obtained in this work had high pollen fertility. These plants may be used for introducing nuclear genes of cauliflower to radish or vice versa. SJÖDIN and GLIMELIUS (1989) produced somatic hybrids resistant to *Phoma lingam* by protoplast fusion between *Brassica oleracea* and *Brassica nigra*. Somatic hybrids obtained in this work are under investigation of resistance to diseases of radish and cauliflower.

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ダイコン (*Raphanus sativus* L.) とカリフラワー (*Brassica oleracea* L.) の
効率的な体細胞雑種作出山中寿子¹⁾・釘貫靖久・菅野紹雄²⁾・西尾 剛³⁾

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これまで報告されたプロトプラスト培養法, 融合処理法, 雑種細胞選抜法等を検討することにより, ダイコン (*Raphanus sativus* L.) と, カリフラワー (*Brassica oleracea* L.) の体細胞雑種を効率よく作出する方法が確立できた。ダイコンは胚軸から, カリフラワーは子葉からプロトプラストを得て細胞融合を行った。こうすることにより, 雑種細胞の識別が可能となると共に, 常に同程度の活性のあるプロトプラストが得られ, 実験の再現性が高められた。細胞融合性は, ポリエチレングリコール (PEG) 法がよく, ジメチルスルホキシド (DMSO) を PEG 液に加えることにより, 更に雑種細胞の作出率が高まった。DMSO の濃度が高くなると細胞融合率は高まるが, プロトプラストの生存率が低下するため, 10% DMSO よりも 5% DMSO の PEG 液で良好な結果が得られた。雑種作出率を高めるため, プロトプラストをプラスチックシャーレの底に沈める必要があったが, 胚軸プロトプラストは沈み難いため, プロトプラスト混合液での胚軸プロトプラストの比率を高めると共に, PEG 処理前の静置時間を長くした。雑種細胞の選抜のために, プロトプラストの混合の前に, カリフラワーのプロトプラストを 10 mM ヨードアセトアミド液で 10 分間処理して不活化した。

8 回の融合実験で, 2965 個のカルスが得られ, それらを再分化培地に移植したところ, 163 のカルスから不定芽が分化した。それらの培養を続け, 111 のカルスから植物体 that 得られた。ダイコンは用いた培養法では不定芽が分化しないため, 得られた植物体はカリフラワーの再分化能を有するものと考えられる。葉の形態観察, リボゾーマル DNA 及び酸性フォスファターゼの分析により, 111 の植物体のうち, 62 が体細胞雑種であることが確認された。残りの 49 個体はこれらの分析でカリフラワーと識別できなかった。体細胞雑種の一部を鉢上げしたところ開花し, いずれの個体もダイコンと同様白花であった。染色体数 36 の個体 (4 倍体) 及び 54 の個体 (6 倍体) とともに異数体が得られ, 4 倍体や 6 倍体は花粉稔性が比較的高く, 種子も得られ, ダイコンやカリフラワーの育種素材として利用できるものと考えられた。