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Culture and fusion of pollen protoplasts of *Brassica oleracea* L. var. *italica* with haploid mesophyll protoplasts of *B. rapa* L. ssp. *pekinensis*

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Summary. Hybrid callus was formed from the successful protoplast fusion between pollen protoplasts of *Brassica oleracea* var. *italica* and haploid mesophyll protoplasts of *Brassica rapa*. The pollen protoplast isolation frequency in broccoli was highly related to the ratio of trinucleate pollens in the male gametophyte population. Large quantities of pollen protoplasts with high vigor could be isolated, and the isolation frequency reached up to 90% in 6.0–7.0 mm long flower buds with about 94.7% trinucleate-stage pollens. Pollen protoplasts could be collected and purified by discontinuous gradient centrifugation. In 1% Na-alginate embedding culture, cell divisions were observed but no further development was found. The haploid mesophyll protoplasts were isolated from in vitro haploid plants of *B. rapa*. Results strongly showed the variability in culturability of mesophyll protoplasts from different haploid lines. Both pollen protoplasts and haploid mesophyll protoplasts retained a stable round shape in the designed prefusion solution with an osmotic pressure of 0.74 osmol/kg. Polyethylene glycol was used for the protoplast fusion, and 40% polyethylene glycol 4000 enabled the highest fusion frequency of about 20%. Some postfusion protoplasts showed cell divisions up to callus proliferation. Calli were screened by random amplified polymorphic DNA analysis for their hybrid character. Results revealed the existence of the hybrid calli. Some of the hybrid calli grew well with green color and shoot primordia. According to our knowledge, this is the first report about a hybrid formation between two haploid protoplasts. Potential comprehensive applications, as well as problems of this technique, are discussed.

Keywords: Pollen protoplast; Haploid mesophyll protoplast; Fusion; Hybrid callus; *Brassica oleracea*; *Brassica rapa*.

Abbreviations: DAPI 4′,6-diamidino-2-phenylindole; FDA fluorescein diacetate; PEG polyethylene glycol; RAPD random amplified polymorphic DNA.

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Introduction

Protoplast fusion provides opportunities for bringing together genomes of taxonomically divergent species that cannot be combined sexually due to crossability barriers. Up to now it has been a promising tool for the transfer of valuable polygenic agronomical traits like resistances from wild to crop species. The interspecific somatic hybridization between *Brassica oleracea* ssp. and *B. rapa* was shown by several groups, demonstrating the totipotency of both the isolated protoplasts from *B. oleracea* and *B. rapa* (Robertson and Earle 1986, Kao et al. 1990) as well as the plant regeneration from the fusion products (Schenck and Röbbelen 1982, Loudon et al. 1989, Yamashita et al. 1989). The attempts were aimed, e.g., at transfer of resistances (Ren et al. 2000), at creation of new nuclear-cytoplasmic combinations (Rosén et al. 1988, Christey et al. 1991, Cardi and Earle 1997), and at formation of new genetic material for fundamental research (Heath and Earle 1996). So far, hybrid plants can be obtained in most of the somatic hybridization experiments performed, but their use has been limited due to the difficulties to overcome the high sterility of fusion plants deriving from the high or aberrant number of chromosomes.

Through fusion of haploid and diploid protoplasts, new forms of intra- and interspecific hybrids with lower level of ploidy have been obtained. The extent of homology between the haploid and diploid homologous chromosome complement influences the chromosome pairing during meiosis and, therefore, the fate of the chromosomes. By repeated backcrossings some chromosomes can be eliminated, resulting in plants harbouring additional chromosomes or introgressed novel genes. Gametosomatic hybrids

have been developed by intra- and interspecific fusion between microspore protoplasts and somatic protoplasts in *Nicotiana* spp. (Pental and Cocking 1985; Pental et al. 1988, 1989; Pirrie and Power 1986; Desprez et al. 1995a, b) and *Petunia* spp. (Lee and Power 1988a, b). In a review, Zhou and Yang (2000) described a successful fusion between immature pollen protoplasts of *Brassica chinensis* and hypocotyl protoplasts of *B. napus*, where one allotriploid and two allotetraploid plants were regenerated.

Since microspores are an unlimited source for haploid protoplasts, they were exploited as the haploid fusion partner. In the early phases of androgenetic sporogenesis, the tetrads offer a unique possibility of isolating haploid protoplasts in a large quantity. Due to the difficulties in protoplast release, only a few research groups have used mature pollens for fusion experiments (Desprez et al. 1995a, b). In *Brassica* species, protoplast isolation from microspores at late unicellular stage and pollen at early bicellular stage has been performed in *B. rapa* and *B. napus*, and the isolation frequency was about 20–40% (Li et al. 1994, Sun et al. 1999).

Cultivation of protoplasts isolated from *B. napus* gametophytic cells has not succeeded as of yet, although in some studies, structures like elongated cells, budding cells (Zhou 1989), and microcalli (Sun et al. 1999) have been observed. Usually the gametophytic protoplasts stopped any further development even if the first cell divisions had taken place, and many protoplasts still showed a strong tendency to produce pollen tubes in vitro (Zhou and Wu 1990, Xia et al. 1996). The totipotency of such a highly differentiated structure has not yet been verified (Sun et al. 1999).

Fusion of male gametophyte protoplasts has been carried out by a few research groups. All of the reports show that the pollen or tetrad protoplasts can be fused; however, further cell division is difficult and no microcalli have been obtained (Deka et al. 1977, Ueda et al. 1990).

Protoplast fusion of two haploid protoplasts can be used to form intraspecific cybrids that are complete diploids with the potential for increased fertility. It will however greatly reduce the time required for the cytoplasm trait transfer (Chuong et al. 1988). The reduced ploidy level may also benefit the fertility of the regenerated plants in interspecific hybrids. The similarity of haploid protoplast fusion to sexual hybridization, with the exception of the combination of the cytoplasm of the donor plants, may also make it a good experimental system for developmental biology research. Despite these considerations, only two reports about a haploid–haploid fusion exist. The first was published during the beginning of the "protoplast era" in 1974. Melchers and Labib (1974) produced 21 putative double heterozygote hybrids after intraspecific fusion of *N. tabacum*. Both fusion partners were haploid mesophyll protoplasts from different anther culture-derived plants. The second report was published fourteen years later, in 1988. Chuong and coworkers carried out the intraspecific fusion between protoplasts from haploid cytoplasmic atrazine-resistant (CATR) and haploid cytoplasmic male sterile (CMS) *B. napus* plants resulting in a diploid CMS-CATR cybrid (Chuong et al. 1988).

The present communication describes a reliable pollen protoplast isolation protocol for *B. oleracea* ssp. *italica*, and the use of these protoplasts for the first attempts in establishing the interspecific protoplast fusion between the pollen protoplasts of *B. oleracea* and haploid mesophyll protoplasts of *B. rapa*. Through the fusion of haploid protoplasts, new forms of intra- and interspecific hybrids can be obtained. The advantage of such material for crop improvement and fundamental research as well as the problems of the proposed approach will be discussed.

Material and methods

Isolation and culture of protoplasts from pollen cells of B. oleracea

Three cultivars ('Corvet' F_1, 'Medway' F_1, 'Calabrese') of *Brassica oleracea* L. convar. *botrytis* (L.) Alef. var. *italica* Plenck were used as the donor plants for pollen collection. 'Corvet' and 'Medway' are commercial varieties sold by the seed company Royal Sluis, Enkhuizen, the Netherlands. 'Calabrese' is a primary variety. All cultivars are diploid ($2n = 2x = 18$). Seeds were sown in the middle of July and the young plants were vernalized for 2 months at 5–10 °C. The plants flowered from the middle of November to early June of the next year in the greenhouse. Lighting from October to the end of March was compensated. The fading flower branches were pruned to promote the growth of new branches.

Flower buds were then collected according to their length. Microspores and pollen were isolated and purified in B5 medium (Gamborg et al. 1968) using procedures described for microspore culture (Cao et al. 1993) except that the sieve used had a pore size of 100 μm. Developmental stages of freshly isolated cells were determined by fluorescence microscopy after staining with DAPI (4′,6-diamidino-2-phenylindole), 1 μg/ml of distilled water.

Isolated microspores were suspended in the enzyme solution (Sun et al. 1999), which contained 1.0% (w/v) cellulase (Onozuka R-10) (Serva, Heidelberg, Federal Republic of Germany), 0.8% (w/v) pectinase (Serva), 0.3% (w/v) macerozyme R-10 (Serva), 0.02% (w/v) pectolyase Y-23 (Duchefa, Haarlem, the Netherlands), 9% D-mannitol, and 7.2% sorbitol in CPW solution (Power and Chapman 1985), pH 5.8. The digestion was carried out at 30 °C for 2–3 h in the dark with gentle shaking. Viability of the isolated protoplasts was tested by staining with fluorescein diacetate (Serva). Drops of a stock solution of fluorescein diacetate (40 μg/ml of acetone) were added to a sucrose solution (17.12 g of sucrose per 100 ml of distilled water) until a light milky discoloration appeared. This freshly prepared mix was then added to the protoplast solution at a ratio 1 : 1. The microscopic observation began immediately after mixing.

Pollen protoplast isolation frequency was assessed under the inverted microscope. It was very easy to distinguish pollen grains and pollen protoplasts by their color and shape. The pollen wall is brown showing some structures of the exine. Pollen protoplasts are light colored and the cytoplasm is visible.

The suspension was centrifuged at 180 **g** for 4 min, and the pellet was layered on the top of 5 ml of 30% sucrose-CPW solution, and then covered with 2 ml of W5 solution (Menczel et al. 1981). The tubes were centrifuged at 40 **g** for 12 min to separate the intact pollen protoplasts from the other parts. Pollen protoplasts were carefully pipetted out and rinsed 3 times with W5 by centrifugation at 120 **g** for 3 min each time. The purified pollen protoplasts were cultured in liquid protoplast medium (in this case 1/2 medium) (Ryschka et al. 2003) directly or first embedded in 1/2 medium with 0.5% low-melting-point agarose (GIBCO BRL, Gaithersburgh, Md., U.S.A.) or 1% Na-alginate to form a liquid-solid two-layer culture system. For Na-alginate embedding, the purified pollen protoplasts were first washed with an osmotic solution consisting of 45.5 g of sorbitol, 45.5 g of mannitol and 2.5 g of glucose per liter to remove the Ca^{2+} ions, then mixed (1 : 1, v/v) with embedding solution (2.0% Na-alginate in osmotic solution as aforementioned) and layered (0.6 ml of mixture per 60 mm diameter petri dish) on the solidified support (1% Bacto agar [Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.] and 2.22 g of $CaCl_2$ per liter in osmotic solution). After 10 min, 2 ml of 1/2 medium were added to the solidified embedding layer. The culture was incubated at 24 °C in the dark.

Isolation and culture of mesophyll protoplasts from haploid plants of B. rapa

Haploid plants of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* [Lour.] Hanelt) were obtained in several varieties by microspore culture following the method of Cao et al. (1994). The ploidy of each plant was identified using a flow cytometer (Partec CA-II) and Partec buffer containing DAPI (De Laat et al. 1987). Individual plants were kept on MS medium (Murashige and Skoog 1962) in vitro and subcultured every month. The cultures were maintained at 25 °C with a 16 h photoperiod (29.9 μmol/s·m^2; Osram L58 W/11, Lumilux daylight; Osram, Munich, Federal Republic of Germany).

Haploid mesophyll protoplasts were isolated from the leaves of the haploid plants and purified following the method described for diploid plants (Ryschka et al. 1996). The protoplasts were adjusted to 1×10^5 per ml and cultured in 1/2 liquid medium directly or embedded in 1/2 medium with 0.5% agarose to form a liquid-solid two-layer culture system as described for the pollen protoplast culture. The protoplasts were incubated at 24 °C in the dark.

On the 6th and 10th days of protoplast culture, cell division frequencies were counted with an inverted microscope. When the cell division became clearly visible, 200 μl of 2/2 dilution medium (composition as medium 1/2, but with 20 g of sucrose per liter instead of mannitol and sorbitol, no additional $CaCl_2 \cdot 2H_2O$) was added per petri dish (diameter, 3 cm). After every 3rd day, the same amount of fresh dilution medium was added.

Protoplast fusion and following culture

Both purified pollen protoplasts and mesophyll protoplasts were stored at 10 °C for about 1–2 h before PEG (polyethylene glycol) fusion treatment. The protoplasts were mixed together in a cell ratio of about 1 : 1 in prefusion solution (45 g of mannitol, 36 g of sorbitol, 4.41 g of $CaCl_2 \cdot 2H_2O$, 5.55 g of KCl, 7.88 g of Tris-HCl per liter, pH 7.2). The cell density was approximately 0.5×10^6 to 1×10^6 cells per ml. Protoplast fusion was carried out with few modifications according to Ryschka et al. (2003) (named Ry-m) or to Li et al. (1994) (named Li-m) as follows.

Method Ry-m. Mixed protoplasts were dropped on a plastic petri dish (diameter, 3 cm), 4 drops per dish, 40 μl/drop, and then kept undisturbed for 5 min to let the protoplasts deposit on the bottom of the dish. 60 μl of PEG solution (400 g of PEG, 54 g of glucose, 7.35 g of $CaCl_2 \cdot 2H_2O$ per liter, pH 7.0) was added gently to each droplet, and 5 min later, 300 μl of W5 solution was supplemented carefully at 5–10 min intervals for the next 20 min. The suspension was mixed gently after each addition. Subsequently, most of the liquid was removed by tilting the petri dishes slightly. Afterwards, 1 ml of W5 solution was added carefully, and the dishes were kept still for another 10–20 min. The solution was pipetted out again, and 1 ml of W5 solution was added once more, and the suspension was kept still for 5 min. Lastly, the W5 solution was removed carefully and 1 ml of 1/2 liquid medium was added for the protoplasts culture.

Method Li-m. The only difference to the method Ry-m was that the 60 μl of PEG solution was dropped in petri dishes first. Thereafter, 40 μl protoplast mixture was added on the top of each PEG drop. Twenty minutes later, the washing procedure was the same as described for method Ry-m.

Postfusion protoplasts were cultured in 1/2 medium at 24 °C in darkness. When multiple cell divisions were observed, 200 μl of dilution medium per petri dish was added every 3 days. These dilution steps made embedding of the fused protoplasts impossible. Two drops per dish of 3% activated charcoal (3% activated charcoal in 0.5% melted agarose) were added after 8 days of culture. Suspensions with colonies consisting of more than 20 cells (about 15 days later) were then transferred to solidified 3/10 medium (B5 salts and vitamins, 150 mg of casein hydrolysate, 30 g of sucrose, and 8 g of agar per liter and plant growth regulators as in 1/2 medium) for callus growth and still cultured in the dark. Five days later, the liquid medium on the top was removed and the petri dishes were kept in a dim light (2.99 μmol/s·m^2). Proliferating calli were cultured in a normal light (29.9 μmol/s·m^2, Osram L58 W/11, Lumilux daylight; Osram). Calli that reached a size between 1 and 5 mm (after ca. 3–4 weeks) were transferred to 4/1 shoot-inducing medium (MS salts, B5 vitamins, 30 g of sucrose, 0.02 mg of naphthaleneacetic acid, 0.2 mg of 6-benzylaminopurine, 10 g of agar per liter) with 3 mg of $AgNO_3$ per liter.

Percentages of gametophytic developmental stages, protoplast isolation frequencies, and cell division frequencies in mesophyll protoplast culture were determined by counting under the microscope. Five fields of the petri dishes were chosen randomly to count the number for one replicate, the average value was calculated from three replicates. Standard deviations (SD) and significances (using SAS) are given in the tables.

Hybrid identification in the callus population by RAPD

Calli were collected and their morphologies were noted. Plant genomic DNA was extracted by a rapid method (Dorokhov and Klocke 1997) and random amplified polymorphic DNA (RAPD) reactions were performed in the thermocycler PE 9600 (Applied Biosystems) in a volume of 12.5 μl containing 2.5 pmol of a single 10-base primer (Operon Biotechnologies Inc., Huntsville, Ala., U.S.A.), 0.25 U *Taq* polymerase (Qbiogene, Heidelberg, Federal Republic of Germany), 1× *Taq* incubation buffer (Qbiogene), 100 μM (each) deoxynucleoside triphosphate, and 20 ng of plant DNA. The cycling program was 94 °C for 3 min, 45 cycles at 94 °C for 20 s, 36 °C for 1 min and 72 °C for 1 min, and finally 36 °C for 1 min and 72 °C for 10 min. Electrophoresis was performed in 2% agarose gel with 0.5× TBE buffer (5× TBE buffer is 54 g of Tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA, pH 8.0, per liter) for about 2 h at a constant voltage of 80 V.

Results

Factors affecting the isolation of protoplasts from pollens

The maturity of the pollen cells was the most important factor affecting protoplast release irrespective of the broccoli varieties used. Pollen cells at the trinucleate stage (about 7 mm long flower bud) had the highest protoplast

Table 1. Relationship between cell developmental stage and protoplast release frequency of pollen in *B. oleracea* var. *italica* 'Medway'

Bud length (mm)	Nr. of buds	% gametophytic cells at stage:[a]				% cells releasing protoplast[b]	Nr. of protoplasts/bud
		MU	LU	BP	TP		
4.0–4.5	131	2.2	71.0	23.7	3.2	0.5 ± 0.4 D	
4.5–5.0	116		6.7	68.5	24.8	10.0 ± 5.8 C	
5.0–6.0	98		2.5	25.3	72.2	29.8 ± 8.3 B	0.3×10^4
6.0–7.0	88			5.3	94.7	90.0 ± 5.4 A	3.9×10^4

[a] MU, mid-uninucleate microspore; LU, late-uninucleate microspore; BP, binucleate pollen; TP, trinucleate pollen. Developmental stages were determined by DAPI staining
[b] Values are means with standard deviations. Values followed by different letters are significantly different at the 0.01 probability level

release frequency up to 95%. To clarify the detailed relationship of pollen developmental stage with the frequency of protoplast isolation, one cultivar of broccoli ('Medway') was examined further. The development of gametophytic cells was shown to be asynchronous, and the protoplast isolation frequency was highly dependent on the ratio of trinucleate-stage pollens in the male gametophyte populations (Table 1).

Purification and culture of pollen protoplasts

Pollen protoplasts have a higher cytoplasm density and a higher osmotic pressure than protoplasts from other plant cells. Therefore, the pollen protoplasts could only be separated from the damaged pollen and the rest of pollen walls after a serious modification of the protocols for hypocotyl or mesophyll protoplasts (Ryschka et al. 1996). 30% (w/v) sucrose solution and W5 solution were used for discontinuous centrifugation with a lower speed of 500 rpm (40 **g**) for 12 min. Such treatment facilitated that most of the pollen protoplasts floated on the interface between the two layers could be collected with a pipette. The vitalities of isolated pollen protoplasts were 95–100% when checked by fluorescein diacetate staining (Fig. 1a, b).

Purified pollen protoplasts were cultured in 1/2 liquid medium directly or first embedded in 0.5% agarose or 1% Na-alginate and then covered with liquid 1/2 medium to form a two-layer culture. In the first two cases, many of the pollen protoplasts formed some tubelike structures, as in pollen germination, in 3–4 days, and almost all of the protoplasts burst within 1 week. However, when embedded in 1% Na-alginate, the pollen protoplasts retained their shape even after 20 days of culture, and 1–2 cell divisions could be observed after 4–5 days in a few cases, although no further divisions were seen.

Variability in culturability of haploid mesophyll protoplasts

Obvious variability in culturability was found in different haploid lines of *B. rapa* derived from different cultivars of donor plants (e.g., C2, C6, and C9), and even in different haploid lines derived from the same donor plants (e.g., C6-1, C6-2, and C6-4). In general, differences in cell division

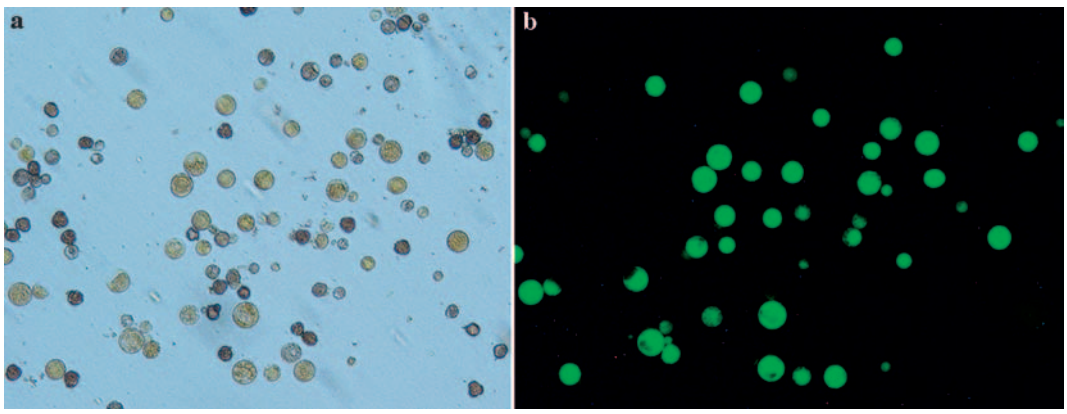

Fig. 1 a, b. Isolation of pollen protoplasts of *B. oleracea* var. *italica*. **a** Isolated pollen protoplasts in the pollen population. **b** Viable protoplasts stained with fluorescein diacetate and observed by fluorescence microscopy (the same field as for panel a). ×200

Table 2. Cell division frequencies of haploid lines of *B. rapa* cultured by different methods

Genotype	% dividing cells in:[a]			
	1/2 medium		Two-layer culture	
	6 days	10 days	6 days	10 days
C2	0.2 ± 1.3 E	ND[b]; brown, structure unclear	0.6 ± 0.8 DE	ND; many vacuolated cells, light brown
C6-1	7.2 ± 2.6 BC	ND; brown, structure unclear	27.2 ± 3.3 A	72.0 ± 5.2; light brown
C6-2	14.3 ± 3.2 B	ND; brown, structure unclear	33.5 ± 4.8 A	78.2 ± 5.8; light brown
C6-4	10.0 ± 3.5 B	ND; brown, structure unclear	7.2 ± 3.5 BC	6.1 ± 2.2; many vacuolated cells, light brown
C9-1	3.3 ± 2.1 C	ND; brown, structure unclear	1.0 ± 0.3 CD	ND; many vacuolated cells, brown, structure unclear
C9-2	0.3 ± 0.5 E	ND; brown, structure unclear	0.3 ± 0.3 E	ND; many vacuolated cells, brown, structure unclear

[a] Values are means with standard deviations. Values followed by different letters are significantly different at the 0.01 probability level
[b] ND, cell divisions not detected

frequency among lines from different genotypes of donor plants (e.g., C2, C6, and C9) were larger than that among lines from the same genotype of donor plants (e.g., C6-1, C6-2, and C6-4). In lines C2 and C9, absolutely no microcalli formed regardless of the culture method used, while many microcalli were formed from lines C6-1 and C6-2 after 10 days of culture embedded in agarose (Table 2).

Protoplast fusion and culture of the postfusion protoplasts

As pollen protoplasts had a higher density of cytoplasm than other plant protoplasts, a new prefusion solution (45 g of mannitol, 36 g of sorbitol, 4.41 g of $CaCl_2 \cdot 2H_2O$, 5.55 g of KCl, 7.88 g of Tris-HCl per liter, pH 7.2) was developed which had a higher osmotic pressure (0.74 osmol/kg) than the solution used by Ryschka et al. (2003) (0.6 osmol/kg). In this solution, both pollen protoplasts and mesophyll protoplasts kept a stable round shape unlike in the other solution, where the pollen protoplasts were distorted in a matter of minutes. W5 solution with an osmotic pressure of 0.74 osmol/kg was also used to substitute the wash solution described by Ryschka et al. (2003).

Several PEG treatments were tested for the fusion frequency in the Ry-m fusion method. The highest fusion frequency of about 20% was observed with 40% PEG 4000 (Fig. 2). The lowest fusion frequency of about 4.0% was seen with 30% PEG 8000.

Fused protoplasts could be distinguished easily from other cells due to the green color of chloroplasts from mesophyll protoplasts and the light brown color from pollen protoplasts (Fig. 2). Quite a few one-to-one fused protoplasts were observed, while some of the fused protoplasts were fusions of 3 or 4 protoplasts. After another 3 days of culture, the different parts (green and light brown) in the cytoplasm of the fused protoplasts diffused, and the cells enlarged, making it difficult to clearly distinguish the fused ones from other cells. At this time, some of the nonfused pollen protoplasts formed a tubelike structure, but many of them burst, as in the pollen protoplast culture described above. Some budding-like structures were formed after 4 days of culture. Therefore, it could be speculated that these derived from the fused heterokaryon protoplasts because such structures were never observed in pure mesophyll protoplast culture or in control dishes of mesophyll protoplast fusion. After about 10 days of culture, some microcalli with dense cytoplasm could be observed. About 1.5 months later, visible callus formed on the solidified 3/10 medium. Calli larger than 4 mm were transferred to 4/1 medium to induce the adventitious shoot regeneration (Fig. 3).

As described for the mesophyll protoplast culture, strong variability in culturability was also found in different haploid lines in fusion and culture experiments. Table 3 displays the results of 40 fusion and postfusion cultures with different lines of haploid Chinese cabbage

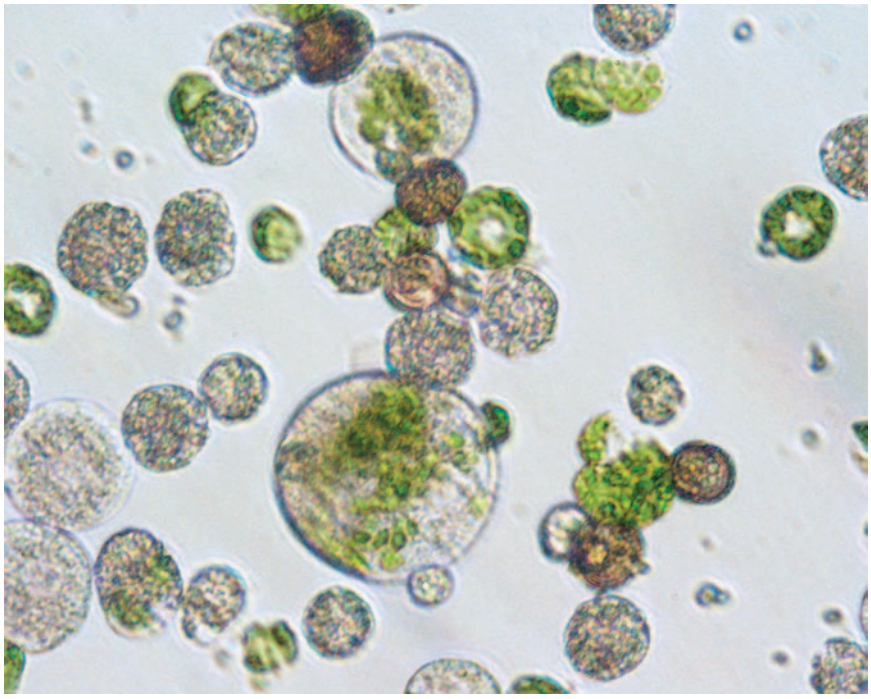

Fig. 2. Protoplast fusion after PEG treatment. ×400

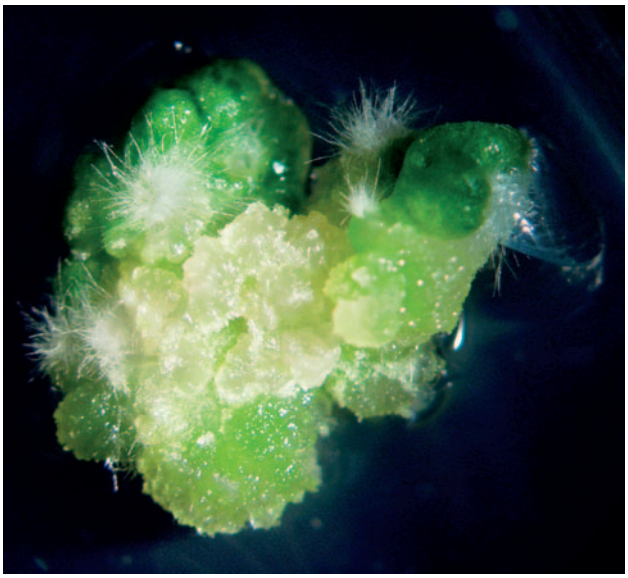

Fig. 3. Hybrid calli with shoot primordia and green color

Table 3. Influence of genotype of mesophyll protoplasts on the postfusion cell division and callus formation

Tested genotype	Nr. of fusion experiments	Nr. of experiments with cell division	Nr. (%) of experiments with callus formation	Total nr. of calli formed
C2	1	1	0	0
C6-1	8	6	5 (62.5)	28
C6-2	7	7	7 (100)	302
C6-3	2	1	0	0
C6-4	9	9	7 (77.8)	397
C9-1	6	1	1 (16.7)	2
C9-2	7	5	1 (14.3)	6

as the donor of mesophyll protoplasts, no matter the donor of pollen protoplasts. It was clearly shown that the donor genotype had a strong influence on the postfusion cell division and callus formation. Line C6-2 seemed to be the most active in cell division and callus formation after fusion treatment, producing callus in all of the 7 experiments.

The PEG fusion treatment was found to promote the cell division and callus formation. In 15 heterokaryon fusion experiments, both fresh and postfusion mesophyll protoplasts cultured in 1/2 medium were set as controls. Multiple cell divisions were never found and the cells finally browned and died. However, multiple cell divisions and callus formation could be observed in postfusion cultures of mesophyll protoplasts after PEG treatment. The promoting effect of fusion treatment on cell division was also demonstrated for several other plant species, although this was following electrical fusion (Ochatt et al. 1988, Keller et al. 1997).

Hybrid identification by RAPD analysis

The callus population was characterized by RAPD. After a primer screening with twenty random 10 bp primers, three primers were selected on the basis of their clear pat-

tern showing the polymorphism between the two parents as well as their reliable reproducibility.

RAPD results clarified that hybrids between pollen protoplasts of broccoli and haploid mesophyll protoplasts of *B. rapa* could divide persistently and callus could be formed. Hybrid callus could be derived from both fusion methods as well as after different PEG treatments (Table 4). Although a higher fusion frequency resulted from the Ry-m method and treatment with 40% PEG 4000, higher frequencies of hybrid callus were not observed in these experiments. It seems that the genotype combination is an important factor for the production of hybrid callus. The frequency of hybrid callus formation from the combination of Chinese cabbage and 'Calabrese' was just 1.7% (3 from 174 calli), while the hybrid frequency was about 23.5% (4 from 17 calli) and 10.2% (5 from 49 calli) for the combinations of Chinese cabbage and 'Corvet' and of Chinese cabbage and 'Medway', respectively.

RAPD analysis revealed that most of the calli came from the mesophyll protoplasts and no callus formed from the pollen protoplasts. This might explain the difficulty of dedifferentiation of such a highly differentiated structure in vitro. The RAPD analyses have shown a large genetic variability among the hybrid calli identified (Fig. 4a, b). Some calli gave a DNA banding pattern containing almost all fragments from the parental lines (e.g., callus 3, 8, and 17) or more from the *B. rapa* donor. Callus 15 was the sole callus showing patterns more like broccoli (donor of pollen protoplast). Some calli had a RAPD pattern lacking some fragments from the parents (e.g., callus 1, 7 and 15). After further investigation with other primers, the same calli could show new bands, i.e., fragments not in the DNA profile of the parents (data not shown). The parental DNA was isolated from leaves. Taking into account that the broccoli varieties 'Corvet' and 'Medway' are F_1 hybrids, the pollen population should be highly heterozygous. This heterozygosity could cause the new or absent fragments in comparison with the RAPD patterns from the parents. However, by using several primers common bands from each parent were found for the hybrid calli.

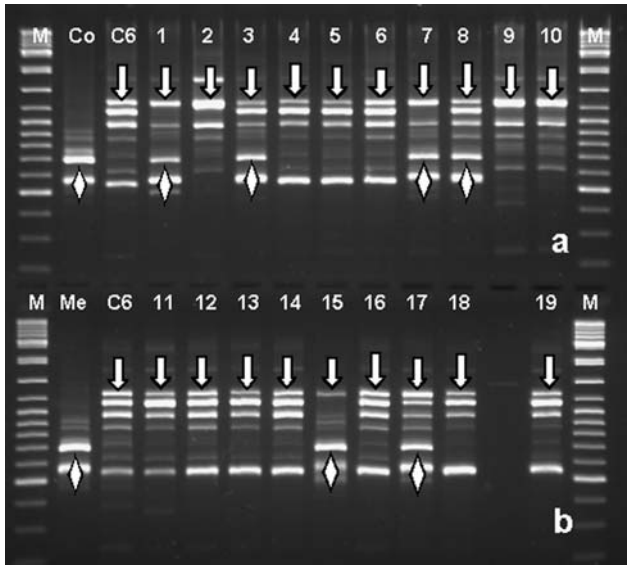

Fig. 4 a, b. RAPD pattern of calli DNA using primer OPA15. **a** Calli (*1–10*) derived from fusion between pollen protoplasts of *B. oleracea* var. *italica* 'Corvet' (*Co*) and haploid mesophyll protoplasts of *B. rapa* line C6-2 (*C6*). **b** Calli (*11–19*) derived from fusion between 'Medway' (*Me*) and C6-2. *M* GeneRuler DNA ladder Mix (MBI Fermentas). Diamond, typical fragment from broccoli parent; arrow, *B. rapa* fragment

It was noted that most of the hybrid calli developed vigorously and were green (Table 4), while calli from mesophyll protoplasts were small and brown. One could reason that the two sets of unpaired chromosomes from the two haploid donor protoplasts did not have a negative influence on the growth of the callus. Some of the hybrid calli formed shoot primordia (Fig. 3), although the regeneration of plants did not succeed till now.

Table 4. Factors affecting the hybrid callus formation and results of RAPD analyses

Fusion partner	Bud length (mm)	Nr. of calli tested by RAPD	Nr. of hybrid calli	PEG treatment	Fusion method	Growth status of hybrid callus
Corvet + C6-1	5.5–6.5	5	2	PEG 6000, 40%	Li-m	large, green
Corvet + C6-2	5.0–7.0	11	1	PEG 8000, 30%	Li-m	large, yellow-green
Corvet + C9-2	5.0–6.5	1	1	PEG 4000, 30%	Ry-m	large, green
Medway + C6-1	5.0–7.0	16	4	PEG 4000, 25%, 30%	Ry-m	small, brown-yellow
Medway + C6-2	5.0–6.5	7	0			
Medway + C6-4	5.0–6.5	26	1	PEG 4000, 30%	Ry-m	large, green
Calabrese + C6-2	5.0–6.5	174	3	PEG 4000, 30%	Ry-m	small, brown

Discussion

Isolation of male gametophytic protoplasts has been achieved at different developmental stages of gametophytic cells. It seems that the protoplast isolation capacity is highly related to the developmental stages of the gametophytic cells. The optimal stage varied among different species. For *Digitalis* sp., isolation was carried out from tetrad cells (Arnalte et al. 1991), but in some species, isolation from tetrads to mature pollen has been realized (Zhou 1989). This may depend on the structure of the gametophytic cells in different species. In our work, the highest protoplast isolation frequency was from the trinucleate-stage pollens of *B. oleracea*, using flower buds 5–7 mm in length. Sun et al. (1999) described similar findings. The authors obtained protoplasts from precultured *B. napus* microspores isolated at late uninucleate to early binucleate stages, indicating that further development of cells occurred during preculture. Their outcomes confirmed that the protoplast isolation frequency increased directly along with the higher number of trinucleate pollens after preculture.

Our work proved that variability in culturability of mesophyll protoplasts of Chinese cabbage was apparent, not only among haploid lines derived from different genotypes of donor plants but also among haploid lines derived from the same genotype of donor plants. This phenomenon was also found in studies of *B. napus*, where protoplasts were isolated from stem peels of 55 microspore-derived haploid plants. Both responding and nonresponding lines were identified in haploids obtained from the same donor genotypes (Chuong et al. 1987). This may be due to residual heterozygosity in the cultivars or somaclonal variation or may indicate that culturability is a complexly inherited trait controlled by several genes on different chromosomes that can become separated during haploid production.

Although pure culture of broccoli pollen protoplasts failed, and difficulties also occurred in the culture of Chinese cabbage haploid mesophyll protoplasts, fusion of these protoplasts was successful up to the callus formation. Some of the green calli formed were identified to be hybrid. This might imply the phenomenon of complementary effect of regeneration capacity through fusion, which has also been observed in other studies (Xia et al. 2001). At the very least, the combination of the two haploid genomes of the chosen species seemed not to interfere seriously the cell division and growth of the hybrid callus tissue. Moreover, the hybrid calli appeared more viable than those from the mesophyll protoplasts. This might be due to the difficulty of the tissue culture of Chinese cabbage (AA genome) as reported by Glimelius (1999). These primary results showed that it may be possible to obtain hybrid plants by haploid protoplast fusion. Such plant material provides an ideal system for the exploration of genetic and cytological problems, such as better understanding of the genus and determining the influence of genome dosage on morphogenesis.

Somatic protoplasts have a uniform germplasm background, while more variation occurs in pollen protoplasts due to the meiosis of the microsporocyte. Although the totipotency of pollen protoplasts has not yet been demonstrated, this enriched genetic background might be utilized for protoplast fusion.

The biggest obstacle for the practical application of somatic hybrids is the disturbed fertility of the regenerated plants (Glimelius 1999), partly caused by a high chromosome number. The haploid protoplast fusion system might be useful for reducing the ploidy level of the regenerated plants and could benefit the increase of fertility.

Chevre et al. (1994) compared the somatic hybrids between *B. napus* (AACC) and *Sinapis alba* with sexually produced hybrids from the same species. It was found that chromosome rearrangements occurred more frequently in the somatic hybrids than in the sexual hybrids. The rearrangements induced by protoplast fusion might facilitate the introgression of new traits from taxonomically distant species to a crop. There are some possibilities for the chromosome pairing and recombination even between different haploid genomes. This might be useful for the gene introgression between two species, enabling the creation of new material for plant breeding. The number of publications about the production of gametosomatic hybrids from haploid protoplasts alone is currently limited to two. The described work is the first concerning the genus *Brassica*, reflecting the difficulties of such an approach. However, the evidence of well growing hybrid callus is a first step towards obtaining unique plant material promising for fundamental research as well as for practical crop improvement.

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