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Production of new CMS *Brassica oleracea* by transfer of 'Anand' cytoplasm from *B. rapa* through protoplast fusion

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Abstract New types of cytoplasmic male sterility (CMS) in *Brassica oleracea* would be useful for F_1 hybrid seed production. The 'Anand' cytoplasm derives from the wild species *B. tournefortii*. Rapid cycling stocks of *B. rapa* and *B. oleracea* were used in cybridization experiments as donor and recipient of 'Anand' (='tour') CMS, respectively. Prior to fusion with PEG, donor protoplasts were inactivated with 30 krad γ-rays and recipient ones with 3 m*M* iodoacetate, respectively. No calli were obtained from the pre-treated protoplasts. The frequency of shoot regeneration was 21–43% in untreated *B. oleracea* controls, but only 0–0.5% in 'Anand' *B. rapa*. Putative cybrids were regenerated from about 3% of the calli from fused protoplasts. Regenerated plants were analyzed for nuclear DNA content, plant and flower morphology, pollen production, female fertility, cold tolerance, and organelle composition. Eighty-one percent of the regenerated controls and 63% of fusion-derived plants were diploid. The rest showed DNA contents corresponding to 2*x*–4*x*, 4*x*, or higher ploidy levels, presumably due to somatic doubling in vitro and/or fusions in which the donor nucleus was not completely eliminated. Sixty-four percent of the cybrids had stamens and petals varying in size and shape and were male-sterile, with indehiscent anthers. Their phenotype was otherwise similar to that of *B. oleracea*. The remaining plants had normal flowers and were male-fertile. Data from crosses with fertile pollinators indicated good female fertility in some of the sterile lines, both after hand and insect pollinations in cages. Mitochondrial (mt) segregation in the cybrids was slightly biased towards 'Anand' mitochondria, and the presence of 'Anand' mtDNA fragments was strongly associated with male sterility. Evidence of mtDNA rearrangements was obtained in some cybrids. Segregation of chloroplasts was slightly biased towards *B. oleracea*. The presence of 'Anand' chloroplasts with a *B. oleracea* nucleus did not result in cold temperature chlorosis, as seen in 'Ogura' CMS plants.

Key words *Brassica oleracea* · *Brassica tournefortii* · Anand · Cytoplasmic male sterility · Protoplast fusion

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Introduction

The genus *Brassica* includes many important species used for oil, condiment, forage, and food production. Most *Brassica* vegetables, such as cauliflower, broccoli, cabbage, and others, belong to the species *B. oleracea,* which is highly polymorphic. In the latter species, as well as in other species within the genus, the commercial production of F_1 hybrids has gained considerable importance in the past few years. At present it generally relies on self-incompatibility systems that are genetically complex and difficult to work with. The breeding of parental lines and production of hybrid seed would both be simplified by using cytoplasmic male sterility (CMS) (Renard et al. 1992).

Several types of CMS are known in *Brassica* spp. and related genera (Shiga 1980; Renard et al. 1992). Nevertheless, the preservation and the enlargement of genetic variability at the cytoplasmic level are highly desirable objectives to provide breeders with new and improved CMS systems and reduce the danger of epidemic diseases such as southern corn leaf blight in T-CMS maize (Levings 1990). In comparison with sexual hybridization, somatic cell fusion is a much more powerful method for increasing cytoplasmic diversity in a population: it is possible to transfer chloroplasts and mitochondria in a single step, even between incongruent species, and to obtain novel combinations of nucleus and cytoplasm organelles (Kumar and Cocking 1987; Earle et al. 1992).

The 'Anand' CMS was originally described in *B. juncea*. Subsequently, *B. tournefortii* was identified as the most likely donor of the cytoplasm, so the male-sterile phenotype is probably due to alloplasmic relationships between mitochondrial genes of the wild and nuclear genes of the recipient species (Rawat and Anand 1979; Pradhan et al. 1991). Recently, *B. tournefortii* or other genotypes with *tournefortii* cytoplasm have been used as donors of nuclear and organelle genes in fusion experiments aimed at the production of somatic hybrids and cybrids with *B. napus* (Stiewe and Röbbelen 1994; Landgren and Glimelius 1994; Liu et al. 1995, 1996) or hybrids with substitution genomic configuration TCBB (Mukhopadhyay et al. 1994). After symmetric hybridization, however, analysis of the effect of the new cytoplasm on plant morphology and fertility is hindered by the higher ploidy levels and chromosome compatibilities related to the bi-parental transmission of nuclear genomes. Several generations of backcross and selection are usually needed to return to the cultivated phenotype. The production of cytoplasmic hybrids (cybrids) is particularly attractive when one is interested in the transfer of cytoplasmic-encoded traits into a particular genetic background. Although spontaneous elimination has been reported in some instances (Earle et al. 1992), donor parental protoplasts must usually be treated in some way to eliminate the nucleus (Kumar and Cocking 1987).

Rapid cycling (RC) *Brassica* stocks have been developed recently (Williams 1985). They show a considerably shortened life-cycle and hence allow much easier and faster study of the genetics of new traits. Subsequently, crosses with cultivated *Brassica* spp. can be easily performed to transfer traits of interest.

This paper describes the transfer of the sterile 'Anand' cytoplasm from RC *B. rapa* to RC *B. oleracea* by protoplast fusion. Cybrids were obtained after inactivation of recipient and donor parental lines by iodoacetate and γ-irradiation, respectively. Regenerated plants have been characterized for plant and floral morphology, male and female fertility (after crosses with fertile *B. oleracea*), nuclear DNA content, cold tolerance, and organelle composition.

Materials and methods

Plant material

Rapid cycling stocks of *B. oleracea* with fertile *oleracea* cytoplasm (CrGC #3–1) and *B. rapa* with sterile 'Anand' cytoplasm (CrGC #1–31) were obtained from the Crucifer Genetics Cooperative (University of Wisconsin-Madison, USA) (Williams 1985).

Seeds were surface sterilized by a 5-min incubation in 70% ethyl alcohol followed by 10 min in 30% Clorox bleach + 0.5% (w/v) PEX detergent. The sterilized seeds were placed in LS medium (Linsmaier and Skoog 1965) supplemented with 3% sucrose and solidified with Bacto-Agar, Gelrite, or Phytagar (LS30 medium). Jars were incubated at 25°C, 16 h light, 60–80 μE m^{-2} s^{-1}. Plants were propagated in vitro under the same conditions by making nodal or shoot-tip cuttings every 1–2 months.

Protoplast isolation and culture

Either seedlings or shoot cultures were used for protoplast isolation. Mesophyll protoplasts were isolated and cultured according to Hansen and Earle (1994) with the following modifications. The enzyme solution was diluted 1 to 4 with W5 instead of SCM medium. Phytagar (7 g l^{-1}) was used for solidifying media B, C, and F, whereas agarose (Type I, Sigma, 8 g l^{-1}) was used for regeneration medium E. The feeder layer (Walters and Earle 1990) consisted of *Brassica napus* cells and was used only on medium B. Protoplasts were plated at 8–10×10^4 ml^{-1} in 200-μl aliquots. The plating efficiency of the protoplasts was estimated as the ratio between the number of calli recovered and the number of protoplasts plated in medium B (×100).

Protoplast fusion

About 2 h after the start of enzyme digestion, donor *B. rapa* protoplasts were irradiated with 30 krad γ-rays from a [^{137}Cs] source. Recipient *B. oleracea* protoplasts were treated for 20–30 min at 30°C with 3–5 m*M* iodoacetate; a concentrated solution of the latter was freshly prepared in W5 medium and added to the protoplasts in enzyme solution to give the final concentration. Protoplasts were fused following the procedure of Thomzik and Hain (1988), as modified by Walters et al. (1992), either at 1 or 2×10^6 ml^{-1} using PEG 8000 (Aldrich) as the fusogenic agent. Both the unfused and the fused mixture were plated together with controls (untreated and treated parents) at twice the plating density used for the latter.

Plant analyses

Regenerated shoots (1–2 cm long) were transferred to LS30 medium for rooting and incubated as described before. Rooted shoots were propagated in vitro by tip culture, while clonal copies were transferred to a growth chamber (24°C, 24 h light, 180–220 μE m^{-2} s^{-1}) for further analyses. Control plants grown from seeds were incubated under the same conditions. Subsequently, another set of regenerated shoots, including clonal copies of plants already analyzed in the growth chamber plus some other regenerants not previously examined, was transferred into a greenhouse.

The nuclear DNA content of young leaves was evaluated by flow cytometry using the procedure described by Arumuganathan and Earle (1991). Chicken red blood cells were used as standard.

Control and regenerated plants were analyzed at maturity for plant and flower morphology, and flowering date. Male fertility was evaluated by examining anthers for pollen production and by staining pollen with acetocarmine. To estimate female fertility, we either backcrossed male-sterile plants grown in the greenhouse to fertile RC *B. oleracea* by hand pollinations or crossed them to broccoli by insect pollinations. In the latter case, the plants were caged with flies.

Cold tolerance tests were carried out by incubating potted plants at 12°C for 5 weeks.

For organellar restriction fragment length polymorphism (RFLP) analysis, total DNA was isolated from a sample of 15 seed-derived parental plants and 38 regenerants using the Dellaporta procedure (Dellaporta et al. 1983) modified as follows. After the first precipitation, the DNA pellet was resuspended in 500 μl TE buffer, treated with RNAse, and consecutively purified with phenol:chloroform:isoamyl alcohol (25:24:1) and with chloroform:isoamyl alcohol. Finally, polysaccharides were removed from the aqueous phase by salt precipitation (1 *M* NaCl) at 4°C. Five micrograms of purified DNA was cut with restriction enzymes, run in a 0.8% agarose gel, and transferred to nylon filters for Southern analysis. *Cox*I and *cox*II genes from the maize mitochondrial (mt) genome (3.95-kb *Bam*HI/*Eco*RI and 2.4-kb *Eco*RI inserts, respectively), and the 12.3-kb *Bam*HI fragment from the potato chloroplast (cp) genome were kindly provided by Prof. C.J. Leaver, Oxford University, U.K., and Dr. S. Heinhorst, University of Southern Missouri, USA. They were labelled with dUTP-digoxigenin (Boehringer Mannheim) and used as probes. After overnight hybridization at 65°C, filters were washed sequentially with 2×SSC, 0.1% SDS (2×5 min, RT), 0.2×SSC, 0.1% SDS

(2×5 min, RT), 0.1×SSC, 0.1% SDS (2× 15 min, 65°C). Southern blotting and detection were carried out as reported in Kreike et al. (1990) with some minor modifications. CSPD (Tropix) was used as the substrate for the chemiluminescent detection.

Results

Protoplast fusion and plant regeneration

The best growth of seedling or nodal cultures used for protoplast isolation was obtained using Phytagar as the gelling agent. On Bacto-Agar roots tended to become necrotic, whereas on Gelrite shoots frequently became hyperhydrated. Protoplast yield in RC *B. oleracea* was 3.1×10^7 protoplasts per gram of fresh tissue (min. 0.7 – max. 5.3×10^7), while in RC 'Anand' *B. rapa* it was somewhat lower (average of 1.8×10^7; range from 0.3 to 3.1×10^7)

A few days after plating on medium B, control parental protoplasts started to divide and form small colonies. After the transfer of filters onto medium C and then to medium E, both RC *B. oleracea* and 'Anand' *B. rapa* showed a layer of contiguous mini-calli. *B. oleracea* protoplasts treated with iodoacetate usually did not show any division, whereas in the γ-rays-treated *B. rapa* protoplasts and in the unfused mixture of treated protoplasts, some colonies were observed, although they stopped growth after a few divisions. In the fused mixture, colonies that showed sustained growth could be transferred singly to regeneration medium along with control parental calli.

The average plating efficiency of protoplasts was 0.15% and 0.29% in parental *B. oleracea* and 'Anand' *B. rapa*, respectively, while in the fused mixture it ranged from 0.01 to 0.1% (Table 1). A total of 1551 calli derived from fused protoplasts were transferred to regeneration medium. The average regeneration frequency (percentage of calli that regenerated at least one shoot) was 3.4%; however, most calli regenerated multiple shoots. In the same experiments, recipient untreated *B. oleracea* protoplasts showed an average regeneration frequency of 33.7% (20.5–43.2%), whereas the untreated *B. rapa* protoplasts regenerated hardly any shoots. No shoots were obtained from the few calli derived from treated protoplasts. Almost all regenerated shoots could be rooted on propagation medium and successfully transferred to in vivo conditions.

Ploidy level

On the basis of results of flow cytometric analysis, the distribution frequencies of individual plants for their DNA content were plotted (Fig. 1). All plants from seeds of the parental genotypes were diploid, with mean DNA contents equal to 1.32 (SD 0.14) and 1.22 (SD 0.13) pg/nucleus for *B. oleracea* and 'Anand' *B. rapa*, respectively. In regenerated control *B. oleracea* plants DNA content ranged from 1.23 to 3.67 pg/nucleus. Eighty-one percent were diploids (average DNA content=1.39 pg±0.12) and 19% were tetraploids (average DNA content=3.35 pg±0.32). Sixty-three percent of the fusion-derived plants (61% of calli) had DNA contents similar to diploid *B. oleracea* (1.46 pg±0.13), whereas the remaining showed DNA values corresponding to 2*x*–4*x*, 4*x*, or higher ploidy levels. Only one callus produced two shoots with different ploidy levels.

Plant morphology and fertility

In the growth chamber, all *B. oleracea* plants grown from seeds showed normal flower phenotype and were male-fertile (Table 2 and Fig. 2). Seed-grown 'Anand' *B. rapa* plants showed some variation for pollen production and anther dehiscence but generally had flowers with reduced anthers that either contained pollen or were empty.

All but 2 (6%) of the regenerated control *B. oleracea* had normal plant and flower phenotypes and were male fertile. Tetraploids were generally similar to diploids, except for reduced plant size in a few cases. Tetraploid flowers had a normal shape but were consistently bigger in size. The phenotype of putative cybrids was similar to that of *B. oleracea*, except for flower morphology and pollen production (Fig. 2). Of 44 putative cybrids 28 (64%) were

Table 1 Plating efficiency and plant regeneration frequency in calli derived from parental protoplasts, and their unfused and fused mixture

Genotype	Protoplast pre-treatments	Protoplast plated (no.)	Plating efficiency (%)[a,b]	Calli on regeneration medium (no.)	Calli regenerated[c]		Shoots recovered (no.)[d]
					(no.)	(%)[b]	
B. oleracea	None	1.7×10^5	0.15±0.07	255	86	33.7	86
B. rapa Anand	None	1.7×10^5	0.29±0.05	501	1	0.2	2
B. oleracea	Iodoacetate (IA)	1.7×10^5	0.0006±0.0010	1	0	0	–
B. rapa Anand	γ-rays	1.7×10^5	0.0024±0.0009	4	0	0	–
B. o. + *B. r.* Anand[e]	IA + γ	3.4×10^5	0.0003±0.0005	1	0	0	–
B. o. (+) *B. r.* Anand[f]	IA + γ	2.7×10^6	0.05±0.04	1551	52	3.4	201

[a] (No. of calli recovered/no. of protoplasts plated)×100±SD
[b] Average of three experiments
[c] Calli with at least one regenerated shoot
[d] Total number of shoots recovered from regenerated calli and transferred on rooting medium. For control *oleracea* only one regenerated shoot per callus was transferred
[e] Pre-treated protoplasts were mixed but not fused
[f] Pre-treated protoplasts were mixed and fused

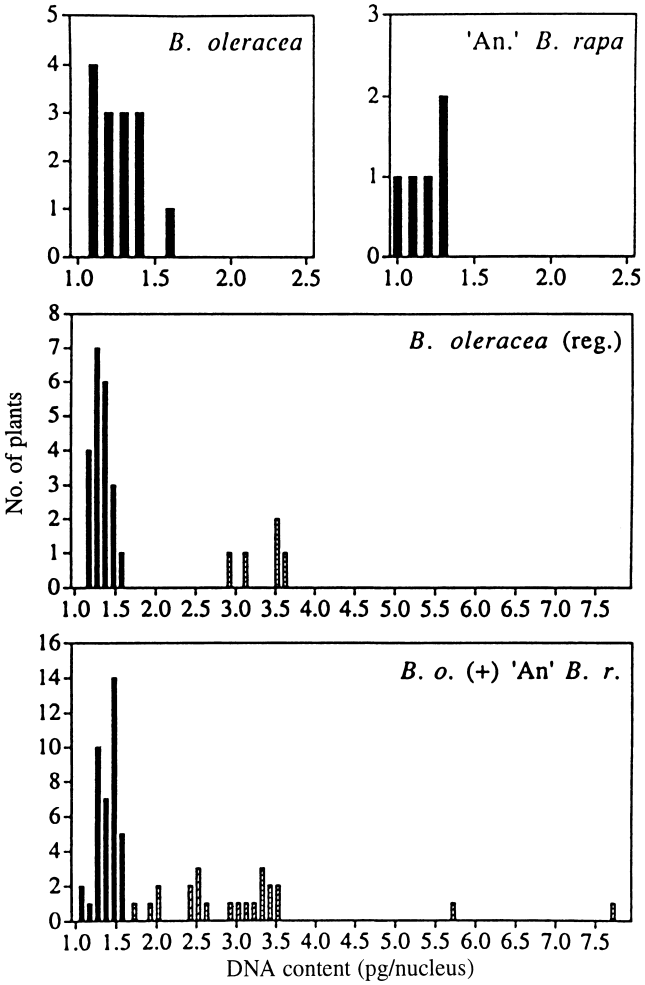

Fig. 1 Distribution frequencies of DNA contents in seed-derived *B. oleracea* and CMS 'Anand' *B. rapa* plants, regenerated *B. oleracea* (*reg.*), and fusion products. *Filled bars* ploidy level = 2*x*, *stippled bars* ploidy level > 2*x*

male-sterile with rudimentary and empty anthers and an array of flower malformations. Some flowers were normal in shape but smaller than in controls; their morphology was rated as good. Others showed ruffled petals, sometimes also funnel-shaped ones, and flowers not completely open (flower morphology=fair). In the rest, petals were narrow like strips and/or funnel-shaped, and sometimes fewer than four petals were present (flower morphology=poor). Sepals were generally normal, but in flowers with major malformations they were yellowish. Pistils were normal, but sometimes bent and/or open longitudinally. Nectaries were usually normal. About two-thirds of the male-sterile plants analyzed had either good or fair flower morphology. The remaining 16 plants regenerated after fusions were male-fertile with normal-looking flowers. In these plants pollen stainability was around 100%, except in a few plants with 4*x* or higher ploidy levels, where it was about 50%.

Only 1 plant showed sterile flowers with different phenotypes on two branches. In all other cases the flower phenotype was uniform on the plant. On the other hand, 7 out of 20 calli with multiple shoots produced plants with different sterile, or even fertile and sterile, phenotypes.

Putative cybrids flowered 29.2 days (SD 3.4) after transplanting, which was not significantly different from the regenerated controls (29.7 days, SD 1.8). Time to flowering was generally similar in diploids and tetraploids and in male-fertile and sterile plants. Four putative cybrids regenerated from 2 calli and showing 4*x* or intermediate ploidy level flowered very early, even in vitro. Under the same conditions, seed-grown parental *B. oleracea* and 'Anand' *B. rapa* showed the first flowers 30.6 (SD 2.7) and 29.4 (SD 2.4) days after sowing, respectively.

In the greenhouse, 64% of the plants analyzed had indehiscent anthers and were considered to be male-sterile, but some variability was present among sterile plants (Table 2). Sixty-three percent of them had empty anthers, as previously observed in the growth chamber, whereas the rest produced some stainable pollen. Only 2 plants were male-sterile in the growth chamber and fertile in the greenhouse, or vice versa. Seven of the sterile plants (23%) had good flower morphology, 8 (27%) were rated as fair, and

Table 2 Male fertility in seed-derived and regenerated plants

Genotype/ Environment	Number of plants analyzed	Number of plants with anthers			
		Dehiscent	Indehiscent		
			with pollen	without pollen	Intermediate[b]
Seedlings/GC					
B. oleracea	15	15 (100)[d]	0	0	0
B. rapa Anand	15	2 (13)	7 (47)	4 (27)	2 (13)
Regenerants/GC					
B. oleracea	34/34[c]	32 (94)	0	2 (6)	0
B. o. (+) *B. r.* Anand	44/26	16 (36)	0	28 (64)	0
Regenerants/GH					
B. oleracea	2/2	2 (100)	0	0	0
B. o. (+) *B. r.* Anand	47/32	17 (36)	6 (13)	19 (40)	5 (11)

[a] GC, growth chamber; GH, greenhouse
[b] Plants showing variable pollen production and pollen stainability
[c] Number of plants/no. of calli from which plants were derived
[d] Percentages in parentheses

Fig. 2 Plant and flower morphology in seed-derived and regenerated plants. **a** *Left B. oleracea*, *Right* CMS *B. rapa*; **b** sterile fusion product; **c**–**f** flowers of fertile *B. oleracea* and sterile fusion products with good, fair, and poor morphology, respectively

15 (50%) were rated as poor. Ratings in the growth chamber and in the greenhouse generally agreed well.

After hand pollinations, all plants except 1 set pods, and no clear effects of flower morphology were detectable for female fertility, based on pod and seed set (Table 3). When insect pollinations were carried out, the flower morphology of the female parents strongly influenced the results. Plants with limited flower malformations had a good seed set, whereas those with extensive malformations generally showed a poor or fair seed set. Plants with intermediate flower morphology had an intermediate attractiveness toward insects.

Cold tolerance

After 5 weeks at 12°C fertile *B. oleracea* and 'Anand' cybrids remained green, while the 'Ogura' CMS *B. oleracea* used as control showed the typical cold temperature-induced chlorosis (Fig. 3).

Organellar DNA analysis

After hybridization with the chloroplast B153 probe, *B. oleracea* and 'Anand' *B. rapa* showed three bands of about 5.3, 4.5, and 2.0, and 5.7, 5.2, and 4.8 kb, respectively (Fig. 4a). The 5 regenerated control *B. oleracea* plants had *ole* chloroplasts, while among the fusion-derived plants 15 had *ole* chloroplasts and 9 'Anand' ones. One out of nine calli with multiple shoots regenerated 2 plants with different chloroplast composition (Table 4).

B. oleracea displayed one band of about 6 kb after probing with *cox*I and four bands (7.8, 6.8, 5.7, 4.0 kb) with *cox*II. 'Anand' *B. rapa* had two bands (14.0 and 7.7 kb) for *cox*I and one (4.0 kb) for *cox*II (Fig. 4b, c). Since the *cox*I 14.0-kb band varied in intensity and presence both among seed-derived 'Anand' and regenerated plants, only the 7.7-kb band was considered to be diagnostic for the presence of the 'Anand' mtDNA sequences in the regenerants. All 5 control plants showed the *B. oleracea* hybridization pattern with both probes, including 1 (F23-0–1A) that was

Table 3 Seed set of diploid CMS cybrids after crosses with fertile rapid cycling *B. oleracea* and broccoli

Flower morphology[a]	Plant[b]	Hand pollination[c]			Insect pollination[d]
		Pod set		Seeds/pod (no.)	
		Number[e]	(%)		
Good	F25-F-1A	60/176	34	2.3	Good
	F25-F-4A	21/33	64	7.8	Good
	F25-F-4B	71/128	55	8.5	Good
Fair	F25-F-2E	26/61	43	5.19	Not tested
	F25-F-3A	47/128	37	1.0	Not tested
	F25-F-5B	24/73	33	3.8	Good
	F25-F-6B	56/65	86	10.0	Fair-good
	F25-F-13B	4/116	3	0.8	Not tested
	F25-F-23D	27/75	36	5.0	Not tested
	F28-F-18A	44/81	54	3.8	Not tested
Poor	F23-F-1A	18/41	44	4.9	Not tested
	F23-F-1B	13/32	41	3.8	Not tested
	F25-F-5A	30/33	91	2.2	Not tested
	F25-F-6C	71/111	64	11.2	Fair-good
	F25-F-12B	25/61	41	5.0	Fair
	F25-F-13C	13/162	8	1.1	Very poor
	F25-F-18C	24/37	73	4.5	Fair
	F25-F-19A	0/47	0	–	Not tested
	F28-F-9A	126/251	50	5.9	Not tested
	F28-F-11A	45/104	43	6.8	Not tested

[a] See text and Fig. 2
[b] Plants that differ only for the last letter were regenerated from the same callus
[c] With rapid cycling *B. oleracea*
[d] With broccoli (2403, SC). Plants were caged with flies
[e] Number of pods harvested/no. of flowers pollinated

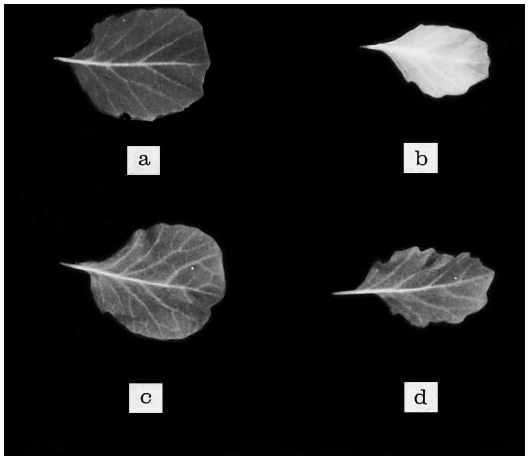

Fig. 3a–d Leaves from plants grown at 12°C for 5 weeks. **a** Fertile *B. oleracea*, **b** 'Ogura' CMS *B. oleracea*, **c** and **d** 'Anand' CMS fusion products

male-sterile. Fertile putative cybrids also generally showed the *B. oleracea* pattern, except for 1 with 'Anand' *cox*I. On the other hand, 13 and 15 sterile cybrids out of 16 analyzed had the 'Anand' *cox*I and *cox*II fragments, respectively. One fertile and 2 male sterile plants displayed mtDNA fragments from different parents in the two loci. The 2 plants

Table 4 Organelle composition of a sample of regenerants based on Southern analyses with one chloroplast and two mitochondrial probes

Genotype	Plant[a]	Ploidy level[b]	Male fertility[c]	cpDNA B153	mtDNA	
					*cox*I	*cox*II
B. oleracea	F23-0-1A	1	S	ole[d]	ole	ole
	F23-0-2A	2	F	ole	ole	ole
	F25-0-1A	1	F	ole	ole	ole
	F25-0-2A	1	F	ole	ole	ole
	F28-0-1A	1	F	ole	ole	ole
B.o.(+)*B.r.* 'An'	F23-F-1A	1	S	ole	An	An
	F25-F-1A	1	S	An	An	An
	F25-F-1B	1	S	An	An	An
	F25-F-2A	1	S	ole	ole	An
	F25-F-2E	1	S	ole	–	–
	F25-F-3A	1	S	An	An	An
	F25-F-3D	1	S	–	An	An
	F25-F-4A	1	S	–	An	An
	F25-F-4B	1	S	–	An	An
	F25-F-5A	1	S	–	An	An
	F25-F-6B	1	S	ole	An	An
	F25-F-8A	2	S	–	ole	An
	F25-F-12A	1	S	An	–	–
	F25-F-12B	1	S	An	–	–
	F28-F-5A	1	S	–	An	An
	F28-F-9A	1	S	An	An	An
	F28-F-9B	1	S	An	An	An
	F28-F-11A	1	S	An	An	An
	F28-F-15D	2	S	ole	ole	ole
B.o.(+)*B.r.* 'An'	F28-F-2A	2	F	ole	ole	ole
	F28-F-2D	2	F	ole	–	–
	F28-F-3A	1	F	ole	ole	ole
	F28-F-3B	1	F	ole	ole	ole
	F28-F-5C	1	F	An	An	ole
	F28-F-8A	2	F	–	ole	ole
	F28-F-10A	1	F	–	ole	ole
	F28-F-11B	1	F	ole	–	–
	F28-F-12A	2	F	ole	ole	ole
	F28-F-12B	2	F	ole	ole	ole
	F28-F-13B	1	F	ole	ole	ole
	F28-F-13D	1	F	ole	–	–
	F28-F-14B	1	F	–	ole	ole
	F28-F-14D	1	F	ole	ole	ole

[a] Plants that differ only for the last letter were regenerated from the same callus
[b] 1=2*x*, 2=>2*x*
[c] F, Fertile, S sterile
[d] ole, *B. oleracea*; An, 'Anand'

regenerated from callus F28-F-5 differed in fertility phenotype and in the origin of the *cox*II region (Table 4).

Four cybrids (22%) showed the reassortment of mitochondria and chloroplasts. Three (F23-F-1A, F25-F-2A, F25-F-6B) had *B. oleracea* chloroplasts and either one or both 'Anand' mtDNA fragments; one (F28-F-5C) had the 'Anand' chloroplasts and *cox*I mtDNA fragment but the *B. oleracea cox*II mtDNA fragments.

Discussion

After protoplast fusion of fertile *B. oleracea* and CMS 'Anand' *B. rapa*, approximately 200 plantlets from 52 calli

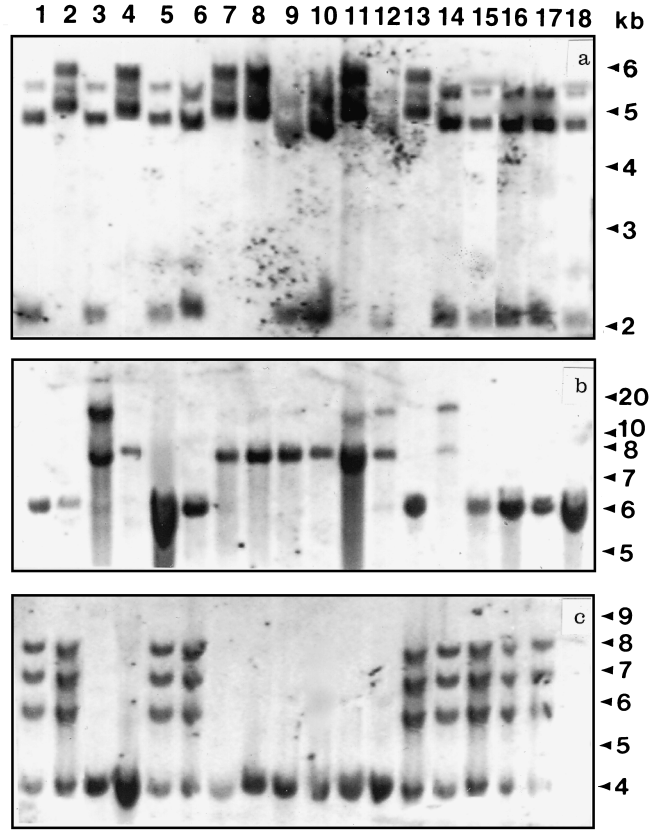

Fig. 4a–c Southern analyses with a chloroplast (a) and two mitochondrial (**b**, **c**) probes. **a** pStB153/*Bgl*II: *1* and *10* fertile *B. oleracea*, *2* and *11* 'Anand' CMS *B. rapa*, *3* and *12* fertile regenerated *B. oleracea*, *4–8*, *13*, and *18* sterile fusion products, *9* and *14–17*, fertile fusion products. **b** *cox*I/*Hin*dIII and **c** *cox*II/*Hin*dIII: *1–2* fertile *B. oleracea*, *3–4* 'Anand' CMS *B. rapa*, *5–6* fertile regenerated *B. oleracea*, *7–12* sterile fusion products, *13–18*, fertile fusion products. (In **c** *18* is blank)

were regenerated in three fusion experiments. Overall, 17 of 41 (42%) fusion-derived plants scored both for male fertility and ploidy level were diploid and male-sterile, suggesting that the 'Anand' phenotype had been effectively transferred into the *B. oleracea* nuclear background. Although more investigations are needed to verify that these plants do not contain nuclear DNA from donor *B. rapa*, on the basis of nuclear DNA content and morphological, molecular, and genetic analyses we considered them to be putative cybrids. Crosses involving some of the putative cybrids showed maternal inheritance of male sterility in the progenies (M.H. Dickson and E.D. Earle, unpublished).

Recipient RC *B. oleracea* showed plant regeneration with an average frequency close to that previously reported by Hansen and Earle (1994), whereas donor RC *B. rapa* with 'Anand' cytoplasm yielded many calli, but only 1 plant. Genotype is known to have a strong influence on the in vitro response of *Brassica* genotypes (Loudon et al. 1989; Jourdan and Earle 1989; Jourdan et al. 1990; Kik and Zaal 1993).

The inability of the donor partner to regenerate helped eliminate unfused escapes. In addition, callus formation and plant regeneration in both parents were strongly inhibited by pretreatments of protoplasts with either iodoacetate or γ-rays. After fusion, complementation between the treated protoplasts allowed callus growth and plant regeneration. However, as noted by Walters et al. (1992), regeneration frequency of calli coming from fused protoplasts was significantly lower than that of control *B. oleracea*. That could be due to: (1) a residual effect of iodoacetate only partially complemented by the donor genotype; (2) an unbalanced chromosome number in fusion products derived by multiple fusions and/or partial elimination of donor nuclear DNA after irradiation treatment (Yamashita et al. 1989); (3) a negative nuclear – cytoplasmic interaction on in vitro performance, as previously shown in alloplasmic lines of *B. oleracea* and other species (Jourdan and Earle 1989; Henry et al. 1994).

About 20% of the regenerated control RC *B. oleracea* plants were tetraploid, as previously noted by Hansen and Earle (1994). This may be due to the spontaneous fusion of protoplasts induced by the enzyme Cellulysin, as demonstrated in maize (Ye and Earle 1991), and/or somatic doubling in culture (Karp and Bright 1985). Plants with ploidy levels higher than 2*x* were regenerated from fusion-derived calli at a frequency close to 40%. They may derive from somatic doubling and/or from fusions in which the donor nucleus was not completely eliminated after γ-irradiation, even though 30 krad were sufficient to prevent unfused protoplasts from forming calli. Similar results with *Brassica* protoplasts have been obtained by other investigators (Menczel et al. 1987; Yarrow et al. 1990; Walters et al. 1992; Kao et al. 1992).

The transfer of 'Anand' mitochondria was confirmed by the analysis of mtDNA in two loci. Although the data obtained do not allow localization of a hypothetical locus for 'Anand' CMS in either of the two mitochondrial regions analyzed, the presence of 'Anand' fragments was strongly correlated with male sterility. When the proportion of sterile and fertile plants and the frequency of plants with 'Anand' fragments in the two groups is considered altogether, it can be seen that the segregation of mitochondria in the regenerated cybrids was slightly biased towards the 'Anand' ones, as has been found by others as well (Mukhopadyay et al. 1994; Liu et al. 1995, 1996). This could be due to an inherent better ability of 'Anand' mitochondria to replicate in the hybrid cytoplasm and/or to a reduced contribution of the *B. oleracea* mitochondria as a consequence of the iodoacetate treatment of the recipient protoplasts. Previous reports, however, seem to indicate such an effect of IA only on hypocotyl protoplasts (Walters and Earle 1993). The male sterility observed in 2 regenerated *B. oleracea* controls was very likely due to tissue culture-induced changes (e.g. Menczel et al. 1987).

In all of the species and hybrids into which it has been transferred, 'Anand' CMS is associated with flower abnormalities (Rawat and Anand 1979; Mathias 1985; Stiewe and Röbbelen 1994; Liu et al. 1995, 1996). In somatic hybrids of *B. napus* (+) *B. tournefortii*, the sterility pheno-

type and abnormal flower morphologies were attributed to their high DNA content, nuclear incompatibilities between the two parental species, or mitochondrial-nuclear interactions (Liu et al. 1995). The latter two are likely the cause of the phenotype of the diploid *B. oleracea* cybrids regenerated in this study, although the effect of small donor nuclear DNA fragments cannot be ruled out. It would be interesting to correlate the degree of flower malformations with the proportion of the donor mitochondrial genome transferred into the fusion-derived plants.

Indications of mtDNA rearrangements were obtained in 3 plants that showed *B. oleracea*-derived bands in one locus and 'Anand'-derived bands in the other one. Some variability among cybrids was observed either for the presence or the intensity of the 14.0-kb *cox*I band from the 'Anand' parent; however, since similar variability was found in seed-derived 'Anand' *B. rapa* plants, it could not be attributed to fusion-induced recombination of mtDNA. Further, no novel hybrid-specific bands were detected in either *cox*I or *cox*II loci, as generally reported in fusions between parents with *B. campestris* and *B. tournefortii* cytoplasms (Stiewe and Röbbelen 1994; Landgren and Glimelius 1994; Liu et al. 1995, 1996), suggesting that these loci are not directly involved in fusion-induced recombination events. Other sites on the mitochondrial genome seem to be preferentially involved in fusion-induced rearrangements of mtDNA in *Brassica* hy(cy)brids (Earle et al. 1992; Temple et al. 1992; Stiewe and Röbbelen 1994; Landgren and Glimelius 1994; Liu et al. 1995, 1996).

In the cybrids studied here, 63% of the plants showed *B. oleracea* chloroplasts and 37% 'Anand' ones, but the small number of plants analyzed did not permit statistical rejection of the hypothesis that chloroplasts are randomly transmitted. In contrast, a preferential retention of *tour* chloroplasts has been found in somatic hybrids or cybrids derived from partners with *B. tournefortii* and either *B. nigra* or *B. rapa* cytoplasms (Mukhopadyay et al. 1994; Landgren and Glimelius 1994; Liu et al. 1995, 1996). In the present investigation, chloroplasts tended to co-segregate with mitochondria, and no plants with *B. oleracea* mitochondria and 'Anand' chloroplasts were obtained. The presence of 'Anand' chloroplasts seems to be fully compatible with normal plant development, since no cold sensitivity or other negative interactions were observed. In contrast, in hy(cy)brids with the 'Ogura' cytoplasm from *Raphanus sativus*, nuclear-plastid incompatibilities determine a strong bias towards *Brassica* chloroplasts and, at the plant level, a chlorosis at temperatures around 10°C (Earle et al. 1992; Walters and Earle 1993).

For both types of organelles, variability between shoots regenerated from the same callus was very rarely observed, indicating that the sorting-out of parental types is usually complete at the time of regeneration.

The population of cybrids obtained in this study could be used in more detailed analyses of the organization of the mitochondrial genome to pinpoint the region(s) responsible for 'Anand' CMS and regulation of flower development in *B. oleracea*. In addition, these cybrids provide a useful starting point for the development of new types of CMS *B. oleracea* vegetable lines for F_1 hybrid production. Male-sterile cybrids selected for good flower morphology, attractiveness to insects, and female fertility have been crossed and back-crossed to cauliflower, broccoli, and cabbage genotypes. Selected progenies are currently being evaluated in the field.

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