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M. A. Sigareva · E. D. Earle

Direct transfer of a cold-tolerant Ogura male-sterile cytoplasm into cabbage (*Brassica oleracea* ssp. *capitata*) via protoplast fusion

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Abstract Cold tolerant cytoplasmic male-sterile (CMS) cabbage (*Brassica oleracea* var. *capitata*) was produced by the fusion of leaf protoplasts from fertile cabbage and cold-tolerant Ogura CMS broccoli lines. The cabbage lines tested showed great variation in plant regeneration from unfused protoplasts; three with high regenerability were selected as the fusion partners. Several procedures for eliminating the nuclear DNA of the broccoli fusion partner were tested. Diploid cabbage plants were identified by flow cytometry and morphological characters. Gamma-irradiation (30 krad) was the most successful procedure; isolation of cytoplasts from broccoli leaf protoplasts, followed by gamma-irradiation of the cytoplast fraction, also produced diploids. UV-irradiation of the broccoli protoplasts was less effective. PCR using primers for an Ogura CMS-specific mitochondrial DNA sequence permitted the identification of cybrids likely to be CMS. Over 200 diploid plants with the CMS-specific sequence were obtained from 66 independent fusion products and three cabbage lines. Plants were ready for transfer into soil within 8 months after fusion. The plants identified as CMS by PCR produced male-sterile flowers. Our procedures permit the transfer of a desirable male-sterile cytoplasm into cabbage much more rapidly than conventional backcrossing procedures.

Key words Cabbage · Cybrid · *Brassica* · Protoplast fusion · Cytoplasmic male sterility

Introduction

Cytoplasmic male sterility (CMS) is a convenient method for the production of hybrid seeds, but its use in *Brassica* crops has been limited because of problems with the available CMS systems. The low-temperature chlorosis seen in Ogura CMS lines (Ogura 1968; Bannerot et al. 1977) has now been eliminated via protoplast fusion (Pelletier et al. 1983; Menczel et al. 1987; Walters et al. 1993); organelle assortment after fusion replaced the radish chloroplasts responsible for the chlorosis with *Brassica* chloroplasts while retaining the mitochondrially encoded CMS trait. The cold-tolerant Ogura CMS is thus an attractive alternative to self-incompatibility for hybrid production in *Brassica* crops. *Brassica oleracea* vegetables are especially suitable for CMS-mediated pollination control since no restorer genes are required (in contrast to *Brassica* oilseed crops).

It is now of interest to transfer the non-chlorotic Ogura CMS to a broad range of the *B. oleracea* lines used as parents for hybrid vegetables. For annuals, such as cauliflower and broccoli, sexual backcrosses may be the simplest approach; however, for biennials, like cabbage, many years of backcrosses are required for the recovery of the original parental type. In such cases, the production of cybrids via protoplast fusion may be a more rapid method for the transfer of CMS to new lines.

The production of cybrid lines that contain a nucleus from one source and cytoplasmic components from another requires efficient elimination of the nucleus of the cytoplasm donor. Treatment with gamma-rays is the most common cybridization method, although it can result in incomplete removal of donor DNA, as well as polyploidization of the final product (Yamashita et al. 1989; Kao et al. 1992). Hall et al. (1992) reported that UV-irradiation causes better fragmentation of donor DNA and proposed it as a substitute for gamma-rays. An alternative to irradiation, developed by Lörz et al. (1981), uses physical elimination of the nucleus from the CMS donor. Their method produces enucleate cytoplasts through ultracentrifugation of protoplasts in a discontinuous percoll/mannitol gradient.

In the present study we tested several methods of obtaining *Brassica* cybrids in order to transfer a non-chlorotic Ogura CMS from broccoli into fertile cabbage lines. The work included the following steps: (1) tests of the re-

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M. A. Sigareva · E. D. Earle (✉)
Department of Plant Breeding and Biometry, Cornell University, Ithaca, NY 14853-1902, USA

generability of protoplasts from the lines of interest; (2) pre-treatment and fusion of selected parental protoplasts; (3) culture and regeneration of fusion products; and (4) screening of regenerates for nuclear DNA content and CMS traits. Our goal was to develop a rapid and reliable procedure that would be useful to *Brassica* breeders.

Materials and methods

Plant materials

Seeds of 40 male-fertile lines of cabbage (*B. oleracea* ssp. *capitata*) and seven lines of cold-tolerant Ogura CMS broccoli (*B. oleracea* L. ssp. *italica*) were provided by the Asgrow Seed Co. (Kalamazoo, Mich.). The broccoli lines had been obtained by previous protoplast fusion experiments conducted at Asgrow. Surface-sterilized seeds were germinated and propagated in vitro on Linsmaier-Skoog (1965) medium without growth regulators (LS-0). Conditions in the culture room were: 16 h photoperiod, 25–50 μE.m^{-2}.sec^{-1}, 24°C.

Protoplast isolation and culture

Protoplasts were isolated from young leaves of 1-month old aseptically grown plantlets as described by Robertson and Earle (1986), with W5 solution (Medgyesy et al. 1980) used in place of SCM. For an assessment of regenerability, protoplasts were cultured using the feeder layer system of Walters and Earle (1990). Calli were plated (approximately 50/plate) on medium E (Pelletier et al. 1983) and scored for shoot formation after several monthly transfers on the same medium. For 29 lines, at least two experiments with 200 calli each were conducted. Eleven additional cabbage lines (A-30 to A-40) were screened in single experiments in which approximately 100–200 calli were scored.

Pre-treatments

Before fusion, parental protoplasts were treated to prevent the division of unfused cabbage protoplasts and to eliminate the nucleus of the CMS broccoli line. Cabbage protoplasts were treated with 3 mM of iodoacetate for 15 min immediately before protoplast isolation (while still in enzyme solution). This concentration of iodoacetate prevented division of the cabbage protoplasts. Broccoli protoplasts in W5 solution were irradiated approximately 10 min after isolation, either with gamma-rays using a ^{137}Cs source (Gammacel) or with UV in a Stratalinker (Stratagene, La Jolla, Calif.) equipped with 254-nm bulbs.

Cytoplast isolation was performed according to Lörz et al. (1981). An iso-osmotic percoll: mannitol gradient was carefully pipetted into 38.5-ml centrifuge tubes as follows (from the bottom to top): 3 ml of a 1:1 mixture of percoll+1.9 M mannitol; 3 ml of 1 part percoll: 4 parts 0.6 M mannitol; 3 ml of 1 part percoll to 19 parts 0.5 M mannitol; 2 ml of 0.5 M mannitol; 2 ml of 0.5 M mannitol with 0.21 M $CaCl_2$; 1 ml of freshly isolated broccoli protoplasts in W5 solution. Protoplasts were centrifuged at 40 000 *g* at 12°C for 1 h. The top fraction of W5 was collected and stained with ethidium bromide (Ye and Earle 1991) to determine the percentage of nucleated protoplasts and cytoplasts. Because many nucleated protoplasts were present, the cytoplast/protoplast fraction was then subjected to gamma-irradiation (20 krad).

Protoplast fusion

After the pre-treatments, protoplasts were washed twice in W5 solution and re-suspended at 0.5×10^6 protoplasts/ml. Cabbage protoplasts were mixed 1:1 with broccoli protoplasts (or with cytoplasts+protoplasts) and placed in drops on the bottoms of plastic Petri dishes. The polyethylene glycol (PEG) fusion procedure was basically that of Negrutiu et al. (1986) with $CaCl_2.2H_2O$ instead of $Ca(NO_3)_2$ in the fusion solution at pH 10.5. After fusion, protoplasts were cultured in liquid medium B (Pelletier et al. 1983) for 2–3 days in the dark before transfer to Millipore filters over *B. napus* feeder cells (Walters and Earle 1990). Calli were grown by a sequential transfer from Medium B to Medium C to Medium E , as described by Walters and Earle (1990). Medium F was omitted. Regenerated shoots were then transferred to LS-0 medium containing 10 g/l of sucrose for rooting. For transfer out of culture, roots of plantlets were washed with water. Plantlets were then placed in small pots of soil mix and covered with plastic bags to prevent wilting. Plants were gradually hardened off by making holes in the bag over a 4-day period. Selected rooted plants in Magenta boxes were shipped by express mail to an Asgrow Field Station in California for further evaluation.

Nuclear DNA content

The nuclear DNA content of leaves of controls and regenerated plants was determined by flow cytometry (Arumuganathan and Earle 1991). Nuclei from rice (cv Taipei 309, 2C=0.87 pg) were used as an internal standard. Samples were analyzed on an EPICS PROFILE cell cytometer (Coulter Electronics, Hialeah, Fla.).

DNA isolation and PCR analysis

Total DNA was isolated from approximately 100 mg of leaf tissue by a miniprep method, either according to Hu and Quiros (1991) or by the leaf squash method described by Langridge et al. (1991). PCR analysis for CMS used *orf*138 primers (kindly provided by Dr. Chris Makaroff, Miami University, Ohio) specific to the mitochondrial DNA (mtDNA) regions correlated with Ogura CMS (Krishnasamy and Makaroff 1993). The sequences of the 5′ and 3′ primers were GTCGTTATCGACCTCGCAAGG and GTCAAAGCAATTGGGTTCAC, respectively. Amplification conditions were as follows: 50 ng of plant DNA, 5 pmoles of primer, PCR buffer with 2.5 mM $MgCl_2$, 5 mM dNTPs, 1.5 u *Taq* polymerase (Boehringer Mannheim) in a 25-μl reaction volume. Three minutes of denaturation at 90°C were followed by 30 cycles of 30 s 92°C, 1 min 46 C, and 2 min 72°C. PCR products were separated on a 2% agarose gel and photographed with Polaroid 667 film.

Cold tolerance test

Cold tolerance of the fusion partners was tested by growing seedlings of three CMS broccoli lines (AB-2, AB-3, and AB-4) and a fertile cabbage (A-5) in a growth chamber at 13°C day/6°C night, 80–150 μE m^{-2}s^{-1}, for 3 weeks. Rapid-cycling *B. oleracea* with the standard cold-sensitive Ogura CMS (Crucifer Genetics Cooperative #3–7) was used as a control. Plants were scored as cold-tolerant if newly formed leaves were not chlorotic (see Fig. 1).

Results

Tests of regenerability

Forty male-fertile cabbage lines and seven CMS broccoli lines were tested for the regeneration of plants from leaf protoplasts. Regenerability was assessed as the percentage of protoplast-derived colonies that formed shoots. The cabbage lines tested showed the whole spectrum of possible responses, from no regeneration to a very high level (Ta-

Table 1 Plant regeneration from leaf protoplasts of 40 cabbage and eight broccoli lines

Level of regeneration	Percentage of calli forming shoots	Number of lines	
		Cabbage	Broccoli
None	0	12	0
Very low	1–4	8	2
Low	5–10	8	0
Intermediate	11–20	4	3
High	20–40	4	0
Very high	>40	4	2

ble 1). In four lines, over 40% of the calli regenerated; two of these (designated A-8 and A-40) had >70% regeneration. However, the majority of the cabbage lines did not respond well: eight had very low regeneration (1–4%), and 12 produced no shoots at all. The CMS broccoli lines also showed a wide range of regenerability (Table 1).

A red cabbage (designated A-5) was selected as the initial recipient partner in our fusion experiments because of its consistently high regeneration capacity (42% + 9.9 in five experiments) and distinct morphology. A-5 plants have purple stems and veins when grown in soil and even darker purple leaves when grown in vitro. The CMS broccoli lines used as the cytoplasm donors were either high regenerating (AB-3) or low regenerating (AB-2, AB-4), but all had green leaves both in soil and in vitro. Their leaves did not show chlorosis when grown at low temperature (Fig. 1), indicating that they contained *Brassica*, rather than radish, chloroplasts.

Irradiation treatments

Gamma-irradiation

CMS broccoli protoplasts (AB-2, AB-4) were treated with 20, 30, 40, 50 or 100 krad of gamma-rays prior to fusion with A-5 cabbage (Table 2). Treatment with 20 krad did not completely prevent the formation of control donor colonies, so this level of irradiation was omitted from further work. At higher levels of irradiation, controls produced no colonies. Many calli were recovered after fusions using cabbage protoplasts irradiated with 30–50 krad, and 3–10% of them formed shoots. The number and percentage of calli that produced plants was substantially higher after 30 krad than after 40 or 50 krad. Very few calli and no plants were obtained after the 100-krad treatment.

The nuclear DNA content of the regenerated plants was determined by flow cytometry and compared to that of seed-grown controls. A-5 cabbage grown from seed contained 1.32±0.045 pg of DNA/nucleus; AB-3 broccoli contained 1.32±0.037 pg DNA/nucleus. Initially one plant from each fusion-derived callus was analyzed. Fusion-derived plants whose DNA content was 1.26–1.4 pg were considered as diploids. This range was selected on the basis of the minimum and maximum values seen in the cabbage controls. After irradiation with 30, 40, or 50 krad, approximately 50% of the plants were diploids (Table 2). The rest were either polyploids or asymmetric hybrids (with a DNA content between 2n and 4n, 4n and 6n, or 6n and 8n; see Fig. 2). Most shoots that formed early and had red

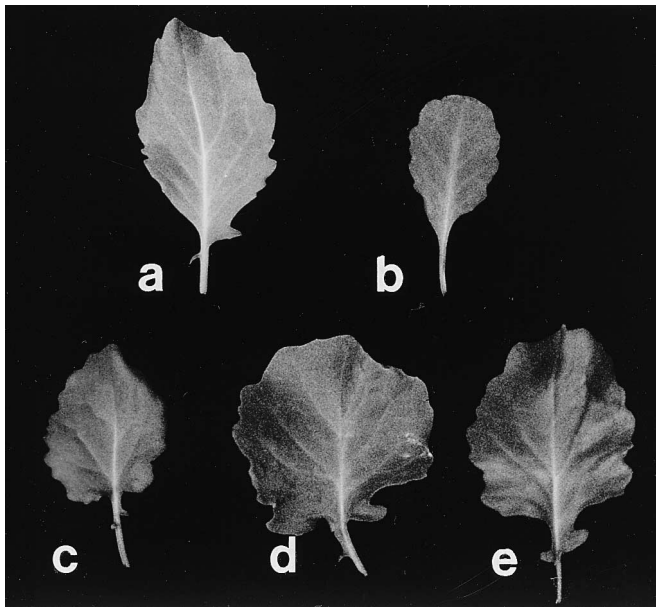

Fig. 1 Leaves of **(a)** rapid cycling *Brassica oleracea* with cold-sensitive Ogura CMS, **(b)** A-5 cabbage, **(c)** AB-2 broccoli, **(d)** AB-3 broccoli, **(e)** AB-4 broccoli after the cold-tolerance test. Only **(a)** shows chlorosis. Broccoli lines AB-2, 3, and 4 are CMS and cold-tolerant

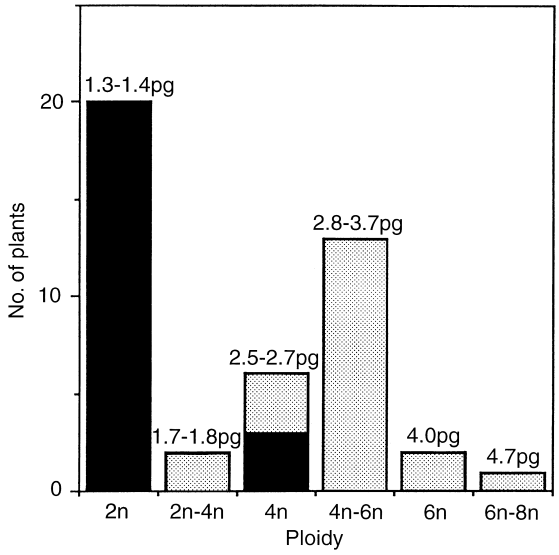

Fig. 2 Distribution of ploidy and nuclear DNA content among plants obtained after fusion of A-5 cabbage and AB-4 broccoli irradiated with 30 krad. *Figures* at the top of each bar are the range of 2C nuclear DNA content (pg DNA/nucleus) for each ploidy class. The *solid bars* represent plants with the morphology and color of A-5 cabbage; the *hatched bars* represent plants with abnormal morphology. The tetraploid plants include ones that looked like cabbage as well as ones that were probably somatic hybrids

Table 2 Recovery of diploid CMS cabbage plants after fusion of protoplasts from fertile cabbage with irradiated CMS broccoli protoplasts or cytoplasts

Krad	CMS broccoli	Fertile cabbage	Protoplasts fused[a]	Calli recovered[b]	Calli with shoots (%)	Calli with plants[c] (% of calli)	Calli producing diploid plants[d] (% of calli with plants)	Calli producing CMS plants[e] (% of diploids)
Irradiation of protoplasts								
30	AB-4	A-5	5.0×10^6	570	57 (10)	44 (8)	20 (45)	18 (90)
40	AB-2	A-5	4.2×10^6	799	22 (3)	15 (2)	7 (45)	1 (14)
50	AB-2	A-5	4.0×10^6	894	69 (8)	19 (2)	10 (63)	5 (50)
100	AB-2	A-5	7.0×10^6	17	0	0	–	–
30	AB-7	A-16	7.0×10^6	425	42 (10)	31 (7)	18 (58)	18 (100)
30	AB-4	A-8	7.2×10^6	120	15 (13)	9 (8)	2 (22)	2 (100)
30	AB-7	A-8	4.9×10^6	90	22 (24)	13 (14)	7 (54)	6 (86)
Irradiation of cytoplasts								
20	AB-3	A-5	5.0×10^6	825	213 (26)	135 (16)	39 (28)	16 (41)

[a] Number of protoplasts used in the fusion experiment
[b] Unfused pre-treated broccoli and cabbage protoplasts did not produce calli so these calli probably result from the fusion of broccoli and cabbage protoplasts
[c] Not all shoots grew into plants. Some calli produced more than one plant
[d] Data are for the first plant analyzed. Other plants from the callus were usually similiar
[e] Only the diploid plants were tested for CMS. Data are for the first plant analyzed. Other plants from the same callus were usually similiar

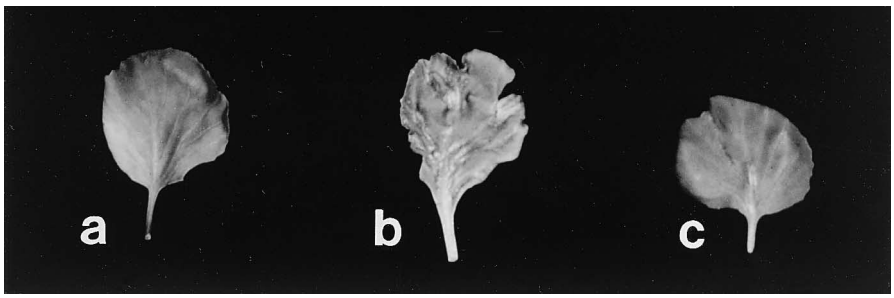

Fig. 3a–c Leaves of plants from fusion of A-16 cabbage and AB-3 broccoli. **(a)** cybrid (2C=1.3 pg); **(b)** asymmetric hybrid (2C=2.3 pg); **(c)** seed-grown A-16 cabbage (2C=1.3 pg)

leaves with normal morphology showed a diploid DNA content. A few were tetraploids that resembled diploid cabbage except for larger and more succulent leaves. These tetraploids are probably cybrids that doubled their chromosomes after fusion. Other plants with a tetraploid nuclear DNA content regenerated more slowly and had a morphology different from that of cabbage. These were probably somatic hybrids of broccoli + cabbage. Some asymmetric hybrids and hexaploids showed abnormalities or new morphological features (Fig. 3).

UV-irradiation

We also pre-treated donor broccoli protoplasts with UV (200 000–800 000 μjoules). Doses from 200 000–600 000 μjoules did not prevent colony formation in unfused controls, so we tested 700 000 and 800 000 μjoules for fusion. At these doses protoplasts still appeared to be viable, i.e., they accumulated fluorescein after staining with fluorescein diacetate. However, they were extremely sensitive to PEG treatment, and no fusion products were recovered. From fusions in which lower doses of UV were applied to the broccoli protoplasts, several plants with the morphology of red cabbage were selected as likely fusion products (four after irradiation with 200 000 μjoules; one each with 250 000 and 300 000 μjoules; three with 350 000 μjoules). Five of these nine plants were diploid.

Cytoplast/irradiation experiment

Cytoplasts were produced from the highly regenerable broccoli line AB-3 by ultracentrifugation of leaf protoplasts in a percoll/mannitol gradient. The highest percentage of cytoplasts was found in the W5 layer at the top of the gradient. This fraction contained 50% cytoplasts and 50% nucleated protoplasts. The high contamination of the cytoplast fraction with protoplasts was probably due to the use of leaf protoplasts; these are smaller than the protoplasts of the *Zea mays* or *Hyoscyamus muticus* cell suspensions from which Lörz et al. (1981) obtained 93–98% cytoplasts. The "cytoplast" fraction was irradiated at 20 krad to inactivate the nuclei of the remaining protoplasts prior

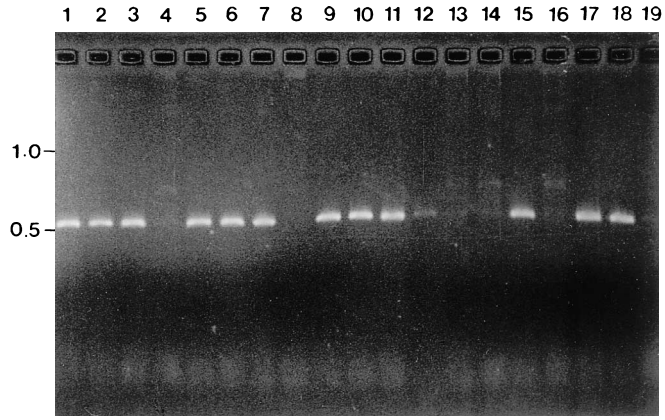

Fig. 4 PCR analysis with CMS-specific primers. *Lanes 1–3* CMS broccoli lines AB-2, AB-3, and AB-4 used as parents showing the characteristic 0.5-kb band; *lane 4* fertile cabbage A-5; *lanes 5–7, 9–12, 15, 17, 18* CMS cybrids; *lanes 8, 13, 14, 16, 19* fertile cybrids

Table 3 Comparison of plant regeneration from unfused cabbage protoplasts and from fusion products[a]

Cabbage line	Regeneration from			
	Control calli (%)		Fusion-derived calli (%)	
A[b]				
A-5	420/990	(42)	57/570	(10)
A-8	205/300	(72)	57/210	(18)
A-16	95/242	(47)	42/425	(10)
B[c]				
A-30	0/92	(0)	23/1129	(2.3)
A-31	1/136	(0.7)	2/613	(0.3)
A-32	0/110	(0)	0/90	(0)
A-33	10/111	(9)	21/356	(6)
A-34	2/214	(0.9)	2/191	(1)
A-36	13/105	(12)	54/870	(9)
A-37	0/135	(0)	0/97	(0)
A-38	0/93	(0)	0/130	(0)
A-39	1/99	(1)	7/251	(2.7)
A-40	83/105	(79)	87/219	(40)

[a] The fusion experiments combined the cabbage line (pre-treated with iodoacetate) with a broccoli line pre-treated with 30 krad of gamma-irradiation
[b] Regeneration from protoplasts was tested in 2–5 experiments for the cabbage lines in Section A. The regeneration experiments were not done at the same time as the fusion experiments
[c] Regeneration from protoplasts was tested in one experiment for the cabbage lines in Section B. The same protoplast preparation was used in both the regeneration and fusion experiments

to fusion with A-5 cabbage protoplasts. Shoots formed on 26% of the 825 calli recovered, and 135 calli formed plants (Table 2). Thirty-nine of the one-hundred and thirty-five plants analyzed (28%) were diploid. The rest were polyploids or aneuploids.

Screening of plants for CMS

PCR with primers specific to the Ogura CMS region in mtDNA simplified the identification of CMS cybrids. Seed-grown CMS plants consistently showed a 0.5-kb band not present in fertile plants (Fig. 4). All fusion-derived plants with a diploid DNA content were analyzed by PCR. The percentage that were scored as CMS varied considerably in different fusion combinations (from 14% to 100%, Table 2), although there was some bias toward the CMS type. None of the five diploids recovered from the UV-irradiation treatments had the CMS-specific PCR band.

Further analyses of plants

When one plant from a fusion-derived callus was found to be diploid and CMS, additional plants from that callus were analyzed to determine whether they had the same ploidy and sterility. Forty two calli formed more than one shoot. For three calli we analyzed all of the plantlets that developed from them (15–20/callus); we tested 2–4 different plantlets from the other 39. For 41 calli, all plants originating from the same callus were diploid. For one callus, two of the four shoots tested were diploids, and two were tetraploids. This callus colony was probably chimeric.

For 3 of the 42 calli where the first plant tested had the CMS-specific PCR band, some of the additional shoots tested failed to show the band. We also tested the stability of the CMS identification over time. Regenerants were first screened 7–9 months after fusion. For 11 lines the PCR analysis was repeated after 2–3 additional months in culture on clonal progeny of the original plants. Ten lines continued to show the CMS-specific band. One line from which the two initial shoots (#1 and #2) were scored as CMS showed changes at the second assay. Four of five additional plantlets recovered from shoot #1 and all 11 new plantlets from shoot #2 did not show the CMS-specific band. Shoots #1 and #2 developed poorly, and all the newer plantlets developed from calli at the base of the stem of the original shoots. Therefore the loss of the CMS-band may have resulted from organelle sorting-out within the callus rather than in the organized tissue of the regenerated plants.

In almost all cases, if one plant from a fusion-derived callus was found to be diploid and CMS, all other regenerates from that callus were similar. For complete assurance that all plants used for horticultural purposes have the desired traits, an analysis of each plant may be appropriate.

Work with additional cabbage lines.

This work suggested that irradiation of broccoli protoplasts with 30 krad was the best treatment for obtaining A-5 cabbage cybrids. We repeated this procedure in fusions using cabbage lines A-8 and A-16, which also had high regenerability (72% and 47%, respectively). Eighteen CMS diploids were identified from fusion experiments with A-16, and eight from experiments with A-8 (Table 2).

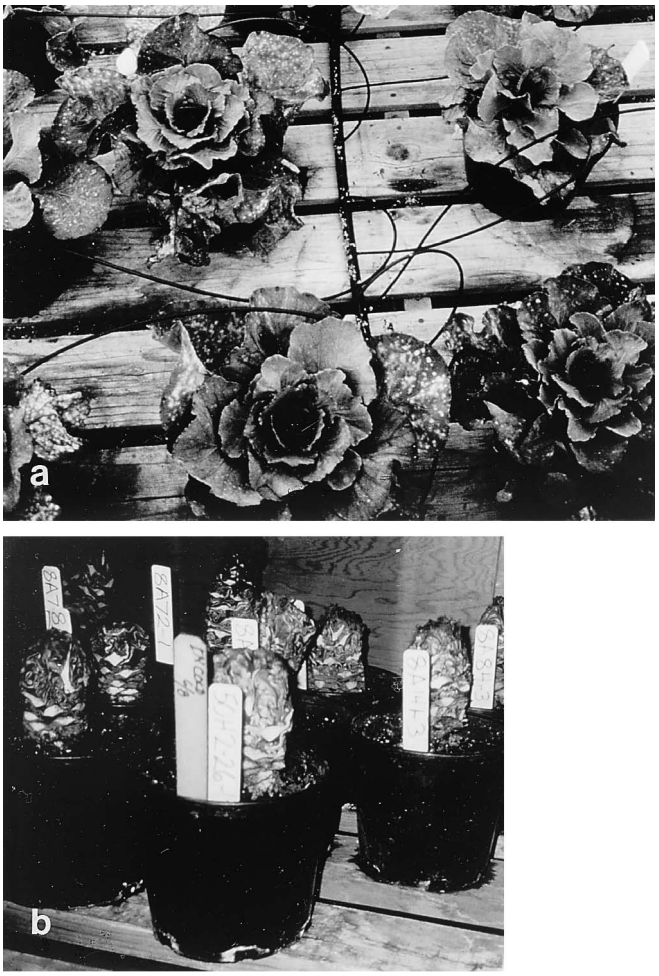

Fig. 5 Protoplast fusion-derived cabbage plants: **(a)** in the greenhouse; **(b)** after vernalization

Additional fusion experiments were done with some of the other cabbage lines. In general, lines with high plant regeneration from control protoplasts also gave the best regeneration from fusion-derived calli, although regeneration after fusion was usually lower (Table 3). A few lines that showed little or no regeneration in initial tests with controls gave better regeneration from the fusion-derived calli obtained in the same experiment (A-30 and A-39, Table 3).

Fertility ratings of the cabbage cybrids

To-date, 210 diploid putative CMS cabbage plants have been recovered from 66 fusion-derived calli and three different cabbage lines. The plants were shipped to an Asgrow field station in California where they were transferred to soil, grown in a greenhouse (Fig. 5a), vernalized (Fig. 5b), and planted in the field. Their horticultural characters as well as their flower morphology are being evaluated. The 26 plants that have flowered to-date are all male-sterile (Fig. 6). These include both red and green cabbage lines. Seed set after hand pollination was normal.

Discussion

The cabbage lines tested showed great variation in plant regeneration from protoplasts, as has been seen in other studies with *Brassica* materials (Jourdan and Earle 1989). Selection of lines with high regenerability probably contributed to the success of our fusion experiments; however, regeneration of control protoplasts was not always correlated with the results after fusion (Table 3). For very important elite lines it may be worthwhile to attempt fusion experiments even if their regenerability initially appears to be poor.

We compared several different techniques for the elimination of the nuclear DNA of the broccoli cytoplasm donor. Irradiation with UV is an attractive concept because it is accessible to more laboratories than either gamma- or X-irradiation. Moreover Hall et al. (1992) reported that UV caused much better fragmentation of DNA than gamma-treatments that give comparable inhibition of cell division. In our experiments, UV-irradiation was not an effective method for obtaining cybrids. Doses that prevented the division of parental protoplasts (700 000 and 800 000 μjoules) left them viable (as judged by their spherical shape and staining with FDA) but unable to complement iodoacetate-treated cabbage protoplasts after fusion. Lower UV-treatments resulted in many broccoli escapes. After such treatments, we were able to identify A-5 cabbage diploids by their red leaf color, but this would not be feasible with most materials.

The efficiency of gamma-irradiation has varied greatly in different *Brassica* cybridization studies. Irradiation has been applied to cells during enzymatic digestion (Kao et al. 1992; Walters et al. 1992) or to protoplasts after isolation (Barsby et al. 1987; Menczel et al. 1987; Yarrow et al. 1990). Kao et al. (1992) found *Brassica* material to be extremely radioresistant, even to a 100 krad treatment; most fusion products contained the sum of the chromosomes from the irradiated donor and the recipient. In contrast, Walters et al. (1992) found that much lower doses (20 krad) prevented control colony growth and eliminated donor chromosomes from 19 fusion products in three experiments. Because of conflicting results in the literature, we tested doses of 20, 30, 40, 50 and 100 krad and treated protoplasts 10 min after isolation to prevent repair processes. Treatment with 20 krad did not prevent division of all unfused controls, and high doses of irradiation were correlated with decreased plant recovery after fusion. The best treatment was 30 krad, which prevented the growth and regeneration of controls and allowed recovery of a reasonable number of regenerants from fusion products. About half of the plants obtained after fusion were diploid. Irradiation with 30 krad also gave good results in experiments using several different broccoli and cabbage lines. We

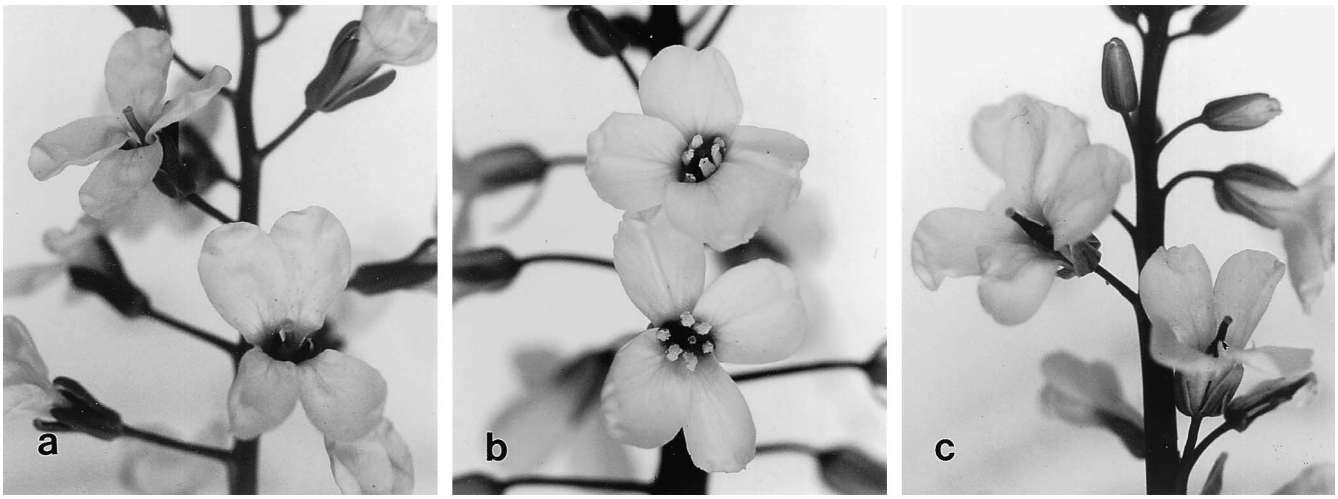

Fig. 6 Flower morphology of **(a)** CMS broccoli; **(b)** fertile cabbage; **(c)** CMS cybrid

scored plants as cybrids on the basis of flow cytometry, so we cannot rule out the possibility that some broccoli DNA sequences are present. The fact that the plants showed normal cabbage morphology suggests that this is not a major concern.

Our attempts to create cybrids by cytoplast+protoplast fusions encountered several problems. In preliminary experiments, similar to those of Sakai and Imamura (1990), we obtained a fraction containing about 90% cytoplasts; however, all putative fusion products obtained proved to be broccoli escapes. This is probably due to the tendency of cytoplasts to float up so that they did not fuse well with the cabbage protoplasts. We had even more problems with escapes using the method of Sundberg and Glimelius (1991) because the cytoplast fraction was heavily contaminated with nucleated protoplasts (40–50%). Additional limitations in our case were insufficient seeds for the use of hypocotyls as starting material and difficulties with establishing cell suspensions from the CMS broccoli lines. For these reasons we used broccoli leaf protoplasts and irradiated the cytoplast/protoplast fraction. This approach gave us more regenerates and cybrids than obtained in previous studies with *Brassica* cytoplasts, perhaps because of the high regenerability of A-5 cabbage. Irradiation of the cytoplast/protoplast fraction with >20 krad might increase the recovery of diploids above the 28% we obtained. Nevertheless, the combined cytoplast-irradiation approach is more complicated and appears to have no particular advantages over irradiation alone. A comparable number of diploid CMS lines were obtained by both approaches.

In our experiments all plants recovered were analyzed for nuclear DNA content by flow cytometry. Plants that resembled the cabbage fusion partner were diploid or occasionally tetraploid. All plants that had abnormal phenotypes in vitro (e.g., had succulent or twisted leaves) proved to be somatic hybrids or asymmetric hybrids. It would therefore be efficient to discard such plants and select only those with normal leaf morphology for further analysis.

PCR for CMS-specific mtDNA sequences was a convenient and reliable way to screen large numbers of regenerants for CMS. This approach has been used by Akagi et al. (1995) to select CMS plants at an early stage of plant regeneration from rice protoplasts. In our work, 80–100% of the cybrids obtained after a 30-krad treatment were scored as sterile by PCR. This is consistent with data for fusion-derived cauliflower calli (Walters and Earle 1993) in which Southern hybridizations showed a bias toward the Ogura CMS-specific mtDNA sequence. Our PCR assay could not detect whether the cabbage cybrids contained sequences from fertile as well as CMS mitochondria. Nevertheless, the value of the assay is confirmed by the fact that all cabbage plants scored as CMS via PCR were male-sterile in the field trial. The PCR selection thus makes it possible to discard fertile plants before transfer to soil, eliminating the need for greenhouse space and lengthy vernalization procedures.

Since we checked only for the presence of the Ogura-specific sequence and not for mtDNA rearrangements, the mitochondrial genomes of the cybrids may vary. Whether this is reflected in variation in female fertility or other horticultural characters remains to be seen. Having a large population of CMS cybrids should enhance the chances that some are suitable for use in hybrid production. We obtained more than 210 CMS cabbage plants from 66 independent fusion events.

In conclusion, our results show that elite fertile cabbage lines can readily be converted to Ogura CMS lines via fusion of iodoacetate-treated cabbage protoplasts and broccoli protoplasts treated with 30 krad of gamma-irradiation. The plants recovered were ready for transfer to soil within 8 months after fusion, a substantial saving of time over standard backcrossing procedures. Because both fusion partners contained *Brassica* chloroplasts, it was necessary only to select diploid fusion products that contained the Ogura-specific mtDNA sequence. Our strategy is simple and efficient in comparison with other methods, including recently published work on the microinjection of mito-

chondria (Verhoeven et al. 1995). The CMS transfer technique described here should facilitate breeding programs for hybrid *Brassica* vegetables.

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