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Production of asymmetric somatic hybrid plants between *Cichorium intybus* L. and *Helianthus annuus* L.

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Abstract In order to obtain male-sterile asymmetric somatic hybrids between chicory (*Cichorium intybus* L.) and a sunflower (*Helianthus annuus* L.) male-sterile cytoplasmic line, mesophyll chicory protoplasts inactivated with iodoacetic acid and hypocotyl sunflower protoplasts irradiated with γ-rays have been fused, using PEG and applying two different procedures. Thirty three plants were regenerated from putative hybrid calli. A cytological analysis of their root-tip cells indicated that most of them had 18 chromosomes, the same number as chicory. Through Southern hybridisation on total DNA using the maize mitochondrial specific gene probes Cox I, Cox II and Cob, three plants were identified as cytoplasmic asymmetric hybrids, as shown by hybridisation bands specific for both chicory and sunflower. One of the regenerated plants produced a novel pattern of hybridisation that was not detected in either parent. When hybridisation of total DNA was carried out with an atpA mitochondrial gene probe the same three cybrids presented both the fertile chicory fragment and the male-sterile sunflower fragment. Finally, Southern hybridisation with an ORF 522 probe, which in sunflower is co-transcribed with the atpA gene, confirmed the hybrid nature of the three plants. The morphology of the cybrids resembled the parental chicory phenotype, and at anthesis their anthers produced fewer pollen grains which could not germinate either ”in vitro” or ”in situ.” Cybrid plants grown in the field produced seeds when free-pollination occurred.

Keywords *Cichorium intybus* L. · *Helianthus annuus* L. · Asymmetric somatic hybrid · Cytoplasmic male sterility · Protoplast fusion

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait which has been observed in many plant species. The CMS phenotype fails to produce functional pollen without loosing female fertility. It can appear spontaneously or be induced either by inter- or intra-specific crosses as a consequence of incompatibility between the nucleus and the cytoplasm (Monéger et al. 1994). CMS is often associated with chimeric mitochondrial open reading frames (ORFs). In some cases transcripts originating from these altered ORFs are translated into unique proteins that interfere with mitochondrial function and pollen development. In the CMS phenotype fertility can be restored by nuclear restorer genes suppressing the effect of CMS-associated mitochondrial abnormalities (Schnable and Wise 1998). CMS systems in which nuclear restoration has been characterised can be exploited for hybrid seed production in grain-crop species. The restoration system might not be needed in crop species where vegetative organs are utilised.

In chicory (*Chicorium intybus* L.) the presence of a naturally occurring CMS system has not been reported, although CMS plants with different levels of female fertility were obtained:

(1) through *Agrobacterium*-mediated transformation using the TA29-BARNASE system (Reynaerts et al. 1993);
(2) through protoplast fusion between chicory and a CMS line of sunflower (Rambaud et al. 1993). In sunflower the CMS trait has been found in interspecific crosses of *Helianthus petiolaris* Nutt. and *Helianthus annuus* L. and can be restored by two dominant nuclear restorer genes (Horn et al. 1991, 1996). This CMS is associated with the insertion into the mitochondrial DNA of a novel ORF (ORF 522) downstream from the atpA gene. This ORF is co-transcribed with atpA and encodes a 16-kDa protein (Horn et al. 1991) probably responsible for the CMS phenotype (Monegér et al. 1994).

Our aim was to produce asymmetric somatic hybrids by protoplast fusion in order to obtain plants with a male-sterile sunflower cytoplasm and chicory chromosomes. For the creation of cybrids, hypocotyl protoplasts of a CMS sunflower line were irradiated with γ-rays, to direct chromosome elimination, prior to their fusion with mesophyll protoplasts of chicory, whose cytoplasm was inactivated with lethal doses of iodoacetate.

Materials and methods

Plant material and protoplast isolation

An in vitro clone of a red chicory genotype, CH363 (Varotto et al. 1997), and seeds of the male-sterile line of sunflower CMSHA89 reproduced by cross-pollination with the isogenic male-fertile line HA68, were used. Chicory protoplasts were isolated from young leaves as described elsewhere (Nenz et al. 2000). Twenty grams of sunflower seed were surface-sterilised in 20% commercial bleach with 6% sodium hypochlorite and rinsed in bi-distilled sterile water overnight at 4°C. After the tegument was removed, seeds were sterilised a second time as described above and rinsed twice for 5 min with bi-distilled sterile water. Ten sterilised seeds were put in 9-cm diameter Petri dishes containing solidified (agar 8 g/l) B5 (Gamborg et al. 1968) medium and grown at 23°C for 5–10 days in the dark. After germination, thin sections from 4–6 cm-long hypocotyls were cut and incubated in 20 ml of an enzyme mixture, WS9M solution (Nenz et al. 2000) plus 0.1% Cellulase Onozuka R10, 0.05% Driselase and 0.02% Macerozyme, at room temperature in the dark for 16–18 h on a rotary shaker (60 rpm).

Inactivation treatment and protoplast fusion

Chicory protoplasts were resuspended in WS9M medium at a density of $2–3\times10^4$ protoplasts/ml and treated with concentrations of iodoacetic acid (IOA SIGMA) ranging from 1 to 4 mM, for 20 min. After this treatment, protoplasts were washed and purified with a 13% (w/v) sucrose solution and used for fusion experiments. Following digestion, sunflower protoplasts were filtered through a stainless steel sieve (83 µm mesh size), and irradiated by ^{135}Cs γ-rays at a dose rate of 330–350 rad/min for various time intervals (15–90 min). Protoplasts were then washed, resuspended in FS20S solution containing 20% (w/v) sucrose and the suspension centrifuged at 120 g for 10 min. Floating protoplasts were washed and cultured in PM1 medium (Varotto et al. 1997) at a density of $1–2\times10^4$ protoplasts/ml and then used for fusion experiments. The viability of protoplasts from both parents was estimated using fluorescein diacetate (FDA) 0, 2 and 4 days after treatment with IOA or γ-rays, as described by Nenz et al. (2000).

PEG (Polyethylene glycol) was used for the fusion of both treated and untreated protoplasts employing two different procedures. With the first one, protoplasts were mixed in a 3 (chicory): 1 (sunflower) proportion to give a density of 2×10^5 protoplasts/ml. One volume of the protoplast suspension was placed in a Petri dish and 3 vol of the PEG solution (30 g of PEG 3350, 150 mg of $CaCl_2 2H_2O$, 10 mg of KH_2PO_4 in a final volume of 100 ml of H_2O at pH 5.5) were added dropwise. After 1 min, 3.5 vol of the KAO solution (735 mg of $CaCl_2 2H_2O$, 375 mg of glycine, 8 g of mannitol in a final volume of 100 ml of H_2O at pH 10.5) were added twice at 1-min intervals. Five minutes later, 10 vol of PM1 medium were added three times at 5-min intervals. After incubation for 10–15 min at room temperature, the fusion mixture was centrifuged (10 min at 100 g) and the protoplasts were washed twice with PM1 medium and then incubated in the same medium at a density of 2×10^4, for 2 days at 28°C in the dark.

With the second procedure protoplasts were mixed in a 3 (chicory) : 1 (sunflower) or a 1:1 proportion to give a total population density of about 1.5×10^6 protoplasts/ml, centrifuged at 120 g for 5 min and resuspended in the fusion solution [4.5 g of PEG 6000, 4.72 g of $Ca(NO_3)_2$, 485 mg of glycine in a final volume of 100 ml of H_2O, pH 9] for 8 min at room temperature. After fusion, protoplasts were centrifuged at 120 g for 5 min, resuspended in PM1 medium at a final density of 2×10^4 protoplasts/ml and cultured for 2 days in the dark at 28°C.

Controls consisted of (1) untreated and fused protoplasts, (2) untreated and unfused protoplasts.

One hour after protoplast fusion, samples for microscopic observations were collected and the fusion frequency (the ratio between heterokaryons from binary fusions and the total number of cultured protoplasts) was calculated. Multiple heterokaryons, recognized by their larger size, were not counted. The heterokaryons can be easily distinguished since they have both the uncoloured cytoplasm of sunflower protoplasts and the chloroplast-rich cytoplasm from chicory. Protoplast viability was assessed with FDA from 1 to 18 h after fusion had occurred.

Protoplast culture and putative hybrid-plant regeneration

According to the procedure described by Nenz et al. (2000), 2 days after fusion, protoplasts were immobilised in drops of Ca-alginate, placed in 1:1 solution of PM1 and PM2 and incubated at 23°C with 14-h light at an intensity of 40 µmol/m^{-2} s^{-1}. Every 10 days the culture medium was replaced with fresh PM2 solution. After 5 weeks, droplets of Ca-alginate were de-polymerized (Nenz et al. 2000) and microcalli were transferred to a solidified (3 g/l agar) PM2 medium. Calli (0.7–1 cm) were cultured on PI medium (Saski et al. 1986) for inducing regeneration via organogenesis and subcultured every 15 days. Regenerated shoots were grown on solidified (8 g/l of agar) B5 medium. Developed plantlets were transferred to soil and grown in a humid chamber where they differentiated roots in 15–20 days. From early spring, plants were grown in the field, and during flowering, which occurred in June–July, some of them were cultivated under cages to prevent insect pollination.

Chromosome counting

For chromosome counting, root tips were fixed in 3:1 ethanol: acetic acid for 15–60 min, hydrolyzed in 1 N HCl at 60°C for 5–10 min, stained with Feulgen solution for 15 min and squashed on a slide in a drop of acetocarmine. Chromosomes were counted in 8–10 metaphase plates of each root tip.

Southern analysis

DNA was isolated from young leaves of chicory and putative hybrids, and from hypocotyls of sunflower seedlings. According to a modified CTAB chloroform/isoamyl extraction protocol (Doyle and Doyle 1990), where the percentage of β-mercaptoethanol in the extraction buffer was increased from 0.2% to 1% (v/v), two chloroform isoamyl alcohol extractions were performed and, finally, DNA was precipitated in 100% ethanol at room temperature. Extracted DNA was quantified using a spectrophotometer (Ultraspec 2600 Pharmacia) and 12 µg digested with *Bam*HI and *Hind*III over night at 37°C. Digested DNA was electrophoresed in a 0.8% agarose gel in TAE buffer. DNA was transferred to a nylon membrane (Hybond–N Amersham) using the capillarity blotting technique. Cox I, Cox II and Cob genes from the maize mitochondrial (mt) genome (3.95-kb of *Bam*HI/*Eco*RI, 2.4-kb of *Eco*RI, 680-bp of *Eco*RI/*Hin*dIII inserts, respectively) and ORF522 and atpA genes (522-bp of *Sma*I/*Eco*RI, 700-bp of *Eco*RI/*Bam*HI, respectively) from the CMS sunflower mitochondrial (mt) genome were kindly provided by Prof. C. J. Leaver, Oxford University, U.K. Probes were labelled with dUTP-digoxigenin (Roche) by PCR following the manufacturer's instructions. Filters were prehybridised at 65°C for at least 3 h and subsequently hybridised over-night at the same temperature. Filters were washed twice for 10 min with 2×SSC, 0.1% SDS and 0.5×SSC, 0.1% SDS sequen-

tially at 68°C. Chemiluminescent detection with CDP-Star (Roche) was carried out following the manufacturer's instructions.

Histological analysis

Putative hybrid plants were either selfed or crossed by hand pollination. The pollinations were carried out in the early morning, covering the receptive surface of each stigma with an excess of pollen. Forty five-min after pollination, styles were fixed for 30 min in 3:1 (v/v) ethanol : acetic acid and stored at 4°C. For microscope observation, the styles were hydrolyzed and stained as reported elsewhere (Varotto et al. 1995).

In vitro germination of pollen was carried out in a solution containing sucrose (450 g/l), $Ca(NO_3)_2$ (350 mg/l) and H_3BO_3 (100 mg/l) at 28–30°C, for 3–5 h in the dark.

Results and discussion

Protoplast isolation and inactivation

The average diameter of the isolated mesophyll protoplasts of chicory genotype CH363 ranged from 22 to 40 µm. The protoplast yield of the CMSHA89 sunflower line was $3–5.6\times10^5$ protoplasts per 50 hypocotyls. Protoplasts of sunflower obtained after incubation in the enzymatic mixture had a mean diameter ranging from 10 to 36 µm. Purification of protoplasts in FS20 S solution increased the fraction of cells having a size between 20 and 25 µm. The efficiency of heterokaryon production was proportional to the uniformity in size of fused pro-

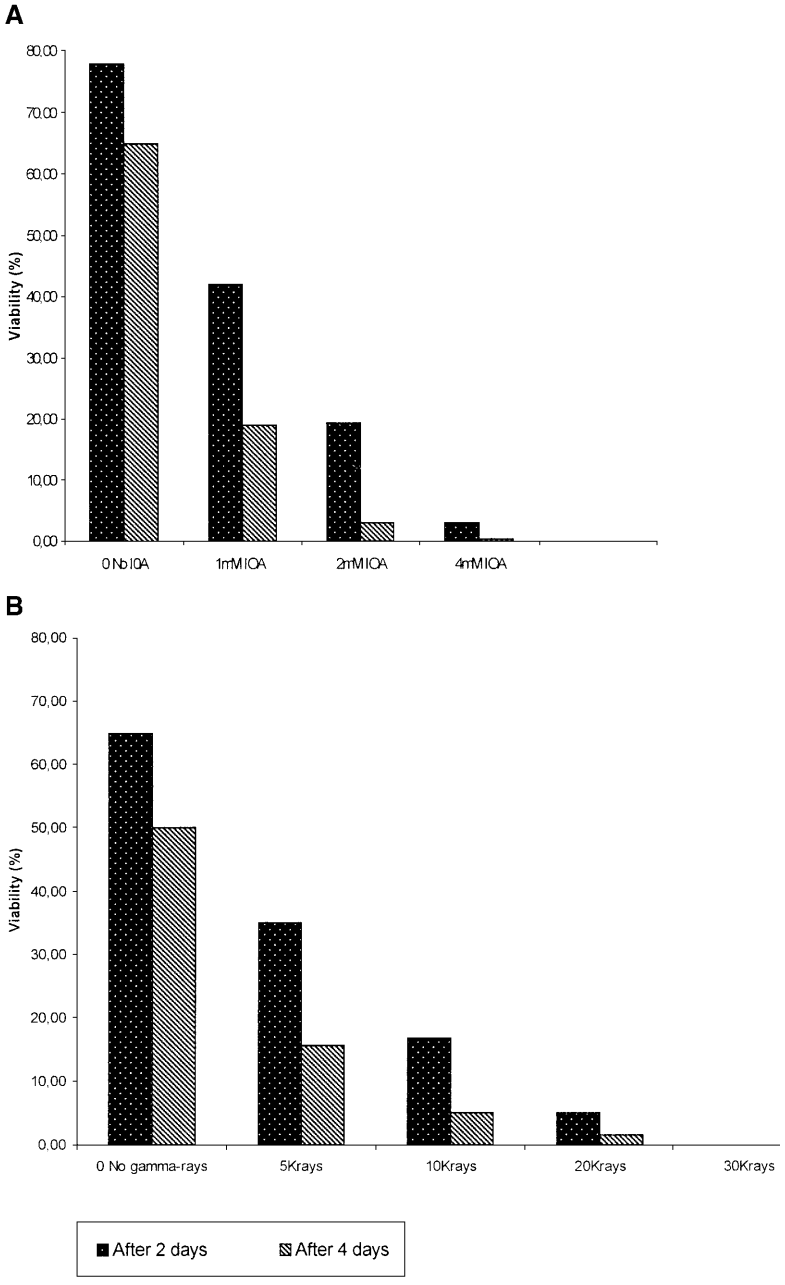

Fig. 1 Iodoacetic acid (IOA) effect on CH363 protoplast viability (**A**) and gamma radiation effect on CMS HA89 protoplast viability (**B**), 2 and 4 days after treatments

toplasts. In sunflower the same degree of purification was obtained in a ficoll gradient (Chanabe et al. 1989). One day after isolation, protoplasts began to divide, producing the septum in an asymmetric position.

The effect of IOA treatment on the viability of chicory mesophyll protoplasts is shown in Fig. 1A. The extent of inactivation is concentration-dependent. Since IOA is an irreversible inhibitor of the mitotic-spindle assembly acting at the prophase of mitosis, cells cannot divide and then degenerate. Four days after the treatment with 1 mM of IOA there was a reduction to 18% of protoplast viability but 6 days later, 2% of the protoplasts reorganised their cytoplasm and divided, showing that this concentration of IOA was not sufficient to prevent cell division. Treatment with 2 and 4 mM of IOA was found adequate to completely inhibit protoplast division and thus these dosages were adopted in the fusion experiments. The effect of γ-ray dosage on sunflower protoplast viability 2 and 4 days after treatment is shown in Fig. 1B. As expected, γ-irradiation affected protoplast viability in a dose-dependent manner. Doses of 30 krads of γ-rays were used to arrest division of the protoplasts employed in the fusion experiments.

Fusion and regeneration of plants

The results of the fusion experiments which allowed the production of somatic hybrids are summarised in Table 1. Five days after fusion, 3% (first procedure) to 5% (second procedure) of the total observed heterokaryons, started to divide, while unfused and untreated protoplasts of the control experiments had a mean division frequency ranging from 30 to 40%. The first consequence of the inactivation treatment was a drastic reduction of the viable protoplast plating-density in the medium: this reduction itself may contribute to lower the frequency of heterokaryon divisions along with the metabolic alteration determined by inactivation treatment and the toxic effect of PEG. These observations are in agreement with the results reported for experiments on somatic hybridisation conducted in other species: after fusion the metabolic complementation between inactivated protoplasts allowed the proliferation of hybrid calli and the regeneration of plantlets with a lower frequency in comparison to the untreated parental protoplasts (Walters et al. 1992; Li et al. 1993). As reported by Cardi and Earle (1997) several hypotheses have been postulated to explain these observations:

(1) a residual effect of IA only partly complemented by the parental donor genotype;
(2) an unbalanced chromosome number in cells derived by multiple fusion or in cells whose nuclear DNA provided by the donor parents was not, or not completely, inactivated (Yamashita et al. 1989);
(3) a negative interaction between nucleus and cytoplasm for the "in vitro" culture of the two parental partners (Jourdan et al. 1989).

Table 1 Summary of the asymmetric hybridisation experiments between CH363 and CMSHA89

Fusion procedure	Parental protoplasts ($\times 10^5$)	IOA (mM) CH363	Gamma rays CMSHA89	Protoplast viability after 18 h	Heterokaryon frequency (%)	Calli obtained	Morphogenetic calli	Regenerated plants	DNA tested plants	Asymmetric hybrids	Chromosome number of the hybrids
1	10.3	2	30	32.0±0.2[a]	1.7±0.3[b]	41	32	21	21	3	18
2	8.4	2	30	56.1±0.3[a]	1.3±0.2[b]	25	18	20	12	1	18
1	15.7	4	–	71.4±0.5[a]	3.1±0.5	–	–	–	–	–	–
2	21.2	2	–	75.9±0.3[a]	3.0±0.3	–	–	–	–	–	–

[a] ±SE
[b] Mean value of five experiments ±SE

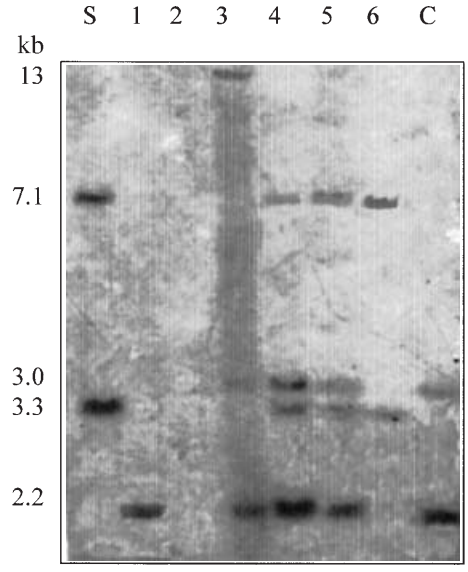

Fig. 2 DNA gel-blot hybridisation of *Bam*HI-digested total DNA with a Cox I probe; *S* CMSHA89; *C* CH363; (*1–6*) putative cybrids

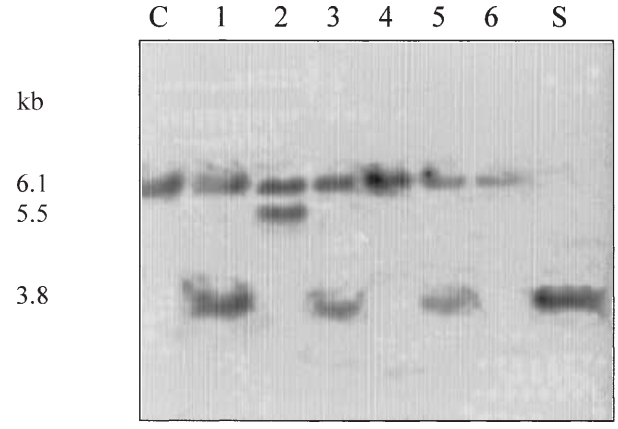

Fig. 3 DNA gel-blot hybridisation of *Hin*dIII-digested total DNA with a Cox II probe; *C* CH363; *S* CMSHA89; (*1–6*) putative cybrids

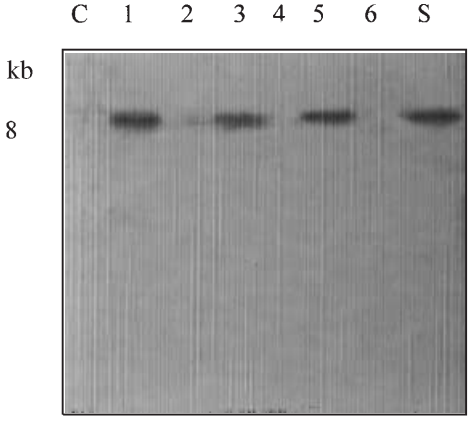

Fig. 4 DNA gel-blot hybridisation of *Hin*dIII-digested total DNA with an ORF522 probe; *C* CH363; *S* CMSHA89; (*1–6*) putative somatic cybrids

To-date, somatic hybrids of chicory have been obtained in two different fusion experiments carried out with "Magdeburg" chicory protoplasts (Rambaud et al. 1992, 1993). In the first case, intraspecific somatic hybridisation produced tetraploid plants from a diploid parent; in the second, interspecific hybrid plants of *C. intybus*× *H. annuus* were regenerated. Both hybridizations were symmetric and thus they did not require parental protoplast inactivation before fusion. Although a comparison between these observation and our results is not possible, the reported experiments indicate that the efficiency of fusion is higher when protoplasts are not inactivated.

Characterisation of regenerated plants

Out of the 33 plants analysed after the asymmetric fusion of CH363 (2n=18) + CMSHA89 (2n=34), six had a chromosome number ranging from 30 to 36; the remaining plantlets had 18 chromosomes. Hybrids with 30–36 chromosomes may be due to a γ-ray induced partial fragmentation of the donor parent nucleus or to multiple fusion events. The hybrid nature of the regenerated plants showing 18 chromosomes was confirmed by Southern analysis of total DNA with the mitochondrial probes Cox I, Cox II and Cob. When *Bam*HI genomic DNA digests were probed with the Cox I gene, chicory was characterized by a 2.2 kb and a 3.0-kb fragment, while the sunflower CMS line used in fusion experiments had two fragments of 3.3 kb and 7.1 kb respectively (Fig. 2, lanes C and S). Two out of all the regenerants examined showed a hybridisation pattern characteristic of both the sunflower CMS line and chicory, indicating that the mitochondria were derived from both parents (Fig. 2, lanes 4 and 5). One regenerant showed the hybridisation pattern of sunflower but the chromosome number of chicory (Fig. 2, lane 6). When *Hin*dIII total-DNA digests were probed with Cox II three plants presented both the sunflower and the chicory hybridisation pattern with fragments of 3.8 kb and of 6.1 kb respectively (Fig. 3, lanes 1, 3 and 5). In both hybridisation experiments one plant showed a peculiar pattern, presenting fragments of 13 kb (Fig. 2, lane 3) and 5.5 kb (Fig. 3, lane 2) respectively: this result provides evidence for recombination of the mitochondrial genomes of *H. annuus* and *C. intybus*. The recombination of mtDNA has been reported to occur very often in somatic hybrids and it has been observed that one recombined genome, among many others, is retained in regenerated cybrids (Pelletier 1993). Southern analysis performed on DNA digested with *Bam*HI and probed with Cob confirmed the hybrid status of two regenerated plants (data not shown).

As confirmed by Southern analysis, the Cox I, CoxII and Cob mitochondrial probes could not be used as male-sterility trait markers on the cytoplasmaic hybrids

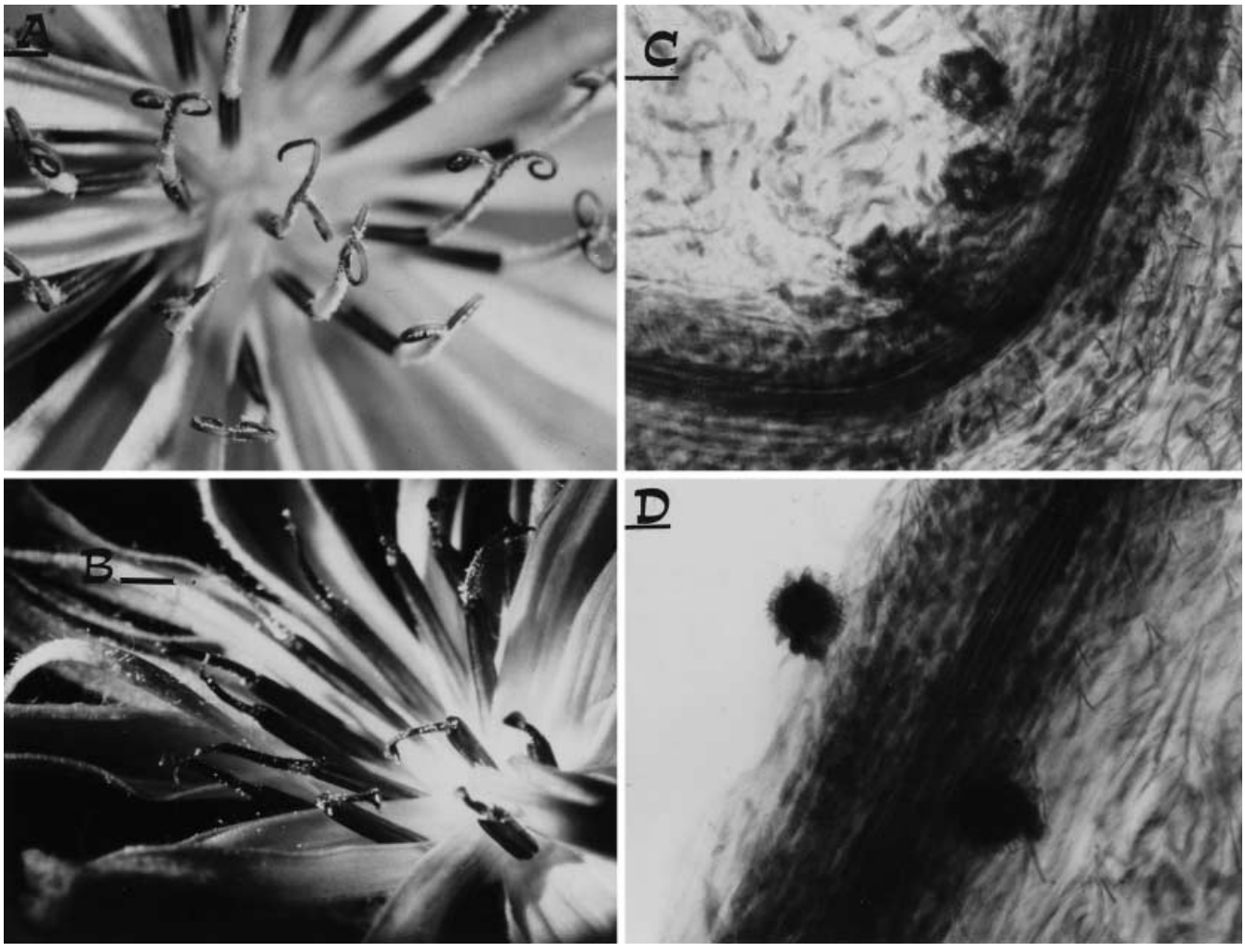

Fig. 5 Flower of a CH363 chicory plant (A) and of a cybrid plant (**B**), bars=500 µm. Germinating pollen of the CH363 genotype on the stigma after a cross-pollination (**C**) and non-germinating pollen of a cybrid (**D**); bars=10 µm

obtained. To verify the introgression of the CMS gene from sunflower, an ORF 522 probe was hybridised to total DNA digested with *Hind*-III. The three hybrid plants showed the presence of the ORF 522 sequence in their DNA and the same hybridisation pattern as the sunflower CMS line (Fig. 4, lanes 1, 3 and 5). Since in male-sterile sunflower ORF 522 is co-transcribed with atpA (Monèger et al. 1994) the hybridisation pattern of atpA was analysed. AtpA was used as a probe on total DNA digested by *Hind*III and the results showed that all the cybrids possessed both the fertile chicory atpA fragment and the sunflower fragment (data not shown).

Plants regenerated from hybrid calli resembled the chicory recipient parent morphologically. They were vernalized and 29 of them produced flowers during summer. Flowers of the cybrid plants differed from those of the CH363 genotype since styles were covered by few pollen grains (Fig. 5, A and B). Moreover, while male-sterile anthers of the sunflower CMSHA89 line do not contain pollen at anthesis, cybrid plants produced a certain number of pollen grains which could not germinate either “in vitro” or on the stigma after self- and cross-pollinations. In Fig. 5 “in situ” germinating pollen of the CH363 genotype (C) and non-germinating pollen of a cybrid plant (D) are depicted. Rambaud et al. (1993) reported that all the plants obtained by symmetric protoplast fusion between chicory and sunflower showed a chicory phenotype seldomly associated with a decrease in plant vigour, while among them three types of sterility could be distinguished: lack of anther dehiscence without pollen or with non-viable pollen; complete absence of the anthers; absence of both anthers and styles or the presence of reduced styles.

Interestingly, all the hybrid plants regenerated after asymmetric fusion experiments showed the same male-sterile phenotype with both male and female organs altered: floral organs were always present, while the number of pollen grains released by the dehisced anthers varied among the cybrids. A decrease in cybrid plant vigour was never observed. Seed yield was lower in cybrids when compared with the CH363 genotype: the seed set observed after self-fertilisation, hand-pollination and free-pollination is reported in Table 2.

Table 2 Seed set (number of germinating seeds per inflorescence) of three cybrids and of a CH363 chicory plant

Genotype	Seed set		
	Self-pollination[a]	Hand-pollination[a]	Free-pollination[b]
Cybrid 1	0	2.6	2.5
Cybrid 2	0	2.6	3.0
Cybrid 3	0	3.2	3.9
CH363	2.0	8.0	7.1

[a] Average of five inflorescences
[b] Average of ten inflorescences

The male sterility trait observed in cybrids may be a consequence of the introgression of the sunflower male-sterile mitochondrial genome into the chicory cytoplasm or of the incompatibility between the nucleus of one species and the cytoplasm of the second species in the regenerated asymmetric hybrids. The new nucleus-cytoplasm combination could have delayed the expression of male-sterility caused by the synthesis of the orf 522 polypeptide, allowing the production of pollen grains which however were not able to germinate. The study of the genetic control of male sterility, carried out in the cybrid plants, may lead to their practical use in chicory breeding.

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References

Cardi T, Earle ED (1997) Production of new CMS *Brassica oleracea* by transfer of "Anand" cytoplasm from *B. napus* through protoplast fusion. Theor Appl Genet 94:204–212

Chanabe C, Burrus M, Aibert G (1989) Factors affecting the improvement of colony formation from sunflower protoplasts. Plant Sci 64:125–132

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. J Exp Res 50:151–158

Horn R, Köhler RH, Zetsche K (1991) A mitochondrial 16-kDA protein is associated with cytoplasmic male sterility in sunflower. Plant Mol Biol 17:29–36

Horn R, Hutstedt JEG, Horstmeyer A, Hahnen J Zetsche K, Friedt W (1996) The CMS-associated 16-kda protein encoded by orfH522 in the PET1 cytoplasm is also present in other male-sterile cytoplasms of sunflower. Plant Mol Biol 30:523–538

Jourdan PS, Earle ED, Mutschler MA (1989) Atrazine-resistant cauliflower obtained by somatic hybridisation between *Brassica oleracea* and atrazine-resistant *B.napus*. Theor Appl Genet 78:271–279

Li YG, Tanner GJ, Delves AC, Larkin PJ (1993) Asymmetric somatic hybrid plant between *Medicago sativa* (alfaalfa, lucerne) and *Onobrychis viciifolia* (sainfoin). Theor Appl Genet 87:455–463

Monegèr F, Smart CJ, Leaver CJ (1994) Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. EMBO J 13(1):8–17

Nenz E, Varotto S, Lucchin M, Parrini P (2000) An efficient and rapid procedure for plantlet regeneration from chicory mesophyll protoplasts. Plant, Cell, Tissue Org Cult 62(1):85–88

Pelletier G (1993) Somatic hybridization. In: Hayward, Bosemark, Ramagosa (eds) Plant breeding: principles and prospects. Chapman and Hall, London

Rambaud C, Dubois J, Vasseur J (1992) The induction of tetraploidy in chicory (*Cichorium intybus* L. var. Magdebourg) by protoplast fusion. Euphytica 62:63–67

Rambaud C, Dubois J, Vasseur J (1993) Male-sterile chicory cybrids obtained by intergeneric protplast fusion. Theor Appl Genet 87:347–352

Reynaerts A, Van de Wiele H, de Sutter G, Janssens J (1993) Engineered genes for fertility control and their application in hybrid seed production. Sci Hort 55:125–139

Saski N, Dubois J, Millecamps JL, Vasseur J (1986) Règeneration de plantes de chicoreè witloof cv Zoom a partir de protoplastes: influence de nutrition glucidique et azoteè. CR Acad Sci Paris 302:165–170

Schnable PS, Wise RP (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci. 3:175–180

Varotto S, Pizzoli L, Lucchin M, Parrini P (1995) The incompatibility system in Italian red chicory (*Cichorium intybus* L.). Plant Breed 114:535–538

Varotto S, Lucchin M, Parrini P (1997) Plant regeneration from protoplast of Italian red chicory (*C. intybus* L.) J Genet Breed 51:17–22

Walters TW, Mutscheler MA, Earle ED (1992) Protoplast fusion-derived Ogura male-sterile cauliflower with cold tolerance. Plant Cell Rep 10:624–628

Yamashita ZQ, Terada R, Nishibayasshi S, Shimamoto K (1989) Asymmetric somatic hybrids of *Brassica*: partial transfer of *B. campestris* genome into *B oleracea* by cell fusion. Theor Appl Genet 77:189–194