Theor Appl Genet (1993) 87:347–352

THEORETICAL AND APPLIED GENETICS

Male-sterile chicory cybrids obtained by intergeneric protoplast fusion

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Received: 18 February 1993 / Accepted: 1 March 1993

Abstract. Male-sterile chicory plants were obtained by fusion of chicory mesophyll protoplasts and hypocotyl protoplasts derived from male-sterile sunflower plants. The protoplasts of both species were fused by the PEG method and the products were selected manually and cultivated at very low density in a liquid medium. Three to twenty percent of the heterokaryocytes divided and evolved into microcalli, then into calli where budding could be induced. The mitochondrial genome of ten male-sterile or totally sterile plants was studied. Restriction endonuclease profiles of mitochondrial DNA and molecular hybridization with specific genes of the mitochondrial genome used as probes indicated that mitochondrial DNA rearrangement had occurred between sunflower and chicory and the intensity of the rearrangements correlated with the degree of sterility of the different plants.

Key words: *Cichorium intybus* – Protoplast fusion – Cytoplasmic male sterility – Mitochondrial DNA

Introduction

Cytoplasmic male sterility (CMS) is the maternally inherited trait observed in many plants which prevents the production of viable pollen but does not affect female fertility. Analysis of the mitochondrial DNAs (mtDNAs) from various CMS plants has shown that CMS seems to be associated with mtDNA modifications (Leaver and Gray 1982; Pring and Lonsdale 1985; Hanson 1991). Structural variations in mtDNA between normal and CMS plants have been observed in more than 170 species (Ichikawa et al. 1989).

In *Cichorium intybus* only nuclear male-sterile plants have been observed and our aim was to produce CMS, which is a valuable trait for commercial production of hybrid seeds, by the protoplast fusion procedure. This method is known to provide a good means of modifying mtDNA organization (Belliard et al. 1979; Chetrit et al. 1985; Ichikawa et al. 1989). Our intention was to fuse chicory protoplast (recipient) with protoplasts of a CMS donor line of sunflower so as to induce CMS in chicory.

We first established a regeneration protocol for chicory protoplasts, yielding a large number of plants (Rambaud et al. 1990) and have subsequently been interested in the formation of cybrid plants by the fusion of chicory and sunflower protoplasts. The obtaining of heterokaryocytes has led us to consider manual selection and a low cell density culture. The analysis of restriction endonuclease patterns of mtDNA has allowed us to demonstrate DNA rearrangements in the sterile plants obtained by protoplast fusion.

Materials and methods

Plant materials

Seeds of *C. intybus* L. cv Magdebourg and *Helianthus annuus* (CMS) were provided by "Ets Florimond Desprez".

Protoplast isolation, fusion and culture

Seeds of *C. intybus* were surface-sterilized and cultivated as described by Rambaud et al. (1990). Seeds of *H. annuus* were sterilized with a solution of 50 g/l of calcium hypochloride, then washed three times in distilled water and transferred to a sucrose (10 g/l)/agar (0.6%) solution.

Communicated by G. Melchers
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Chicory leaf protoplasts were obtained as described by Rambaud et al. (1992). For sunflower seedlings, hypocotyls were removed 6–10 days after germination and incubated in the same maceration solution.

Protoplasts were incubated for 5 h 30 min at 30 °C in the dark without agitation, purified by filtration through 50-µm steel mesh, collected and washed three times by low-speed centrifugation (100 g) for 15 min. After the removal of the upper layer, they were mixed in the ratio 1:3 (sunflower/chicory) so as to obtain a suspension containing 7–11 × 10^6 protoplasts/ml.

Protoplasts were fused according to the method of Kao (1982) with modifications as notified by Rambaud et al. (1992), except that the PEG-protoplast mixture was gently homogeneized for 3 min instead 1 min.

Fused protoplasts were cultured at 30 °C on the MC1 liquid medium of Rambaud et al. (1990). After 1 or 2 days of culture in this medium at a density of 2 × 10^4 protoplasts/ml, the isolated heterokaryocytes were cultured at a low density (12/100 µl) at 30 °C in a modified MC1 medium (0.5 mg/l NAA) to which 2-(N-morpholino)ethanesulphonic acid (MES) (5 mM), casein hydrolysate (150 mg/l) and coconut milk (2%; v/v) were added.

One month later, the colonies derived from heteroplasmic protoplast fusions were transferred onto a proliferation medium and then onto a medium for regeneration (Rambaud et al. 1990). After rooting, plants were transferred to a greenhouse for a few weeks and then transplanted to fields.

Isolation of mitochondria and mt DNA

Mitochondria were obtained from whole plants of chicory grown in the greenhouse and from 6-day-old hypocotyls of sunflower. They were isolated according to the method of Rode et al. (1985) and modified as described by D'hondt et al. (1987). The purified DNA was recovered as described by Rode et al. (1985).

Isolation of total DNA

Total DNA was extracted from leaves of chicory and hypocotyls of sunflower according to procedure of Dellaporta et al. (1983).

DNA digestion with restriction endonuclease, agarose electrophoresis

DNA (1–2 µg) was digested with *Sal*I, *Eco*RI, *Hin*dIII and *Bam*HI in the presence of 4 mM of spermidine and in a total volume of 20–30 µl. The restriction fragments were separated by electrophoresis in 0.8% (w/v) agarose vertical slab gels in Tris-EDTA acetate (TEA) buffer and photographed under UV light. A 1-kb ladder (Bethesda Research Laboratories) was used as the molecular weight standard.

Southern transfer and hybridization

Gels were treated as described by Southern (1975). DNA was transferred onto nylon filters (Hybond N, Amersham) and prehybridized and hybridized as described by Anderson and Young (1985) with labelled probes. Washing was done in 2 × SSC, SDS 0.1% (2 × 5 min at room temperature and 2 × 30 min at 50 °C) and then in 2 × SSC (1–3 times at room temperature). After hybridization, the filters were autoradiographied at least 12 h at −80 °C. Probes were radiolabelled by random priming with the T7QuickPrime Kit (Pharmacia) and purified with Quiagen tip-5 (Diagen). Mitochondrial genes of *Triticum aestivum* were used as probes.

Results

Protoplast fusion and regeneration of heterokaryocytes

Chicory protoplasts were able to divide and to regenerate buds on the defined medium with a plant yield of 6%; under the same conditions, no buds could be regenerated from sunflower protoplasts.

The fusion process gave 20 to 25% of heterokaryocytes easily identified by the fact that the sunflower hypocotyl protoplasts are colourless and the mesophyll protoplasts of chicory are green. The heterokaryocytes were isolated under a microscope using a thin mouth Pasteur pipette after 24 h of culture and then placed on a microwell Nunc plate in which each well contained 100 µl of culture medium. In the MC1 medium used for the chicory protoplasts cultured at high density (2 × 10^4 protoplasts/ml), the heterokaryocytes became dark and died. It was the addition of casein hydrolysate (150 mg/l), coconut milk (2%, v/v) and MES (5 mM) that allowed the division of some cells. The concentration of NAA in the previously formulated medium for culture at high density (Rambaud et al. 1990) had inhibitory effects and had to be diluted from 2 to 0.5 mg/l to promote the development of microcalli.

Under these conditions the percentage of microcalli formed varied from 3 to 20%. After 60 to 90 days of culture, when they reached the size of 1 mm, they were transferred to two media which respectively led to either proliferation or budding (Rambaud et al. 1990). The yield of regenerants from heterokaryocytes was about 25% instead of the 90% generally found for the calli from chicory protoplasts which have not been treated with PEG.

All the plants obtained by protoplast fusion showed a chicory phenotype, and among the 600 regenerated plants, 16 presented either male sterility or a total sterility differently characterized according to the plants. Eleven among the sixteen sterile plants have been multiplied by in-vitro culture, in which we could distinguish three types of sterility. In one case, male sterility was characterized by a lack of anther dehiscence, and without pollen or with non-viable pollen as revealed by the Alexander (1969) test; this was the case for plants I1, CT52/2, CT52/3, CT45/1 and CT35/3. Another type of male sterility was the complete absence of anthers, as was found for plants CT41/1 and CT30/1. The third type was characterized either by the absence of both anthers and style or else by the presence of a reduced style; these sterile plants included CT35/2, CT39/1, CT39/2 and CT52/1.

It was possible to obtain descendants with all the male-steriles, but the yield of the seeds was very different according to the plants. Generally, few seeds were obtained except for the case of CT41/1.

To determine whether the origin of the male sterility was nuclear or cytoplasmic, progenies were studied. The male-sterile plant CT41/1 was the one chosen to examine the sterility of progeny plants, because it showed better vigour than the other male-steriles. After the first cross, CT41/1 showed progeny with a variety of sterility phenotypes based on an observation of 373 plants: we found fertile plants (21.7%), totally sterile plants (13.7%) and two types of male-sterile plants, either without anthers (34%) or with brown non-dehiscent anthers (30.6%). In the second generation, only the male-sterile plants have been crossed with pollinators. Of 5689 progeny plants observed only 4.3% were fertile; 15.3% were totally sterile, 44.4% were male sterile without anthers and 36% were male sterile with brown anthers. In the third generation, though only with some pollinators, it was possible to observe 100% male-sterile plants in the progeny. Among 2595 plants analyzed, 2.2% were fertile, 8.7% were totally sterile and 89.1% were male sterile (52.75% without anthers and 36.35% with brown anthers).

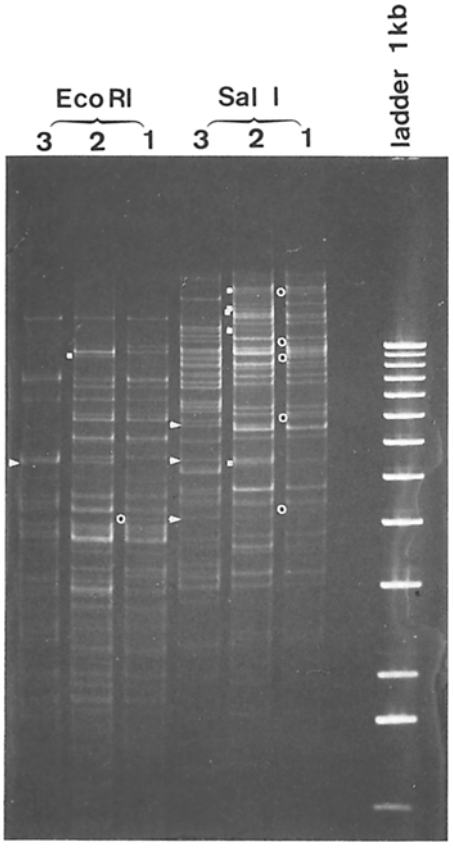

Fig. 1. *Sal*I and *Eco*RI mtDNA restriction patterns of the parental lines *C. intybus* fertile (*1*), *H. annuus* (*3*) and the male-sterile plant (*2*). ○ bands specific to the parent chicory; ■ new bands present in the male-sterile plant; ▲ bands specific to the parent sunflower found in the male-sterile plant

Analysis of mitochondrial DNA

Mitochondrial genomes, which have been reported to be associated with CMS in many plant species, were analyzed. Figure 1 shows the *Sal*I and *Eco*RI restriction endonuclease patterns of the male-sterile plant I1 and the parental species. Electrophoresis of restricted mtDNA yielded a complex pattern of bands in fertile and male-sterile chicories. Digestion of the mtDNA of each plant yielded at least 40 bands with *Sal*I or *Eco*RI, the size of which ranged from more than 20 kb to less than 1 kb. We estimated the mt genome size by the summation of bands to be between 300 and 400 kb. *C. intybus* and *H. annuus* showed different banding patterns.

With *Sal*I, the male-sterile plant had three distinctive fragments of approximately 6.6, 5.5 and 4.2 kb which were specific to *H. annuus* and five new bands of approximately 18, 17, 14, 11 and 5.4 kb which were specific to this male-sterile chicory. Five other bands of approximately 25, 12, 11, 7 and 4.3 kb which are present in fertile chicory disappeared from the male-sterile plant. With *Eco*RI, differences were less numerous but there seemed to be a greater difference in the intensity of the bands; only one band of approximately 10 kb was specific to male-sterile chicory, one other band (5.5 kb) present in male-sterile chicory was specific to sunflower and only one band of fertile chicory (4 kb) was not present in the male-sterile plant.

Six specific genes of the mtDNA were used as probes against the total DNA of the two parents and ten sterile and male-sterile plants digested by *Eco*RI, *Hin*dIII and *Bam*HI. The results showed that all the sterile or male-sterile plants tested were different both from the two parents and from each other. Hybridizations of the *cox*II gene with *Eco*RI-digested DNA (Fig. 2a) and the *atp6* gene with *Bam*HI-digested DNA (Fig. 2c), have been sufficient to demonstrate this. When total DNA of the plants digested by *Hin*dIII was hybridized with the *cob* gene, a fragment of 10.9 kb, specific for sunflower mtDNA was present in all the male-sterile or sterile chicories obtained by protoplast fusion (Fig. 2d).

By contrast, as Fig. 2b shows, the *cox*I gene hybridized with the same fragment in both fertile chicory and the fusion products. Evidently, that part of the mt genome containing the *cox*I gene has not been rearranged in any of the fusion products. Our results show that CT41/1 (no. 6, Fig. 2 a–d) was the only male-sterile plant which retained all the fertile chicory genes (all data not shown). The plants characterised by complete sterility or by a low vigour have lost one or several of the fertile chicory genes. The 2.6-kb *Bam*HI fragment (Fig. 2c) which hybridized to *atp6* in the fertile chicory is found in male-sterile chicory only in CT4/1 (no. 6) and CT52/3 (no. 10), though CT52/3 had lost the 8-kb

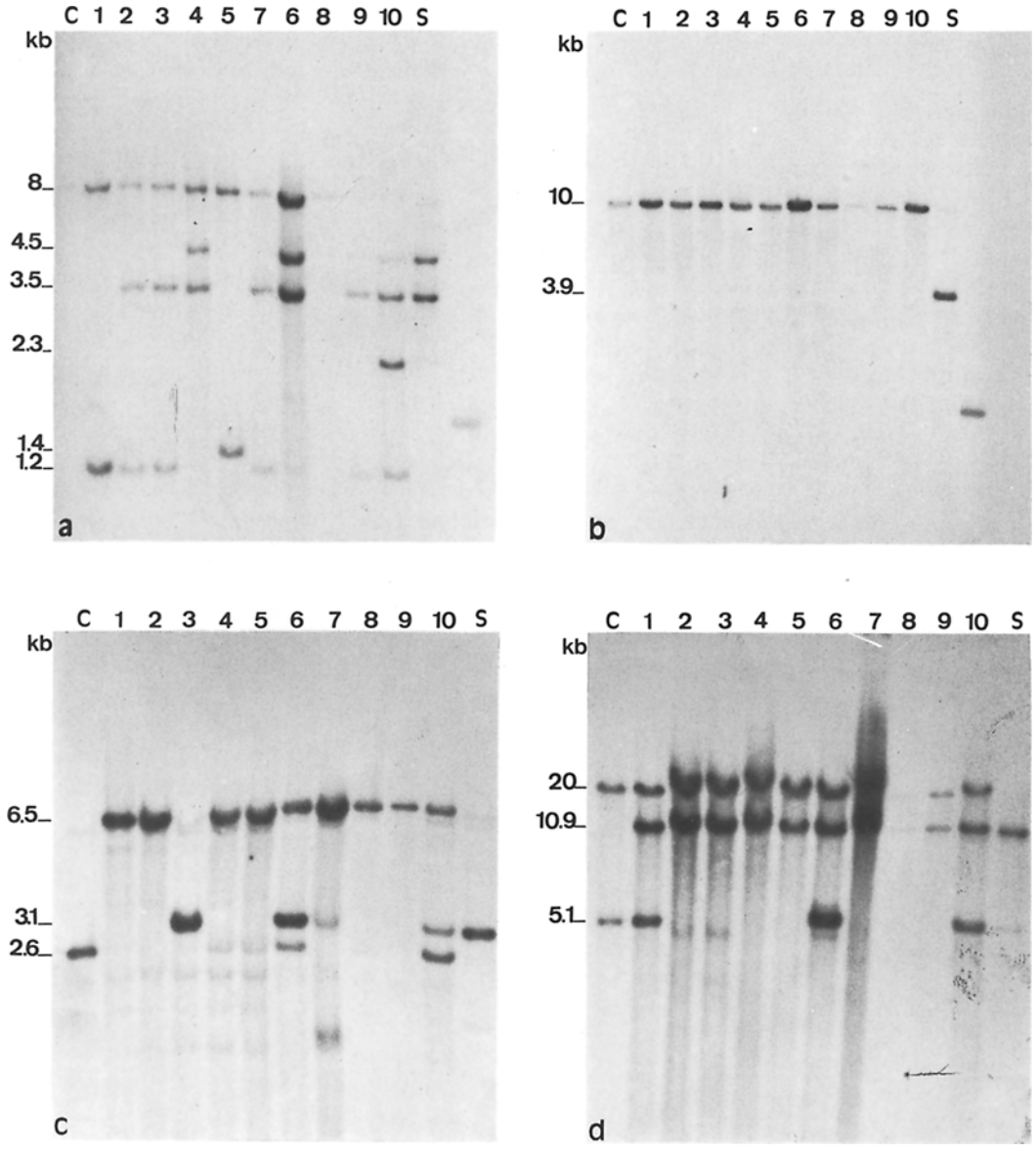

Fig. 2a–d. Southern-blot analysis of total DNAs from the parental species (chicory, *C*; sunflower, *S*) and the sterile plants obtained by protoplast fusion (*1–10*: CT30/1, CT35/2, CT35/3, CT39/1, CT39/2, CT41/1, CT45/1, CT52/1, CT52/2, CT52/3) with mt gene-specific probes. **a** Total DNA digested by *Eco*RI and hybridized with *cox*II. **b** Total DNA digested by *Eco*RI and hybridized with *cox*I. **c** Total DNA digested by *Bam*HI and hybridized with *atp6*. **d** Total DNA digested by *Hin*dIII and hybridized with *cob*

*Eco*RI fragment (Fig. 2a) which hybridized with *cox*II and which was specific to fertile chicory.

Discussion

By the fusion of chicory protoplasts and sunflower protoplasts, a wide range of chicories with sterile or male-sterile phenotypes has been regenerated. Molecular hybridization with specific mtDNA genes used as probes, showed that a large part of the mtDNA of sunflower has been incorporated in the mtDNA of the chicory. When rearrangements affected the sequences of the chicory mt genes, chicory flowers presented more abnormalities and were often totally sterile; the vigour of these plants could also be affected. Finally, only one plant among the ten studied could be used for the production of hybrids whose yields were equal to or higher than those of the traditional varieties and the hybrids obtained by using genic male sterility.

A large part of the mtDNA of sunflower, but not all of the genome, must have been incorporated into the

chicory mtDNA because, of the six genes studied, only one (*cox*I) was not modified.

The fact that the fusion of chicory and sunflower protoplasts (sexually incompatible species) has brought about the emergence of male sterility in chicory suggests that there is a high degree of homology between the mitochondrial genome of chicory and those of related (sexually compatible) species, which can explain the absence of major recombination of the mtDNA and also the absence of a CMS-type wild chicory.

Fusion with protoplasts of lettuce (*Lactuca scariola* var. *sativa*) has also been achieved but did not produce male-sterile chicory. As reported by Melchers et al. (1992), we are of the opinion that fusion partners for the production of male sterility need to be phylogenetically distant to generate sufficient mt rearrangements.

Study of the stability of male sterility in CT41/1 has shown that the mitochondrial genome is likely to take a long time to stabilize. In the first generation, no totally male-sterile plants were obtained. In the third generation, fertile plants could still be observed (only 2.2%) though in some cases with specific pollinators totally male-sterile plants have been obtained in the progeny, thus confirming the cytoplasmic origin of the male sterility; however, the persistence of totally sterile plants in all of the generations studied cannot be explained. Moreover, preliminary analysis of the mtDNA of the progeny have shown that the mt genome has still not stabilized (data not shown) so that a more detailed study of the mtDNA in the progeny seems to be necessary in order to confirm that the mt genome of CT 41/1 is going to stabilize.

A large number of studies on tobacco (Belliard et al. 1979; Galun et al. 1982), alfalfa (D'hont et al. 1987), and carrot (Tanno-Suenaga et al. 1988) have shown that it was possible to transfer CMS by protoplast fusion. There are, however, only two reports relating the induction of CMS by somatic hybridization. Kumashiro et al. (1988) has shown that it was possible to create CMS somatic hybrids from two sexually incompatible fertile *Nicotiana* sp. which have different mtDNAs (Breiman and Galun 1990). More recently, Melchers et al. (1992) reported the regeneration of tomato plants with a variety of CMS phenotypes by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. We were unable to determine whether the appearance of sterility in chicory is due to a transfer of the sunflower gene responsible for this characteristic, as suggested by Köhler et al. (1991) and by Laver et al. (1991), or whether the fusion process has given rise to a new chimeric gene which could induce a new type of male sterility specific to chicory.

It would certainly be interesting to study the mitochondrially encoded *in-organello* translation products from fertile and male-sterile chicories. This would allow us to determine whether or not a new polypeptide was synthetised by the mitochondria from male-sterile chicory and whether some correspondence with the 16-kDa protein of the male sterile sunflower (Horn et al. 1991) was evident.

Acknowledgements. We thank Prof. F. Quetier and Prof. B. Lejeune for kindly providing the mt gene probes. In addition, we thank L. Delesalle and C. Dhellemmes for the field trials. This work was supported by the Florimond-Desprez Co and by the "Association pour la Recherche et le Développement de la Chicorée de France".

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