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New mitochondrial genome organization in three interspecific somatic hybrids of *Medicago sativa* including the parent-specific amplification of substoichiometric mitochondrial DNA units

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Abstract Three somatic hybrid plants produced by protoplast fusion between *Medicago sativa* and each of the three species *Medicago coerulea*, *Medicago falcata* and *Medicago arborea* have been analysed for the composition of their mitochondrial DNA. Restriction fragment length polymorphism (RFLP) analysis of mitochondrial genes in somatic hybrids and their parental lines showed various degrees of rearrangement. The *M. sativa+M. coerulea* hybrid retained all of the *M. coerulea*-specific bands but lost all the major *M. sativa*-specific bands. The *M. sativa+M. falcata* hybrid showed only *M. sativa*-specific bands together with non-parental bands, and the *M. sativa+M. arborea* hybrid showed a partial incorporation of bands from both parents together with non-parental bands. The three different outcomes were attributed mainly to differences in the genetic distance between the parents of each hybrid. Analysis of the sexual progeny of the *M. sativa+M. coerulea* hybrid showed that a residual mitochondrial DNA subunit of *M. sativa* was retained in the hybrid cytoplasm. This subunit was amplified and inherited in a mutually exclusive, allelic-like fashion with its *M. coerulea* homologous counterpart in the sexual progeny of the hybrid. Possible mechanisms for the partitioning of mitochondrial DNA in the generative lineage of the somatic hybrids are discussed in relation to the creation of new nucleus-cytoplasm assortments otherwise impossible to obtain by a sexual cross in *Medicago*.

Keywords *Medicago sativa* · Somatic hybrids · Rearrangements · Substoichiometric mtDNA

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Introduction

Somatic hybridization has long been considered an effective tool for transferring genes from wild germplasm to cultivated plants beyond the limits of sexual compatibility (Pelletier 1993). A unique advantage of somatic hybridization over conventional crossing procedures is the possibility of exploiting new cytoplasmic combinations which in some cases have led to the successful transfer of important traits such as cytoplasmic male sterility into a new genetic background (Rambaud et al. 1993; Liu et al. 1996). Although recombination of chloroplast DNA (cpDNA) is very rare, mitochondrial DNA (mtDNA) in somatic hybrids is unstable, often resulting in the appearance of non-parental molecular markers in the hybrid pattern (Pelletier 1986). In our laboratory three somatic hybrids were obtained which combined the genome of alfalfa (*Medicago sativa* L.) with those of two related species *Medicago coerulea* and *Medicago falcata* and with that of the more-distantly related *Medicago arborea*. These plants were thoroughly examined at the molecular, cytological and morphological levels (Arcioni et al. 1997). Analysis of the nuclear composition of the somatic hybrids revealed large-scale as well as small-scale rearrangements, which were detected as RFLPs of both ribosomal and random loci. The chromosome elimination detected in *M. sativa+M. arborea* (S+A) became even more dramatic in *M. sativa+M. falcata* (S+F) somatic hybrids where only one *M. falcata* chromosome was retained in the hybrid genome. S+A showed the most-striking morphological features, with a plant habit intermediate between a shrub and a herb and the inability to flower. An analysis of the cytoplasmic DNA would be necessary to complete the characterization of these somatic hybrids. The availability of sexual progenies in the *M. sativa+M. coerulea* somatic hybrid (S+C) gives an opportunity to study the consequences of somatic hybridization on the structure of the organellar DNA of these progenies. The aim of the present work was, therefore, to assess the organization of mtDNA in three *Medicago* somatic hybrids. Specifically,

our objectives were: (1) to evaluate the contribution of each parent to the genetic make up of the mitochondrial genomes, (2) to investigate the relationships between nuclear and cytoplasmic rearrangements, and (3) to study the evolution of mtDNA in the sexual progeny of S+C.

Materials and methods

Plant material

The three somatic hybrids S+C, S+A and S+F were obtained by symmetrical electrofusion of mesophyll protoplasts from a highly regenerable genotype of *M. sativa* (R-15) with those isolated from cell lines induced from one regenerable genotype for each of the three species *M. coerulea* (Pupilli et al. 1992), *M. arborea* (Nenz et al. 1996) and *M. falcata* (Crea et al. 1997). A single hybrid cell line was isolated for each parent combination from which several plants were regenerated. In the case of S+C, two regenerated plants were propagated by cuttings, kept isolated from any other source of compatible pollen and left to open-pollinate. Since the two regenerated S+Cs were identical for both nuclear (Pupilli et al. 1995) and mtDNA RFLPs (this study), their open-pollination offspring is genetically equivalent to a self progeny.

Molecular techniques

Total DNA was isolated from leaves of one, two and nine first generation somatic hybrids for S+F, S+C and S+A respectively, of their parental lines and of ten progenies of S+C. The procedure was that of Saghai-Maroof et al. (1984) with minor modifications. The plant material (0.5 g, fresh weight) was ground in liquid nitrogen and the fine powder obtained re-suspended in 5 ml of pre-warmed (65°C) extraction buffer (2×CTAB containing 2% β-mercaptoethanol) and incubated at 65°C for 1 h under gentle agitation. After centrifugation (3,700 *g* for 20 min at room temperature) an equal volume of chloroform:octyl alcohol (24:1) was added to the supernatant and the mixtures agitated on a rotary shaker (100 r.p.m.) for 25 min at room temperature. After a further centrifugation (3,700 *g* for 20 min at room temperature), 50 µl of RNaseA (50 mg/ml) was added to the aqueous phase and the tubes were incubated for 30 min at room temperature. The nucleic acids were precipitated with a volume of isopropanol, washed with 10 mM of ammonium acetate and re-suspended in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Eight micrograms of DNA were restriction-digested with 20 units of each of the following restriction endonucleases: *Bam*HI, *Sac*I, *Xho*I, *Sma*I and *Hin*dIII, (New England Biolabs) according to the supplier's instructions. Digested fragments were electrophoresed through a 1% agarose gel and blotted onto Hybond-N^+ membranes (Amersham) according to the standard capillary procedure (Southern 1975). Blots were hybridized with ^{32}P-labelled probes, washed according to the membrane users' guide avoiding high stringency washes and replacing SSPE with SSC, and exposed to X-ray films. Probed blots were stripped, when necessary, in 50% (v/v) formamide, 6×SSC (1×SSC: 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (w/v) sodium dodecyl sulphate (SDS) buffer at 74°C for 1 h. Recombinant plasmids were amplified through transformation of competent *Escherichia coli* cells, and inserts were isolated from plasmids according to standard procedures (Maniatis et al. 1982).

Mitochondrial gene clones

The mitochondrial clones used in this study were: the 6-kb *Bam*HI fragment encompassing the 18S-5S genes of *Zea diploperennis* (*18S-5S*, Gwynn et al. 1987), the 2.7-kb *Hin*dIII fragment of the mtDNA of maize containing ATPase subunit 6 (*atp6*, Dewey et al. 1985), the 3.95-kb *Bam*HI/*Eco*RI fragment of the subunit 1 (*cox*I, Isaac et al. 1985) and the 2.4-kb *Eco*RI fragment of subunit 2 (*cox*II, Fox and Leaver 1981) of the cytochrome *c* oxidase of *Zea mays*, and the 680-bp *Eco*RI/*Hin*dIII fragment of the coding region of the apocytocrome *b* gene of maize (*cob*, Dawson et al. 1984).

Results and discussion

RFLPs for mitochondrial genes were surveyed across the four parental lines of the three *Medicago* somatic hybrids using five restriction endonucleases in combination with the probes described in the Materials and methods section to identify the most-informative probe/enzyme (P/E) combination(s). The informativeness of each P/E was measured as the capacity to reveal parent-specific bands in each of the three species *M. coerulea, M. arborea* and *M. falcata* in comparison with *M. sativa*. The *cox*I probe did not show any polymorphic bands among the parents with any of the enzymes used, indicating a highly conserved structure across species boundaries in *Medicago*. *Cox*II showed the highest number of parent-specific fragments (30), followed by *cob* (22), *atp6* (14) and *18S-25S* (5), and in general *Bam*HI, *Sac*I and *Xho*I produced the most discriminating patterns among the parents and were used extensively in subsequent analyses. Figure 1 reports the hybridizing banding pattern of *cob* with the DNA of the four parental lines digested with four enzymes. All of the enzymes produced a single major band together with other bands of lower intensity with the exception of *Sac*I whose restriction products in *M. falcata* and *M. coerulea* resulted in an unresolved smear of high-molecular-weight. Although these two species were not polymorphic to each other with any of the enzymes used, all the species considered showed at least one specific band when compared with *M. sativa*, the common parent of the somatic hybrids. This fact made the *cob* locus very informative for investigating the contribution of each parent to the hybrid mitochondrial genome.

The RFLP patterns of the parental lines were then compared with those of their corresponding somatic hybrids using all the informative P/Es. In total, two S+Cs, nine S+As and only one S+F were analysed; when more

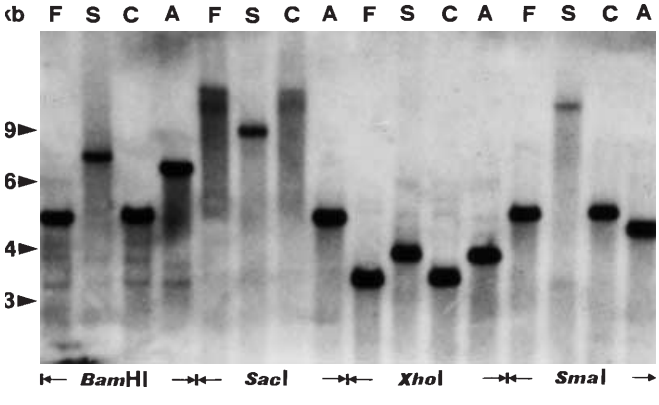

Fig. 1 Hybridizing banding pattern of the *cob* probe with the DNA digests of the parental lines of the somatic hybrids. *F=M. falcata, S=M. sativa, C=M. coerulea, A=M. arborea.*

than one plant was considered for the same parent combination, these were different regenerants originating from the same fusion event. Unfortunately, as all but one of the somatic hybrids that showed the most-dramatic somaclonal rearrangements (S+F, Cluster et al. 1996) were no longer available, no comparison between nuclear and cytoplasmic somaclonal variation could be made. In general, no somaclonal variants for mtDNA were observed among plants regenerated from the same hybrid callus, as already shown for rDNA (Cluster et al. 1996) and random nuclear loci (Pupilli et al. 1995; Nenz et al. 1996). The mitochondrial RFLP band-composition of somatic hybrids was either uniparental or biparental and, in some cases, the appearance of non-parental bands was detected (Table 1). S+C showed the same hybridizing banding pattern as the *M. coerulea* parent while losing all the *M. sativa* major bands with *cob*, *cox*II and *18S-5S* probes. S+F showed *M. sativa*-specific bands together with non-parental bands, but no *M. falcata*-specific bands, and S+A displayed a biparental hybridization pattern together with non-parental bands (Table 1). Figure 2 reports the hybridizing banding pattern of the P/E *cob*/*Bam*HI of the DNAs of S+F, nine somaclones of S+A and their parents. S+F showed a unique non-parental band of about 5.4 kb, and S+A likewise showed a non-parental band of about 5 kb together with a 7.8 kb band inherited from *M. sativa*; no somaclonal variants were detected among the nine S+As analyzed. S+C exhibited the sum of the parental chromosomes but small-scale rearrangements were detected in that nearly 30% of *M. coerulea*-specific nuclear alleles were lost (Pupilli et al. 1995). That is, at the level of the mtDNA, the parental contribution to the hybrid genome differs from that of the nucleus, indicating that the nuclear and organellar genome uptake and evolution during the tissue-culture phase are independent from each other. S+A showed an incomplete integration of the chromosomes from both parental genomes as revealed by the differential FISH hybridization pattern of a highly repeated *M. sativa*-specific probe (Calderini et al. 1997). Similarly, some mtDNA RFLPs were inherited from both parents, while others were lost without any parental bias and, in addition, the occurrence of non-parental bands was noted in the hybrid pattern (Table 1). The appearance of non-parental bands was also detected in this hybrid by means of either nuclear (Nenz et al. 1996) or rDNA (Cluster et al. 1996) RFLPs. Although the lack of some parental bands was attributed to chromosome loss, other intensive mutational events, such as gene conversion, unequal crossing-over and gene duplication, have been suggested as possible causes of the gain or loss of restriction sites that are responsible for the appearance of new bands (Nenz et al. 1996). Hence, in this case, the same extent of rearrangements as was noted in the nuclear genome was also detected at the level of the mtDNA, probably as a consequence of the large genetic distance between the parents. Of the three somatic hybrids surveyed, S+F was the most asymmetric in that, although it originated from a symmetric somatic fusion experiment, only one highly rearranged *M. falcata* chromosome was retained (Cluster et al. 1996; Crea et al. 1997). Similar to what was observed in S+A, the mtDNA composition of S+F markedly reflected its nuclear composition in that the strong asymmetry of the nucleus was also evident at the level of the mtDNA. Although chloroplast genome recombination is very rare, and has actually been reported to occur at a very low frequency in only a few *Nicotiana* (Medgyesy et al. 1985) and *Nicotiana*+*Solanum* somatic hybrids (Thanh and Medgyesy 1989), extensive mtDNA rearrangement and/or recombination is a common phenomenon in the somatic hybrids of several plant species (Earle 1995). The causes of such rearrangements are usually attributed to: (1) pre-existing somatic variability in the plants used as a protoplast source, (2) stress induced by the fusion process itself and by the union of distantly related species, and (3) mutational events in-

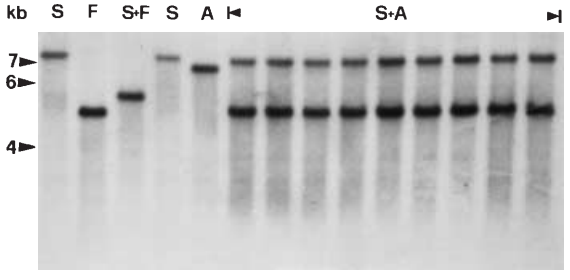

Fig. 2 Hybridizing banding pattern of the *cob* probe with the *Bam*HI DNA digests of the parental lines *M. sativa*, *M. falcata* and *M. arborea*, and their somatic hybrids. *S*=*M. sativa*, *F*=*M. falcata*, *A*=*M. arborea*, *S*+*F*=1 *M. sativa*+*M. falcata* somatic hybrid, *S*+*A*=9 *M. sativa*+*M. arborea* somatic hybrids

Table 1 Mitochondrial DNA compositions of three *Medicago* somatic hybrids on the basis of the origin of parent-specific bands. C=*M. coerulea*-specific band, S=*M. sativa*-specific band, A=*M. arborea*-specific band N=non-parental band

Probe/enzyme combination	S+F		S+C		S+A	
	Total bands	Parental origin	Total bands	Parental origin	Total bands	Parental origin
cob/*Sma*I	–	–	1	C	–	–
cob/*Xho*I	–	–	1	C	–	–
cob/*Bam*HI	1	N	1	C	2	SN
*cox*II/*Bam*HI	2	SN	3	CC	6	SAAAAN
*cox*II/*Sac*I	1	N	2	CC	4	AAA
18S-5S/*Bam*HI	3	S	2	C	3	S

duced by tissue culture. Although heteroplasmy has been demonstrated for cpDNA in alfalfa (Johnson and Palmer 1989; Fitter and Rose 1993; Fitter et al. 1996), no evidence for somatic heteromorphisms of the mtDNA has been reported to-date in this species. Moreover, cross-pollination and the biparental plastid inheritance characteristic of *M. sativa* (Zhu et al. 1993) have contributed to maintain heteroplasmy for cpDNA while mtDNA was stably inherited in a uniparental maternal fashion. Taken together, these data allowed us to rule out the mtDNA heteroplasmy of parental lines as a primary cause of the variability in the mtDNA rearrangements observed in *Medicago* somatic hybrids. The genetic distance between parents has been claimed to affect the extent of mtDNA rearrangements in terms of the occurrence of non-parental bands in potato (Cardi et al. 1999) and between potato and tomato (Wolters et al. 1995). A comparison of the extent of mtDNA rearrangements in S+C and S+A shows a good agreement between parental genetic distances and the degrees of mtDNA rearrangement. However, since *M. falcata*, *M. coerulea* and *M. sativa* are genetically closer to one another than are *M. sativa* and *M. arborea*, we expected *M. falcata* to combine better than *M. arborea* with *M. sativa* in the somatic hybrids. Probably, as suggested by Crea et al. (1997), non-genetic factors contributed to the dramatic rearrangements noted in S+F at the nuclear level; for example, involving differences in the cell cycles between parental nuclei. According to this hypothesis the genome of the parent with the slower cell cycle (*M. falcata*) is eliminated through a mechanism known as premature chromosome condensation (Szabados and Dudits 1980). As a matter of fact, the division rate of parental protoplasts, expressed as a percentage of the dividing protoplasts over the initially plated protoplasts, differed markedly between the parental lines being 72% and 7% in *M. sativa* and *M. falcata* respectively (Pupilli et al. 1992; Crea et al. 1997). This factor could also be responsible for the lack of *M. falcata* mtDNA in S+F. The mtDNA of another somatic hybrid between *M. sativa* and *M. falcata* also showed varying degrees of rearrangement such as the appearance of non-parental bands and a different assortment of parent-specific bands (D'Hont at al. 1987). The occurrence of non-parental bands could be ascribed to the inter-parental exchange of mitochondrial genomes, as described early in the story of somatic hybridization (Belliard et al. 1979) and demonstrated later in *Petunia* (Rothenberg et al. 1985) and *Brassica* (Landgren and Glimelius 1994) with the aid of molecular tools. Tissue culture has been reported to induce rearrangements in somatic embryo-derived plants in wheat (Hartmann et al. 1989), *Hordeum marinum* (Shimron-Abarbanell and Breiman 1991), triticale (Weigel et al. 1995) and *Coffea arabica* (Rani et al. 2000). Kane et al. (1992) showed that protoplast isolation itself is an additional source of somaclonal variation in the cell culture of *Sorghum*. However, no variation in mtDNA was detected among plants regenerated from mesophyll protoplasts of alfalfa (Rose et al. 1986) and among the different regenerants of S+A and S+C (this study). The intensive mtDNA rearrangements detected in the three somatic hybrids are likely to be due to a combination of two main factors: the genetic distance of the parents and differences in the cell-cycles of the parental protoplasts.

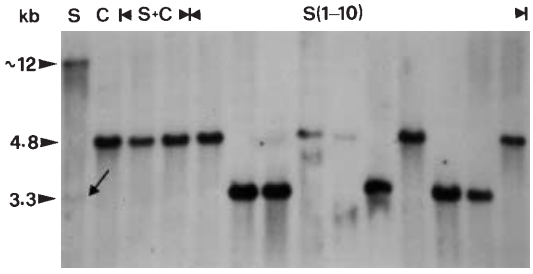

Fig. 3 Hybridizing banding pattern of the *cob* probe with the *Sma*I DNA digests of the parental lines *M. sativa*, *M. coerulea*, their somatic hybrids and ten self-pollination progenies of the somatic hybrids. *S=M. sativa*, *C=M. coerulea*, *S+C=2 M. sativa+ M. coerulea* somatic hybrids, S(*1–10*) (from left to right)=ten self-pollination progenies of S+C

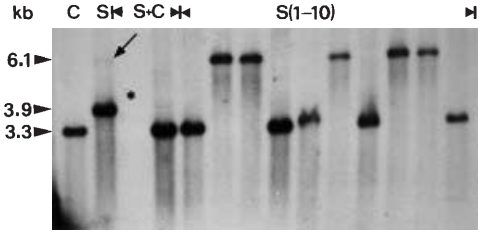

Fig. 4 The same as in Fig. 3 with the DNA digested with *Xho*I. *=missing sample

The evolution of the mtDNA in the sexual progenies of S+C deserves separate consideration. Figures 3 and 4 document the hybridizing banding pattern of the *cob* probe with the DNAs of two somatic hybrids S+C (one is missing in Fig. 4), their parents and ten of their self progenies. As shown previously, the two first-generation hybrids retained the *M. coerulea*-specific bands but lost the major *M. sativa*-specific band with both the enzymes shown in the figures. In the self-mating progeny of S+C two classes of genotypes were detected in the proportion of 1:1. When the DNAs were digested with *Sma*I, progenies 1,4,5,7 and 10 displayed the 4.8-kb band typical of *M. coerulea*, and progenies 2,3,6,8 and 9 another band of 3.3 kb that was different from the major *M. sativa*-specific band. The two bands segregated in a mutually exclusive and allelic-like fashion that ruled out their nuclear origin (Fig. 3). A similar situation emerged when the DNA was digested with *Xho*I (Fig. 4) and *Bam*HI (data not shown). This indicated that the polymorphism detected with the *cob* probe is likely to be due to rearrangements involving a relatively large portion of the DNA surrounding the corresponding gene rather than to point mutations which, on the other hand, were seen to be responsible for cpDNA heteroplasmy in alfalfa (Fitter et al. 1996). The segregation of mtDNA in the self progeny of S+C was noted only with the *cob* probe, whereas

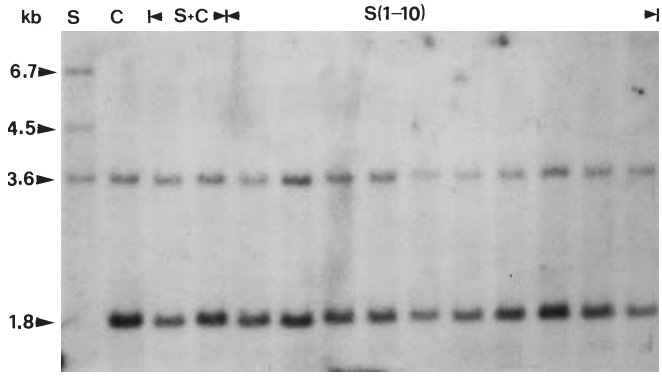

Fig. 5 The same as in Figs. 3 and 4 with the DNA digested with *Sac*I and hybridized with the *cox*II probe

*cox*II (Fig. 5) and *18S-5S* (data not shown) showed a unique maternal inheritance originating from the *M. coerulea* parent. This would indicate that in alfalfa some mitochondrial genes are more susceptible than others to rearrangement as already reported in other species (Kemble and Shepard 1984; Brears et al. 1989; Shirzadegan et al.1989) or, alternatively, that the presence of a *cob* homolog as the only portion of the mtDNA of *M. sativa* retained in S+C could have triggered such rearrangements (see below).The P/E *cob*/*Sma*I pattern of the *M. sativa* parent displayed a weak band (arrow, Fig. 3) which the same molecular weight as the 3.3-kb fragment segregating in the self progeny. A similar 6.1-kb band was also present in the *Xho*I digest of the same plant (arrow, Fig. 4). This indicates that the apparently non-parental bands detected in the self progeny of S+C are most likely due to the amplification of pre-existing parental mtDNA regions which are present in substoichiometric forms in the mother plant, rather than to the de-novo synthesis of new DNA regions. The fact that the substoichiometric units (subunits) were not detectable in the S+C pattern can be explained by the dilution of the *M. sativa* cytoplasm with the *M. coerulea* cytoplasm, which lowered the concentration of the template DNA to below the detection level with Southern hybridization. The differential amplification of substoichiometric mtDNA has already been reported as a consequence of protoplast isolation and culture in *Sorghum* (Kane et al. 1992) and *Brassica* (Shirzadegan et al. 1991), and of protoplast fusion in potato (Lössl et al. 1999). According to these last authors, the subunits of mtDNA coexist with the main mtDNA in potato and can be inherited independently from each other. A similar picture was observed in our somatic hybrids. The *cob* gene homologs in *M. sativa* could be located both on the main chromosome and on a subunit of the mtDNA: the subunit was transmitted to the somatic hybrid but the main chromosome was not. The allelic-like mode of segregation of the two parent-specific bands suggests the existence of a mutually exclusive mechanism for a parent-biased sorting out of the mtDNA units carrying *cob* homologs in somatic hybrids. If we assume a complete maternal inheritance of the mtDNA in S+C then, on the basis of our results, we should hypothesize some sort of incompatibility between the parental cytoplasms, or between the *M. sativa* cytoplasm and the *M. sativa*/*M. coerulea* nuclear composition, as a result of which the mitochondrial genomes of the two species cannot coexist in the same cell. This nuclear-cytoplasmic incompatibility could also explain why in *M. coerulea*×*M. sativa* sexual crosses viable seeds are obtained only when *M. coerulea* is used as the female parent (Mariani 1968). Therefore, the sorting out of parent-specific mtDNA in S+C is a way to overcome the incompatibilities resulting from the "artificial" heteroplasmic state of this plant. A probable mechanism dealing with the 1:1 allelic-like segregation of sexual progenies of S+C could be similar to that responsible for the biparental plastid inheritance in alfalfa (see Mogensen 1996 for references). According to this mechanism, the parental unfused mitochondria would have segregated at the apical or basal part of the egg cell, depending on the parental origin, leaving the embryo with the mitochondria of either one or the other parent. The last point at issue is the reason why only the portion of *M. coerulea* mtDNA carrying the *cob* homolog was sorted out in an allelic fashion with its *M. sativa* counterpart, while all the other mitochondrial genes of *M. coerulea* were inherited maternally in the same sexual progeny of S+C. Two possible explanations are that: (1) the *cob* homolog of *M. coerulea* was located on a mitochondrial subunit that was independent from the other genes analysed, or (2) the presence of a *M. sativa* residual mitochondrial DNA has triggered the excision of the corresponding *M. coerulea* homolog from the main chromosome through homologous recombination. In either case the two alternative mitochondrial DNAs have been compartmentalized in specific mitochondria and sorted out by the process described above. However, given the multiple copies of the 'normal' mitochondrial chromosome in a single cell, it is difficult to understand how a single recombinant mtDNA subunit can assert itself and transmit its unique phenotype in a segregating population.

To sum up, somatic hybridization has induced various degrees of mtDNA rearrangement in *Medicago* somatic hybrids: only minor units of the *M. sativa* genome were transmitted in S+C; these units were not detected in the first-generation hybrids, but they were in subsequent sexual generations. S+A and S+F suffered a higher rate of mtDNA alteration than S+C probably due to a larger genetic distance between their parents. However, in all three cases, but in particular in S+C, new nuclear-cytoplasmic combinations were established in the first-generation hybrids and became stable after one cycle of sexual reproduction. Whether and how this new genetic material can be useful for alfalfa breeding has yet to be established, but beyond any doubt somatic hybridization has generated new gene assortments that would have been difficult (if not impossible) to obtain by conventional methods.

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