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A study of the mitochondrial DNA rearrangements in three interspecific somatic hybrids of *Medicago sativa*

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SUMMARY – Three somatic hybrid plants produced by protoplast fusion between *Medicago sativa* and each of the three species *M. coerulea*, *M. falcata* and *M. 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 to differences in the genetic distance and cell cycles 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 sexual cross in *Medicago*.

Key words: *Medicago sativa*, somatic hybrids, rearrangements, substoichiometric mtDNA.

RESUME – *"Etude des réorganisations de l'ADN mitochondrial chez trois hybrides somatiques interspécifiques de* Medicago sativa". *On a produit trois hybrides somatiques interspécifiques de* Medicago sativa *avec chacune des trois espèces* M. coerulea*,* M. falcata *et* M. arborea*. L'analyse du polymorphisme de restriction (RFLP) des gènes mitochondriaux chez les hybrides somatiques et leurs lignées parentales a montré plusieurs degrés de réarrangements. L'hybride* M. sativa + M. coerulea *a gardé toutes les bandes spécifiques de* M. coerulea *et a perdu la majorité des bandes spécifiques de* M. sativa*. L'hybride* M. sativa + M. falcata *montre seulement des bandes spécifiques de* M. sativa *avec des bandes non détectées chez les parents et l'hybride* M. sativa + M. arborea *montre une incorporation partielle des bandes des deux parents avec des bandes non parentales. Les différences entre les résultats obtenus chez les trois hybrides sont attribuées à des différences entre les parents et les hybrides au niveau des distances génétiques et au niveau des cycles cellulaires. L'analyse des descendants issus de la reproduction sexuée de l'hybride* M. sativa + M. coerulea *a montré qu'une sous-unité résiduelle de l'ADN mitochondrial de* M. sativa *a été retenue dans le cytoplasme de l'hybride. Cette sous-unité a été amplifiée et héritée chez la descendance sexuée de l'hybride, de façon mutuellement exclusive et comparable à un allèle-like avec son homologue chez le partenaire* M. coerulea*. Des mécanismes possibles pour la répartition de l'ADN mitochondrial à travers les trois générations d'hybrides somatiques sont discutées en relation avec la création de nouveaux assortiments cytoplasme-noyau qui sont impossibles à obtenir par croisement sexuel chez* Medicago*.*

Mots-clés : Medicago sativa*, hybride somatique, réarrangement, mtADN substoïchiométrique.*

Introduction

A unique advantage of somatic hybridization over conventional crossing procedures is the possibility of exploiting new cytoplasmic combinations though either nuclear-cytoplasmic or cytoplasmic-cytoplasmic recombination. In our laboratory three somatic hybrids, were obtained which combined the genome of alfalfa (*Medicago sativa* L.) (S) with those of two related species *M. coerulea* (C) and *M. falcata* (F) and with that of the more distantly related *M. arborea* (A). 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 (Arcioni *et al.,* 1997). The aim of the present work was therefore to investigate the cytoplasmic composition in the 3 *Medicago* somatic hybrids by analysis them mitochondrial DNA (mtDNA). Specifically, our objectives were: (i) to evaluate the contribution of each parent to the genetic make up of the mitochondrial genomes; (ii) to investigate the relationships between nuclear and cytoplasmic rearrangements; and (iii) to study the evolution of mtDNA in the sexual progeny of S+C.

Material and methods

The three somatic hybrids S+C, S+A and S+F were obtained by symmetrical electrofusion of mesophyll protoplasts of 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. Total DNA was isolated from leaves of 1, 2 and 9 first generation somatic hybrids for S+F, S+C and S+A respectively, of their parental lines and of 10 progenies of S+C. Details about experimental procedures of RFLP analysis and mitochondrial probe DNA origin are described in Pupilli *et al.* (2001).

Results and discussion

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. S+C showed the same hybridizing banding pattern as the *M. coerulea* parent while losing all the *M. sativa* major bands with cob, coxII 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.

In a previous study (Pupilli *et al*., 1995) 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. So 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. Calderini *et al.* (1997) showed that S+A exhibited 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.

Similarly, in the same hybrid, 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. So 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 a large genetic distance between the parents.

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). So 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 a large genetic distance between the parents.

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: (i) pre-existing somatic variability in the plants used as a protoplast source; (ii) stress induced by the fusion process itself and by the union of distantly-related species; and (iii) mutational events induced by tissue culture.

Although heteroplasmy has been demonstrated for cpDNA in alfalfa (Johnson and Palmer, 1989), no evidence for somatic heteromorphisms of the mtDNA has been reported to date in this species. Moreover, no variation for the 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). Therefore, the intensive mtDNA rearrangements detected in the three somatic hybrids are likely due to a combination of two main factors: the genetic distance of the parents and differences in the cell-cycles of the parental protoplasts. The evolution of the mtDNA in the sexual progenies of S+C deserves separate consideration. Figure 1 documents the hybridizing banding pattern of the cob probe with the DNAs of two somatic hybrids S+C, their parents and 10 of their self progenies.

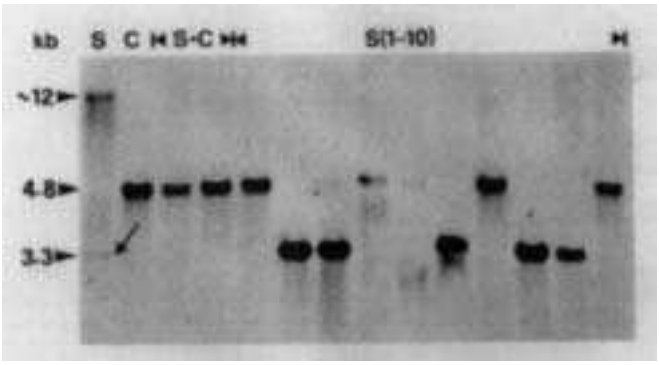

Fig. 1. Hybridizing banding pattern of the cob probe with the SmaI DNA digests of the parental lines *Medicago sativa, Medicago coerulea*, their somatic hybrids and 10 self pollination progenies of the somatic hybrids. *S* = *M. sativa, C* = *M. coerulea,* S+C = 2 *M. sativa* + *M. coerulea* somatic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

The first generation hybrid (S+C) retained the *M. coerulea*-specific bands but lost the major *M. sativa*-specific band. In the self-mating progeny of S+C two classes of genotypes were detected in the proportion of 1:1. 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. 1). A similar situation emerged when the DNA was digested with XhoI and BamhI (not shown) indicating 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. The segregation of mtDNA in the self progeny of S+C was noted only with the cob probe whereas coxII and 18S-5S (not shown), showed a unique maternal inheritance originated from the *M. coerulea* parent. This would indicate that in alfalfa some mitochondrial genes are more susceptible than others to rearrangement or, alternatively, that the presence of a cob homologue as the only portion of mtDNA of *M. sativa* retained in S+C could have triggered such rearrangements. The P/E cob/SmaI pattern of the *M. sativa* parent displayed a weak band (arrow, Fig.1) of the same molecular weight as the 3.3 kb fragment segregating in the self progeny. This indicates that the apparently non-parental bands detected in the self progeny of S+C are 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 *M. coerulea* cytoplasm, which lowered the concentration of the template DNA to below the detection level of Southern hybridization. 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 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 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.

Conclusions

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 3 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 is 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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