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A repetitive and species-specific sequence as a tool for detecting the genome contribution in somatic hybrids of the genus *Medicago*

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Abstract A highly repeated sequence (C300) was cloned from *Medicago coerulea* and its organization in the *M. sativa-coerulea-falcata* complex, *M. arborea*, and three somatic hybrids involving *M. sativa*, was investigated. Southern-blot analysis revealed a tandemly repeated array and a species-specificity of the sequence to those species belonging to the complex. Various degrees of amplification of C300 were detected among the species of the complex and the outcome in the somatic hybrids was dependent on parental composition. Sequence analysis revealed strong homology (96%) of C300 with a clone (E180) previously isolated from *M. sativa*. As FISH analysis showed that C300 was dispersed along the chromosomes of *Medicago* spp., it should prove a valid tool for establishing the chromosome origin of somatic hybrids.

Key words Alfalfa · Repetitive DNA · FISH · Parentage identification

Introduction

Repetitive sequences form an important part of the eukaryotic genome. In higher plants they may account for from 20 to more than 90% of the genome (Flavell 1980). Between-species variations in the size of the genome (Bennett and Smith 1976) are attributable to the amount of repetitive DNA (Flavell 1980). Repetitive sequences are not uniformily distributed throughout the genome; some are clustered at certain chromosomal domains (subtelomeric or paracentromeric), others are widely dispersed.

Moreover the repeat unit may vary in length from 2 bp (microsatellites) to 10 kbp (rDNA), the most common repetition being 160–180 bp long (Harrison and Heslop-Harrison 1995). The biological function of these sequences is still a matter of debate. Although it has been suggested that they play no role (John and Miklos 1979) there is mounting evidence that they may influence chromosome structure and recombination events (Lapitan 1992). They have also recently proven useful for enhancing the rate of transformation in petunia and alfalfa protoplasts, so facilitating the transfer of alien genes (Meyer et al. 1988). Not only are repetitive sequences useful for studying the evolution and behaviour of plant genomes but, since they have undergone rapid evolution and become species-specific, they help to differentiate closely related species and to detect genomic introgression following sexual or somatic hybridization (Saul and Potrykus 1984; Anamthawat-Jonsson and Heslop-Harrison 1993).

The cultivated germ plasm of alfalfa (*Medicago sativa* L.) is restricted to a few species belonging to the *sativa-coerulea-falcata* complex. Although these species are sexually compatible at the same ploidy level there is interest in employing protoplast fusion to increase the ploidy level and so maximize the degree of heterozygosity (Chase 1963). In the genus *Medicago*, the isolation of highly repetitive sequences should provide new and pertinent information on genome organization, the genetic affinity of the species belonging to the *M. sativa* complex, and the genome evolution of their somatic hybrids. Moreover, once a repetitive sequence has proven to be specific for the complex, it could be used to quantify the genome introgression of *M. sativa* into somatic hybrids with other, not sexually compatible, *Medicago* species; for example, *M. arborea*, which could act as donor for desirable traits (for references see Nenz et al. 1996). There are but few reports on the genome structure of *Medicago*, and the application of molecular

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cytogenetics to these species has always been hampered by the small size and uniform morphology of the chromosomes. However, highly and middle repetitive sequences have recently been cloned in *M. sativa* (Xia and Erickson 1993; Xia et al. 1996) and ribosomal genes have been physically mapped in the *M. sativa-coerulea-falcata* complex and in *M. arborea* (Calderini et al. 1996; Cluster et al. 1996).

Here we report the isolation of a repetitive sequence from *M. coerulea*, a species representative of the basic chromosome complement of the complex, as well as the organization and copy number of this sequence, named C300, in *M. sativa*, *M. coerulea*, *M. falcata* and their somatic hybrids, its physical distribution along the chromosomes, and its specificity with respect to *M. arborea*.

Materials and methods

Plant material

The genotypes used in this study were three somatic hybrids *M. sativa* + *M. coerulea* (S + C, Pupilli et al. 1992), *M. sativa* + *M. arborea* (S + A, Nenz et al. 1996) *M. sativa* + *M. falcata* (S + F, unpublished data), and their parental lines. These were: *M. sativa* ($2n = 4x = 32$) cv Rangelander genotype 15, kindly supplied by Dr. Phil Larkin (CSIRO, Plant Industry Canberra); *M. coerulea* ($2n = 2x = 16$) genotype C1 and *M. falcata* ($2n = 2x = 16$) genotype F2, both selected for high regeneration capability and provided by the All Union of Plant Industry, St. Petersbourg, and Prof. E.T. Bingham (University of Wisconsin), Madison respectively; and an experimental strain of *M. arborea* ($2n = 4x = 32$) derived from mesophyll protoplasts (Mariotti et al. 1984). All somatic hybrids and *M. sativa*, *M. coerulea* and *M. falcata* were propagated by cuttings, while *M. arborea* was seed-propagated.

DNA preparation

DNA was isolated according to Saghai-Maroof et al. (1984) with minor modifications. The plant material (2 g of fresh weight) was ground in liquid nitrogen, re-suspended in 15 ml of cTAB buffer at double strength and incubated at 65°C for 30 min. After chloroform-isoamylic extraction, the aqueous phase was collected, the nucleic acids precipitated with cold isopropanol and collected by centrifugation. The pellet was recentrifuged overnight in a CsCl gradient (Maniatis et al. 1982). The band corresponding to genomic DNA was collected, dialyzed for 3 h at room temperature against TE buffer and ethanol-precipitated.

Construction of a partial genomic *Eco*RI library and screening for repeated and species-specific sequences

Twenty micrograms of DNA isolated from *M. coerulea* were digested overnight with *Eco*RI and the resulting fragments fractionated by electrophoresis on 1% agarose. Fragments with a length of between 300 and 2500 bp were electroeluted from the gel using a Biotrap (Schleicher and Schüll) apparatus and cloned into the corresponding *Eco*RI site of the pUC19 (Boehringer) plasmid vector. The recombinant plasmids were used to transform the JM 109 strain (Promega) of *E. coli* and the resulting colonies identified with a blue/white screen on LB plates containing ampicillin, x-Gal and IPTG (Maniatis et al. 1982). The recombinant colonies were stored at −80°C on LB medium containing 15% v/v glycerol.

The screening of recombinant colonies was performed according to the protocol of Costanzi and Gillespie (1987) modified as follows: after the freeze-thawing procedures, the bacterial DNA preparations were denatured in 0.4 M NaOH and 10 mM EDTA at 100°C for 10 min and then loaded onto Hybond-N^+ membranes using a Bio-Dot Microfiltration Apparatus (Bio Rad) according to the supplier's instructions. Each blot was done in duplicate and hybridized to 25 ng of genomic, undigested *M. coerulea* and *M. arborea*, DNA used as a probe; labelling, hybridization and washing procedures were those reported below for Southern blots. Clones showing high intensity signals when hybridized with *M. coerulea* DNA, and no signal with *M. arborea*, were selected.

Southern-blot and hybridization procedures

Five micrograms of DNA were restriction-digested with 10 units of *Dra*I (New England Biolabs) according to the supplier's instructions. Digests were electrophoresed in 1.2% agarose and blotted onto Hybond-N^+ membranes (Amersham) according to the standard capillary procedure (Southern 1975). The plasmid containing the C300 insert was isolated from recombinant colonies (Maniatis et al. 1982), digested with *Eco*RI, and the insert electroeluted from the gel slices after electrophoresis with the Unidirectional Electroelution Apparatus (UEA, IBI). Twentyfive nanograms of insert were labelled with [^{32}P]-dCTP using a Ready-To-Go kit (Pharmacia) according to the supplier's instructions. Membranes were hybridized overnight at 65°C and washed at high-stringency conditions (0.1 × SSC; 0.1% SDS 65°C, three changes of 15 min each).

Estimation of copy number

Genomic DNA of the parental species and the three hybrids were serially diluted in stepwise concentrations ranging from 2 ng/10 µl to 25 pg/10 µl. Similarly, the C300 insert was diluted from 20 pg/10 µl to 0.25 pg/10 µl. The DNA solutions were denatured in 0.4 M NaOH and 10 mM EDTA at 100°C for 4 min, then spotted onto Hybond-N^+ membranes and hybridized with labelled C300. Hybridization and washing procedures were the same as reported above for Southern blots. After an overnight exposure to X-ray films the membranes were cut into pieces and the radioactivity of the hybridized DNA measured in a liquid scintillation apparatus (Beckmann). Exposed X-ray films were developed for visual comparison with the scintillation counts. Estimation of the copy number of genomic DNA was performed against a linear range of cpm/pg of DNA obtained using C300 as a standard. The DNA content per haploid genome in *M. arborea* and *M. sativa* was from Bennett and Smith (1976). As the chromosomes of the species investigated are uniform in size (Bauchan and Campbell 1994) it was assumed that they had the same DNA content (Furuta and Nishikawa 1991). The DNA content per haploid genome of the somatic hybrids and diploid parents was therefore estimated by multipling the average DNA content per chromosome by the number of chromosomes.

DNA sequencing

The insert C300 was excised from pUC19 and cloned into the *Eco*RI site of the plasmid vector pGEM11 bearing the SP6 and T7 promoter primers. The sequencing was carried out using the Thermo Sequenase Sequencing Kit (Amersham) according to the supplier's instructions. The DNA sequence was analyzed using the PC Gene program, and sequence-homology searches in the data base were performed with the FASTA program (Pearson and Lipman 1988). The accession number of C300 at Gene Bank is U83398.

Fluorescent *in situ* hybridization (FISH)

Chromosome spreads for *M. sativa*, *M. coerulea*, *M. falcata* and the somatic hybrids were obtained from root tips of cuttings, whereas for *M. arborea* seedling roots were utilized. Pre-treatment was carried out in ice-water for 24 h followed by 3 h in α-bromonaphtalene at 4°C. FISH was performed basically as described by Orgaard and Heslop-Harrison (1994). The probe C300 was directly labelled with rhodamine-4-dUTP (Amersham) and hybridization performed in 50% formamide, 2 × SSC. Six post-hybridization washes were carried out, two being the most stringent (20% formamide 0.2 × SSC at 42°C 5 min) followed by three washes in 2 × SSC at room temperature for 5 min and a final wash in 4 × SSC, 0.2% Tween 20, at room temperature for 5 min. Slides were examined with a Zeiss Axiophot epifluorescence microscope with filter sets 01 for DAPI and 15 for rhodamine-4-dUTP. The number of signals was averaged in ten metaphases for each species and somatic hybrid.

Results

About 800 clones were screened with the dual hybridization procedure and eight selected on the basis of their strong hybridization signal with *M. coerulea* DNA and a background signal only with *M. arborea* DNA. Due to their cross-hybridization (data not shown) all eight clones bore the same inserted sequence. C300, therefore, appears to be a highly repetitive sequence of *M. coerulea* which differentiated it from *M. arborea*. The Southern hybridization of this clone to the *Dra*I-digested DNA of the four parental species and the three somatic hybrids is shown in Fig. 1. *M. arborea* gave no signal and so confirmed the results of the dot-blot analysis. The pattern displayed by the other species and the somatic hybrids is a typical tandem-repeat ladder with a basic unit of around 180 bp. As the signal was much weaker in *M. falcata* than in either *M. sativa* or *M. coerulea*, C300 was less abundant in the former than in the latter. The somatic hybrids S + C and S + F gave stronger signals than S + A. The copy number varied among the three species considered (Table 1); C300 was most abundant in *M. coerulea* with 3×10^5 copies per haploid genome, a value almost twice that found in *M. sativa*, even though the latter has a double chromosome complement. Conversely, the number of copies was markedly lower in *M. falcata*, thereby confirming the results shown in Fig. 1 . There was also variation in the C300 copy number in the somatic hybrids which was dependent on the contribution of each parent. The copy number of S + F was very similar to that of *M. sativa*, indicating that most chromosomes (probably 32 out of 33) were derived from that parent. The lower number of copies in S + A than in *M. sativa* is consistent with the observation that this hybrid lacked some *M. sativa* chromosomes (Nenz et al. 1996). Although both parental chromosome complements were present in S + C, the copy number recorded in this hybrid was lower than the expected one.

Sequence analysis (Fig. 2) of C300 revealed high homology with a repetitive sequence, E180, recently cloned from *M. sativa* DNA (Xia and Ericksson 1993). C300 is an AT-rich sequence of 746 bp containing four repeats of 187 bp. As Fig. 2 A shows there were few differences among the four repeats, mainly point mutations and single base insertion/deletion events. A comparison of the consensus sequences of C300 and E180 showed 96% homology. No "hot spots" for mutations were observed along the sequence and the DraI site internal to the repeated unit explained the ladder pattern of Fig. 1.

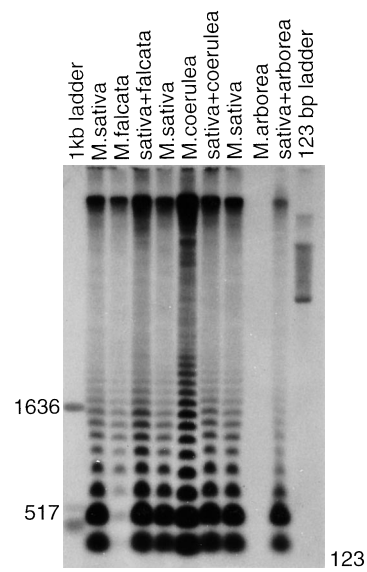

Fig. 1 Southern analysis of four *Medicago* spp. and three somatic hybrid DNAs digested with *Dra*I and probed with C300. Molecular weights are expressed in bp

Table 1 Chromosome number, the DNA content per haploid genome, and the copy and signal number of clone C300 in three *Medicago* species and three somatic hybrids

Item	*M. sativa*	*M. coerulea*	*M. falcata*	(S + C)[a]	(S + A)	(S + F)
Chromosome number	32	16	16	48	56	33
DNA per haploid genome (p g)	3.5	1.75	1.75	5.25	6.1	3.6
Copy number	1.8×10^5	3.0×10^5	0.8×10^5	2.1×10^5	1.2×10^5	1.7×10^5
Signal number[b]	44–46	24–26	8–10	–	26	–

[a] (S + C) = *M. sativa* + *M. coerulea*; (S + A) = *M. sativa* + *M. arborea*; (S + F) = *M. sativa* + *M. falcata*
[b] The values reported are the ranges of variation recorded in ten metaphases

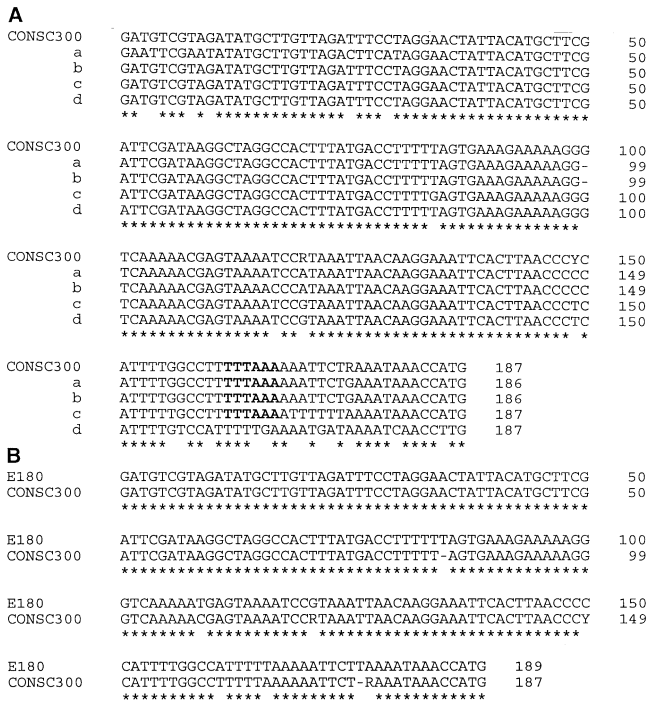

Fig. 2A, B Sequence of the repeated fragment C300. **A** Alignment of the four repeating units (*a*, *b*, *c*, *d*) of C300 and its deduced consensus sequence (CONSC300). The *Dra*I restriction site is in *bold letters*. (*) same nucleotide; (−) nucleotide not present; (*R*) G or A; (*Y*) C or T. **B** Alignment of the consensus sequences of C300 and of E180. (*) same nucleotide. (*R*) and (*Y*) as in **A**

FISH of C300 on metaphase chromosomes of *M. sativa*, *M. coerulea* and *M. falcata* is reported in Fig. 3 A–C. As in the Southern analysis, physical mapping of this sequence revealed that the average number of signals and the level of repetition was higher in *M. sativa* (Fig. 3 A) and *M. coerulea* (Fig. 3 B) than in *M. falcata* (Fig. 3 C). In general, no more than two signals per chromosome were observed, the signals could have different intensities and the satellited chromosomes carried two signals in *M. sativa* and *M. coerulea* and one in *M. falcata*. In particular, the latter exhibited the lowest number of signals, namely 8–10 (Table 1), and the chromosomes displayed one or no signal. The chromosome distribution of C300 was similar in *M. coerulea* and *M. sativa*, and chromosomes with one or two signals were detected in both species. When one signal was visible, it was located either close to the centromere (solid arrow, Fig. 3 A) or in subtelomeric regions (solid arrow, Fig. 3 B). When two signals were present on the same chromosome they were located either pericentromerically (open arrow, Fig. 3 A) or on the same chromosome arm (arrowhead, Fig. 3 A). A total of 24–26 signals were visible in *M. coerulea* while in *M. sativa* the number was 44–46 (Table 1). In addition, the C300 probe hybridized with all *M. coerulea* chromosomes, whereas in *M. sativa* the signal was not detected in 1–3 chromosomes. Such variability could be due to the difficulties of detecting low copy number repeats in the small chromosomes typical of these species. The species-specificity of C300 revealed by dot-blot and Southern analysis was confirmed by FISH, since no signal was detected in *M. arborea* chromosomes (data not shown). In the S + A somatic hybrid, the C300 probe hybridized to 26 out of 56 chromosomes (Fig. 3 D), indicating incomplete integration of both parental genomes in the hybrid. In fact, if *M. sativa* is considered to have a maximum of three chromosomes without a signal, a maximum of 29 chromosomes would be attribuable to *M. sativa* and a minimum of 27 to *M. arborea*. There was, therefore, no dramatic imbalance of the parental chromosome complements in this somatic hybrid. Because *M. sativa*, *M. coerulea* and *M. falcata* differed for the number of copies and signals of C300, but not for its specificity, FISH was not performed on S + C and S + F.

Discussion

The physical mapping and genome organization studies we performed on a highly repeated sequence (C300) isolated from *M. coerulea* DNA in four perennial *Medicago* species yielded new and important findings. Both FISH and Southern analysis demonstrated that the sequence was not present in *M. arborea*, but was specific for the *M. sativa-coerulea-falcata* complex. The C300 sequence was therefore able to detect genome introgression in somatic hybrids that involved the species complex and *M. arborea*, which is considered an interesting source of traits, such as winter growth and drought resistance, that are lacking in cultivated alfalfa (Corleto et al. 1980). As far as we know only one other species-specific sequence has been reported for the genus *Medicago*, that specific for *M. granadensis* (Skinner 1992), and it has not yet been used for the identification of interspecific hybrids. Southern analysis revealed that the organization of C300 in the *Medicago* complex is typical of a tandemly repeated sequence and its sequencing showed the presence of a repeating unit of 180 bp, rich in AT. A sequence not unlike it has been reported in *M. sativa* by Xia and Erickson (1993). The cloning of almost identical sequences from *M. sativa* and *M. coerulea* DNA, and the near similar organization of C300 in their genomes, provides further evidence in favor of the very close origin of these species, which has also been inferred from morphological (Quiros and Bauchan 1988) and cytological (Calderini et al. 1996) observations. Repetitive sequences are known to have undergone cycles of amplification or deletion during evolution through mechanisms such as unequal crossing over and gene conversion (Smith 1975; Dover 1982; Arnheim 1983). Polyploid species are often considered to be more recent in evolution

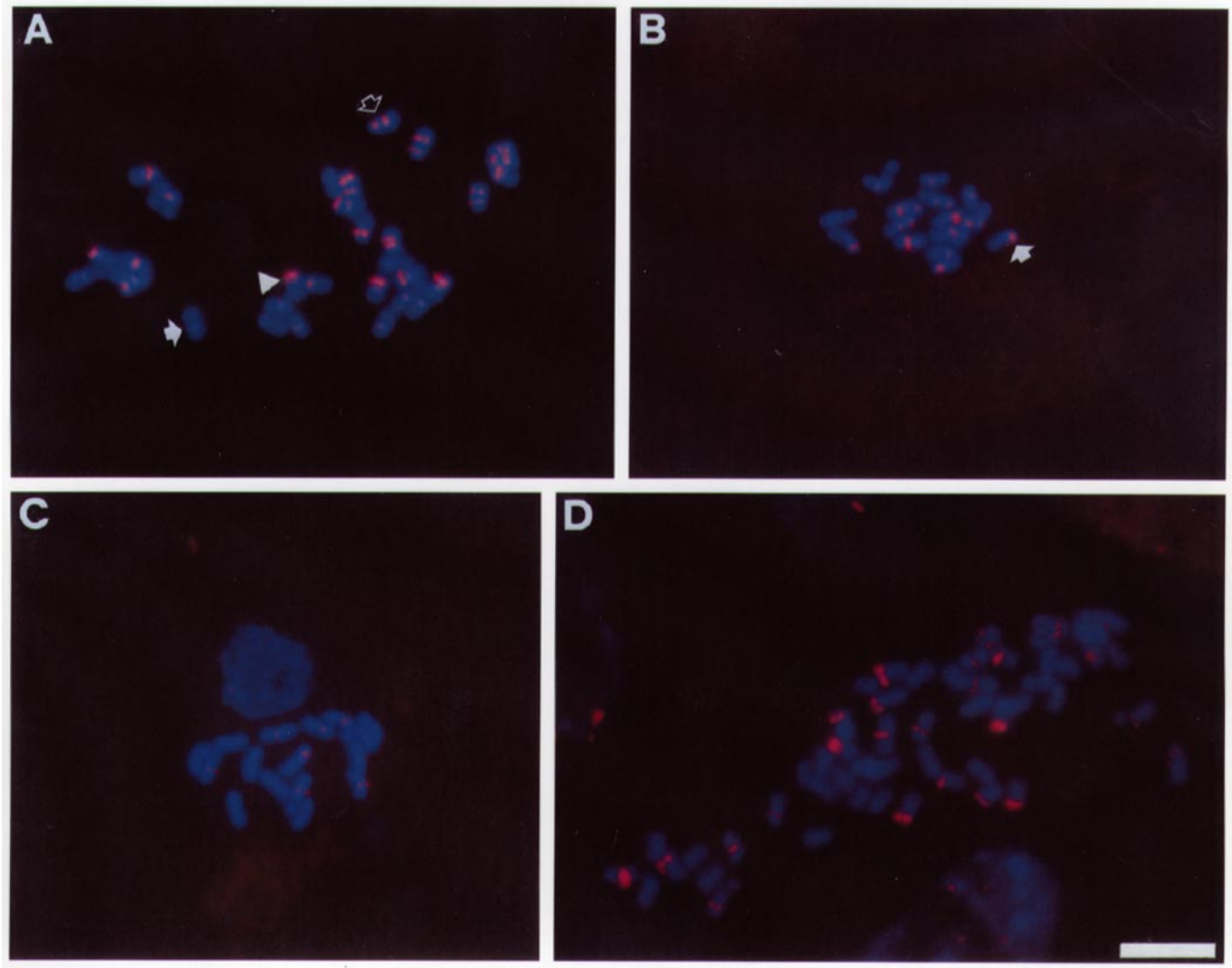

Fig. 3A–D Fluorescent *in situ* hybridization to somatic metaphases of three *Medicago* spp. and the somatic hybrid S + A. The chromosomes were stained with the fluorochrome DAPI (blue) and the *pink spots* correspond to the hybridization sites of rhodammine-labelled C300. **A** *M. sativa* metaphase in which the *solid arrow* indicates a single signal close to the centromere, the *open arrow* indicates two signals in a pericentromeric position, and the *arrowhead* indicates a pericentromeric and a subtelomeric signal. **B**, a *M. coerulea* metaphase in which the *solid arrow* indicates a single subtelomeric signal. **C**, a *M. falcata* and **D**, a S + A metaphase. The bar represents 10 μm

than diploids; in particular, tetraploid *M. sativa* is thought to have originated through sexual polyploidization from the diploid species *M. coerulea* and *M. falcata* (Quiros and Bauchan 1988). The marked differences in the copy number of C300 among species belonging to the complex may be due to the different degrees of amplification of the sequence during evolution from a common ancestor. Likewise, somatic hybridization seems to have triggered major changes in the level of repetition of C300 due either to chromosome loss (S + A and S + F) or minor chromosome rearrangements (S + C). It is worth noting that the same somatic hybrids exhibited alterations for another class of tandemly repeated sequences, which involved the loss of one NOR of the *M. arborea* parent in S + A and the rapid amplification of portions of the rDNA genes in S + F (Cluster et al. 1996).

Other than chromosome loss, in vitro culture has been reported to induce small-scale rearrangements in DNA that mainly affect heterocromatic regions of the chromosomes (Lee and Philips 1987). Such rearrangements involve chromosome breakage and successive fusion of the broken segments which give rise to either loss or gain of chromatin (Kidwell and Osborne 1993). This mechanism is likely to be responsible for the marked reduction in C300 copy number detected in S + C with respect to the parents. Pupilli et al. (1995) noted that in this somatic hybrid about one-third of RFLP alleles specific to *M. coerulea* were lost, whereas almost all those of *M. sativa* were retained. They suggested that such asymmetrical incorporation of parental alleles in the hybrid was due to chromosome

rearrangements occurring more frequently in *M. coerulea* than in *M. sativa* because it was subjected to a longer phase of tissue culture. Therefore the loss of repeated units belonging to *M. coerulea*, rather than to *M. sativa*, probably accounts for the unexpectly low copy number of C300 in S + C. Although translocations and/or inversions have been hypothesized to be responsible for the disappearence in S + C RFLP patterns of *M. coerulea*-specific low-repeated sequences, the decrease in C300 copy number we documented is unlikely to be due to these mechanisms. However, translocation/inversion and breakage/fusion mechanisms are not mutually exclusive. Since single or low-copy sequences are likely to be coding, and repetitive sequences non-coding and selectively neutral regions, selective pressure could have eliminated those cells where coding-region rearrangement was dramatic during tissue culture. In contrast, those cells in which both major and minor rearrangements involved the repetitive, presumably non-coding, regions were able to regenerate plants. The findings reported here are further evidence that during the regeneration process of somatic hybrids extensive DNA rearrangments occur at random and may involve any portion of the genome irrespective of the ploidy level or the genetic affinity of the parents. The changes documented in genome architecture may therefore be attributable only to those rearrangements that did not compromise the viability of the regenerated plants. The regeneration process appears to act as a sieve to eliminate unsuccessfull genomic combinations and create new variability.

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