Theor Appl Genet (1995) 90:347–355

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Molecular, cytological and morpho-agronomical characterization of hexaploid somatic hybrids in *Medicago*

Received: 9 May 1994 / Accepted: 28 July 1994

Abstract Somatic hybrid plants produced by protoplast fusion between tetraploid *Medicago sativa* (2n = 4x = 32) and the diploid species *Medicago coerulea* (2n = 2x = 16) have been RFLP fingerprinted to establish their nuclear composition. Although all of the chromosomes were present, molecular analysis revealed an incomplete incorporation of the alleles of the diploid parent in the fusion products. In the polycross progeny the alleles of both parents segregated in a Mendelian mode. Cytological observations indicated that in the somatic hybrid population minor abnormalities are present; these are restricted mainly to the formation of univalents and lagging chromosomes. Meiosis appeared to be more stable than has been previously reported in the hexaploids of alfalfa. The somatic hybrids grown in the field had a rather vigorous aspect, particularly with respect to the vegetative organs. Forage yield was comparable to that of the more productive parent. The results are discussed with a view to utilizing the somatic hybrids as starting material for breeding alfalfa at the hexaploid level.

Key words *Medicago* · Somatic hybrids · RFLP analysis · Meiotic analysis · Field evaluation

Introduction

In the plant kingdom, the information present in two different genomes can be combined using sexual or asexual strategies. When sexually incompatible individuals contribute to the creation of new living forms, a new type of gene flow allows the enrichment of a cultivated germ plasm with useful traits. Somatic hybridization protocols have been established for several species (Gleba and Shlumukov 1990), and progenies derived from protoplast fusions are used in plant breeding programs, mainly in *Solanum* (Austin et al. 1985; Hegelson et al. 1986; Gleddie et al. 1986) and *Brassica* (Sjodin and Glimelius 1989).

Alfalfa (*Medicago sativa* L.) is the most important protein source in animal fodder. In principle, desirable traits such as disease resistance and stress adaptation could be transferred through protoplast fusion from wild species to the cultivated varieties of alfalfa. However, in spite of the fact that it is relatively easy to produce plants from callus and protoplasts, only a few examples of interspecific somatic hybrid plants have been reported in the genus *Medicago* (Arcioni et al. 1994). Recently, hexaploid hybrids have been obtained by somatically combining tetraploid *M. sativa* with diploid *M. coerulea* (Pupilli et al. 1992). In alfalfa, hexaploidy is more desirable than tetraploidy because of its potential higher level of heterozygosity and the consequent slower decline in vigor due to inbreeding (Julén 1944; Lesins et al. 1975). However, a major deterrent to the practical utilization of alfalfa at the hexaploid level is its chromosomal instability (Lesins et al. 1975; Yen and Murphy 1979; Smith et al. 1984). Before the introduction of somatic fusion, hexaploid *Medicago* plants were either identified after a spontaneous occurrence (Mariani 1975; Bingham and Binek 1969) or produced by inter- and intra-specific crosses between parents at different ploidy levels (McCoy and Bingham 1991). They have also been recovered from tissue culture (Hartman et al. 1984; Latunde-Dada and Lucas 1983).

Somatic hybrids may, however, be of more interest than sexual ones for the following reasons: (1) their heterozygosity is higher than that in sexual hybrids derived from colchicine-treated parents; and (2) the

This paper was supported by the National Research Council of Italy, Special Project RAISA, Sub-project No.2 paper No. 1911

Communicated by G. Wenzel

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combination of the cytoplasms of both parents could be an additional source of variability.

The use of somatic hybrids in plant breeding requires some knowledge of the mode of inheritance of the two genomes during subsequent sexual generations. In the investigation reported here, restriction fragment length polymorphism (RFLP) markers were used to study the genetic make-up of the hexaploid somatic hybrids and the transmission of both parental genomes to the progenies. A microsporogenesis analysis was also carried out to compare the chromosome stability of somatic hybrids with that of cross-derived and spontaneous hexaploids. Furthermore, morpho-agronomical traits were evaluated to investigate the performance of somatic hybrids under field conditions.

Materials and methods

Plant material

Somatic hybrid plants were produced between mesophyll protoplasts of plant R15 of *M. sativa* (2n = 4x = 32) cv 'Rangelander' and callus protoplasts of plant C1 of *M. coerulea* (2n = 2x = 16), All Union of Plant Industry, St. Petersburg, Russia (Pupilli et al. 1992). Hybrids were propagated through cuttings and maintained in the greenhouse. A population of 15 hybrid plants with the correct hexaploid chromosome number (48), all derived from the same fusion event, was selected and used for molecular analysis. This population was intercrossed by hand to generate a polycross population of 40 individuals. A subset of 10 somatic hybrid plants, out of 15 analyzed by RFLPs, was studied for their microsporogenesis and intercrossed by hand to generate a polycross population of 35 individuals. A segregating population of 55 individuals was generated by crossing plant C1 of *M. coerulea* with a diploid plant of *M. sativa* (cv 'CADL').

DNA Isolation

DNA was isolated according to Saghai-Maroof et al. (1984). The plant material was ground in liquid nitrogen, resuspended in 15 ml of cTAB (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 then 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.

Source of probes, electrophoresis and hybridization procedures

Of the 27 probes used, 19 were selected from a *Pst*I genomic alfalfa library for their capacity to reveal polymorphism between *M. coerulea* and diploid *M. sativa* (Businelli et al. 1993). Plasmids were purified from recombinant clones according to standard procedures (Maniatis et al. 1982), and the inserts were electroeluted from agarose els after electrophoretic separation with a unidirectional electroelution apparatus (IBI). Inserts were labelled with [^{32}P] dCTD using a random primer procedure (Feinberg and Vogelstein 1983). Probes Xugac085, Xugac281, Xugac083, Xugac109, Xugac118, Xugac553, Xugac540 and Xugac769, chosen to represent each of the eight largest linkage groups of the alfalfa map of Brummer et al. (1993), were also used.

Genomic DNA was digested with enzymes *Rsa*I, *Alu*I and *Hae*III, electrophoresed in 4% denaturing polyacrylamide gels and electroblotted onto Hybond-N$^+$ nylon membranes according to Gebhardt et al. (1989) as modified by Businelli et al. (1993). Hybridization, washing and exposition procedures were those of Gebhardt et al. (1989).

Linkage analysis

Analysis of linkage of the *M. coerulea*-specific DNA fragments was according to Ritter et al. (1990). For each fragment pairwise comparison, the configuration AB/00 × 00/00 (coupling) was considered, where one allele of each of two segregating loci (A and B) is identified by a single segregating fragment present only in the *M. coerulea* parent. The second parent contributed the two null alleles 00.

Cytological analysis

For microsporogenesis analysis young flower buds were fixed in ethanol-acetic acid (3:1) for 16 h. The material was then hydrolyzed in 1 *N* HCl for 7 min at 60 °C and stained in Feulgen. Anther squashes were counterstained in 1% Orcein.

Field evaluations

Twenty-nine hybrid plants were maintained through cuttings and field transplanted in May 1993 according to a randomized block design with four replications and 2 plants per replication. In July 1993, blooming time (days from the first of May), weight of leaves and stems per plant (g), weight (g) of trifoliate leaf present at the fifth node (starting from the apex) of the main stem, length and width (cm) of the central leaflet of the above-mentioned trifoliate leaf, diameter (mm) and length (cm) of the main stem, number of stems per plant and growth habit [on a scale of 1 (prostrate) to 5 (erect)] were recorded. Data were submitted to ANOVA using the GLM procedure of the SAS (1988) program.

Results

Molecular analysis

Of 27 probes, 12 detected bands specific to the *M. sativa* parent (MSSBs), 4 detected bands specific to the *M. coerulea* parent (MCSBs), 7 revealed fragments derived from both parents and only 4 were monomorphic. In total, 77 *M. sativa*- and 38 *M. coerulea*-specific bands were detected (Table 1).

The genome of the somatic hybrids was expected to display all of the polymorphic fragments because it is the

Table 1 RFLP analysis of parental lines and somatic hybrids

Parents	Number of				
	Probes	Probe/enzyme combinations	Total bands	Polymorphic bands between the parents	Missing bands in the somatic hybrids
M. sativa	27	73	405	77	2
M. coerulea	27	73	366	38	11

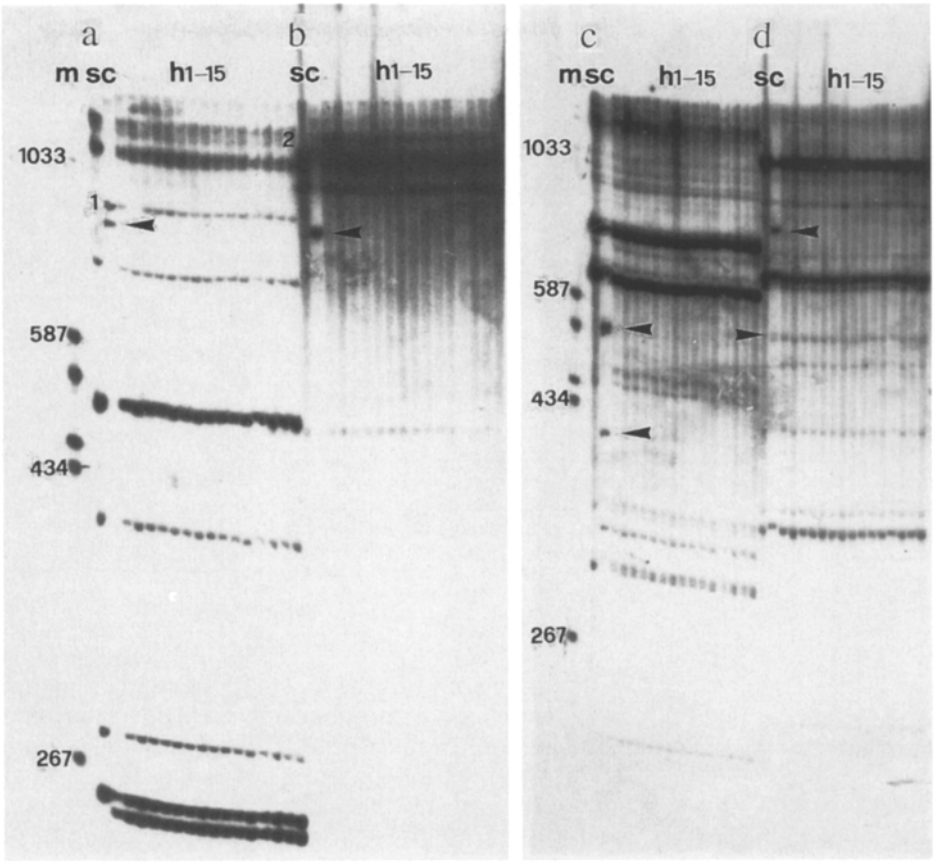

Fig. 1a–d Hybridizing banding patterns of the probes 1H11 (**panels a and b**) and 1D2 (**panels c and d**) with the DNA digests of *M. sativa* (s), *M. coerulea* (c), and 15 somatic hybrids (h_{1-15}). The restriction enzymes were *Alu*I (**panels a and c**) and *Rsa*I (**panels b and d**). *Arrows* indicate the MCSBs missing in the hybrid patterns, and the molecular weights (*m*) are indicated in base pairs

symmetric combination of the parental genomes. However, we noted that 11 out of 38 (29%) MCSBs were absent in the RFLP patterns of the somatic hybrids, while 2 out of 77 (2.6%) MSSBs were missing. The two probes 1H11 and 1D2 hybridized with the DNA of *M. sativa* (S), *M. coerulea* (C) and 15 somatic hybrids (H_{1-15}) digested with *Alu*I and *Rsa*I gave the RFLP patterns reported in Fig. 1. The hybrid nature of the regenerated plants was confirmed by the presence of bands specific to both parents, although some MCSBs (arrows in the figure) were absent. Variability among the 15 regenerated hybrid plants considered was low: of the 430 bands scored, only 2 showed polymorphism. One example is given in Fig. 1 (panel a, hybrid no. 2, band no. 1).

To assess the map position of the genes with the alleles corresponding to the lost fragments in the *M. coerulea* genome, their linkage was studied in an F_1 population obtained by crossing genotype C1 of *M. coerulea* with a diploid *M. sativa* plant polymorphic for the fragments missing in the RFLP patterns of hybrids. All of the fragments segregated in a 1:1 ratio (presence versus absence) without distorted segregations ($P \leq 0.05$, Table 2). By scoring the recombination frequencies between each pairwise combination of these fragments, we were able to identify 6 loci segregating independently

Table 2 Segregation analysis of probes detecting MCSBs missing in the somatic hybrid patterns

Probe	Enzyme	Individuals with bands		$\chi^2_{1:1}$
		Present	Absent	
1H11	*Rsa*I	16	28	3.27 ns
1H11	*Alu*I	19	29	2.08 ns
1D2	*Rsa*I	20	24	0.36 ns
1D2a	*Alu*I	18	28	2.17 ns
1D2b	*Alu*I	18	29	2.57 ns
1D2	*Hae*III	20	24	0.36 ns
1B10	*Rsa*I	20	24	0.36 ns
1C12	*Alu*I	23	24	0.02 ns
2H9	*Hae*III	18	30	3.00 ns
1C5	*Alu*I	19	29	2.08 ns
1C5	*Rsa*I	20	24	0.36 ns

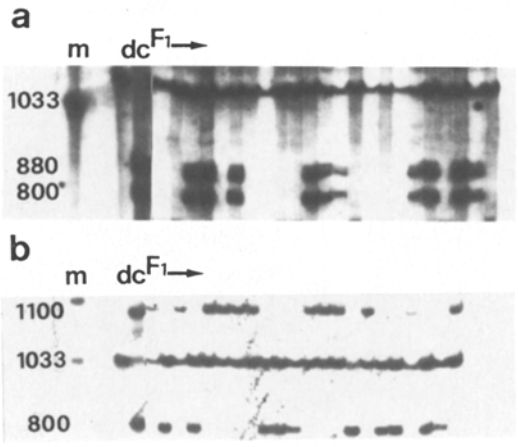

Fig. 2 Hybridizing banding patterns of probe 1H11 with the DNA digests of *M. coerulea* (c), diploid *M. sativa* (*d*) and 23 (**panel a**) and 22 (**panel b**) F_1 individuals. The DNAs were digested with *Alu*I (**panel a**) and *Rsa*I (**panel b**), and the molecular weights (*m*) are indicated in base pairs

and 5 loci associated in two linkage groups. In the first case, 7 cM separated 2 loci; in the second case a locus was flanked by 2 others at a distance of 27 and 29 cM.

Probe/enzyme combination 1H11/*Alu*I detected 2 MCSBs of 880 and 800 bp, of which only the longer one was retained in the hybrids (Fig. 1, panel a). As these 2 fragments co-segregated in a testcross F_1 population, (Fig. 2a), we concluded that they belong to the same *M. coerulea* allele and are either 2 repeated sequences with an *Alu*I site in between or 2 fragments resulting from the cleavage of *Alu*I inside the hybridizing sequence. These results provide evidence for a deletion event which occurred in the DNA of the *M. coerulea* genome present in the somatic hybrid plants. Probe 1D2, which also revealed 2 allelic fragments (1100 and 800 bp) in *M. coerulea* (Fig. 2b), showed that only the larger one was retained in the somatic hybrids (Fig. 1, panel b, band no. 2). The cases reported in Fig. 2a and b are based on 23 and 22 individuals, respectively, of a testcross progeny and are representative of more extensive data reported in Table 2.

Fig. 3 Cytological analysis of somatic hybrids. **a** Metaphase chromosomes of somatic hybrids, **b** metaphase I with laggards (*arrows*), **c** Metaphase II with parallel spindles, **d** Metaphase II with tripolar spindles, **e** dyad, **f** triad, **g** Anaphase II with laggards (*arrows*), **h** pentad. *Bar* 5 μm

The segregation of the MCSBs retained in the hybrid genome was recorded in the polycross progeny of somatic hybrid plants. In a survey based on five probe/enzyme combinations, 5 segregating fragments of *M. coerulea* and 6 of *M. sativa* were identified. They all segregated in a 3:1 ratio (presence versus absence) as was expected for a locus in simplex condition Aaaaaa (Table 3). No distorted segregation ratios were detected. All of the MCSBs were heterozygous and retained this condition in the hybrid genome.

Cytological analysis

Microsporogenesis was analyzed in 10 out of 15 somatic hybrid plants with 48 chromosomes characterized with RFLPs (Fig. 3a). The meiotic abnormalities recorded were: the formation of univalents at Metaphase I (MI) (Fig. 3b) resulting in the formation of laggards at Anaphase I (AI); abnormal orientation of spindles either as parallel (Fig. 3c) or tripolar (Fig. 3d); and the occurrence of lagging chromosomes at Anaphase II (AII) (Fig. 3g). Different sporad types such as dyads (Fig. 3e), triads (Fig. 3f) and pentads (Fig. 3h) were also observed. The percentage of cells with univalent and lagging chromosomes and of cells with abnormal spindle orientations was slightly lower in the parents than in the somatic hybrids (Table 4). By scoring a number (60–75) of pollen mother cells (PMCs), we were able to detect a variable number of cells with unpaired chromosomes at MI in the population of somatic hybrids. Plant no. 9 was the most stable, with only 4% of the cells having 1–4 univalents, while the most unstable was plant no. 22, with 30% of the cells having unpaired chromosomes (Table 4). The proportion of lagging chromosomes at AI was correlated with the percentage of unpaired chromosomes at MI and ranged from 5.3% to 25.9%. In plants

Table 3 Segregation analysis of RFLP fragments in the polycross population of somatic hybrids

Probe	Enzyme	Fragment deriving from		Polycross individuals with fragments		$\chi^2_{3:1}$
		M. coerulea	*M. sativa*	Present	Absent	
2B9	*Alu*I	+	−	21	12	2.27 ns
	*Alu*I	−	+	25	8	0.01 ns
1H11	*Alu*I	+	−	24	9	0.09 ns
	*Alu*I	−	+	23	10	0.49 ns
	*Alu*I	−	+	21	9	0.40 ns
	*Alu*I	−	+	24	9	0.09 ns
3F3	*Rsa*I	+	−	23	9	0.25 ns
	*Rsa*I	−	+	22	9	0.27 ns
2H9	*Rsa*I	−	+	23	9	0.25 ns
	*Rsa*I	+	−	23	10	0.49 ns
3E3	*Rsa*I	+	−	22	11	1.23 ns

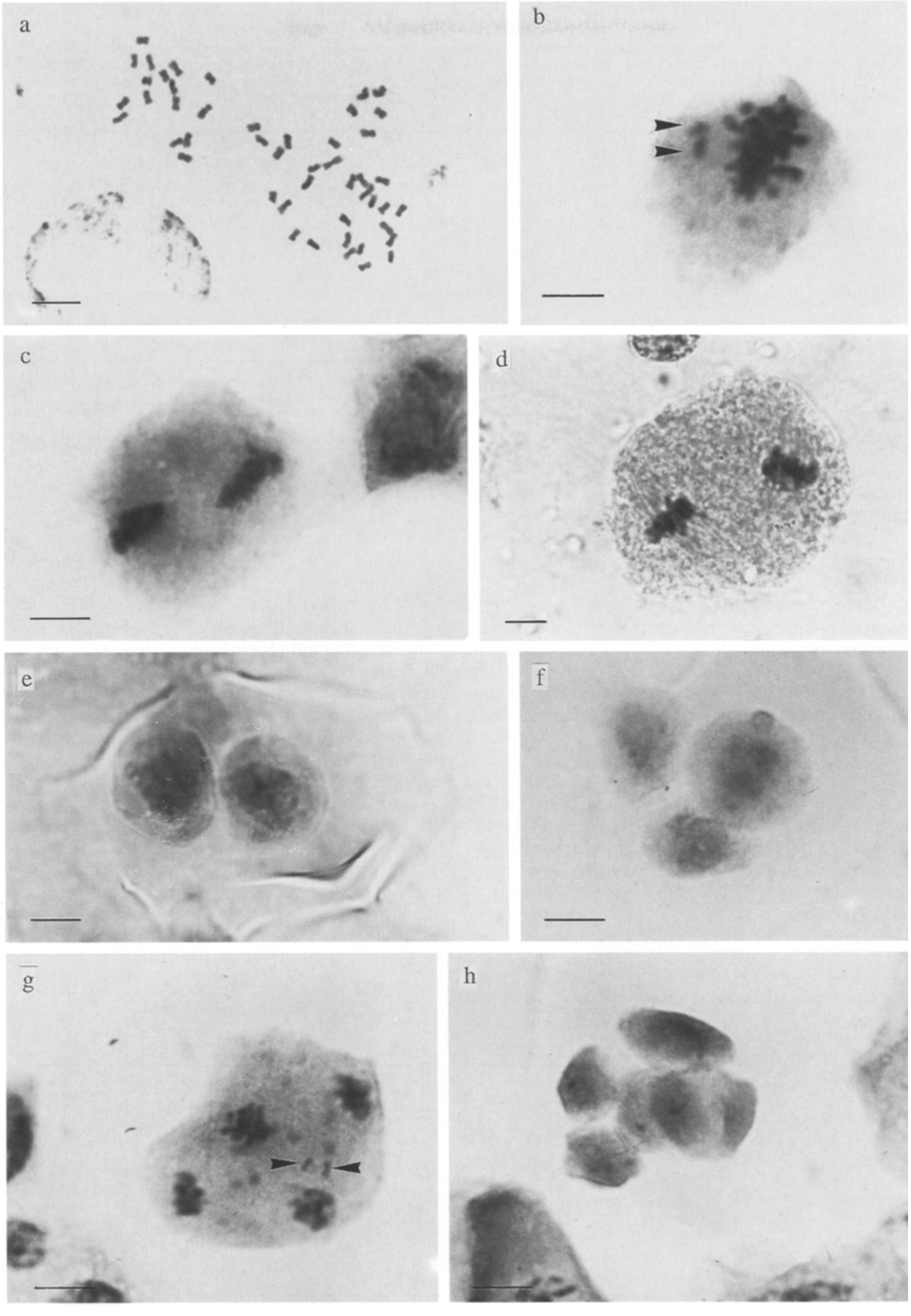
a
b
c
d
e
f
g
h

Table 4 Microsporogenesis analysis in 11 somatic hybrids *M. sativa* + *M. coerulea* (*PS* parallel spindles, *TS* tripolar spindles, *nd* not determined)

Plant	Metaphase I[a] (% of cells with 1–4 univalents)	Anaphase I[b] (% of cells with 1–4 laggards)	Metaphase II[c] (% of cells)		Anaphase II[d] (% of cells with 1–4 laggards)
			PS	TS	
98	19.6	10.5	9.6	6.4	9.4
9	4.1	5.3	20.8	4.16	9.3
89	6.9	5.4	3.4	0.0	6.9
54	6.6	6.7	5.4	5.4	5.8
20	9.4	6.1	3.8	7.6	2.5
22	30.0	25.9	15.2	13.0	3.5
19	14.3	10.0	17.7	11.7	nd
13	12.5	24.1	nd	nd	8.7
33	19.6	13.2	nd	nd	nd
57	10.0	9.6	11.7	11.7	9.1
M. coerulea	4.1	0.0	9.2	10.1	0.0
M. sativa	6.2	2.1	8.0	7.9	3.3

[a] Between 65 and 75 PMCs were observed
[b] Between 25 and 80 PMCs were observed
[c] Between 45 and 93 PMCs were observed
[d] Between 25 and 37 PMCs were observed

98, 89, 20, 22, 19, 33 and 57 the percentage of lagging chromosomes at AI was slightly lower, and in 2 cases (plants no. 9 and 13) higher, than that of unpaired chromosomes at MI, indicating that not all of the univalent chromosomes evolved as laggards at AI and, conversely, that some lagging chromosomes derived from the paired ones.

In some cases, the spindles were not oriented at right angles to define the poles of a tetrahedron and at MII parallel spindle (ranging from 3.4% to 20.8%) and tripolar spindle (ranging from 0% to 13%) orientations were present at similar frequencies. In the somatic hybrids the percentage of cells with abnormal spindle orientations was not different from that of the parental plants. As a consequence of spindle disorientation, sporad types with reduced and unreduced nuclei were detected at various frequencies: the frequency of dyads and triads ranged from 0% to 9.5% (Table 5). At AII the large majority of PMCs with lagging chromosomes displayed 1–4 laggards, but in a few cases 10 or more laggards were detected that could have originated pentads.

The chromosome number of 20 offsprings was analyzed: only 2 plants, with 45 and 44 chromosomes, had a ploidy different from the expected one (48 chromosomes).

Morpho-agronomical analysis

The evaluation of morphological characters in the somatic hybrids revealed considerable variability for the 11 traits considered. For the trait blooming time, the hybrids were found to be similar to the *M. sativa* parent, while for growth habit they were intermediate to, and significantly different from, both parents (Table 6). The latter trait was useful for the identification of hybrids at first sight when they were grown together with the parental plants: as shown in Fig. 4, the stems of *M. sativa* always stood upright, while those of *M. coerulea* were creeping and those of the hybrids were intermediate. Average length, width and weight of the trifoliate leaf were significantly higher in the somatic hybrid population than in the parents. In particular, width and length values were always higher in the hybrid leaflet than in *M. coerulea*, and in 80% of the cases, they were also higher than in *M. sativa*. Leaflet weight was higher in 81% of the hybrids than in *M. coerulea*; the difference

Table 5 Type of sporads produced by the somatic hybrids

Plant	Number of sporads	Dyads(%)	Triads(%)	Pentads(%)
98	97	2.94	3.92	3.92
9	392	1.02	2.04	6.8
89	107	0	0.9	3.7
54	214	0	2.8	6.5
20	286	0.6	2.1	6.5
22	231	4.3	6.9	4.7
19	167	0.6	1.2	5.9
13	241	0.4	1.7	7.1
33	221	9.5	4.5	2.8
57	124	4.8	0.8	0.8
M. coerulea	85	0.2	0.1	0.0
M. sativa	74	0.0	0.0	0.1

Table 6 Field evaluation of agronomic traits of the somatic hybrid population (*MS M. sativa, MC M. coerulea*)

Trait	Mean SE	Minimum	Maximum	MS	MC
Main stem diameter	2.24 ± 0.039	1.81	2.65	2.10**	2.15*
Blooming time	16.98 ± 0.627	10.14	25.00	16.33 ns	19.87**
Node number	12.12 ± 0.122	10.85	13.50	14**	13**
Leaflet width	1.17 ± 0.019	0.96	1.38	1.02**	0.57**
Leaflet length	2.35 ± 0.037	1.93	2.76	2.03**	1.70**
Leaflet weight	0.16 ± 0.004	0.10	0.19	0.12**	0.14**
Stem length	56.04 ± 1.10	44.59	67.50	55.77 ns	60.96**
Stem number	18.91 ± 0.941	9.93	23.72	24.67**	25.62**
Stem weight/plant	33.21 ± 1.191	22.70	37.53	34.39 ns	23.21**
Leaf weight/plant	34.41 ± 2.271	11.18	36.36	35.72 ns	19.94**
Growth habit	3.36 ± 0.131	1.38	4.57	4.67**	1.00**

* Values not significantly different for $P \leq 0.05$
** Values not significantly different for $P \leq 0.01$

Fig. 4 Somatic hybrid phenotype: *M. sativa* (*left*), somatic hybrid (*middle*), *M. coerulea* (*right*)

was, however, significant for 5 hybrids only. It is concluded that in the somatic hybrids stem diameter and width, and length and weight of the intermediate leaf showed heterosis.

In contrast, the average number of stems per hybrid plant and the number of nodes on the main stem were observed to be significantly lower than those of both parents. The effect of the reduced number of stems and nodes on the forage yield of the hybrid plants was counterbalanced by their higher leaf weight and stem diameter, so that leaf and stem weight per plant exhibited average values not significantly different from those of *M. sativa*, the more productive parent.

Discussion

RFLP analysis of somatic hybrids revealed a biased incorporation of parental alleles. The tetraploid parent was almost always entirely represented, while some DNA fragments specific to the diploid parent were missing in the hybrid genome. Chromosome loss is a frequent event in inter-specific sexual crosses (Lang 1971; Ramanna and Hermsen 1971), and in somatic hybrids chromosome elimination is enhanced by tissue culture (D'Amato 1985). In inter-specific somatic hybrids the spontaneous loss of chromosomes has been reported in *Solanum* (Pehu et al. 1989; Austin et al. 1986), *Brassica* (Sundberg and Glimelius 1991) and *Medicago* (Pupilli et al. 1992). As we focused our analysis on *M. sativa* (2n = 4x = 32) + *M. coerulea* (2n = 2x = 16) somatic hybrids, which were selected on the basis of their chromosome number being equal to the sum of the parental chromosomes, the loss of some DNA fragments is unlikely to result from chromosome elimination. However, due to the low resolution of the cytological analysis, any eventual loss of chromosome segments cannot be totally excluded. The absence of the 1H11/*Alu*I MCSB (Fig. 1a) in our hybrids provides evidence for the occurrence of a deletion event. Since no other flanking sequences were available as probes for further analyses, no information could be obtained on the extent of the deletion. Similar results have been reported in potato, where the loss of a fragment located between two retained markers spanning 16 cM was observed (Williams et al. 1990). Scowcroft et al. (1983) suggested that chromosome rearrangements in the form of deletions and consequent translocations or inversions could be the causes of the phenotypic variation that is frequently observed in regenerated plants. In our material, the loss of MCSBs could have occurred during the growth as callus of the *M. coerulea* parent before fusion, thereby explaining the asymmetrical allele composition of the somatic hybrids. The MCSBs missing in the hybrids were located on different linkage groups, indicating that the rearrangements were not restricted to a particular chromosome region but occurred at random in the *M. coerulea* genome. In *M. coerulea*, probe/enzyme combination 1H11/*Rsa*I revealed the existence of 2 alleles represented by fragments of 1100 and 800 bp (Fig. 1b); only 1 of them was still present in a hybrid plant, suggesting that the rearrangement concerned only 1 of the 2 homologous chromosomes.

The fate of the *M. coerulea* genome in the somatic hybrids could only be assessed for those DNA fragments polymorphic with respect to *M. sativa*. To enhance the probability of detecting polymorphism between the parents, we used the four-cutter enzyme/polyacrylamide gel system capable of resolving DNA fragments differing for

only 12–15 bases (Gebhardt et al. 1989). In our study the evolution of the MCSBs present in the hexaploid hybrids was recorded in their polycross progeny using five probes in a population of 33 individuals. As the 15 somatic hybrid plants intercrossed were almost identical from a molecular point of view, their polycross progeny could be considered to be a selfed offspring. As expected, this population segregated in a Mendelian fashion (3:1) for RFLP alleles derived from both parents.

Williams et al. (1990) studied the sexual segregation of a hexaploid somatic hybrid derived from fusion of a tetraploid *S. tuberosum* with a diploid *S. brevidens*. The *S. brevidens*- derived RFLP bands (SBBs) did not segregate because this parent was highly homozygous and the nulliplex individuals (lacking all copies of a marker) for SBBs were expected to derive from inter-genomic chromosome pairing. In our material, considering the high level of heterozygosity of both parents, the presence of nulliplex individuals for MCSBs in the polycross progeny of somatic hybrids is likely due to the segregation of *M. coerulea* alleles. The fact that few non-segregating MCSBs did not show any nulliplex individual indicated that no inter-genomic pairing took place for these loci.

A common feature of spontaneous and cross-derived hexaploid *M. sativa* plants is chromosomal instability (McCoy and Bingham 1991) due to the high percentage of univalent chromosomes that lag at successive anaphases, subsequently producing gametes deficient in 1 or a few chromosomes. In our somatic hybrid population the average percentage of univalent chromosomes at MI was 13%, a remarkably lower value than those reported by Mariani (1975) for a spontaneous hexaploid and by Bingham and Binek (1969) for cross-derived hexaploids. Meiotic abnormalities at frequencies similar to those recorded in cultivated alfalfa varieties (Mariani et al. 1978) were found in our somatic hybrids. Analysis of the chromosome number in the polycross progeny of somatic hybrids confirmed these observations: only 10% of the plants showed a chromosome number lower than 48, a value slightly higher than the one scored in the tetraploid cultivars 'Saranac' and 'Vernal' (Bingham 1968). Conversely, the progenies of hexaploid plants from sexual origins exhibited chromosome numbers ranging from $2n = 33$ to $2n = 51$ (Yen and Murphy 1979) and from $2n = 21$ to $2n = 52$ (Smith et al. 1984). In our materials, chromosomes were almost completely organized in bivalents at meiosis and only occasionally were quadrivalents observed at diakinesis (data not shown). There was a slightly higher percentage of abnormal spindles in the somatic hybrids than in the parents: the percentages of triads and tripolar spindles were of the same magnitude, while the percentage of dyads was remarkably lower than the value expected on the basis of the frequency of parallel spindles. These findings can be explained by considering the fact that parallel spindles at MII can produce either normal tetrads or dyads (Ramanna 1979). In particular, in alfalfa it has been reported (Tavoletti et al. 1991) that dyads were the consequence of parallel, or nearly parallel, spindles at MII. In tetraploid alfalfa, lagging chromosomes at AII can originate irregular quartets with micronuclei (McCoy and Bingham 1991). In our somatic hybrids, a few pentads, in addition to micronuclei, were detected as the final product of meiosis; this type of microspore could derive from the formation of an extra pole at AII when a relatively high number of lagging chromosomes occur. A relatively good meiotic stability of somatic hexaploids may result from a tendency of *M. coerulea* chromosomes to preferentially pair.

Forage yield in alfalfa has long been reported to be positively correlated with the level of heterozygosity (Dunbier and Bingham 1975) and with the presence of favorable allele combinations (Demarly 1963). Under field conditions the proportion of selfing is not negligible and depends on environmental conditions (Lesins 1961). A yield decrease in the next generation is to be expected when self-fertilization occurs during seed multiplication and, from this point of view, utilization of hexaploid varieties of alfalfa could be more profitable. Their higher ploidy level may in fact minimize inbreeding depression: decline of hybrid vigor is expected to be slower in hexaploids than in tetraploids over subsequent selfing generations. For a long time the production of hexaploid alfalfa was discouraged due to its poor seed set (Nilsson and Andersson 1941; Armstrong 1955). More recently, Lesins et al. (1975) reported that seed yield was not a problem when the plants were derived from parents pre-selected for seed production. The most important requirement for an alfalfa variety is high forage yield. Our hexaploid somatic hybrids, despite a high degree of heterozygosity, a high ploidy level and high values of stem diameter, width, length and weight of leaves, did not outperform the tetraploid parent in leaf and stem weight, the traits most correlated to forage production. The somatic hybrids showed a forage production per plant significantly higher than that of the diploid parent and similar to that of *M. sativa*. The number of stems per plant, which was about the same in both parents (25.67 and 25.62 for *M. sativa* and *M. coerulea*, respectively; Table 6), was, however, unaccountably reduced in the somatic hybrids. Our results are in agreement with those of Lesins et al. (1975), who found that forage yields of some hexaploid progenies were comparable with that of a tetraploid cultivated variety.

In conclusion, in the genus *Medicago* inter-specific somatic hybrids seem to be an interesting material for plant breeding since the parental genomes are almost completely transmitted to the progeny, the frequency of meiotic alterations is low, the sexual progeny displays chromosome stability and the morphological traits are similar or superior to those of the tetraploid parent.

Acknowledgements The authors wish to thank Prof. F. Salamini for his critical reading of the manuscript. Thanks are also due to Dr. A. Mariani for her helpful comments and to Prof. J. Bouton, Dept. of Crop and Soil Sci., Athens, Georgia 3062-7272 USA for providing the alfalfa clones. The technical assistance of A. Bolletta in preparing the photographic plates is also acknowledged.

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