Theor Appl Genet (1996) 93: 801–808
© Springer-Verlag 1996

P. D. Cluster · O. Calderini · F. Pupilli · F. Crea
F. Damiani · S. Arcioni

The fate of ribosomal genes in three interspecific somatic hybrids of *Medicago sativa*: three different outcomes including the rapid amplification of new spacer-length variants

Received: 4 April 1996 / Accepted: 26 April 1996

Abstract We have characterized the genetic consequences of somatic hybridization within the ribosomal DNA (rDNA) of three interspecific hybrids, each involving *M. sativa* as one of the parents. Restriction-fragment-length-polymorphisms (RFLPs) of rDNA spacers and fluorescent-in-situ-hybridization (FISH) of an 18S-gene probe to mitotic chromosomes were used to compare parental and hybrid species. The *M. sativa-coerulea* hybrid retained all six parental nucleolar-organizing regions (NORs) and all parental RFLPs representing a complete integration of rDNA. The *M. sativa-arborea* hybrid retained five of six parental NORs while losing half of the *arborea*-specific RFLPs, indicating that simple chromosome loss of one *arborea* NOR accounted for the RFLP losses. Dramatic alterations occurred within the *M. sativa-falcata* hybrid where five of six parental NORs were retained and new rDNA RFLPs were created and amplified differentially among somaclonal-variant plants. The molecular basis of the new RFLPs involved increased numbers of a 340-bp subrepeating element within the rDNA intergenic spacer (IGS), suggesting that recurrent cycles of unequal recombination occurred at high frequency within the rDNA in somatic lineages.

Key words Alfalfa rDNA · Fluorescent in situ hybridization · rDNA IGS variation · Somaclonal variation · Unequal recombination

Communicated by R. Hagemann

P. D. Cluster[1] · O. Calderini · F. Pupilli F. Crea · F. Damiani
S. Arcioni (✉)
CNR Istituto di Ricerche sul Miglioramento Genetico della Piante Foraggere, Via Madonna Alta 130, I-06128 Perugia, Italy

Present address:
[1] Plant Breeding Laboratory, Faculty of Agriculture, University of Kyoto, 606-01 Kyoto, Japan

This paper was supported by the National Research Council of Italy, Special Project RAISA, Sub-project No. 2, Paper No. 1077

Introduction

Somatic hybridization holds great potential for recombining important traits beyond the boundaries of sexual compatibility. Genetic characterization of the somatic hybrids regenerated to date has shown that genome rearrangements occur from the chromosomal to the molecular levels (Williams et al. 1990; Sundberg and Glimelius 1991; Pupilli et al. 1995). They include large-scale chromosome losses, deletions and translocations and small-scale gains and losses of restriction fragment length polymorphisms (RFLPs) and allozymes. Similar mutational variations can also result simply from the regeneration of genetically uniform lines through tissue culture (see Kidwell and Osborn 1993 for discussion and references).

Three interspecific somatic hybrids have combined the genome of tetraploid alfalfa (*M. sativa* L.; 2n=4x=32) with other *Medicago* species by protoplast electrofusion. Two of the hybrids were formed with closely related diploids from the *M. sativa* species complex, namely *M. coerulea* and *M. falcata*, both of which are capable of sexual hybridization and gene exchange with *M. sativa* in nature (McCoy and Bingham 1988). *M. coerulea* (2n=2x=16) combined with *M. sativa* to form the *S+C* hybrid (Pupilli et al. 1992), which contained a complete 48-chromosome complement and displayed nearly-normal meioses. However, 29% of the *coerulea*-specific genomic and cDNA RFLPs (vs. less than 3% from *sativa*) were missing, indicating that numerous small-scale deletions and/or rearrangements occurred in the *coerulea* portion of the hybrid genome. *M. falcata* (2n=2x=16) combined with *M. sativa* to form the *S+F* hybrid (unpublished data), showing a loss of 15 of 48 chromosomes (31%). The third somatic hybrid involved the distantly related tetraploid shrub *M. arborea*, which is incapable of sexual hybridization with *M. sativa* and is the sole species of a separate section of the *Medicago* genus (McCoy and Bingham 1988). *M. arborea* (2n=4x=32) combined with *M. sativa* to form the *S+A* hybrid (Nenz et al. 1996). It lost 7 of 64 chromosomes (11%) and more than 33% of the species-specific RFLPs from each parent, in-

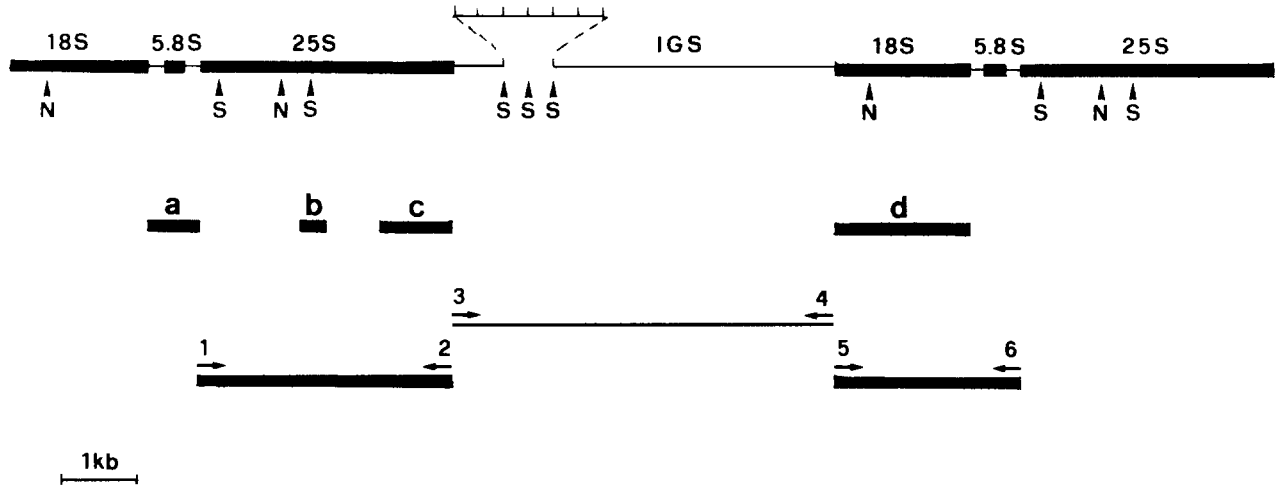

Fig. 1 Diagram of the *M. sativa* IGS and flanking rDNA gene regions. Coding sequences are represented by *thick lines* and noncoding by *thin lines*. The primary length-variable region of the IGS is shown as an expanded group of subrepeated elements. *Nco*I sites (*N*) were determined from genomic Southerns and *Ssp*I sites (*S*) from PCR products. Probes are labelled *a–d*: *a* 5.8S, *b* 25S-B, *c* 25S-C, *d* 18S. PCR primers are shown as *arrows above* their respective products and labelled *1–6*: *1* 25S1, *2* 25S2, *3* CLN12, *4* SP2, *5* NS1, *6* ITS4

dicating that both large- and small-scale rearrangements occurred.

The ribosomal DNA (rDNA) gene family encodes the 18S, 5.8S and 25S ribosomal RNAs. Plants typically contain thousands of tandemly repeated rDNA genes organized into one or a few loci where the highly conserved coding sequences of adjacent repeat units are separated by sequence- and length-variable intergenic spacer (IGS) regions (for reviews see Long and Dawid 1980; Jorgensen and Cluster 1988).

IGS sequences evolve rapidly yet the IGS regions have maintained secondary structural similarities in all eukaryotes; they contain small (100–400 bp) subrepeating elements (Fig. 1) that may act as transcription enhancers (Reeder 1989) and possibly terminators. The number of subrepeats within the IGS can vary widely between individuals and thus form the basis for RFLP studies of variation (Saghai-Maroof et al. 1984; Flavell et al. 1986) and adaptation (Cluster et al. 1987; Allard et al. 1990; Rocheford 1994; Cluster and Allard 1995). Both the length of IGS variants and rDNA gene copy number can change rapidly within the somatic lineages of some plants (Rogers and Bendich 1987).

Fluorescent-in-situ-hybridization (FISH) cytology studies with a labelled 18S rDNA probe among the species of the *M. sativa* complex showed that the diploids *M. coerulea* and *M. falcata* each contain one rDNA locus (two homologous rDNA sites) while the tetraploid *M. sativa* contains two loci (Calderini et al. 1995 and unpublished data). All of the rDNA sites in these three species were found to be transcriptionally active by means of positive silver-nitrate staining (Hubbell 1985) and also to be capable of organizing nucleoli independently.

We have investigated both the small- and large-scale genetic consequences of somatic hybridization in the rDNA gene family. The results include an RFLP analysis of IGS-length variation in four parental *Medicago* species and three pairwise somatic hybrids. Also presented are the results of cytological FISH analyses (to identify chromosomal rDNA sites) and silver-nitrate staining (to determine the transcriptional activity at rDNA sites) in mitotic cell preparations from the *M. arborea* parent and the three hybrids.

Materials and methods

Plant materials

Parental lines *M. sativa* L. (4x; cv 'Rangelander 15'), *M. coerulea* (2x; one seed from All Union of Plant Industry, St. Petersburg, Russia) and *M. falcata* (2x: WD88 from Prof. E.T. Bingham, University of Wisconsin) were propagated by cuttings. *M. arborea* (4x) lines came from seeds of a protoplast-derived plant of Mariotti et al. (1984). Three plants of each parent studied were uniform for rDNA RFLPs. Six *S+C* somaclonal plants (denoted SC1–SC6). 4 *S+A* (SA1–SA4) plants and 9 *S+F* (SF2–SF10) plants were studied. All somaclones within hybrid groups came from one protoplast fusion event.

DNA preparation

The following protocol was modified from a combination of the CTAB and potassium acetate-based procedures of Murray and Thompson (1980), Dellaporta et al. (1983) and Saghai-Maroof et al. (1984). Removal of the polysaccharides was enhanced when CTAB extraction preceded alcohol precipitation of the DNA. Yields were 80–100 µg of high quality DNA from 2 g of leaves. The procedure was effective in monocots, dicots and difficult floral tissues.

Two grams fresh tissue were ground in liquid nitrogen, transferred to an Oakridge tube with 30 ml extraction buffer (100 m*M* Tris pH 8; 50 m*M* EDTA; 100 m*M* NaCl; 500 m*M* sucrose), mixed and centrifuged 15 min at 2000 *g* to pellet chromatin. The pellet was resuspended in 12 ml resuspension buffer (extraction buffer without sucrose) plus 600 ul 20% SDS and incubated 15 min at 65°C. One-third volume 5 *M* KOAc was added, mixed, chilled on ice 30 min and centrifuged 15 min at 15 000 *g*. The supernatant was transferred to a 50-ml Falcon tube, one-half volume CTAB buffer (100 m*M* Tris pH 8; 50 m*M* EDTA; 2.25 *M* NaCl; 3% w/v CTAB) was added, followed by incubation for 20 min at 65°C with mixing. CTAB was extracted twice with equal volumes of chloroform/isoamyl alcohol

Table 1 Heterologous rDNA PCR primers

Primer	Sequence[a]	Reference
NS1	GTAG TCAT ACGC TTGT CTC	White et al. 1990
NS8	TCCG CAGG TTCA CCTA CGGA	White et al. 1990
ITS1	TCCG TAGG TGAA CCTG CGG	White et al. 1990
ITS4	TCCT CCGC TTAT TGAT ATGC	White et al. 1990
25S1	GCAT ATCA ATAA GCGG AGGA	White et al. 1990
25S2	CCGT CGCG TATT TAAG TCGT C	Polanco and Perez De La Vega 1994
CLN12	CTGA ACGC CTCT AAGT CAG	Duchesne and Anderson 1990
SP2	GAGA CAAG CATA TGAC TAC	White et al. 1990

[a] Primer sequences are written 5′ to 3′

(24·1). A two-thirds volume of isopropanol was added, mixed and DNA was pelleted at 2000 *g* for 20 min. The pellet was soaked in 70% EtOH for 1 h, dried, resuspended in 300 µl TE, transferred to a 1.5-ml tube and reprecipitated with 100% EtOH.

Southern blots

DNAs (1 µg) were restriction-digested with 10 U of 11 enzymes (New England Biolabs): *Ase*I, *Bcl*I, *Dra*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Nco*I, *Nsi*I, *Sph*I, *Ssp*I and *Xho*I. Digests were electrophoresed in 1% agarose, blotted onto Hybond N+ membrane (Amersham) and hybridized to [^{32}P]-dCTP-labelled (Ready To Go kit, Pharmacia) probes at 65°C according to the manufacturer's instructions. Four rDNA probes were used (Fig. 1). The 18S probe (1.8 kb) was polymerase chain reaction (PCR)-amplified (see below) from *M. sativa* DNA with heterologous fungal NS1/NS8 primers, and the 5.8S probe (700 bp) was a PCR product of ITS1/ITS4 (Table 1). Amplifications of 18S and 5.8S rDNA were specific to the rDNA. PCR products were cross-hybridized to appropriate fragments of flax rDNA from pBG35 (Goldsbrough and Cullis 1981) to verify them. Probes 25S-B (800 bp) and 25S-C (850 bp) were *Eco*RI/*Bgl*II double-digest fragments of the flax 25S gene of pBG35 isolated as bands cut from low-melting agarose (LMA; FMC BioProducts) and labelled directly in LMA. A 400-bp sequence of 25S-B had homology to the 25S gene (the other 400 bp were pAT153 cloning-vector sequences). All 850 bp of 25S-C had homology to distal 3′ 25S sequences.

PCR amplification of the *M. sativa* rDNA gene

The rDNA repeat unit of *M. sativa* was PCR-amplified in three segments using heterologous primer pairs (Fig. 1). The 18S-5.8S segment (2.5 kb) was amplified with NS1/ITS4 primers, the 25S segment (3.5 kb) with 25S1/25S2 and the IGS (5.3 kb) with CLN12/SP2 (Table 1). 25S1 was the reverse complement of ITS4, 25S2 came from distal 25S *Avena* sequences. CLN12 was from fungal sequences and SP2 was the reverse complement of NS1.

PCR conditions for each 50 µl reaction were: 1.5 ng DNA, 12 pmol of each primer, 5 µl of 10x PCR buffer minus $MgCl_2$ from Gibco BRL (1 µl of *Taq* extender and 5 ul of extender buffer from Stratagene were substituted for PCR buffer for IGS amplification), 200 µ*M* of each dNTP, 1 m*M* $MgCl_2$ and 5 U *Taq* DNA polymerase (Gibco BRL). Reagents were mixed and amplification done on an MJ Research Thermal Controller by "touchdown" PCR (Don et al. 1991) as follows: (1) hot start at 96°C for 1 min 30 s, (2) melting at 96°C for 40 s, annealing at 60, 59, 58, 57 and 56°C for the first 5 cycles and annealing at 55°C for subsequent cycles for 35 s, and extension at 72°C for 3 min for a total of 40 cycles; and (3) final extension at 72°C for 10 min. PCR products were purified through Wizard PCR Prep columns (Promega).

Cytological procedures

For mitotic preparations, root tips were pretreated in ice water for 24 h followed by 3 h in alpha-bromonaphthalene at 4°C, fixed in alcohol:glacial acetic acid (3:1), washed in enzyme buffer (10 m*M* citric acid/sodium citrate, pH 4.8) and softened in 20% (v/v) pectinase (Sigma) plus 2% (w/v) cellulase (Calbiochem) in enzyme buffer for 1 h at 37°C. Root tips were squashed in 45% (v/v) acetic acid. Silver staining was carried out following the protocol of Stack et al. (1991) and FISH followed that of Orgaard and Heslop-Harrison (1994). The 18S probe was labelled with rhodamine-4-dUTP (FluoroRed; Amersham) using a nick translation kit (Boehringer Mannheim). Signals were examined with a Zeiss epifluoresence microscope.

Results and discussion

Variation in parental *Medicago* species

IGS RFLPs were surveyed among the four parental *Medicago* species by Southern blots using 11 restriction enzymes and two rDNA probes to identify the most informative enzyme/probe combinations. The 25S-C probe hybridized to conserved sequences immediately adjacent to the 5′ end of the IGS, and the 18S probe hybridized to sequences adjacent to the 3′ end. This strategy allowed the comparison of RFLPs accross species boundaries where IGS sequences are known to diverge.

*Ase*I, *Bcl*I, *Dra*I, *Eco*RI, *Nco*I, *Nsi*I and *Ssp*I produced species-specific RFLP patterns with both probes. The patterns produced between parental species with different enzymes were consistent; RFLPs were generally longer or shorter depending on the enzyme used, but their positions relative to one another often remained the same indicating that the variation was due primarily to different IGS lengths. *Eco*RI, *Nco*I and *Ssp*I produced the most discriminating patterns among the parents and were used extensively in subsequent analyses.

*Nco*I cut the rDNA of all species at two conserved sites (once in the 18S gene and once in the 25S) producing intact IGS fragments with partial homology to both the 18S and 25S-C probes plus a conserved coding-sequence band at 3.4 kb. *Sativa*, *coerulea* and *falcata* each contained the same major (i.e. high-copy-number) IGS RFLP at 7.9 kb, establishing that these three species share in common a complete 11.3-kb gene that constitutes the larger part of their rDNA. Each of these species also contained approximately 5 minor (lower-copy-number) alternative IGS RFLPs, and these were sufficient to differentiate the species from one another. In contrast, *arborea* had a distinctly different RFLP pattern in two respects: its prominent

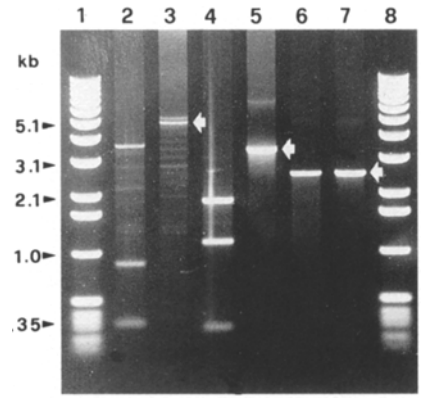

Fig. 2 IGS, 25S and 18S-5.8S PCR products (*arrows*) and their *Ssp*I-digested fragments *Lanes 1, 8* size markers, *lanes 2, 3* IGS cut and uncut, *lanes 4, 5* 25S cut and uncut, *lanes 6, 7* 18S-5.8S cut and uncut

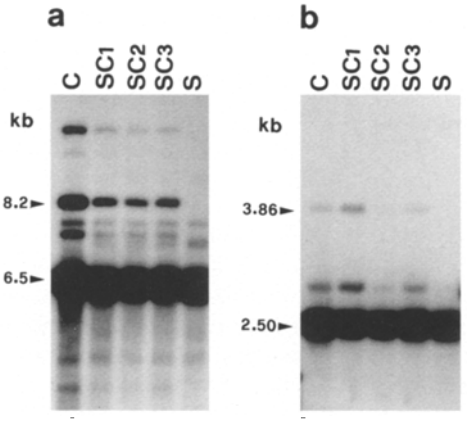

Fig. 3a, b IGS RFLP patterns from the *S+C* analysis with *Ssp*I. **a** Patterns with the 18S probe. **b** patterns with the 25S-C probe. *Lanes*: *C coerulea*, *SC1–SC3*. *S+C* somaclones. *S sativa*

IGS RFLP was 8.5 kb (reflecting a complete gene size of 11.9 kb), and the overall pattern contained more than 15 minor variants.

In all species, the sizes of the RFLPs generally differed in a step-ladder-like fashion characteristic of IGS length variation based on differing numbers of subrepeating elements. Although the observed variation indicated that more than one size class of subrepeats was present, most of the major variants differed by approximately 340 bp. This suggests that the *Medicago* IGS contained 340-bp subrepeating elements and that the number of elements per IGS was moderately variable.

PCR-based *Ssp*I restriction-site mapping

*Ssp*I restriction sites in the rDNA of *M. sativa* were mapped by PCR amplification of the entire gene in three segments followed by digestion of the products (Fig. 2). The resulting *Ssp*I fragments were analysed in Southern blots with four probes to determine their relative order. Comparisons between the digested PCR products and RFLP patterns in genomic Southerns confirmed the map in Fig. 1.

The 2.5-kb 18S-5.8S PCR product contained no *Ssp*I sites and hybridized to both the 18S and 5.8S probes. The 3.5-kb 25S product contained two *Ssp*I sites and produced three fragments of 2.0, 1.2 and 0.3 kb; the 2.0-kb fragment hybridized to both the 25S-B and 25S-C probes, the 1.2-kb fragment hybridized to the 25S-B probe and the 0.3-kb fragment did not hybridize to either probe. Amplification of the IGS produced 1 major 5.3-kb product plus several minor bands, possibly from alternative IGS variants and non-specific amplification. The major 5.3-kb IGS product contained at least two (and probably three) *Ssp*I sites resulting in fragments of 3.8 and 0.8 kb plus a band of 340 bp. From comparisons with genomic Southern patterns it was determined that the 3.8-kb fragment corresponded to the 3′ end of the IGS adjacent to the 18S gene and the 0.8-kb fragment to the 5′ end. The 340-bp band apparently contained at least two copies (per IGS) of an internally subrepeating element. Further analysis confirmed the presence of 340-bp subrepeats in the *Medicago* IGS that were variable for *Ssp*I sites (i.e. some subrepeats contained the *Ssp*I site and others did not).

rDNA in the *sativa-coerulea* hybrid

In genomic DNA digests, *Ssp*I cleaved the IGS within the subrepeating elements producing 2 IGS end-fragments per gene copy: 1 was contiguous with the downstream 18S gene and another contiguous with the upstream 25S. Thus, the 18S and 25S-C probes revealed variation from their respective ends of the IGS.

With the 18S probe, both *sativa* and *coerulea* contained major *Ssp*I RFLPs at 6.5 and 6.2 kb (Fig. 3a) but were differentiable by minor IGS variants. Hybrid *S+C* plants contained the complete combination of all parental RFLPs.

With the 25S-C probe, both *sativa* and *coerulea* contained a major *Ssp*I RFLP at 2.5 kb and *coerulea* alone contained 2 additional minor RFLPs (Fig. 3b). Again, all hybrid *S+C* plants contained all parental bands. Additional Southerns of *Eco*RI and *Nco*I digests gave similar results.

Signal intensities from the minor variants contributed by *coerulea* appeared diminished and sometimes variable in intensity in *S+C* patterns. Much of this effect reflected that *coerulea* contributed only one-third of the hybrid rDNA. However, the variable signal intensities of some bands suggest that minor copy-number changes may have occurred in *S+C* plants. No somaclonal variation for the qualitative presence or absence of RFLPs was detected.

When a fluorescent-labelled 18S gene was hybridized *in situ* (FISH protocol) to mitotic chromosomes of *S+C* six signals per nucleus were visualized (Fig. 4a). The rDNA sites were located at the secondary constrictions of 6 satellited chromosomes in a 48-chromosome complement. Silver nitrate stained positively at all six secondary constrictions (not shown), indicating transcriptional activity at all rDNA sites. This result reflected a complete unal-

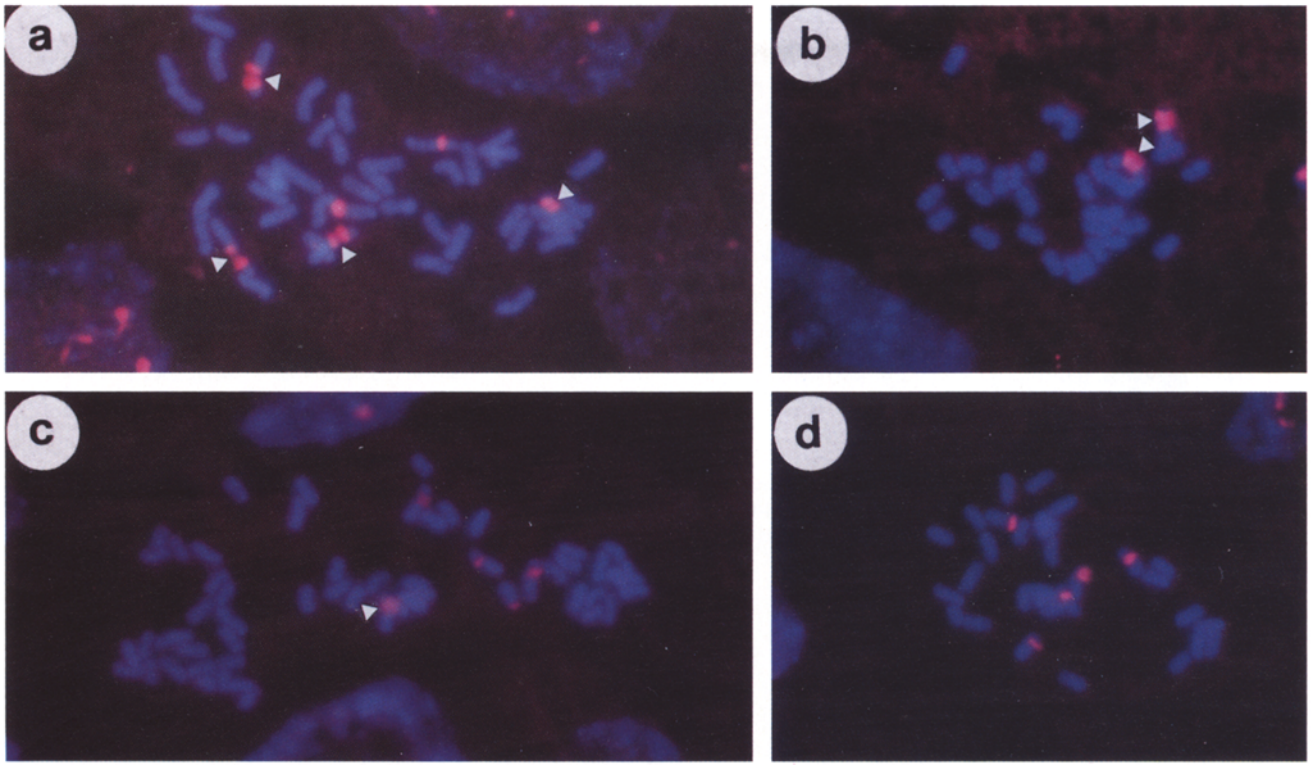

Fig. 4a–d 18S FISH to mitotic chromosomes. **a** *S+C* mitosis showing six FluorRed NORs. Four of the NORs (*arrows*) are stretched at secondary constrictions producing signal doublets. **b** *M. arborea* mitosis showing two FISH signals (*arrows*). The signal at the extreme right came from a separate interphase nucleus. **c** *S+A* mitosis. The large NOR (*arrow*) is suspected to have originated from the *arborea* parent. The signal at the extreme top of the figure belongs to a separate nucleus. **d** *S+F* mitosis

tered integration, structurally and functionally, of both parental karyotypes with *sativa* contributing four NORs and *coerulea* two.

We cannot rule out the possibility that limited rDNA copy-number changes could have occurred in *S+C*. However, based on these data we conclude that few, if any, significant genetic alterations occurred within the rDNA as a consequence of the somatic hybridization of *sativa* and *coerulea*.

rDNA in the *sativa-arborea* hybrid

With the 18S probe, *Ssp*I digests of *arborea* rDNA contained 1 major IGS RFLP at 6.5 kb as did *sativa* (Fig. 5a). The pattern of minor variants in *arborea*, however, was highly heterogeneous; numerous minor RFLPs formed a nearly complete 340-bp step ladder ranging from 4.5 to 14 kb. This extreme variability appears to have originated from different numbers of subrepeating elements within those IGS fragments downstream from the internal IGS *Ssp*I sites.

Hybrid *S+A* plants contained all of the RFLPs of both parents shown by the 18S probe. Also, 3–4 new *Ssp*I bands not present in either parent appeared in *S+A* plants. They were minor in signal intensity and greater than 14 kb, suggesting that limited changes may have occurred in the rDNA. Similar minor alterations were observed in *Eco*RI and *Nco*I digests. These minor alterations in *S+A* plants were less conclusive than those found within the *sativa-falcata* hybrid (see below).

The 25S-C probe for upstream *Ssp*I IGS fragments in *S+A* plants showed a different result (Fig. 5b): *arborea* contained a major RFLP at 2.84 kb (vs. 2.5 kb for *sativa*) plus 5 minor variants. The *S+A* hybrids contained only the 2 major RFLPs (one from *sativa* and the other from *arborea*), while all minor *arborea*-specific variants were lost. No somaclonal variation was detected in the rDNA among *S+A* hybrid plants.

FISH of the 18S probe to parental *arborea* chromosomes showed two rDNA sites (one chromosome pair) in this tetraploid (Fig. 4b) in contrast to tetraploid *sativa* that contains four sites. Both *arborea* NORs consistently produced FISH signals approximately twice as large as those from *sativa*. The *S+A* hybrid contained only 57 of 64 parental chromosomes and only five of six rDNA sites which were located at the secondary constrictions of 5 satellited chromosomes. One of the five FISH signals was about twice the size of the other four, indicating that it may have come from the *arborea* parent (Fig. 4c). Silver nitrate clearly stained both NORs in the *arborea* parent. The NORs in the *S+A* hybrid were, however, relatively recalcitrant to silver nitrate staining. This was possibly due to the large cytoplasm and high chromosome number in *S+A*. Nevertheless, one large well-stained NOR plus two to four weakly-stained NORs were observed in many *S+A* mitoses (not shown). On this basis we conclude

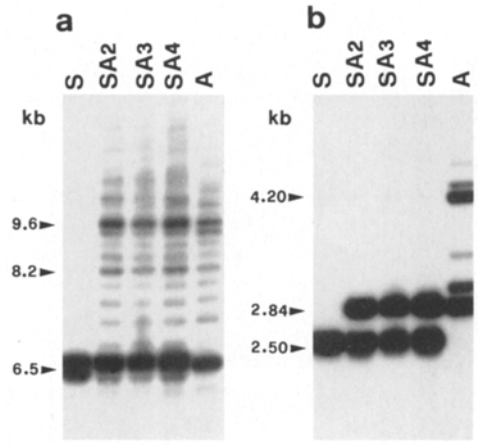

Fig. 5a, b IGS RFLP patterns from the *S+A* analysis with *Ssp*I. **a** Patterns with the 18S probe, **b** patterns with the 25S-C probe. *Lanes*: *S sativa*; *SA2–SA4*, *S+A* somaclones, *A arborea*

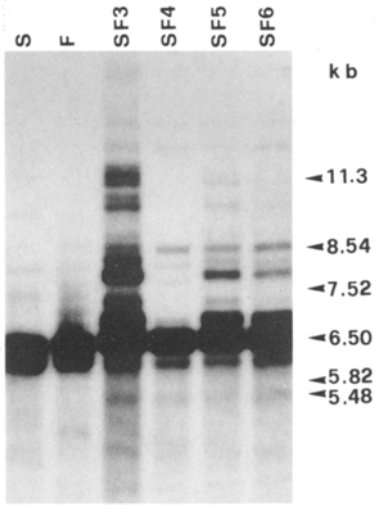

Fig. 6 IGS RFLP patterns from the *S+F* analysis with *Ssp*I and the 18S probe. *Lanes*: *S sativa*, *F falcata*, *SF3–SF6*, *S+F* somaclones

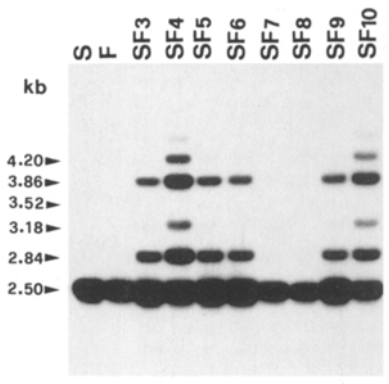

Fig. 7 IGS RFLP patterns from the *S+F* analysis with *Ssp*I and the 25S-C probe *Lanes*: *S sativa*, *F falcata*, *SF3–SF10 S+F* somaclones

that all five *S+A* NORs retained some transcriptional activity.

Taken together, these data suggest the most likely explanation is that the *S+A* hybrid retained only 1 of 2 NOR-containing chromosomes from *arborea*. Inherent is the conclusion that 1 *arborea* NOR chromosome contained all the minor *Ssp*I variants seen with the 25S-C probe and was among those chromosomes lost during hybrid formation. The other *arborea* NOR chromosome was retained; it contained all the minor variants seen with the 18S probe and apparently underwent minor rDNA alterations.

rDNA in the *sativa-falcata* hybrid

IGS RFLPs from the *sativa* and *falcata* parents were very similar with all enzyme/probe combinations. These parentals were differentiable only by a few weak-signal-intensity bands, and both contained the major *Ssp*I RFLPs at 6.5 and 2.5 kb with the 18S and 25S-C probes, respectively.

RFLP patterns among *S+F* hybrid plants showed numerous deviations from those of the parentals; *S+F* plants contained new IGS length-variant RFLPs not seen in either parent, and the signal intensities of many of them suggested that, once created, their copy numbers were multiplied (Figs. 6 and 7). Somaclonal variation existed among different *S+F* plants for both the presence of new IGS variants and for the extent of their copy-number amplification. These results were confirmed in *Eco*RI, *Nco*I and *Ssp*I digests with both probes.

The new length variants appeared primarily at 340-bp intervals, indicating that most of them contained altered numbers of the 340-bp subrepeating element. With the downstream 18S probe, some new IGS variants were shorter than the major parental RFLPs, while others were longer, indicating that the mechanism involved in their creation was capable of decreasing as well as increasing the number of IGS subrepeats (Fig. 6). With the upstream 25S-C probe, only longer new variants were found (Fig. 7). Several predictable size classes of new variants in the 340-bp step-ladder were never seen in *Ssp*I digests (note that no new variants appeared at 5.82 or 7.52 kb in Fig. 6 nor at 3.52 kb in Fig. 7). The variable presence of *Ssp*I sites among subrepeats is suspected to be responsible for at least some of the missing classes. Because new variants were found in *Ssp*I digests with both probes it appears that they originated by changes in the IGS both upstream and downstream from the internal IGS *Ssp*I sites.

Another type of new RFLPs ranging in size from 10 to 20 kb was observed with the 18S and 25S-C probes in *Eco*RI, *Nco*I and *Ssp*I digests in several *S+F* plants (Fig. 6). These large RFLPs probably did not simply contain long IGSs because they also hybridized to the internal-coding-sequence 5.8S probe in *Nco*I digests (not shown). Conserved *Nco*I sites in coding regions must be lost for this to occur. It is also unlikely that DNA methylation was responsible because *Eco*RI and *Nco*I are not sensitive to normal plant methylation patterns and *Ssp*I is not known to be sen-

sitive to any methylation. It appears therefore that this group of new variants resulted from the loss of normal restriction sites through coding and IGS regions, which could be due to sequence disruptions or complex rearrangements, possibly relegating some gene copies to a nonfunctional state.

18S FISH to mitotic *S+F* chromosomes showed five signals within the 33-chromosome complement (Fig. 4d). Thus, one NOR region of the six parentals was lost. Silver nitrate also stained positively at five secondary constrictions (not shown) demonstrating that all *S+F* NORs were transcriptionally active. We cannot determine from these data if the missing NOR originated from *sativa* or *falcata*.

The mechanism(s) responsible for the new rDNA variability in *S+F* plants must explain four consequences: (1) creation of new IGS variants, (2) copy-number amplification of the new variants, (3) differential amplification of new variants within and between plants and (4) the disruption of normal restriction-site periodicity extending through one or more complete gene copies at a time.

Unequal recombination operating at a high frequency in recurrent cycles is the mechanism most likely responsible because it is capable of producing all of the alterations. It is known to create copy-number variation in tandemly repeated gene families (Smith 1973, 1976), and its action in somatic lineages between chromatids and homologous and nonhomologous chromosomes is considered prominent in gene-family evolution (for review see Flavell 1982).

The rDNA contains two levels of tandem repetition: subrepeated IGS elements are nested within the larger gene-family repeats. Presumably, unequal exchanges among IGS subrepeats created most of the new variants observed, and recurrent misalignment and exchanges among the larger gene-family repeats amplified their copy numbers. Ongoing cycles of exchange in independent cell lineages would have created the differential amplification of new variants within and between plants. The observed sequence disruptions were likely a secondary consequence where recombined, but misaligned, strands became sequence altered by the correction of base-pair mismatches. Cycles of unequal exchange may have begun early within the callus tissue since all eventual shoots contained some rDNA changes.

Alterations in the rDNA are not novel, and unequal (or abnormal) exchanges are usually considered possible sources of variation. The data presented here, however, indicate that unequal exchanges are predominantly responsible. The clear step-ladder-like addition of progressively greater numbers of subrepeats in upstream IGS regions (Fig. 7) are a precise prediction of such a model. New 340-bp variants were created with precision in localized upstream IGS regions and a cascade of related alterations were also manifested extending through downstream IGS regions (Fig. 6) and coding regions (i.e. sequence disruptions) in significant portions of the gene family, as was expected from unequal exchanges.

In the legume *Vicia faba*, a 325-bp subrepeated rDNA IGS element was found to have approximately 75% homology to a related repetitive DNA family located in heterochromatic regions throughout the karyotype (Maggini et al. 1991). In the case of *V. faba* and others, rDNA subrepeats and related elements may thus be capable of interacting and migrating in the genome. While it is conceivable that the new variants in *S+F* could have involved interactions between IGS subrepeats and a related gene family, the data do not support this alternative. All probes were homologous to rDNA coding regions, not to the IGS subrepeats, thereby eliminating the possibility that the variants in Southerns were hybridization products from a separate subrepeat-related gene family. The sizes of all rDNA variants were consistent with predictions from the rDNA restriction map, and in situ hybridization to mitotic chromosomes showed no signals other than those from secondary ribosomal constrictions. If a separate gene family had been involved in exchanges or interactions with the rDNA, it is likely that rDNA-homologous sequences would have been translocated or redistributed in the karyotype. This did not occur.

Somatic hybridization creates formidable stresses that hybrid cells, and eventually plants, must overcome. The *S+F* hybrid endured a particularly stressful period during its formation as evidenced by the fact that the emergence of *S+F* plantlets from tissue culture required nearly twice the time as did the *S+C* and *S+A* hybrids (unpublished data). The *S+F* hybrid thus required a longer than usual period of genetic and metabolic adjustment, and one outcome appears to have been the induction of high-frequency somatic recombination in the rDNA.

Several examples of stress-induced changes of rDNA copy number have been reported in plants. Different environments induced changes in flax (Cullis 1977, 1981), and tissue culture resulted in changes in potato (Landsmann and Uhrig 1985), triticale (Brettell et al. 1986) and alfalfa (Kidwell and Osborn 1993). Most of these reported changes involved reductions in rDNA copy number, but some featured increases that should also be a natural consequence of unequal exchanges.

It is unclear as to which specific metabolic or physiological conditions within tissue-cultured cells induce the variety of genetic alterations that have been observed. However, if abnormal DNA exchanges (abnormal either in terms of increased frequency or propensity for misalignment) are induced routinely among tandemly repeated sequences throughout the genome, such exchanges could be responsible for many diverse mutational events. One possibility leading to abnormal exchanges, for example, could be that DNA replication proceeds normally and that mitosis is delayed, allowing an inordinately long period for replicated strands to interact.

Contrasting results were evident when correlating taxonomic distances between the parental species with the severity of genetic alterations. Although at different ploidy levels, *sativa* and *coerulea* are very closely related and *falcata* is perhaps only slightly more distant, the *S+C* and *S+F* hybrids showed the greatest disparity with respect to rDNA changes. The *S+A* hybrid, formed with the distantly related *arborea*, was intermediate. We attribute this result

to random factors operating during hybrid formation and find that taxonomic relatedness did not necessarily predict the stability of rDNA integration.

Acknowledgements We are grateful to Dr. A. Mariani for assistance and discussions and to Sig. A. Bolletta for photography. This work was supported in part by a fellowship to PDC from the Italian CNR-PF Ricerche Avanzate Per Innovazioni Nel Sistema Agricolo (RAISA).

References

Allard RW, Saghai-Maroof MA, Zhang Q, Jorgensen RA (1990) Genetic and molecular organization of ribosomal DNA (rDNA) variants in wild and cultivated barley. Genetics 126:743–751

Brettell RIS, Pallotta MA, Gustafson JP, Appels R (1986) Variation at the NOR loci in triticale derived from tissue culture. Theor Appl Genet 71:637–643

Calderini O, Pupilli F, Cluster PD, Mariani A, Arcioni S (1995) A study of the nucleolar organizer regions of *Medicago sativa* chromosomes. Chromosome Res 3 [Suppl]:44–45

Cluster PD, Allard RW (1995) Evolution of ribosomal DNA (rDNA) genetic structure in colonial Californian populations of *Avena barbata*. Genetics 139.941–954

Cluster PD, Marinkovic D, Allard RW, Ayala FJ (1987) Correlations between development rates, enzyme activities, ribosomal DNA spacer-length phenotypes and adaptation in *Drosophila melanogaster*. Proc Natl Acad Sci USA 84:610–614

Cullis CA (1977) Molecular aspects of environmental induction of heritable changes in flax Heredity 38:129–154

Cullis CA (1981) Environmental induction of heritable changes in flax: defined environments inducing changes in rDNA and peroxidase isozyme band pattern. Heredity 47:87–94

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1 19–21

Don RH, Cox ET, Wainwright BJ, Baker K, Mattick JS (1991) "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008–4011

Duchesne LC, Anderson JB (1990) Location and direction of transcription of the 5S rRNA gene in *Armillaria* Mycol Res 94:266–269

Flavell RB (1982) Sequence amplification, deletion and rearrangement: major sources of variation during species divergence. In Dover GA, Flavell RB (eds) Genome evolution. Academic Press, London, pp 301–324

Flavell RB, O'Dell M, Sharp P, Nevo E, Beiles A (1986) Variation in the intergenic spacer of ribosomal DNA of wild wheat, *Triticum dicoccoides*, in Israel. Mol Biol Evol 3:546–558

Goldsbrough PB, Cullis CA (1981) Characterization of the genes for ribosomal RNA in flax. Nucleic Acids Res 9:1301–1309

Hubbell HR (1985) Silver staining as an indicator of active ribosomal genes. Stain Technol 60:285–294

Jorgensen RA, Cluster PD (1988) Modes and tempos in the evolution of nuclear ribosomal DNA. new characters for evolutionary studies and new markers for genetic and population studies. Ann Mo Bot Gard 75:1238–1247

Kidwell KK, Osborn TC (1993) Variation among alfalfa somaclones in copy number of repeated DNA sequences. Genome 36:906–912

Landsmann J, Uhrig H (1985) Somaclonal variation in *Solanum tuberosum* detected at the molecular level. Theor Appl Genet 71:500–505

Long EO, Dawid IB (1980) Repeated genes in eukaryotes. Annu Rev Biochem 49:727–764

Maggini F, Cremonini R, Zolfino C, Tucci GF, D'Ovidio R, Delre V, DePace C, Scarascia Mugnozza GT, Cionini PG (1991) Structure and chromosomal localization of DNA sequences related to ribosomal subrepeats in *Vicia faba*. Chromosoma 100:229–234

Mariotti D, Arcioni S, Pezzotti M (1984) Regeneration of *Medicago arborea* L. plants from tissue and protoplast cultures of different organ origin. Plant Sci Lett 37:149–156

McCoy TJ, Bingham ET (1988) Cytology and cytogenetics of alfalfa. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and alfalfa improvement. Agronomy, Crop Science, and Soil Science Societies of America, Madison, Wisconsin, pp 737–776

Murray MG, Thompson WF (1980) Isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nenz E, Pupilli F, Damiani F, Arcioni S (1996) Somatic hybrid plants between the forage legumes *Medicago sativa* L. and *Medicago arborea* L. Theor Appl Genet 93:183–189

Orgaard M, Heslop-Harrison JS (1994) Investigations of genome relationships between *Leymus*, *Psathyrostachis* and *Hordeum* inferred by genomic DNA:DNA *in situ* hybridization. Ann Botany 73:195–203

Polanco C, Perez De La Vega M (1994) The structure of the rDNA intergenic spacer of *Avena sativa* L.: a comparative study. Plant Mol Biol 25:751–756

Pupilli F, Scarpa GM, Damiani F, Arcioni S (1992) Production of interspecific somatic hybrid plants in the genus *Medicago* through protoplast fusion. Theor Appl Genet 84:792–797

Pupilli F, Businelli S, Caceres ME, Damiani F, Arcioni S (1995) Molecular, cytological and morpho-agronomical characterization of hexaploid somatic hybrids in *Medicago*. Theor Appl Genet 90:347–355

Reeder RH (1989) Regulatory elements of the generic ribosomal gene. Curr Opinions Cell Biol 1:466–474

Rocheford TR (1994) Change in ribosomal DNA intergenic spacer-length composition in maize recurrent selection populations. 1. Analysis of BS13, BSSS, and BSCB1. Theor Appl Genet 88:541–547

Rogers SO, Bendich AJ (1987) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol Biol 9:509–520

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Smith GP (1973) Unequal crossing over and the evolution of multigene families. Cold Spring Harbor Symp Quant Biol 38:507–513

Smith GP (1976) Evolution of repeated DNA sequences by unequal crossover. Science 191:528–535

Stack S, Herickoff L, Sherman J, Anderson L (1991) Staining plant cells with silver. I. The salt-nylon technique. Biotechnic Histochem 66:69–78

Sundberg E, Glimelius K (1991) Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within *Brassicaceae*. Theor Appl Genet 83:81–88

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Williams CE, Hunt GJ, Helgeson JP (1990) Fertile somatic hybrids of *Solanum* species. RFLP analysis of a hybrid and its sexual progeny from crosses with potato. Theor Appl Genet 80:545–551