Genomic organization of rDNA loci in natural populations of *Medicago truncatula* Gaertn.

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Medicago truncatula Gaertn. is an annual self-pollinating species characterized by a diploid complement 2n = 16 and low DNA content. It responds very well to transformation methods so it is used as a model species for Leguminosae. In contrast with the advanced studies in molecular biology, cytogenetic research has remained limited even though it is an extremely valuable approach to the analysis of the genome structure. In the present study we examined the chromosomal distribution of rDNA sequences in five natural populations of *M. truncatula*, explored the genomic diversity of this species and found markers for chromosome identification. FISH experiments revealed three distribution patterns of rDNA sequences, distinguished by one, two and three loci of 5S genes; 18S-5.8S-25S genes were always localized at a single locus. The results add information to the genome structure of *M. truncatula*, revealing a pattern of distribution of rDNA genes unobserved previously, which consists of 5S genes clustered at a single locus. The physical mapping of rDNA sequences is a first contribution towards the construction of a detailed molecular karyotype of *M. truncatula*.

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Annual species constitute the most numerous component of the genus *Medicago*. They are native to the Mediterranean basin from which they spread to areas of the world with mediterranean climate. These species, commonly named medics, are extremely heterogeneous in morphology, environmental adaptation and in chromosome number. The greater part are diploid with 2n = 16 and 2n = 14 and two are polyploid with 2n = 30 (QUIROS and BAUCHAN 1988). Because of some interesting characteristics such as rapid growth, large production of pods, viability of the seeds on soil for long periods of time and nitrogen fixation by means of the bacterial activity, medics received an increasing attention in the last decade (CRAWFORD et al. 1989; RUSSI et al. 1992). At present they are regarded as very promising species for sustainable agricultural systems as well as for environmental uses (BAUCHAN 1998). Numerous studies have also been carried out to understand their phylogenesis and to explore their germplasm (MARIANI and FALISTOCCO 1990; BRUNNER et al. 1995; DIWAN et al. 1997). One of the most studied species is *M. truncatula*, a diploid (2n = 16) self-pollinating plant with a small genome size (0.9–1.1 pg) and which responds very well to regeneration and transformation methods (BENNETT and LEITCH 1995; CHABAUD et al. 1996). Such characteristics make *M. truncatula* a very suitable species for basic genetic studies, and it has been considered a model species for Leguminosae, in general, and of alfalfa (*M. sativa* L.) in particular (BARKER et al. 1990).

The analysis of the genetic diversity and the construction of a linkage map based on recombinant lines have been the principal objectives of the research programmes using this species. At present, more then 200 genetic markers have been mapped over 6 linkage groups (BONNIN et al. 1996).

In contrast with the advances in the field of molecular biology, cytogenetic research has remained very limited even though it can offer an extremely valuable approach to the analysis of the genome structure of the species and to the understanding of intraspecific variations.

In recent years molecular cytogenetic techniques have opened new possibilities for chromosome study, particularly in those species that, like medics, have small and similar chromosomes, thus yielding scarce information when treated with traditional methods (MALUSZYNSKA and HESLOP-HARRISON 1993; SCHMIDT et al. 1994).

DNA fluorescent in situ hybridization (FISH) used in conjunction with fluorochrome staining has turned out to be a crucial tool for effective progress in the study of animal and plant chromosomes by overcoming limitations due to size and morphology. The FISH technique enables the location of genes or other DNA sequences to be visualized directly on metaphase chromosomes. The positions of the la-

belled sequences may provide useful information in studies of genome organization, chromosome evolution and cytotaxonomy (JIANG and GILL 1994).

The sites of the ribosomal genes (18S-5.8S-25S rDNA and 5S rDNA) constitute reliable landmarks for chromosome identification, so the detection of rDNA sequences by FISH is generally the first step toward the physical mapping of chromosomes (LEITCH and HESLOP-HARRISON 1992). Such genes represent two highly conserved families of repetitive DNA sequences in the eukaryotic genome. The 18S-5.8S-25S genes are present in hundreds of repeated units arranged in tandem arrays at the nucleolar organizer regions (NORs) and sometimes at other sites not associated with the NORs (LAPITAN 1992). The 5S rRNA have a similar organization, even though their position along the chromosome is not revealed by any morphological characteristics and their detection is possible only by applying in situ hybridization. In the higher eukaryotes such genes constitute a cluster independent from the 18S-5.8S-25S genes.

In the present study we analyzed the distribution of 18S-5.8S-25S and 5S ribosomal genes on chromosomes of *M. truncatula* by using double target in situ hybridization. Our purpose was to add more information on the genome structure of this species and to provide cytological markers useful for chromosome identification. The physical position of rRNA genes in *M. truncatula* has been previously investigated by CERBAH et al. (1999) who examined two selected lines, Jemalong J5 and R-108-1 and found a difference in the the number of 5S sites. We extended the FISH analysis to five natural populations from different areas of origin in order to better explore the genome variability of this species.

MATERIAL AND METHODS

Plant material

The material used for this study consisted of five natural populations collected and multiplied by the United States Department of Agriculture Plant Introduction Station, Pullman, Wash. (USA). The number of accessions and the countries of collection are the following: PI 295607 (Turkey), PI 384656 (Morocco), PI 442892 (Australia), PI 493295 (Portugal) and PI 493296 (Portugal).

Chromosome preparations and FISH

Seeds of each accession were germinated in petri dishes at room temperature. Actively growing root tips were excised when they were about 1 cm in length, pretreated in a saturated aqueous solution of α-bromonaphthalene for about 4 h and then fixed in ethanol acetic acid (3:1) overnight.

For chromosome preparations, the dropping method described by LEITCH et al. (1994) was followed.

Two heterologous probes were used for identification of ribosomal gene sites. Clone pTa71 contains a 9-kb *Eco*RI fragment of *Triticum aestivum* L. consisting of the 18S-5.8S-25S rRNA genes and nontranscribed spacer sequences (GERLACH and BEDBROOK 1979). Clone pXVI contains the complete gene of 5S rRNA and the spacer region of *Beta vulgaris* L. (SCHMIDT et al. 1994). Clone pTa71 was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation, while pXVI was labelled with biotin-11-dUTP (Sigma) using the polymerase chain reaction.

FISH was accomplished in the following way. Slides were pretreated with 100 μg/ml of RNase A in 2 × SSC (0.3 M Na Cl, 0.03 M sodium citrate) for 1 h at 37°C and washed three times in 2 × SSC. After incubation with 80 units/ml of pepsin (Sigma) in 10 mM HCl for 15 min at 37°C, the chromosome preparations were stabilized in freshly depolymerized 4 % (w/v) paraformaldehyde in water, washed in 2 × SSC, dehydrated in a graded ethanol series and air dried.

The hybridization mixture, consisting of 150 ng/μl of each labelled probe, 50 % (v/v) formamide, 10 % (w/v) dextran sulphate, 0.1 % (w/v) SDS (sodium dodecyl sulfate) and 300 ng/μl sheared salmon sperm DNA, was incubated for 10 min at 70°C and chilled on ice. Forty μl of hybridization solution were applied to each chromosome preparation and these were covered with a plastic coverslip. The hybridization mixture and the chromosomes were denaturated together at 70°C in a modified thermocycler for 5 min and the temperature was gradually decreased to 37°C. The hybridization was carried out overnight at 37°C, alternatively the time of hybridization was reduced to 3–5 h. Stringent washing consisted of 10 min in 20 % formamide (v/v) in 0.1 × SSC at 42°C and 10 min in 2 × SSC at room temperature. Detection of the probes labelled with digoxigenin and biotin was carried out simultaneously. The slides were transferred to detection buffer (4 × SSC/0.1 % Tween 20) for 5 min, treated with 5 % (w/v) BSA (bovine serum albumin) in detection buffer for 5 min and incubated in 20 μg/ml of sheep anti-digoxigenin antibody conjugated with FITC (Boehringer Mannheim) and 5 μg/ml of streptavidin conjugated with Cy3 in detection buffer containing 5 % (w/v) BSA for 1 h at 37°C. After incubation the slides were washed at 37°C in detection buffer three times for 8 min each. The slides were counterstained with 2 μg/ml of DAPI (4′,6-diamidino-2-phenylindole) and

then mounted in antifade solution Vectashield (Vector Laboratories). Slides were examined with a Microphot Nikon epifluorescence microscope. Photographs were taken on Fujichrome 400 color slide film, digitized with a film scanner (Polaroid). Contrast optimization and superimposition of images were performed with the software Adobe Photoshop.

RESULTS AND DISCUSSION

Double target in situ hybridization carried out with probe pTa71 labeled with digoxigenin and probe pXVI labeled with biotin revealed the organization of rDNA sequences in all the five populations of *M. truncatula*.

The results of the FISH experiments showed that the number of sites of 18S-5.8S-25S genes was constant among the accessions whereas that of the 5S genes was remarkably variable. On the basis of the different number of red fluorescent signals associated with the probe pXVI, three patterns of hybridization were distinguished. Metaphase chromosomes from the root tips of accessions PI 493296, PI 493295 and PI 384656, hybridized with the two probes and counterstained with DAPI, are shown in Fig. 1 as an example of distribution pattern of rDNA sequences detected.

The probe pTa71 revealed that, in each accession, ribosomal genes 18S-5.8S-25S are clustered in two sites. Metaphase chromosomes not excessively contracted showed green fluorescent signals associated with the secondary constrictions of the satellited chromosome pair identified with DAPI staining. The pair of loci of these rDNA sequences was always easily identified due to the large size and the high intensity of the signals. Additional minor sites were never observed.

The hybridization of the probe pXVI showed a wide range of distribution of 5S rDNA sequences. In accessions PI 442892 and PI 493296 two sites were detected, clearly visible in all metaphases analyzed with strong signals of considerable size. In accession PI 493295 the probe labelled four sites; they differed in signal intensity, in a pair of which being weaker than in the other. In accessions PI 295607 and PI 384656 six 5S rDNA loci were found. In both accessions the additional pair of signals was localized on chromosomes with the nucleolar organizer regions, in close proximity to the site mapped by the probe pTa71.

The chromosomes of *M. truncatula*, similarly to those of other annual *Medicago* species, are of small size and a rather uniform morphology. Such characteristics make the identification of individual chromosomes quite difficult, thus limiting possibilities for further cytogenetic investigations. Recent studies have demonstrated that in situ hybridization is a very effective method for improving chromosome analysis in the genus *Medicago*. By using repetitive sequences or total genomic DNA as probes it has been possible to examine the chromosome organization of some species and resolve cytotaxonomic and phylogenetic problems (CALDERINI et al. 1996; FALISTOCCO 2000).

In this study the chromosomal distribution of rDNA sequences was investigated to improve our knowledge of the genome structure of *M. truncatula* and to find markers useful for chromosome identification.

FISH experiments revealed three different patterns of distribution of rDNA sequences which were characterized by the presence of one, two and three loci of 5S genes. On the contrary 18S-5.8S-25S genes were always localized at a single locus. In plants with the highest number of rDNA loci, one of the 5S sites was located on the nucleolar chromosome in close proximity to the NOR. The presence of both 18S-5.8S-25S and 5S loci on the same chromosome is not rare. Among species of Triticeae this situation occurs frequently with the two loci carried by the same chromosome arm (CASTILHO and HESLOP-HARRISON 1995). In some species of *Hordeum* and *Silene* the two rDNA families were both localized in the same position (TAKETA et al. 1999; SIROKY et al. 2001). In *M. truncatula* the two loci appeared very close to each other but non juxtaposed since the hybrid signals did not overlap. A significant difference in the intensity of the hybridization signal was observed in the plants characterized by two 5S loci. This suggests that there is a difference in the copy number of the repeat unit between the loci. In fact, even though FISH is not a quantitative method, the strength of hybridization signal is generally considered related to the copy number of genes (MALUSZYNSKA and HESLOP-HARRISON 1993).

Patterns of hybridization similar to those described above have been reported in lines Jemalong J5 and R-108-1 of *M. truncatula* (CERBAH et al. 1999); however in R-108-1 with two sites of 5S genes, no differences in the dimension of these sites was observed. During this study a further variation of the distribution of ribosomal genes in *M. truncatula* was discovered, consisting of 5S genes clustered at a single locus.

Organization of rDNA sequences in plant genomes has been extensively investigated. The wide variation in the number and size of rRNA sites found in many species suggests that ribosomal genes have undergone substantial quantitative changes during the evolution of related taxa. Changes may affect the number of loci within the genome and the copy number of the repeat units at single loci (FLAVELL 1989). On the

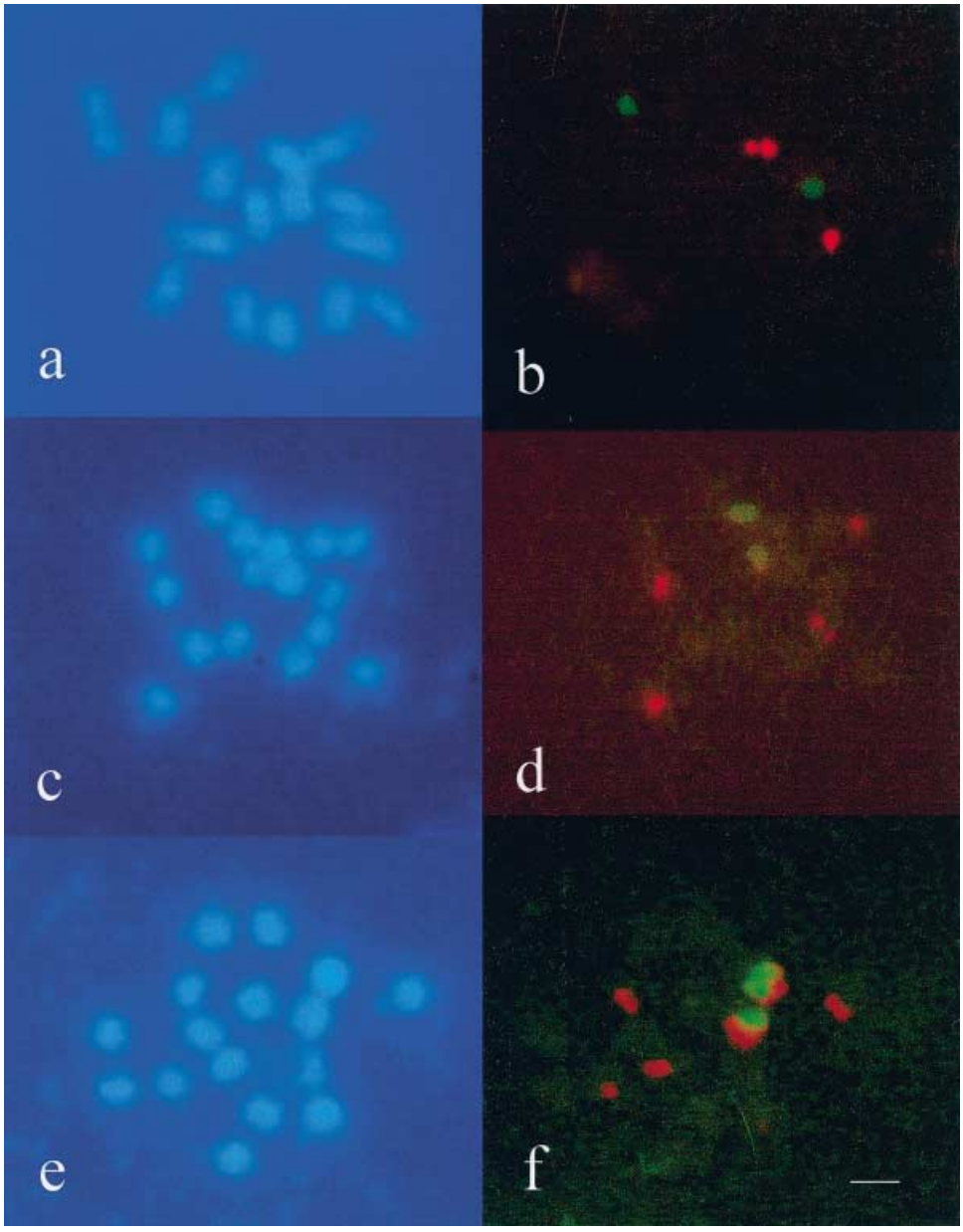

Fig. 1a-f. Chromosomal distribution of rDNA sequences in natural populations of *M. truncatula* after simultaneous in situ hybridization with 18S-5.8S-25S and 5S rDNA probes. Metaphase chromosomes of accessions PI 493296 (**a**, **b**), PI 493295 (**c, d**) and PI 384656 (**e**, **f**) have been chosen as an example of three patterns of hybridization detected. Chromosomes counterstained with DAPI (**a, c, e**) and hybridized with pTa71 (green) and pXVI (red) probes (**b, d, f**). The bar represents 2 μm.

other hand, the loss of rDNA sequences can also occur very rapidly within the same species (LANDSMANN and UHRIG 1985). This could be the case with *M. truncatula*. The results reported by CERBAH et al. (1999) seem to support this hypothesis since they found a higher number of loci 5S in the line J5 characterized by a genome of larger size (2C = 1.15 pg) than R-108-1 (2C = 0.98 pg). However, further analyses are needed to verify whether the different number of sites is the consequence of the loss of genes or, instead, is due to mechanisms of chromosome reorganization.

The heterogeneous distribution of rDNA sequences observed in *M. truncatula* is sharply in contrast with that found in diploid (2n = 16) and tetraploid (2n = 32) subspecies of *M. sativa* and in its putative ancestor *M. glomerata*. The FISH analysis revealed that the organization of ribosomal gene sites has been

conserved during the evolutionary steps passing from the primitive diploid form to the cultivated alfalfa (FALISTOCCO 2000).

The results of this study add futher information on the genome structure of *M. truncatula* since they revealed a pattern of distribution of rDNA genes not observed before. Moreover, the detection of rDNA sites provides the basis for the continuation of this research. The isolation and the characterization of repeated DNA sequences are currently in progress and we intend to use these sequences together with heterologous DNA probes in multicolor FISH to realize a detailed molecular karyotype of *M. truncatula*. FISH experiments will be also performed by probing molecular markers specific of single chromosomes in order to assigne genetic linkage groups to chromosomes and integrate physical and genetic recomination maps.

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