Technical Advance

FISH Chromosome Mapping Allowing Karyotype Analysis in *Medicago truncatula* Lines Jemalong J5 and R-108-1

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Two lines of *Medicago truncatula*, R-108-1 and Jemalong J5, exhibit differences in their symbiotic properties, morphological markers, and capacity for regeneration and transformation. Using cytogenetic techniques, we detected differences in chromomycin A₃ staining on the two sides of the secondary constriction of the nucleolar chromosome pair of J5 and R-108-1, while DAPI (4', 6-diamidino-2-phenylindole) staining revealed no differences between the two lines. FISH (fluorescent in situ hybridization) analysis showed a difference between J5 and R-108-1 for the same 5S rRNA genes; three loci were found in J5 but only two loci in R-108-1. However, for the 18S-5.8S-26S rDNA genes only a single locus was found in both lines. Based on these analyses, a karyotype for the two lines was established.

The tetraploid *Medicago sativa* (alfalfa) is a major forage crop all over the world. Due to its capacity to establish symbiosis with *Sinorhizobium meliloti* and mycorrhizal fungi, *M. sativa* can have a direct impact both on ecology and agriculture. Recently, a detailed genetic map has been constructed for alfalfa and about 1,300 markers (RFLP [restriction fragment length polymorphism], RAPD [random amplified polymorphic DNA], cDNA, isozyme, and morphological markers) have been mapped on the eight linkage groups representing the haploid chromosome set of the diploid *M. sativa* (Kiss et al. 1993). However, the out-crossing nature of *M. sativa* and the relatively large genome size make genetic studies difficult in this species. Therefore, *M. truncatula*, an annual autogamous, diploid species with a small genome size (0.98 to 1.15 pg; Blondon et al. 1994) was proposed as a model plant for the Leguminosae (Barker et al. 1990). Efficient and reproducible regeneration and transformation methods have been worked out for two lines of *M. truncatula*, Jemalong J5 and R-108-1 (Chabaud et al. 1996; Trieu and Harrison 1996; Trinh et al. 1998). These two lines exhibit differences in several morphological markers, such as the shape and pigmentation of

leaves and flowers, the form of seed pods, and also in their symbiotic properties (Hoffmann et al. 1997). In addition, in various *Medicago* spp. only a few karyological data are available, like the C-banded karyotype in *M. sativa* subsp. *coerulea* (Bauchan and Hossain 1997). In the same species, cytological studies on the nucleolar organizer regions (NORs) have been conducted by chromomycin banding and in situ hybridization of ribosomal DNA (Calderini et al. 1996). However, until now no karyotype has been available for *M. truncatula*. Therefore, we carried out cytogenetical studies on the *M. truncatula* lines J5 and R-108-1 with fluorochrome banding and in situ hybridization to learn if there are differences in genome organization between the two lines. This molecular cytogenetic approach may serve as a basis for the integration of genetic and physical maps and for the fine analysis of the chromosome structure including mutations as well as individual genes.

For the cytological studies, young root meristems excised from plants cultivated in the greenhouse (Trinh et al. 1998) were pretreated with α -bromonaphtalene at 4°C for 2.5 h and fixed in 3:1 ethanol-acetic acid. Chromosome spreads were obtained by isolation of protoplasts after incubation in an enzyme mixture of 4% hemicellulase (Sigma, St. Quentin Fallavier, France), 1% pectolyase Y23 (Seishin, Tokyo), and 4% cellulase R10 Onozuka (Yakult Honsha, Tokyo), by the air-drying technique of Geber and Schweizer, with light modifications described in Cerbah et al. (1998). Protoplasts were obtained after sequential centrifugations of 5 min in microvolumes of 100 μ l in 0.01 M citrate buffer and fresh fixative (3:1 ethanol-acetic acid).

For fluorochrome banding, chromomycin A₃ (CMA₃) and DAPI (4', 6-diamidino-2-phenylindole) were used. The GC-rich heterochromatin was stained with CMA₃ according to Schweizer (1976). The AT-rich heterochromatin regions were revealed by DAPI counterstaining of chromosomes after in situ hybridization. CMA₃ staining revealed large GC-rich heterochromatin blocks in both sides of the secondary constriction (SC) of the nucleolar chromosome pair. In J5, the two CMA₃ bands were the same size (Fig. 1A), while in R-108-1 they were asymmetric—one band was smaller than the other (Fig. 1B). Another difference was that in J5 a thin band at the

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centromeric region of each chromosome was detected, while in R-108-1 these bands were visible only occasionally and only in certain chromosomes. In the interphase nuclei, two bright spots, corresponding to GC-rich chromocenters, were

observed in both lines. In contrast to CMA₃, DAPI staining revealed no obvious differences between the two lines. The AT-rich bands were found at the centromeric position of each chromosome. Exceptionally, for the nucleolar chromosomes,

Fig. 1. Analysis of *Medicago truncatula* chromosomes in lines J5 and R-108-1 by fluochrome staining and in situ hybridization. **A**, Bright spots in the nucleolar chromosome pair (large arrow) and thin centomeric bands (thin arrow) in J5. **B**, Bright spots in the nucleolar chromosome pair (large arrow) in R-108-1. **C** and **D**, DAPI (4', 6-diamidino-2-phenylindole) banding in centromeric position of all chromosomes and at each side of the secondary constriction of nucleolar chromosome (thin arrows) in (C) J5 and (D) R-108-1. **E** and **F**, In situ hybridization with 18S-5.8S-26S rDNA probe (green fluorescence, large arrows) and 5S rDNA probe (red fluorescence) in (E) J5 and (F) R-108-1. Bar = 5 μm. **G** and **H**, Ideograms showing a representative structure of chromosomes in (G) J5 and (H) R-108-1. Yellow: GC rich sequences. Blue: AT rich sequences. Green: 18S-5.8S-26S rDNA. Red: 5S rDNA.

they were found not only at the centromeric position but also on each side of the SC (Fig. 1C, D).

For in situ hybridization, two ribosomal DNAs (18S-5.8S-26S and 5S rDNA) were used because they present the highly conserved families of repeated sequences in plants (Lapitan 1992). Therefore, their loci can be visualized on the chromosomes with the use of heterologous probes. The 18S-5.8S-26S rDNA allows the localization of NORs in nucleolar chromosomes. The 5S rDNA was used as tandemly repeated units with a high copy number (1,000 to 50,000 copies per haploid genome for 200 to 900 bp long; Goldsbrough et al. 1982). To localize these two ribosomal genes (18S-5.8S-26S and 5S rDNA) on the chromosomes, fluorescent in situ hybridization (FISH) was carried out with two probes simultaneously according to the protocol of Heslop-Harrison et al. (1991) and with the modifications of Cerbah et al. (1998). One of the probes was a 4-kb *EcoRI* fragment containing a part of the 18S-5.8S-26S rDNA as well as intergenic spacers from *Arabidopsis thaliana*. The second probe was the pTA 794 clone (Gerlach and Dyer 1980), which contains a 410-bp *BamHI* fragment of the 5S wheat rDNA, recloned in pUC. Both probes (18S-5.8S-26S and 5S rDNA) were labeled by the polymerase chain reaction (PCR) with digoxigenin-11-dUTP (Boehringer Mannheim, Meylan, France) for the 18S-5.8S-26S rDNA and Fluoro-Red-dUTP (Amersham, Les Ulis, France) for the 5S rDNA. The slides containing the chromosome spreads were incubated with 100 µg of DNase-free RNase per ml in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 1 h at 37°C, then with 5 µg of pepsin per ml in 0.01 M HCl for 15 min at 37°C, followed by a wash in 2× SSC and dehydration with an ethanol series. Approximately 0.5 µg/ml of the rDNA probes was mixed with 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, 0.1% (wt/vol) sodium dodecyl sulfate, 250 µg of salmon sperm per ml, and 2× SSC. The mixtures were denatured at 80°C for 10 min and immediately chilled on ice. After addition of the hybridization mix, the slides were also denatured at 80°C for 10 min (for chromosome denaturation), and hybridization was carried out overnight at 37°C. Post-hybridization washing consisted of a 5 min immersion in 2× SSC at 42°C followed by two stringent washes in 20% formamide in 0.1× SSC at 42°C. Slides were washed in 0.1× SSC for 5 min at 42°C, in 2× SSC for 3 × 5 min and in 4× SSC, 0.2% Tween 20 (4× SSCT) for 5 min at room temperature. For detection of the 18S-5.8S-26S signal, the slides were treated with 5% (wt/vol) bovine serum albumin (BSA) in 4× SSCT for 5 min, incubated for 1 h at 37°C in 20 µg of anti-digoxigenin-fluorescein per ml (Boehringer Mannheim) in the same buffer, and rinsed with 4× SSCT three times for 5 min at room temperature. Chromosomes were counterstained for 30 min with 2 µg of DAPI per ml in McIlvaine solution at pH 7 (0.11 M Na₂HPO₄ plus 0.006 M citric acid). Preparations were mounted in anti-fade solution (AF1 citifluor) and examined with a Zeiss Axio-phot microscope. Forty metaphase chromosomes isolated from root tips of different individuals were analyzed for each line—J5 and R-108-1. These metaphase chromosomes were digitized with a highly sensitive CCD camera (Princeton Instruments, Evry, France) and an image analyzer system (Graftek; Fluograb, CITY, France), allowing superimposed images. Photographs were obtained with a Sony color printer (Sony, Paris). In situ hybridization of rRNA genes is shown in

Figure 1E and F. The 18S-5.8S-26S rRNA genes (green) were found on the nucleolar chromosome pair in both lines and co-localized with the GC-rich heterochromatin at the SC. In contrast, the 5S rRNA gene (red) was detectable in three chromosome pairs of J5 while it was found on only two chromosome pairs of R-108-1. In R-108-1, the 5S and 18S-5.8S-26S rRNA genes were carried by different chromosomes while in J5 one of the three 5S rRNA gene loci was observed near the 18S-5.8S-26S rRNA genes in the chromosome pair 1. The remaining two 5S rRNA gene loci were carried by two metacentric chromosome pairs in both lines (pairs 6 and 8). The 5S loci were co-localized with DAPI bands.

As shown in Figure 1G and H, most of the *M. truncatula* chromosomes have similar morphology, especially the chromosome pairs 2, 3, 4, and 5, which were difficult to distinguish from each other. The karyotype of *M. truncatula* is constituted by metacentric (chromosome pairs 6 and 8) and submetacentric chromosomes. The length of the largest chromosome pair (1) was about 4.5 µm, while that of the smallest pair (8) was about 3 µm. An SC, which is the site of the NORs, was observed adjacent to the centromere on the short arm of chromosome pair 1. Despite the difficulties of the karyotype analysis, an ideogram was built for the two lines with the aid of an image analyzer by measuring chromosome length, centromere position, detection of DAPI and CMA₃ bands, and rDNA localization. Other cytogenetic markers, like C-bands and FISH experiments with other probes, are needed for further specific marking of the chromosome.

The major difficult step of in situ hybridization is the spreading of the chromosomes directly on slides. It is rare to obtain metaphases with all chromosomes well separated (Fig. 1E and F): in most cases, some chromosomes are always stuck together (Fig. 1A and B).

Our results show that in addition to differences in symbiotic properties and morphological markers, the lines Jemalong J5 and R-108-1 also exhibit some distinct cytogenetical properties as well as differences in the location of the 5S rDNA. However, no differences were found in the number and location of the 18S-5.8S-26S rDNA between the two lines, as was also observed in the diploid *M. sativa* subsp. *coerulea* and subsp. *falcata* (Calderini et al. 1996). The additional locus of 5S in J5 and the larger CMA₃ band observed in the same location might reflect a larger genome size in J5 (2C = 1.15 pg) than in R-108-1 (2C = 0.98 pg) and differences in base composition (38.6% GC and 38.1% GC, respectively; Blondon et al. 1994). The absence of the third 5S locus in the R-108-1 line seems to correlate with the small CMA₃ band observed in this chromosome pair. Nevertheless, from these results, it is difficult to know whether the chromosomal locations of 5S rDNA observed are due to differences in the copy number of rDNA sequences between the two lines, followed by translocation mechanisms. Comparison of the number and location of the 18S-5.8S-26S and 5S rDNA genes in related species of *Medicago* may shed some light on the chromosome evolution in this genus.

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LITERATURE CITED

- Barker, D. G., Bianchi, S., Blondon, F., Dattée, Y., Duc, G., Essad, P., Flament, P., Galuxi, P., Génier, G., Muel, X., Tourneur, J., Dénarié, J., and Huguët, T. 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of *Rhizobium*-legume symbiosis. *Plant Mol. Biol. Rep.* 8:40-49.
- Bauchan, G. R., and Hossain, M. A. 1997. Karyotypic analysis of C-banded chromosomes of diploid alfalfa: *Medicago sativa* ssp. *coerulea* and ssp. *falcata* and their hybrid. *J. Hered.* 88:533-537.
- Blondon, F., Marie, D., Brown, S., and Kondorosi, A. 1994. Genome size and base composition in *Medicago sativa* and *M. truncatula* species. *Genome* 37:264-275.
- Calderini, O., Cluster, P. D., Mariani, A., and Arcioni, S. 1996. Cytological studies of the nucleolus organizing regions in the *Medicago* complex: *sativa-coerulea-falcata*. *Genome* 39:914-920.
- Cerbah, M., Coulaud, J., and Siljak-Yakovlev, S. 1998. rDNA organization and species relationships in the genus *Hypochaeris*. *J. Hered.* 89:312-318.
- Chabaud, M., Larssonneau, C., Marmouget, C., and Huguët, T. 1996. Transformation of barrel medic (*Medicago truncatula* Gaertn.) by *Agrobacterium tumefaciens* and regeneration via somatic embryogenesis of transgenic plants with the MtENOD12 nodulin promoter fused to the GUS reporter gene. *Plant Cell Rep.* 15:305-310.
- Gerlach, W. L., and Dyer, T. A. 1980. Sequence organization of the repeated units in the nucleus of wheat which contains 5S-rRNA genes. *Nucleic Acids Res.* 8:4851-4865.
- Goldbrough, P. B., Lane, C., and Lomonosoff, G. P. 1982. Sequence variation and methylation of the flax 5S RNA genes. *Nucleic Acids Res.* 10:4501-4514.
- Heslop-Harrison, J. S., Schwarzacher, T., Ananthawt-Jonsson, K., Leitch, A. R., Shi, M., and Leitch, I. J. 1991. In situ hybridization with automated chromosome denaturation. *J. Methods Cell Mol. Biol.* 3:109-116.
- Hoffmann, B., Trinh, T. H., Leung, J., Kondorosi, A., and Kondorosi, E. 1997. A new *Medicago truncatula* line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection. *Mol. Plant-Microbe Interact.* 10:307-315.
- Kiss, G. B., Csanadi, G., Kalman, K., Kalo, P., and Okresz, L. 1993. Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. *Mol. Gen. Genet.* 238:129-137.
- Lapitan, N. L. W. 1992. Organization and evolution of higher plant nuclear genomes. *Genome* 35:171-181.
- Schweizer, D. 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* 58:307-324.
- Trinh, T. H., Ratet, P., Kondorosi, E., Durand, D., Kamaté, K., Bauer, P., and Kondorosi, A. 1998. Rapid and efficient transformation of diploid *Medicago truncatula* and *Medicago sativa* ssp. *falcata* in vitro lines improved in somatic embryogenesis. *Plant Cell Rep.* 17:345-355.
- Trieu, A. T., and Harrison M. J. 1996. Rapid transformation of *Medicago truncatula*: Regeneration via shoot organogenesis. *Plant Cell Rep.* 16:6-11.