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Molecular cytogenetic studies in rubber, *Hevea brasiliensis* Muell. Arg. (Euphorbiaceae)

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Abstract: This paper reports the start of a molecular cytogenetics programme targeting the genome of the angiosperm tree species *Hevea brasiliensis* Muell. Arg. (rubber, $2n = 36$), a major world crop about whose genetics very little is known. A metaphase karyotype of rubber is presented. In situ hybridization with the probe pTa71 for ribosomal DNA (rDNA) shows that there are four sites of probe hybridization occurring on two pairs of chromosomes called chromosomes 6 and 7 carrying sites NOR-1 and NOR-2, respectively. An examination of meristematic interphase nuclei shows that all four loci have the potential to be partially decondensed at interphase, although in many nuclei one or more loci appear fully condensed and apparently inactive. The probe pXVI revealed a single pair of chromosomes carrying 5S rDNA. The probes pTa71 and pXVI provide cytogenetic markers for three pairs of chromosomes that will be of use in genetic mapping programmes. The rubber chromosomes also have telomere sequences that hybridize with the *Arabidopsis* consensus sequence TTTAGGG. With the exception of the satellite region containing rDNA, which fluoresces brightly with chromomycin A₃, fluorescence banding showed that there is no strong demarcation of the genome into GC- and AT-rich regions, as occurs in mammalian genomes.

Key words: rubber, *Hevea*, genetic mapping, cytogenetics, ribosomal DNA, rDNA fluorescence banding.

Résumé : Les auteurs rapportent l'initiation d'un programme de recherche en cytogénétique moléculaire portant sur l'étude du génome de l'angiosperme *Hevea brasiliensis* Muell. Arg. (arbre à caoutchouc, $2n = 36$), une culture importante sur le plan mondial mais dont la génétique est très mal connue. Un caryotype en métaphase de l'hévéa est présenté. Une hybridation in situ à l'aide de la sonde pTa71, laquelle contient l'ADN ribosomique (ADNr), montre qu'il y a quatre sites d'hybridation situés sur deux paires de chromosomes, désignés chromosomes 6 et 7, lesquels portent les sites NOR-1 et NOR-2 respectivement. Un examen des noyaux en interphase méristématique montre que les quatre loci peuvent se trouver dans un état de décondensation partielle à l'interphase, bien que dans plusieurs noyaux un ou plus des loci semblent complètement condensés et sont apparemment inactifs. La sonde pXVI a révélé qu'une seule paire de chromosomes porte l'ADNr 5S. Les sondes pTa71 et pXVI constituent des marqueurs cytogénétiques pour trois paires de chromosomes et pourront ainsi contribuer aux efforts de cartographie génétique. Les chromosomes de l'hévéa comptent également des séquences télomériques qui hybrident avec la séquence consensus des télomères de l'*Arabidopsis*, TTTAGGG. À l'exception de la région d'ADN satellite, laquelle contient l'ADNr et émet une fluorescence intense après marquage à la chromomycine A₃, les autres régions ne montrent pas une démarcation nette du génome en régions riches en GC ou en AT tel que cela s'observe chez les mammifères.

Mots clés : arbre à caoutchouc, *Hevea*, cartographie génétique, cytogénétique, ADN ribosomique, ADNr bandes fluorescents.

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Introduction

Little research has been conducted on the genome of *Hevea*

brasiliensis Muell. Arg. (rubber, Euphorbiaceae), despite the commercial importance of the crop. Cultivated rubber is known to have a very narrow genetic base (Tan 1987) and a chromosome number of $2n = 36$ (Ong 1976). This paper examines the karyotype of rubber and reports on the occurrence and physical location of rDNA sequences, telomere sequences, and fluorochrome bands.

The origin of *Hevea* spp. is the Amazon basin and parts of the Matto Grosso, Upper Orinoco, and the Guianas, where nine species and natural hybrids of these species have been recognised (Wycherley 1976). In genetic experiments, there appear to be no genetic barriers between the species (Wycherley 1976). Chromosome counts suggest that all species are $n = 18$, although there are no confirmed counts for three species (Ma-

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jumder 1964). It is possible that *H. brasiliensis* and other species in the genus are allopolyploid, with a basic chromosome number of 9, i.e., $2n = 4x = 36$. Ong (1976) measured the lengths of pachytene chromosomes of *H. brasiliensis* and named the chromosomes I to XVIII in decreasing order of size. He noted that there were four secondary constrictions, one each on chromosomes VI, VII, XIII and XV, suggesting four pairs of rDNA loci.

Only a few angiosperm tree species have been explored using molecular cytogenetic techniques: these include *Populus* (Prado et al. 1996), *Betula* (Anamthawat-Jónsson and Heslop-Harrison 1995), *Malus* (Zhu and Gardiner 1995; Schuster et al. 1997), and *Citrus* (Matsuyama et al. 1996). This paper presents an examination of the angiosperm tree genus *Hevea*, from the plant family Euphorbeaceae, by molecular cytogenetics. It also addresses the need to obtain physical maps of the rubber genome for breeding programmes, to determine the ancestry of rubber, and to facilitate the understanding of rubber genetics as a whole.

Materials and methods

Plant material

All rubber material was obtained from newly germinated seeds growing on moist filter paper. Young roots were excised and fixed without pretreatment in ethanol – glacial acetic acid 3:1.

In situ hybridization

Cell and chromosome spreads were prepared following the methods described previously by Schwarzacher et al. (1989). Three probes were used: (i) pTa71 from wheat, which contains the 9 kb rDNA gene unit (18S–5.8S–25S and intergenic spacer; Gerlach and Bedbrook (1979)); (ii) pXVI, a 349-bp fragment of a 5S rDNA subunit isolated from *Beta vulgaris* (Schmidt et al. 1994); and (iii) a PCR-generated synthetic probe (Cox et al. 1993), which is a concatenation of 5'-(TTTAGGG)-3', the *Arabidopsis* telomere consensus sequence. DNA was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) or biotin-11-dUTP by nick translation. In situ hybridization and probe detection with either anti-digoxigenin-FITC (fluorescein isothiocyanate) IgG (Boehringer Mannheim) or avidin Cy3 (Amersham) and counterstaining with DAPI (4',6-diamidino-2-phenylindole; $2 \mu\text{g}\cdot\text{mL}^{-1}$) was as in Leitch et al. (1992).

Fluorescence banding

Chromomycin A₃ and DAPI staining were carried out by incubating material in $0.2 \mu\text{g}\cdot\text{mL}^{-1}$ DAPI and $2 \mu\text{g}\cdot\text{mL}^{-1}$ chromomycin A₃ in McIlvaine's buffer (0.1 M citric acid plus 0.2 M Na₂HPO₄) for 10 min prior to a brief wash in buffer and mounting in Vectashield antifade solution.

Image analysis

Photographs were taken, using appropriate epifluorescence filters, with Fuji400 colour print film. Negatives were electronically scanned and the contrast, brightness, and colour enhanced using Adobe photoshop, by making uniform adjustments to the whole image.

Results

Chromosomes carrying nucleolar organizing regions (NORs)

A karyotype of rubber was prepared using chromosome size and arm ratio. Chromosomes were metacentric, submetacentric, or acrocentric, with a fourfold range in size from chromo-

some 1 to chromosome 18 (Fig. 1A). The genome size of *H. brasiliensis* is $4C = 8.3 \text{ pg}$ (Bennett and Leitch 1997). Thus the average chromosome is small (only 0.2 pg of DNA) compared with species such as rye (*Secale cereale*), where chromosomes are about 13.5 times larger (about 2.7 pg of DNA). In situ hybridization with digoxigenin- or biotin-labelled pTa71 revealed four sites of probe hybridization on two pairs of medium-large chromosomes called chromosomes 6 and 7. The hybridization sites occur at the distal end of the short arm of each chromosome pair and the signals were approximately equal in size (Figs. 1A and 1B). The loci on chromosomes 6 and 7 were designated NOR-1 and NOR-2, respectively. Usually chromosome 6 can be distinguished after in situ hybridization, because during the denaturation process the long arm becomes demarcated into a DAPI bright and DAPI dull fluorescent region, the transition point being midway along the chromosome arm (as shown by arrows in Figs. 1C and 1D). Both NOR-1 and NOR-2 can be expressed at interphase, as many metaphases showed very elongated constrictions at all rDNA loci. Secondary constrictions occurred at the proximal end of rDNA loci (Figs. 1A–1C), as in *S. cereale* (Leitch et al. 1992).

At interphase, the rDNA probe labelled condensed chromatin, which appeared as large brightly fluorescent spots, as well as decondensed dispersed chromatin, which appeared as a diffuse network of signal (Fig. 1E). All nuclei showed at least some condensed labelled chromatin. An examination of many hundreds of nuclei revealed many with one, two, three, or even four distinct condensed rDNA loci with no indication of decondensation. Sometimes individual rDNA loci had condensed regions from which highly elongated and extended rDNA fibres emanated. Thus there is differential condensation of rRNA genes both within and between loci at interphase. The presence of condensed rDNA in all meristematic nuclei suggests that there is gene redundancy in this cell type.

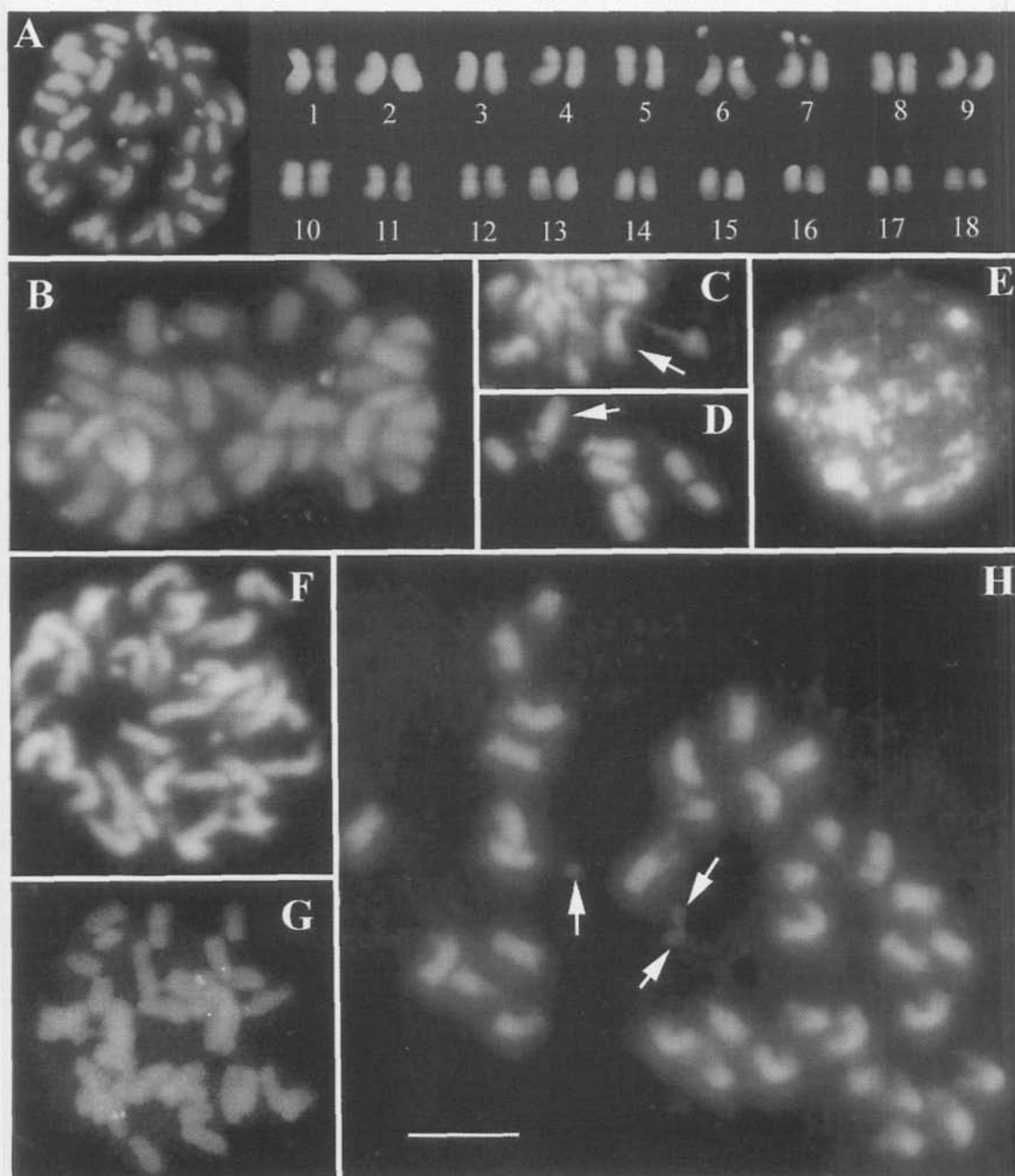
DAPI and chromomycin A₃ fluorescence banding

Rubber chromosomes stained simultaneously with DAPI and chromomycin A₃ showed no bright fluorescent bands except for four chromomycin A₃ positive regions at the satellite of chromosomes 6 and 7 (Fig. 1F). The chromomycin A₃ positive regions were also visible at interphase (results not shown). Interphase nuclei also had large blocks of condensed chromatin that stained with DAPI and chromomycin A₃ (as in Fig. 1E). An examination of cells in early prophase to metaphase showed that the condensed chromatin was predominantly centromeric in location.

Distribution of 5S rDNA and telomere sequences

The probe pXVI for 5S rDNA revealed two sites of probe hybridization to a medium-sized pair of metacentric chromosomes (Fig. 1G). The telomeric probe revealed sites of probe hybridization at the ends of chromosomes, including the satellite regions of chromosomes 6 and 7 (Fig. 1H). The signal strength varied among chromosome arms in all metaphases. Reduced levels of telomere labelling may reflect a low copy number of the sequence or poor hybridization of the sequence to that particular arm. Variable signal strength also occurs in other plant species (e.g., *Hordeum vulgare*; Schwarzacher and Heslop-Harrison 1991). The three distinct satellites shown in Fig. 1H (indicated by arrows) that overlay DAPI positive chro-

Fig. 1. Dividing and interphase nuclei from *H. brasiliensis* root tip meristem cells. (A–E) Double-exposure photographs showing in situ hybridization with digoxigenin-labelled pTa71 for rDNA detected with FITC (yellow–green fluorescence; A and B) or Cy3 (red fluorescence; C–E) and simultaneous staining with DAPI (blue fluorescence): A, metaphase showing 4 rDNA loci and to its right the karyotype of the cell; B, metaphase; C and D, partial metaphases showing (C) chromosome 6 carrying NOR-1, which has an extended secondary constriction, and (D) chromosomes 6 and 7 carrying NOR-1 and NOR-2, respectively; the arrows represent the point of transition between a DAPI bright and DAPI dull region on chromosome 6; E, interphase showing labelled rDNA that is condensed (fluorescent foci) and decondensed (fine network of signal). (F) Late prophase/early metaphase stained with fluorochromes DAPI and chromomycin A₃ (double-exposure). There are 4 chromomycin bright bands at the satellite of chromosomes 6 and 7. (G) Digoxigenin-labelled pXVI detected with FITC, overlaid electronically with the DAPI image of the same metaphase. There are two hybridization sites (cyan dots). (H) Digoxigenin-labelled telomere probe detected with Cy3 (pink dots), electronically overlaid with the DAPI image of the same metaphase. Arrows point to the satellite regions of chromosomes 6 and 7 that carry telomere sequences. Scale bars = 5 (A), 10 (B–D, F, and G), and 15 (E and H) μ m.



matin (DAPI only image not shown) are separated from the remainder of the chromosome by highly decondensed chromatin. These satellites arise by decondensation of rDNA at individual loci to form an elongated secondary constriction as

shown in Fig. 1C. Such constrictions are thought to represent loci that were active at the preceding interphase. That three satellites are seen in Fig. 1H and not four, as might be predicted, is suggestive of a silent locus at the preceding inter-

phase of this cell; condensed, and presumed silencing of entire loci was also observed with rDNA probes (above).

Discussion

Fluorescence staining with DAPI and chromomycin A₃ showed that there is not a strong demarcation of the genome into AT- and GC-rich regions, as occurs in mammalian genomes (cf. Bickmore and Sumner 1989). This is probably due to dispersed repeats, which are thought to be in great abundance in plant genomes (Fuchs et al. 1996). Rubber also has telomeric sequences that are homologous to the *Arabidopsis* consensus sequence 5'-TTTAGGG-3', although this is not the case in all plant species, for example, members of the Alliaceae (Pich et al. 1996).

The probes pTa71 and PXVI have provided cytogenetic markers to three chromosome pairs, and these will be of use in genetic mapping programmes. The 5S rDNA sequence occurs on a small pair of metacentric chromosomes and the 18S-5.8S-25S rDNA sequence occurs at loci designated NOR-1 and NOR-2 on chromosomes 6 and 7, respectively. Chromosomes 6 and 7 correspond to Ong's (1976) pachytene chromosomes VI and VII, which have secondary constrictions. The secondary constrictions reported on the smaller chromosomes XIII and XIV at pachytene (Ong 1976) did not carry rDNA signal.

Besse et al. (1993) reported two rDNA units in rubber that they called type I and type II. It is not known if these rDNA repeat units are found separately at NOR-1 and NOR-2 or whether they are in a mixed array of type I and type II at each NOR. The presence of two different rDNA unit types and of two NORs on separate chromosomes may have arisen by the hybridization of two unknown diploid species, probably with chromosome numbers of $n = 9$. It is generally considered that plant species with chromosome numbers higher than $n = 13$ are probably polyploid (Stebbins 1971). It is also possible that rubber evolved following an autopolyploidization event, and if so, the ancestral diploid may have had the two rDNA unit types, I and II, at a single rDNA locus. We detected only one pair of chromosomes carrying 5S rDNA, which suggests that if *Hevea* is of polyploid origin, then one locus at least must have been lost. The ancestry of rubber needs to be explored further, using genomic in situ hybridization, RFLP (restriction fragment length polymorphism), and isozyme analyses.

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