Karyotype analysis of *Psophocarpus tetragonolobus* (L.) DC by chromosome banding and fluorescence in situ hybridization

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Abstract — Detailed karyotype of *Psophocarpus tetragonolobus* (L.) DC was constructed on the basis of chromosome lengths, DAPI and CPD banding, silver staining, together with fluorescence in situ hybridization (FISH) with 45S and 5S rDNA sequences as probes. FISH of the 45S rDNA probe on mitotic chromosomes revealed three pairs of 45S rDNA sites including one major and two minors. The major pair of sites was located in the unique secondary constrictions of the satellite chromosome pair, while the minors were located on two pairs of non-satellite chromosomes. For 5S rDNA, only one pair of sites was detected using FISH. Distinct DAPI and CPD banding patterns were shown simultaneously after combined PI and DAPI (CPD) staining. CPD bands were shown at all 45S rDNA sites and centromeric regions, suggesting the feature of GC-rich sequences in the centromeres. Each chromosome pair could be identified with DAPI and/or CPD banding patterns in combination with chromosome measurements. Silver staining showed variable number of Ag-NORs on mitotic chromosomes, revealing that the major sites were active in every cell, while the minors were activated only in a proportion of the cells, and the level of expression differed between two pairs of minor NORs.

Key words: Ag-NORs, combined PI and DAPI staining, karyotype, *Psophocarpus tetragonolobus*, rDNA.

INTRODUCTION

The winged bean, *Psophocarpus tetragonolobus* (L.) DC, as a member of the Legume family, is cultivated throughout most of South-East Asia, from Burma to Melanesia. In most regions, it is grown only on a small scale, often as a backyard vegetable, but in the Highlands of Papua New Guinea it may occupy up to one-fifth of the total cultivated area of native gardens and is the major leguminous crop (Khan et al. 1977). Its green pods, dried seeds, fresh leaves, flowers and the tuberous roots may all be eaten and all are rich in protein. Its potential economic importance has attracted worldwide attention in the last decades because of its high nutritional value that equals to that of soybean (May 1977; Claydon 1978; Prakash et al. 2001).

So far, more attentions have been paid on the studies of its nutritional value, seed albumins and yield trials. The cytological and cytogenetic data, which are useful to inter- and intra-specific crossing programs and genome analysis, are rare. Earlier cytological studies (Pickersgill 1980) confirmed its chromosome number (2n=18) and described its karyotype morphology, but could not give the chromosome measurements and accurately identify homologous chromosome pairs because of the limitation of conventional squash and staining methods. Previously, using the enzymatic maceration/flame-drying method and ASG (Acetic acid/Saline/Giemsa) banding procedure, we obtained its fine chromosome spreads and Giemsa bands (She et al. 1998). But more effective cytogenetic and molecular-cytogenetic methods are needed for identification of all chromosome pairs and some centromeric positions to establish an exact karyotype of the winged bean.

Detailed karyotypes generally display chromosomes ordered in sequence of decreasing length, and include the distribution of characteristic chromatin along chromosomes based on chromosome differentiation techniques. Several fluorochrome banding techniques have been developed for plant chromosome analysis. One of these is combined PI (propidium iodide) and DAPI (4', 6 diamino-2-phenylindole dihydrochloride) staining (called CPD stain-

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ing), which has been proven to be able to differentially stain NOR and visualize local variation in DNA and (or) chromatin composition (Hanson et al. 1995; Ji et al. 1997; Peterson et al. 1999; Andras et al. 2000; Lim et al. 2001; Kato et al. 2003). Fluorescence in situ hybridization (FISH), which maps repetitive or single-copy sequences on the chromosomes, now complements banding technologies. The most common application of FISH is the localization of rDNA families on the chromosomes, not only providing information regarding genome evolution and chromosome structure, but also providing landmarks for chromosome identification (e.g. Leitch and Heslop-Harrison1992; Moscone et al. 1999; Brown et al. 1999; Hasterok et al. 2001).

The aim of this study was to establish an exact and detailed karyotype of *P. tetragonolobus* using the combined data of chromosome measurements, DAPI and CPD banding patterns, Ag-NORs and FISH mapping of rDNA families, and provide a primary molecular-cytogenetic characterization of this potentially important plant species. In addition, the expression patterns of the mapped NORs were analyzed based on the data of silver staining on mitotic chromosomes.

MATERIALS AND METHODS

Plant material and slide preparation. - Seeds of *P. tetragonolobus* (L.) DC were obtained from the germplasm collection of the Institution of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences (No. K0000010). Seeds were germinated in Petri dishes with moisture filter and the seedlings were planted in soil. Mitotic chromosomes were prepared from young leaves according to Desel et al. (2001). Young leaves (0.5-1.0 cm) were immediately fixed in 3:1 (v/v) methanol/glacial acetic acid without any pretreatment. The enzyme solution consisted of 1% cellulase RS (Yakult Honsha Co. Ltd.), 1% pectolyase Y23 (Yakult Honsha Co. Ltd.) and 1% cytohelicase (Sigma) in citric buffer (4 mM citric acid, 6 mM sodium citrate, pH 4.5).

CPD staining. – CPD staining was performed according to Andras et al. (2000). Preparations were examined with an Olympus BX60 epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera (SenSys 1401E, Photometrics). The CCD camera was controlled using V^{++} software (Roper Scientific Digital Optics Ltd.). Observation and photography were conducted using a green filter for PI and a UV filter for DAPI. The exposure time through two filters were adjusted to get similar fluorescence intensities. Gray scale images

produced by PI staining (called PI images) or those produced by DAPI staining (called DAPI images) were digitally captured and converted into inverts with Adobe Photoshop (version 6.0; Adobe Inc. USA). DAPI and PI gray scale images of the same chromosome plates were merged to produce CPD images. The final images were adjusted with Adobe Photoshop.

Probe labeling and FISH. - The DNA clones, pTa71 (45S rDNA), pTa794 (5S rDNA) were used as probes. Plasmid pTa71 and pTa794 were labeled with digoxigenin-dUTP and Biotin-dUTP respectively using nick translation. The FISH procedure followed that described by Schubert et al. (2001). Biotin-dUTP labeled probe was detected by Streptavidin-Cy3 (Sigma). Signals were amplified by biotinylated anti-streptavidin (Sigma) and Streptavidin-Cy3. Digoxigenin-dUTP labeled probe was detected by sheep-anti-digoxigenin- FITC and amplified by rabbit-anti-sheep-FITC (Boehringer Mannheim, Germany). DNA was counterstained with DAPI solution in which appropriate quantity of Vectashield (Vector Laboratories) was added. FISH with 45S rDNA probe was performed sequentially on the slides previously stained by CPD. After washing in TN buffer (100 mM Tris-HCl, 150 mM NaCl, pH7.5) for 3×5 min, the slides were dehydrated through an ethanol series and used for FISH experiments. Preparations were examined using the epifluorescence microscope mentioned above. Gray scale images were captured digitally with appropriate filters and then merged. The final images were adjusted with Adobe Photoshop.

Silver staining. - Silver staining was performed according to the method described by Howell (1980). In order to exactly identify whether NORs were stained by silver or not, sequential CPD and silver staining was carried out on the same slides. Metaphase and prometaphase plates in which NORs could be clearly distinguished by CPD staining were recorded. Then the slides were washed in 2×SSC for 3×5 min, dehydrated through an ethanol series and used for silver staining. The plates previously recorded in CPD staining experiments and Ag-NORs could be clearly identified were photographed and used for analysis.

Karyotyping. - Five metaphase and prometaphase plates, whose homologous chromosomes were identified using CPD banding and FISH mapping of 45S rDNA, were selected for measurements of karyotype with Adobe Photoshop. The chromosome relative lengths (% of haploid complement) and arm ratios were calculated.

RESULTS

Six signals, one pair of large signals and two pairs of small signals, were detected on three different pairs of mitotic chromosomes after FISH with 45S rDNA probe (Figs. 1d and 1h). The large signals were very strong in every chromosome plate and corresponded to the unique secondary constrictions of chromosome 9 designated by karyotyping (see below). Two pairs of small signals were located proximally on the long arm of chromosome 1 and the short arm of chromosome 8, respectively. No secondary constrictions were observed in the chromosomal regions in which small signals were shown. The detection rates of the two pairs of small signals were 70% and 100%, respectively. It is obvious that the rDNA sites (NORs) in *P. tetragonolobus* can be distinguished as one major and two minor pairs. In addition, the minor sites on chromosome 1 occurred between the first and second pericentromeric DAPI-positive banded regions on the long arm (Figs. 1i, 1j and 1k). Only one pair of FISH signals of 5S rDNA probe was detected, located at a position near the centromere on the long arm of chromosome 8 (Fig. 1l) and being syntenic to one pair of 45S rDNA minor sites (Fig. 2c).

Distinct DAPI and CPD banding patterns were shown simultaneously after CDP staining. As revealed in the inverts of DAPI images, distinct DAPI banding patterns were clearly shown on prometaphase and prophase chromosomes instead of metaphase chromosomes (Figs. 1a, 1e and 1i). All DAPI-positive bands occurred in pericentromeric regions, displaying as black bands in the inverts of DAPI images (Figs.1e and 1i). The number of DAPI-positive bands per cell ranged from 32 to 36, and 12-16 bands were very bright. The band number and intensity varied a little with states of chromatin condensation. For example, on the long arm of chromosome 1, three very bright bands shown at prophase (Fig. 1i) became one band at prometaphase (Fig. 1e). Nevertheless, the banding patterns of various chromosome pairs were rather stable, so chromosome pairs can be identified easily with their DAPI banding patterns (Fig. 2b).

A number of red CPD bands were shown on mitotic chromosomes of all stages (Figs. 1c, 1g and 1k). As demonstrated by the sequential FISH with 45S rDNA probe, the regions in which 45S rDNA located were all shown as CPD bands constantly (Figs. 1c, 1d; 1g, 1h; 1k), and the CPD bands corresponded to the FISH signals in size and intensity. Other CPD bands all appeared in centromeric regions. The fact that these CPD banded regions belong to the centromeres was confirmed by visualiza-

tion of their positions on mitotic anaphase chromosomes. At anaphase these CPD bands were occurred consistently on the tips (the most poleward positions) of the being segregated chromatids (image not shown). The size and intensity of centromeric CPD bands varied among different chromosome pairs. Chromosome 6 showed the largest centromeric CPD bands whose length was about 20 percent of the chromosome length on average. Larger bands were shown on chromosome 4 and 7, while chromosome 1, 2, 5 and 8 showed small and/or weak centromeric bands that were obvious at prometaphase and prophase but sometimes invisible at metaphase. Weak bands on chromosome 3 were clearly seen only at prophase (Fig. 1k). On chromosome 9 only a very large CPD band corresponding to the strongest FISH signal was shown and the primary constriction could not be recognized. Out of the band no chromatin reflecting centromeric features was observed, excluding the possibility that the centromere located outside this banded region. So most probably, the primary constriction located in this CPD banded region and is closely juxtaposed with the secondary constriction and adjacent to the long arm. In addition, the intervals between centromeric and 45S rDNA CPD bands were too small to be resolved on chromosomes 1 and 8 at metaphase and prometaphase. In consequence, 12, 16, 22 CPD bands were distinguishable in metaphase, prometaphse and prophase plates, respectively (Figs. 1c, 1g and 1k). Using CPD banding patterns in combination with chromosome measurements, each chromosome pair could be readily identified, even at metaphase (Figs. 1c, 1g and 1k). Comparing PI images with DAPI images of the same plates, we found that both rDNA and centromeric CPD bands occurred in the regions that heavily stained by PI but lightly stained by DAPI (Figs. 1a, 1b, 1c; 1e, 1f, 1g; 1i, 1j, 1k). In contrast, most DAPI banded regions were heavily stained by DAPI but relatively lightly stained by PI (Figs. 1e, 1f; 1i, 1j).

Using DAPI and/or CPD banding patterns together with FISH signals of 45S rDNA chromosome pairs of five metaphase and prometaphase plates were exactly identified. Data of measurements from these plates were listed in Table 1. The fluorescent karyotypes and idiogram displaying DAPI and CPD banding patterns as well as the locations of 45S and 5S rDNA sites were constructed and presented in Fig. 2. The haploid karyotype formula is n=4m+5sm (1SAT).

Silver staining revealed variable number of Ag-NORs on mitotic chromosomes (Fig. 3; Tab. 2). One pair of Ag-NORs of the major rDNA sites was observed in every mitotic cell, but the Ag-NORs of the
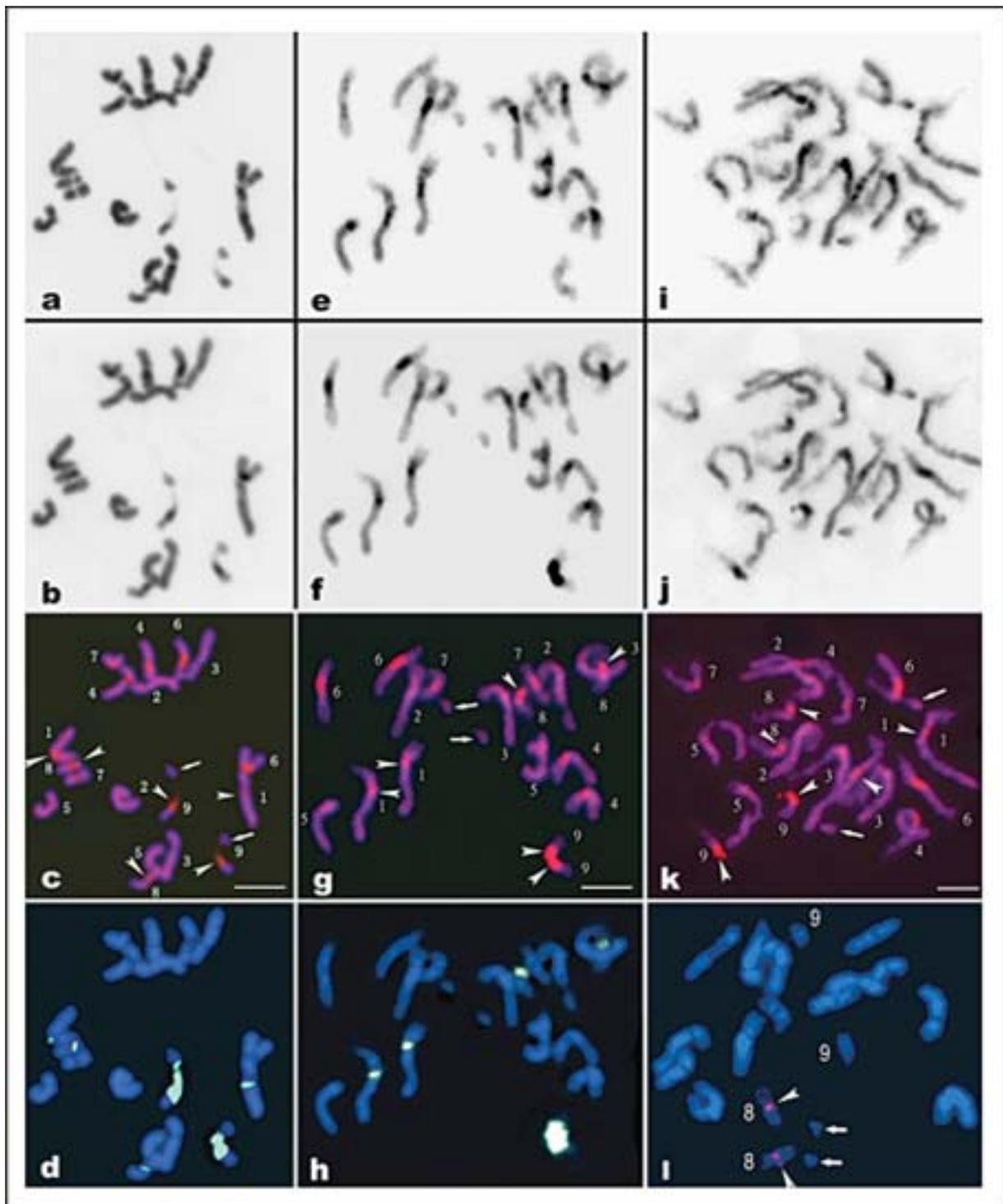
Fig. 1 — Metaphase (a, b, c, d), prometaphase (e, f, g, h) and prophase (i, j, k) chromosomes of *P. tetragonolobus* after sequential CPD staining and FISH with 45S rDNA probe and metaphase chromosomes after FISH with 5S rDNA probe (l). (a, e, i) Inverts of DAPI images; (b, f, j) Inverts of PI images; (c, g, k) CPD images; (d, h) FISH images. FISH image of the prophase plate is not shown. Chromosome numbers are designated by means of karyotyping. Arrowheads indicate 45S rDNA CPD bands. Arrows indi-cate satellites separated from the rest of chromosome 9 in the preparation. Bars = 5μm.

Table 1 — Chromosome measurements of *P. tetragonolobus.*

Chromosome No.	Relative length	Arm ratio (L/S)	Chromosome type
1	15.78±0.92	2.38±0.25	sm
2	14.56±0.51	2.35±0.37	sm
3	13.57±1.64	1.97±0.13	sm
4	12.59±1.80	1.63±0.20	m
5	11.89±1.23	2.44±0.36	sm
6	10.70±0.81	1.47±0.24	m
7	7.60±0.49	1.21±0.12	m
8	7.16±0.63	2.31±0.53	sm
9	6.14±0.70	1.23±0.08	m*

m: metacentric (arm ratio=1-1.7), sm: submetacentric (arm ratio=1.7-3.0). * Satellite chromosome. The length of satellite is included in the chromosome length.

Table 2 — Frequency of Ag-NORs of three pair of NORs in leaf meristematic cells (%).

The major NORs	NORs on chromosome 1	NORs on chromosome 8	No. of cells analyzed
100.0	48.6	85.7	70

minor sites on chromosome 8 appeared in the cells showing four Ag-NORs (Fig. 3b).

DISCUSSION

For the first time the karyotype of *P. tetragonolobus* (L.) DC was characterized in detail based on chromosome lengths, DAPI and CPD banding patterns, Ag-NORs as well as FISH with 45S and 5S rDNA probes. Previously, it was difficult to unambiguously identify all nine pairs of its somatic chromosomes on the basis of chromosome morphology and Giemsa bands (Pickersgill 1980; She et al. 1998). The most difficulties in conventional karyo-

minor sites only occurred in a proportion of the recorded cells. Consequently, cells showing six, four and two Ag-NORs, respectively, were observed (Figs. 3a, 3b and 3c). The occurrence frequency of Ag-NORs of the minor sites on chromosome 8 was much higher than that of the minor sites on chromosome 1 (Tab. 2). Moreover, only the Ag-NORs of the
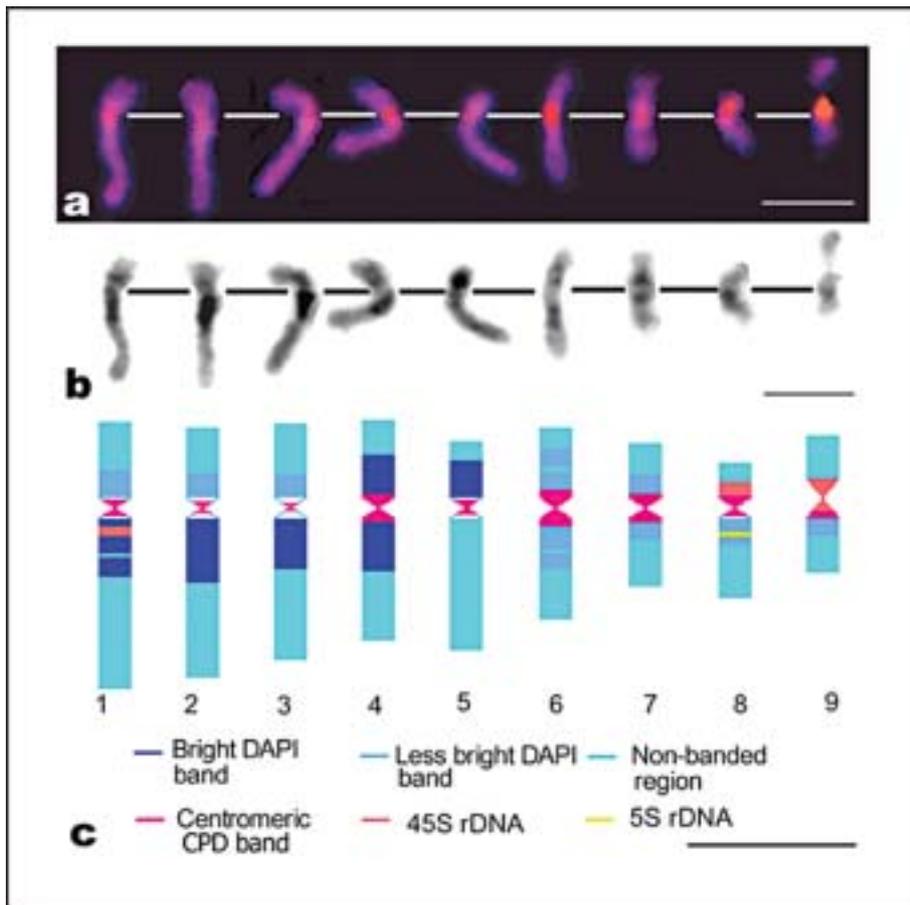

Fig. 2 — Karyotype and idiogram of *P. tetragonolobus*. (a) Karyotype of CPD bands. (a) Karyotype of DAPI bands. (c) Idiogram displaying DAPI and CPD banding patterns and the locations of 45S and 5S rDNA sites. Bars = 5µm.
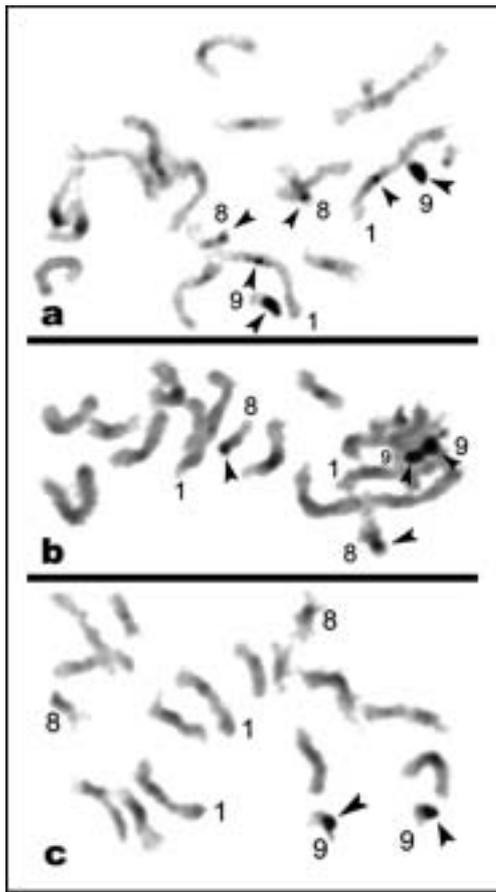
Fig. 3 — Mitotic chromosomes of *P. tetragonolobus* after silver staining. (a, b, c) cells showing six, four and two Ag-NORs (arrowheads), respectively. Numbers mark the NOR chromosomes distinguished by CPD banding performed before silver staining.

typing for the winged bean were to identify the positions of centromeres (e.g. those of chromosome 5, 6, 7) and to distinguish chromosome pairs with similar relative length and/or arm ratio (e.g. chromosome 1, 2, 3). Our results demonstrated that DAPI and CPD banding patterns as well as rDNA FISH signals all are available markers for identifying chromosomes and their centromeres in the winged bean. Using these markers and standard karyotyping procedures, we are able to distinguish each chromosome pair and establish an exact karyotype of *P. tetragonolobus*. As revealed by our results, at prophase and prometaphase each chromosome pair exhibited its distinct DAPI and CPD banding patterns, so different chromosomes could be distinguished more easily than at metaphase. Actually, even though DAPI bands could not be shown on metaphase chromosomes, the distinctive CPD bands in combination with conventional measurements also could distinguish each chromosome pair at metaphase. In addition, FISH

signals of 45S and 5S rDNA alone could mark three different chromosome pairs (Fig. 2c). Although the centromeric position of the chromosome pair with secondary constriction was roughly determined by CPD staining, an exact identification will not be possible before any centromere specific sequence is isolated.

It has been well documented that NORs, i.e. 45S rDNA sequences, are GC-rich compared to most other DNA regions (MACGREGOR and KEZER 1971; YASMINEH and YUNIS 1971; INGLE et al. 1975). CPD staining has been shown to differentially stain NORs in chromosomes of cotton (HANSON et al. 1995; JI et al. 1997), two *Lilium* species (LIM et al. 2000) and rice (KATO et al. 2003). Here, we demonstrated that all NORs in the winged bean could also be efficiently shown by CPD staining. Our experiments also revealed that both rDNA and centromeric CPD banded regions in the winged bean exhibited the same PI and DAPI staining features. Such staining features would reflect the interactions of PI and DAPI with GC-rich DNA sequences (PETERSON et al. 1999). So it is suggested that the centromeric regions of the winged bean contain GC-rich DNA sequences.

The rRNA genes occur in tandem arrays at the nucleolar organizer regions (NORs). The active ribosomal genes can be detected by silver staining technique, which allows the visualization of Ag-NORs that were transcribed in the previous interphase (HUBBELL 1985; MORAIS-CECÍLIO et al. 2000; CARPETA et al. 2002). The size of the in situ hybridization signal of a particular NOR is proportional to the number of ribosomal cistrons it contains (LEITCH and HESLOP-HARRISON 1992; ZURITA et al. 1999). Obviously, in the winged bean the major NORs have much more copies of ribosomal cistrons than the minors. Moreover, the NORs on chromosome 1 should have less copy of ribosomal cistrons than the NORs on chromosome 8 since the detection rate of the FISH signals of these NORs was lower. Silver staining on chromosomes in the winged bean demonstrated that the transcription of rRNA genes of the major NORs took place in every mitotic cell, but that of the two minors only occurred in a proportion of the observed cells, and even between the two pairs of minor NORs the level of expression differed, the NORs with relatively higher amount of ribosomal cistrons had higher activity and were activated preferentially. Similar case has been found in a mammal (ZURITA et al. 1999) but has not been found in any natural diploid plant genome so far. ZURITA et al. (1999) demonstrated that the relative amount of rDNA in a NOR of the multiple NOR system determines its level of expression and its probability of be-

coming active. The expression patterns of three pairs of NORs in the winged bean are in favor of the hypothesis that competition between NORs for essential transcription factors in limiting concentration result in differential activity between different NORs (ZURITA et al. 1998; ZURITA et al. 1999). However, in the winged bean other factors may also be related to the lower activity of the minor NORs. For example, the NORs on chromosome 1 situated on the long arm and between two DAPI-positive heterochromatic domains. The positioning on the long-arm itself or/and protein-protein interactions between the rDNA chromatin and the adjacent heterochromatic domains probably partially lead to decrease of the activity of the NORs (LIMA-DE-FARIA 1976; CARPETA et al. 2002).

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