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F. Ahmad · S. N. Acharya · Z. Mir · P. S. Mir

Localization and activity of rRNA genes on fenugreek (*Trigonella foenum-graecum* L.) chromosomes by fluorescent in situ hybridization and silver staining

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Abstract Fenugreek (*Trigonella foenum-graecum* L.), an annual legume crop grown mainly for seed at present, has the potential to be a high-quality forage crop in western Canada. A cytological survey of germplasm stored at the USDA germplasm center demonstrated a somatic karyotype with a chromosome number of 2n = 16. Structural details of two pairs of chromosomes (numbers 1 and 2) showed secondary constrictions that were presumed to be the site of rRNA genes. Fluorescent in situ hybridization (FISH) with a heterologous rRNA gene probe from wheat was utilized to physically map their location for the first time on fenugreek chromosomes. Strong hybridization signals of apparently similar copy-number sequences were detected primarily near the centromere on the metacentric chromosome 1 and near the telomere on the acrocentric chromosome 2. Silver staining of the nucleolar organizing region provided evidence that both the sites were transcriptionally active and independently capable of forming a nucleolus. However, the site on chromosome number 2 was relatively more active than the one on chromosome number 1.

Key words Trigonella · Fenugreek · NORs · Fluorescent in situ hybridization · Genome mapping

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F. Ahmad (✉)
Botany Department, Brandon University, Brandon, Manitoba R7A 6A9, Canada
Fax: + 1-204-728-7346
E-mail: ahmad@brandonu.ca

S. N. Acharya · Z. Mir · P. S. Mir
Agriculture and Agri-Food Canada, Lethbridge, Alberta, Canada

Introduction

Trigonella foenum-graecum (commonly known as "Methi" or "Fenugreek") of family Leguminosae and subfamily Papilionaceae is extensively grown in tropical and sub-tropical regions of India for its vegetable value and seeds, which are medicinally important (Pandey 1993). It is also traditionally grown in parts of Europe and Africa and represents a potentially new crop for Canada. Besides the condiment value of the seed, there is also interest in developing this crop as high-quality forage for cattle (Mir et al. 1993, 1996).

Cytogenetic studies in the genus have been limited to chromosome counts and preliminary karyotype descriptions of the cultivated species and a few of the wild related species (Singh and Roy 1970; Singh and Singh 1976a; Wanjari 1976; Ladizinsky and Porath 1977; Lavania and Sharma 1980; Agarwal and Gupta 1983; Ladizinsky and Vosa 1986). A few euploid and aneuploid cytogenetic stocks, primarily in the form of tetraploids, triploids and simple primary trisomics, have also been reported (Gopinath 1974; Singh and Singh 1976b,c; Raghuvanshi and Singh 1977). The relatively small number (2n = 16) of chromosomes in the chromosome complement of fenugreek makes it a suitable candidate for cytogenetic studies. With the exception of satellited chromosomes, distinct cytological markers for chromosome identification have not yet been identified. No genetic linkages are known, and no gene(s) have been placed onto their respective chromosomes. Also, differential chromosome staining techniques have not been successfully applied, and DNA probes for in situ hybridization have not been tried for the identification of fenugreek chromosomes.

The ribosomal RNA genes (rRNA genes or rDNA sequences) are a cluster of three genes (18S-5.8S-26S) and are present as many hundreds of tandemly repeated units (Appels et al. 1980). Together with the intergenic spacers, they are localized at one or more chromosomal sites within a genome (Rogers and Bendich 1987). Thus, for example, rRNA genes have been located simultaneously on one

chromosome pair in lentil (*Lens culinaris* Medik, 2n = 14; Abbo et al. 1994), three pairs in oats (*Avena sativa* L., 2n = 42; Linares et al. 1996), four pairs in apple (*Malus* × *domestica* Borkh., 2n = 34; Schuster et al. 1997) and five pairs in wheat (*Triticum aestivum* L. Thell, 2n = 42; Mukai et al. 1991). If active, the rRNA genes form a nucleolus and nucleolar organizing regions (NORs). The secondary constrictions on chromosomes are a definite site for rRNA genes, although the presence of these genes may not necessarily be associated with a secondary constriction (Ahmad et al. 1998). Because of their universal occurrence and redundancy, the ribosomal genes are a valuable model for karyotypic analysis and are also useful for comparative studies of genome organization. It is thus important to know the number of sites of major and minor gene clusters within genomes in order to understand gene expression, aspects of phylogeny and evolution (Lavania 1998).

In situ hybridization of nucleic acids has proven to be an important and versatile technique in modern plant genetics. It provides a unique link between the established methods of classical cytogenetics and a molecular approach to genome analysis (Lavania 1998). In the field of plant molecular cytogenetics, this technique has been used to differentiate parental genomes in hybrids or amphiploids (Mukai and Nakahara 1993; Chen et al. 1995), alien chromosomes added or substituted in cultivated plants (Islam-Faridi and Mujeeb-Kazi 1995; Miller et al. 1996), chromosomal translocations (Friebe et al. 1995; Islam-Faridi and Mujeeb-Kazi 1995) and to assess intergenomic chromosome pairing in wide hybrids (King et al. 1994; Chen et al. 1995; Miller et al. 1996). Additionally, it has also been used to physically locate specific DNA sequences on plant chromosomes (Abbo et al. 1994; Busch et al. 1995; Fominaya et al. 1995; Linares et al. 1996).

In the present paper, we have applied the fluorescent in situ hybridization (FISH) technique to the physical genome mapping of fenugreek using the genes encoding 18S-5.8S-26S rRNA as a model and successfully localized them on metaphase chromosomes as well as interphase nuclei. We have also deliberately given a detailed protoplast procedure for fenugreek chromosome slide preparation which gives clear chromosome preparations that are devoid of cellular debris and explained in detail the FISH procedure for fenugreek to facilitate the transfer of this technique between laboratories. Transcriptional activity of rDNA genes in fenugreek was also studied for the first time.

Materials and methods

Seeds of two fenugreek accessions, L-3053 and PI 244060, were used in this study. Accession L-3053 is cv 'Dilba' from Yemen and PI 244060 is cv 'Amber' from Canada. Seeds were kindly supplied to us by the USDA-Plant Introduction Station (Pullman, Wash., USA).

Fast-growing, healthy root tips about 1.0–1.5 cm in length were collected and pretreated with ice-cold distilled water for 48 h to arrest the cells in metaphase. Pretreated roots were fixed in 0.1% acetocarmine for 2 h at room temperature and then overnight in the same solution at −20°C. Prior to slide preparation by the protoplast method, roots were rinsed a few times for 5 min each in 0.01 *M*, pH 4.5 citric acid-sodium citrate buffer (CA-SC). Only the 1-mm (or smaller) meristematic region of the roots was used as tissue for enzyme digestion for producing protoplasts. The enzyme mixture consisted of 5% cellulase "Onozuka R-10" and 1% pectolyase "Y-23" in CA-SC buffer. After enzyme digestion (30 min at 37°C, the tissue was again rinsed gently and thoroughly with four to five washes in CA-SC buffer. It was further rinsed with Farmer's fixative (3 parts 95% ethyl alcohol : 1 part glacial acetic acid) and finally it was macerated in the same fixative to yield the protoplasts. A drop of this protoplast suspension was dropped onto an alcohol-cleaned slide, air-dried for a few minutes and mounted under a coverglass with 45% acetic acid. The slides were screened using phase contrast microscopy and the coverglass removed from acceptable slides after they were frozen. Slides prepared the same way were used both for FISH as well as silver staining, as described below.

Labelling of probe DNA

Heterologous ribosomal DNA sequence was used for in situ hybridization. Clone pTa71 (Gerlach and Bedbrook 1979) contains a 8.9-kb genomic fragment of bread wheat consisting of the 18S-5.8S-26S rRNA genes and the transcribed and non-transcribed spacer regions and is cloned in the *Eco*RI site of pUC 19 vector. The plasmid was kindly supplied to us by Dr. K. C. Armstrong (Agriculture&Agri-Food Canada, Canada). For labelling purposes, the entire uncut plasmid containing the rRNA gene insert was used, since it was not found necessary to isolate the insert from the plasmid for the in situ experiment reported here. DNA was labelled by nick translation using biotin-14-dATP of the "BioNick Labelling System" kit (GIBCO-BRL). Three micrograms of DNA was labelled per 50 µl of labelling reaction (using 5 µl of 10 × enzyme mix) for 2 h at 16°C, and the reaction stopped with 5 µl of 300 m*M* EDTA (pH 8.0) solution. Unincorporated nucleotides were removed by passing the labelling reaction through a sephacryl S-400 HR resin column (Pharmacia).

Fluorescent in situ hybridization

Prepared slides were immersed in 45% acetic acid solution for 10 min at room temperature, following which they were air-dried for 1–2 h at room temperature. The slides were then treated with RNAse (30 µg/ml in 2 × SSC) for 1 h in a humid chamber at 37°C. After draining the slides, chromosomal DNA was denatured by immersing the slides in 70% formamide (deionized) solution in 2 × SSC at 70°C for 2 min. The slides were then dehydrated at −20°C for 5 min each in a graded series of 70%, 80% and absolute alcohol. After the slides were air-dried at room temperature, the hybridization mixture was applied.

The hybridization mixture per slide consisted of 100 ng labelled probe DNA, 10 µg of autoclave-sheared (20 psi, 20 min) calf thymus carrier blocking DNA, 50% deionized formamide, 10% dextran sulfate, 2 × SSC and 0.1% SDS. The hybridization mixture was denatured for 10 min at 80°C and then immediately quenched on ice for 10 min or longer. For each slide 40 µl of the hybridization mixture was applied and overlaid with a 24 × 30-mm plastic coverslip (cut from an autoclavable plastic bag). The slide, with the hybridization mixture, was incubated at 80°C for 8 min and then overnight at 37°C in a humid chamber.

Following overnight hybridization the coverslips were removed, the slides drained and washed in 2 × SSC at 40°C for 2 × 5 min. They were then washed 1 × 10 min in 50% formamide in 2 × SSC at 40°C to remove the unhybridized and weakly hybridized probes. The stringency was such so as to allow sequences with at least 80–85% homology to bind. After another two washes of 5 min each in 2 × SSC at 40°C, the slides were washed for 5 min each in 2 × SSC and then in detection buffer (4 × SSC, 0.2% Tween-20) at room temperature. They were then incubated with 175 µl of 5% BSA (w/v)

in detection buffer (blocking solution) for 5 min at room temperature in a humid chamber. The blocking solution was drained off, and the preparations were covered with 100 µl of FITC (fluorescein isothiocyanate)-conjugated fluorescein avidin-D (15 µg/ml in detection buffer with 5% BSA). A plastic coverslip was placed over the preparation and the slides incubated in a humid chamber at 37°C for 1 h. After incubation, the slides were washed for 3 × 5 min at 37°C in detection buffer. The preparations were then blocked with 175 µl of 5% normal goat serum (v/v) in detection buffer. They were then drained and covered with 100 µl of biotinylated anti-avidin D (15 µg/ml) in detection buffer with 5% normal goat serum) and incubated in a humid chamber at 37°C for 1 h. Following that they were again washed 3 × 5 min in detection buffer at 37°C. The preparations were blocked again with 5% BSA as above for further amplification of the signal with another layer of 100 µl FITC-conjugated fluorescein avidin-D and incubated as above. Following 3 × 5 min washes in detection buffer, the chromosomes were counterstained with 125 µl of propidium iodide (20 µg/ml in 2 × SSC) for 20 min at room temperature in the dark. The slides were then briefly rinsed in 2 × SSC at room temperature, drained and mounted in antifade. An epifluorescent module with the appropriate triple-band-pass filter set was used for UV illumination to simultaneously visualize the propidium iodide-counterstained chromosomes (red to orange-red fluorescence) and fluorescein signal (yellow to greenish-yellow fluorescence). Representatitive cells were photographed on Fuji G400 color print film.

Silver staining

Freshly prepared slides by the "protoplast" procedure were used for the silver satining protocol of Stack et al. (1991). Briefly, the coverslip was removed from frozen slides and the slides air-dried at room temperature for 24 h or less. They were then immersed in 2 × SSC solution (pH 7.0) for 10 min at room temperature. After the slides were rinsed briefly in distilled water and air dried two drops of 50% silver nitrate aqueous solution were applied to the cellular mass. A plastic coverslip (cut out of autocavable plastic bags) was used to evenly spread and cover the solution. Slides were incubated 6 h to overnight at 60°C under humid conditions, rinsed a few times with distilled water, air-dried and mounted in immersion oil. They were observed with bright field microscopy, and photomicrographs were taken on TechPan 2415 black and white print film.

Feulgen staining

For Feulgen staining, fixed root tips were rinsed in a few changes of distilled water, hydrolyzed in 1 *N* HCl at 60°C for 11 min, rinsed again with distilled water and stained in Schiff's reagent for 45 min at room temperature in the dark. After staining, squashes were made in 45% acetic acid. For later observations, the slides were made permanent by removing the coverslip after freezing the slides, air drying them and subsequently immersing them in absolute alcohol, air drying and finally immersing them in xylene; each step was 30 min long. Air-dried slides were then mounted in Permount and made permanent. Slides were observed with bright field microscopy, and photomicrographs taken on TechPan 2415 black and white print film.

Results and discussion

Feulgen staining of the tip chromosomes revealed that the somatic chromosome number in both lines of fenugreek was 2n = 16 (Fig. 1a). The same chromosome number has been observed for other accessions by other researchers (Singh and Roy 1970; Singh and Singh 1976a; Wanjari 1976; Ladizinsky and Porath 1977; Lavania and Sharma 1980; Agarwal and Gupta 1983; Ladizinsky and Vosa 1986; Ahmad et al. unpublished results). Contrary to some studies reporting one pair of satellited chromosomes in fenugreek (Wanjari 1976; Ladizinsky and Porath 1977; Lavania and Sharma 1980; Agarwal and Gupta 1983) we found that there were two pairs of chromosomes showing a satellite at metaphase (Fig. 1a) and prometaphase (Fig. 1b). This was in accordance with only two other studies (Singh and Roy 1970; Singh and Singh 1976a). The presence of two pairs of satellited chromosomes in fenugreek has also been confirmed in 23 other accessions of diverse origin from an array of eco-geographical areas of the world (Ahmad et al. unpublished results). The longest chromosome, chromosome 1, which is also metacentric, has a near-median secondary constriction proximal to the primary constriction, while the acrocentric chromosome 2 has a near-telomeric secondary constriction. The secondary constriction of chromosome 1 is the one which has been detected and reported in all previous studies, while the latter is more difficult to detect and, therefore, largely gone undetected in many previous studies. The various pretreatment chemicals used and the level of chromosome contraction are the key elements which translate into the capability of being able to detect the secondary constrictions and other fine karyological details. Of the various pretreatments studied by us for fenugreek, only a 48-h ice-cold water pretreatment of the root tips reproducibly gave well-spread chromosomes with good morphology and clear structural details (Fig. 1a,b).

In situ hybridization using the 18S-5.8S-26S rDNA probe pTa71 indicated, as expected, the nucleolar organizing region of the two pairs of satellited chromosomes to be the primary site of rDNA sequences in both lines of fenugreek studied. The cytological observation of these structures in the karyotype can be a reliable indicator of the presence of genes in question. However, the cytological absence of the secondary constriction does not necessarily mean that the rDNA cistrons are not present, as has been shown in wheat (Mukai et al. 1991) and pearl millet (Ahmad et al. 1998). No additional sites of rDNA sequences were detected on any of the non-satellited chromosomes. Approximately 50 cells from many different slides were observed for each of the two lines and the two pairs of satellited chromosomes showed a labelled site in every single cell (Fig. 2a,b). The hybridization signal was clearly visible not only in the secondary constriction but also adjacent to the secondary constriction and proximal to the centromere on one side and extended onto the other side of the constriction to cover almost the entire satellite portion of the short arm of the acrocentric chromosome (Fig. 2b). In all cases, both chromatids of the homologous pair were clearly labelled. In addition to the acrocentric chromosome, another pair of

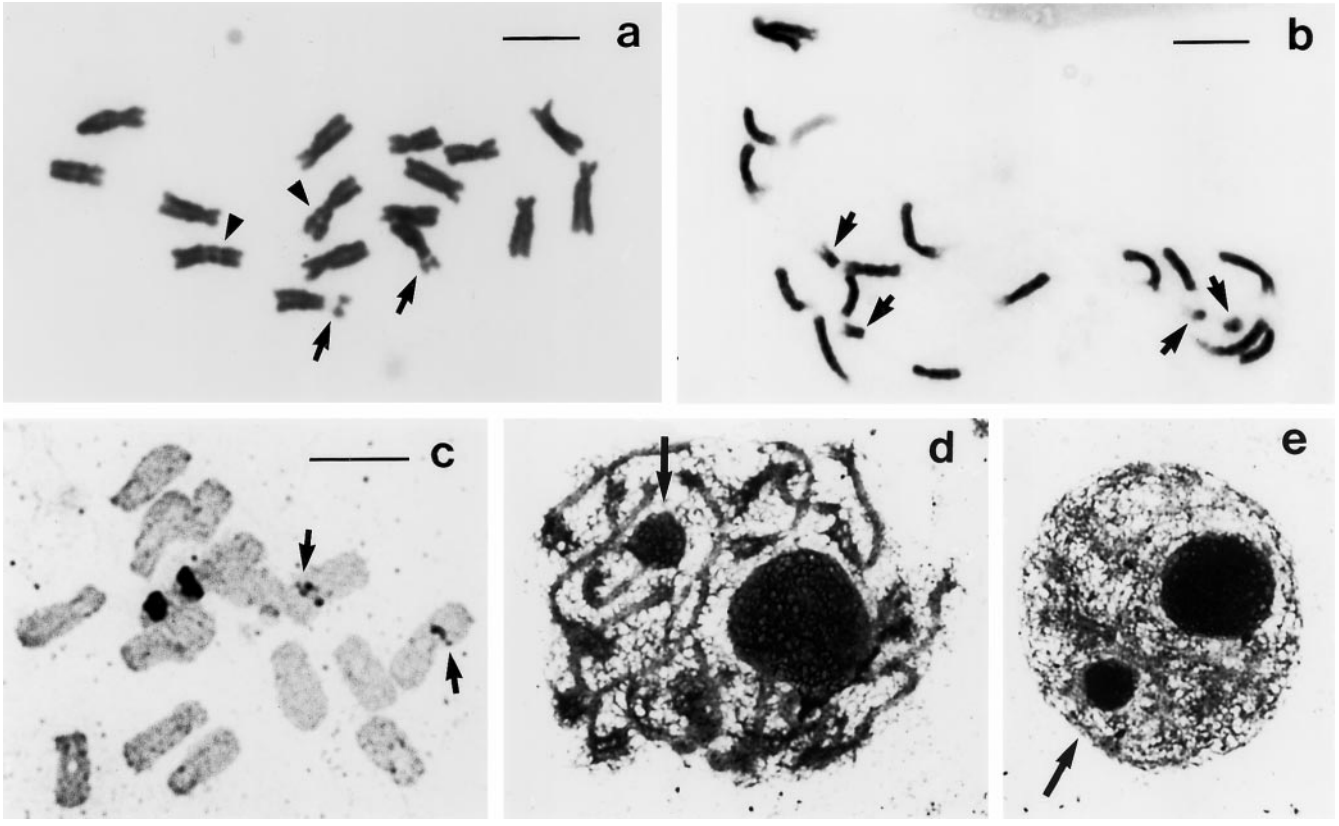

Fig. 1a–e Feulgen-stained (**a, b**) and silver-stained (**c, d, e**) mitotic cells of fenugreek. **a** Metaphase plate showing morphology of the 16 chromosomes. Note the intercalary location of secondary constrictions on the metacentric chromosome pair (*arrowheads*) and the near terminal location on the acrocentric chromosome pair (*arrows*). **b** Prometaphase cell clearly showing four satellites (*arrows*) in the chromosome complement. **c** Silver-stained metaphase plate showing *dark* transcriptionally active NOR bands on two pairs of satellited chromosomes.The NOR site on the metacentric chromosome (*arrows*) has less activity than the one on the acrocentric chromosome. **d** Prometaphase and **e** interphase nucleus showing two different sizes of nucleoli. The smaller one is marked by an *arrow Bar* (**a–c**): 5 μ

metacentric chromosomes also showed a clear signal. Again, as in the satellited acrocentric chromosome, the signal was visible in all cells, and in both chromatids, and was present in an intercalary position (Fig. 2a,b). At a highly decondensed chromatin stage of interphase, more than two pairs of hybridization signals were detected (Fig. 1a), but as the chromatin condensation progressed only two pairs of clear signals were visible (Fig. 1d). Additionally, in a spontaneous tetraploid seed, somatic cells showed four pairs of chromosomes with a distinct signal (Fig. 1c). We propose that of the two rRNA loci detected in fenugreek, the one on the metacentric satellited chromosome should be referred to as *Nor-1*, since this chromosome has been identified to be satellited in all reports. The second locus, on the acrocentric chromosome, which has not shown an obvious secondary constriction in many studies, should be referred to as *Nor-2*.

There are no available reports specifying rDNA sequence localization in fenugreek chromosomes by in situ hybridization or of the activity of NORs by silver staining. The silver staining procedure is commonly used to visualize the activity of rRNA genes since it has been established that only those NORs of metaphase chromosomes that are functionally active during the preceding interphase are capable of staining (Hubbell 1985). In our study silver staining revealed two pairs of dark bands in metaphase chromosmes at the site of the secondary constrictions in both of the accessions studied. However, there was a marked difference in the size of the band on the two chromosome pairs. While the acrocentric chromosome, on which the secondary constriction had a more telomeric location, had a larger band, the metacentric chromosome, with an intercalary secondary constriction, had a relatively much narrower band (Fig. 1c). This may indicate that there is differential activity of the rRNA genes at these two sites. This is further evident in the interphase/late prophase stage of cell division where two nucleoli of markedly different sizes were clearly visible (Fig. 1d,e). We can therefore conclude that both pairs of secondary constricted chromosomes in feugreek contain transcriptionally active rDNA, albeit showing differential activity, and that each of these sites is able to independently organize a nucleolus.

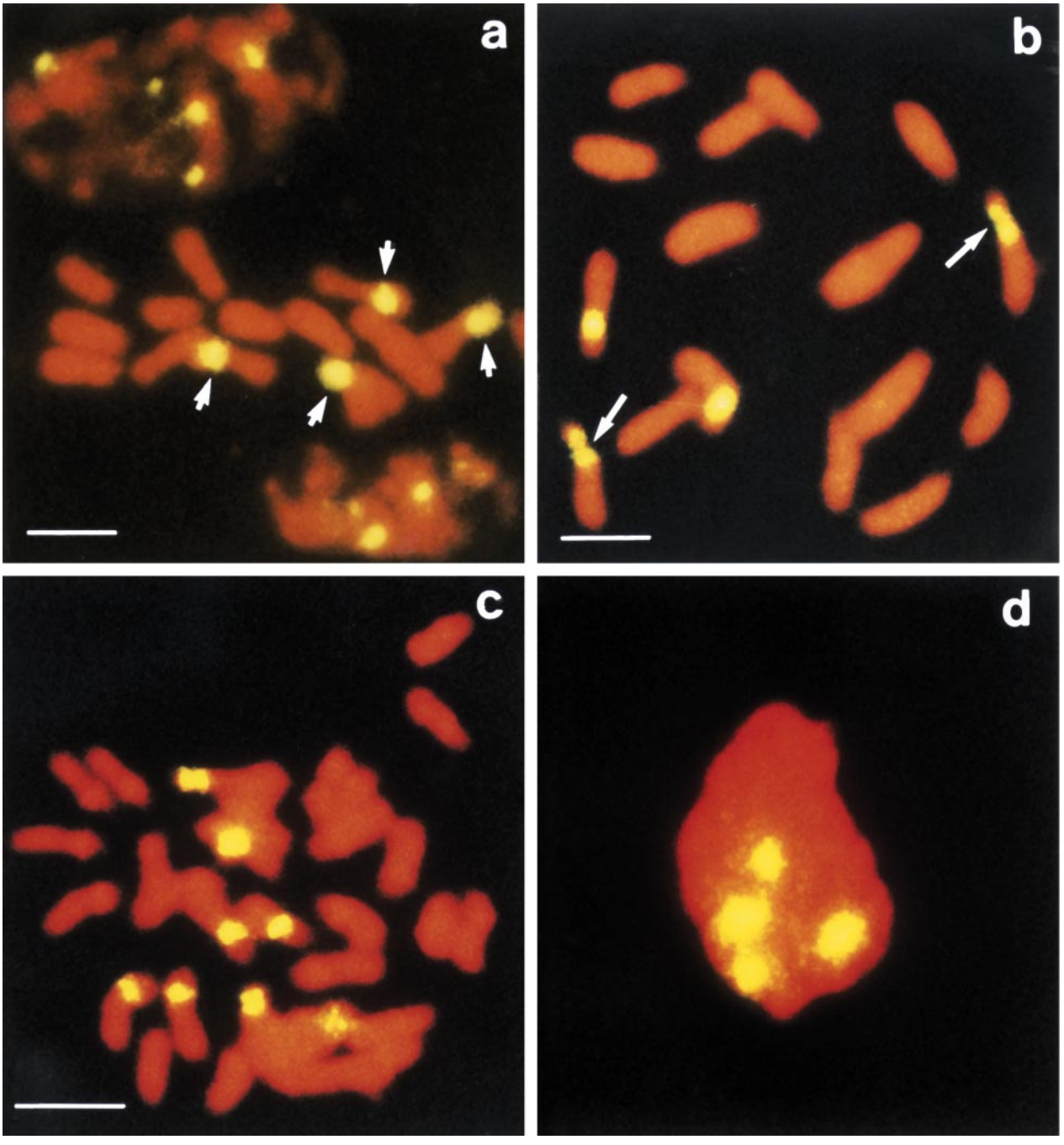

Fig. 2a–d Fluorescent in situ hybridization photmicrographs showing the physical location of rRNA genes (*yellow fluorescence*) on fenugreek chromosomes and interphase nuclei. **a** Mid-to late-metaphase plate showing four hybridization signals (*arrows*, *yellow* color). Also note the decondensed interphase nuclei, above and below the metaphase, with more than four signals. **b** Pro- to early-metaphase showing more structural detail in the acrocentric chromosome (*arrows*) and four hybridization sites. **c** A tetraploid cell with eight hybridization signals. **d** A condensed interphase nucleus showing four clear sites of probe hybridization (*yellow color*). *Bar* (**a–c**): 5 μ

The presence of an additional rDNA site and two chromosome pairs with cytologically detectable and distinct secondary constrictions in a supposedly diploid plant species like fenugreek with only eight pairs of chromosomes is somewhat intriguing. The genetics of fenugreek remains largely unknown (Pandey 1993), and it is also not known if any of the structural genes are also duplicated. In certain other diploid plant species, various levels of gene duplication have been reported (Ellstrand et al. 1983; Wendel et al. 1986; Kazan et al. 1991) and at least in the case of corn an allopolypoidization event has been implicated (Wendel et al. 1989).

The precise origin of the two satellited chromosome pairs in fenugreek is not known, but the involvement of two species/cytotype hybridization could be speculated. This in turn could have been followed by a lack of amphiplasty (suppression of NOR activity), resulting in transcriptional activity of rRNA genes at both of these loci. If this speculative suggestion is at all feasible, then the polyploidization event during the evolution of *Trigonella* species must have taken two different evolutionary lineages, since other *Trigonella* species with fenugreek-like seeds tend to show two pairs of satellited chromosomes, while still other species with little or no seed morphology resemblance show only one pair of satellited chromosomes (Ahmad et al. unpublished results). Further research on these lines could prove helpful in understanding evolution in the genus *Trigonella*.

In situ hybridization of repetitive probes has proven to be a valuable method for examining chromosomal evolution in plants and gives a complementary view to that obtained from plant morphology and chromosome pairing (Lavania 1998). Furthermore, FISH opens up new ways for detailed karyotype analysis by providing chromosomal markers for mapping DNA sequences and gene(s) to chromosomes, for correlating linkage groups of genetic and restriction fragment length polymorphism (RFLP) maps to chromosomes and for the positive identification of alien translocated lines that are used to transfer resistance genes from wild to cultivated plants (Miller et al. 1996; Lavania 1998). The results reported here show that in situ hybridization is the method of choice to map the number and physical location of sites of highly repeated sequences in fenugreek because of its sensitivity, accuracy and simplicity. The characteristic location of the rDNA sites in fenugreek makes them valuable reference points on the chromosomes and are likely to help chromosome identification when physically mapping in combination with other sequences by in situ hybridization. It is anticipated that the isolation of single-low-copy sequences in combination with pachynema cytology (Xu and Earle 1996) will allow a more complete picture of their physical organization on fenugreek chromosomes.

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References

Abbo S, Miller TE, Reader SM, Dunford RP, King IP (1994) Detection of ribosomal DNA sites in lentil and chickpea by fluorescent in situ hybridization. Genome 37:713–716

Agarwal K, Gupta PK (1983) Cytological studies in the genus *Trigonella* Linn. Cytologia 48:771–779

Ahmad F, Comeau A, Collin J, St.-Pierre CA (1998) Fluorescent in situ hybridization (FISH) with 18S-5.8S-26S rRNA genes reveal a novel site on chromosome 6 in addition to the satellited chromosome in pearl millet. J Genet Breed (in press)

Appels R, Gerlach WL, Dennis ES, Swift H, Peacock WJ (1980) Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. Chromosoma 78:293–311

Busch W, Hermann RG, Martin R (1995) Refined physical mapping of the Sec-1 locus on the satellite of chromosome 1R of rye (*Secale cereale*). Genome 38:889–895

Chen Q, Conner RL, Laroche A (1995) Identification of the parental chromosomes of the wheat alien amphiploid Agrotana by genomic in situ hybridization. Genome 38:1163–1169

Ellstrand NC, Lee JM, Foster KW (1983) Alcohol dehydrogenase isozymes in grain sorghum (*Sorghum bicolor*): evidence for gene duplication. Biochem Genet 21:147–155

Friebe B, Zhang W, Raupp JW, Gill BS, Porter DR (1995) Non-homoeologous wheat-rye chromosomal translocations conferring resistance to green bug. Euphytica 84:121–125

Fominaya A, Hueros G, Loarce Y, Ferrer E (1995). Chromosomal distribution of a repeated DNA sequence from C-genome heterochromatin and the identification of a new ribosomal DNA locus in the *Avena* genus. Genome 38:548–557

Gerlach WL, Bedbrook JR (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7:1869–1885

Gopinath PM (1974) A case of rare triploidy in excised root culture of *Trigonella foenum-graecum* L. Curr Sci 43:524–525

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

Islam-Faridi MN, Mujeeb-Kazi A (1995) Visualization of *Secale cereale* DNA in wheat germplasm by fluorescent in situ hybridization. Theor Appl Genet 90:595–600

Kazan K, Kusmenoglu I, Muehlbauer FJ, Weeden NF (1991) Duplication of aldolase and esterase loci in *Cicer* (Cicereae Alef.). J Hered 82:58–63

King IP, Reader SM, Purdie KA, Orford SE, Miller TE (1994) A study of the effect of a homoeologous pairing promotor on chromosome pairing in wheat/rye hybrids using genomic in situ hybridization. Heredity 72:318–321

Ladizinsky G, Porath N (1977) On the origin of fenugreek (*Trigonella foenum-graecum* L.). Legume Res 1:38–42

Ladizinsky G, Vosa CG (1986) Karyotype and C-banding in *Trigonella* section *Foenum-graecum* (*Fabaceae*). Plant Syst Evol 153:1–5

Lavania UC (1998) Fluorescence in situ hybridization in genome, chromosome and gene identification in plants. Curr Sci 74:126–133

Lavania UC, Sharma AK (1980) Giemsa C-banding, somatic association and orientation of interphase chromosomes in *Trigonella foenum-graecum* (L.). Caryologia 33:17–23

Linares C, Gonzalez J, Ferrer E, Fominaya A (1996) The use of double fluorescence in situ hybridization to physically map the positions of 5S rDNA genes in relation to the chromosomal location of 18S-5.8S-26S *r*DNA and a C genome specific DNA sequence in the genus *Avena*. Genome 39:535–542

Miller TE, Reader SM, Purdie KA, King IP (1996) Fluorescent in situ hybridization – a useful aid to the introduction of alien genetic variation into wheat. Euphytica 89:113–119

Mir PS, Mir Z, Townley-Smith L (1993) Comparison of the nutrient content and in situ degradability of fenugreek (*Trigonella foenum-graecum*) and alfalfa hays. Can J Anim Sci 73:993–996

Mir Z, Acharya SN, Mir PS, Taylor WG, Zaman S, Mears G, Goonewardene LA (1996) Nutrient composition, in vitro gas production and digestibility of fenugreek (*Trigonella foenum-graecum*) and alfalfa forages. Can J Anim Sci 77:119–124

Mukai Y, Nakahara Y (1993) Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. Genome 36 : 489–494

Mukai Y, Endo T, Gill BS (1991) Physical mapping of the 18S.26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma 100 : 71–78

Pandey SC (1993) Improvement of leafy vegetables. In: Chadha KL, Kalloo G (eds) Advances in horticulture, vol 5: vegetable crops part I. Malhotra Publ House, New Delhi, pp 325–342

Raghuvanshi SS, Singh AK (1977) Polyploid breeding in *Trigonella foenum-graecum* L. Cytologia 43 : 5–19

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

Schuster M, Fuchs J, Schubert I (1997) Cytogenetics in fruit breeding-localization of ribosomal RNA genes on chromosomes of apple (*Malus* × *domestica* Borkh.). Theor Appl Genet 94 : 322–324

Singh A, Roy RP (1970) Karyological studies in *Trigonella*, *Indigofera* and *Phaseolus*. Nucleus 13 : 41–54

Singh A, Singh D (1976a) Karyotype studies in *Trigonella*. Nucleus 19 : 13–16

Singh D, Singh A (1976b) Double trisomics in *Trigonella foenum-graecum* L. Crop Improv 3 : 125–127

Singh D, Singh A (1976c) Primary trisomics in *Trigonella foenum-graecum* L. Nucleus 19 : 91–95

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

Wanjari KB (1976) Karyotype studies in *Trigonella foenum-graecum* from anther somatic cells. College Agril Nagpur Mag 49 : 51–53

Wendel JF, Stuber CW, Edwards MD, Goodman MM (1986) Duplicated chromosome segments in *Zea mays* L.: further evidence for hexokinase isozymes. Theor Appl Genet 72 : 178–185

Wendel JF, Stuber CW, Goodman MM, Bechett JB (1989) Duplicated plastid and triplicated cytosolic isozymes of triosephosphate isomerase in maize (*Zea mays* L.). J Hered 80 : 278–282

Xu J, Earle ED (1996) Direct FISH of 5S rDNA on tomato pachytene chromosomes places the gene at the heterochromatic knob immediately adjacent to the centromere of chromosome 1. Genome 39 : 216–221