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RESEARCH NOTE

Chromosomal localization of rDNA and DAPI bands in solanaceous medicinal plant *Hyoscyamus niger* L.

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Introduction

The genus *Hyoscyamus* comprises of medicinally important species rich in tropane alkaloids. Its cultivated species have been subjected to genetic enhancement through chromosomal manipulation. Linear differentiation of chromosomes is quite important to facilitate chromosome identification, and inter-chromosomal and intra-chromosomal alterations for complementing cytogenetic analysis. Therefore, two-colour simultaneous fluorescence *in situ* hybridization was performed on *Hyoscyamus niger* ($2n = 34$) that exhibits two prominent 45S rDNA and one weak 5S rDNA site in the chromosome complement. DAPI (4',6-diamidino-2-phenylindole) banding pattern observed on the same metaphase revealed that 15 of the 17 chromosomes have DAPI-positive bands, of which 13 show DAPI-positive bands in the telomeric region of short arm. The two chromosomes that support secondary constriction had only weak DAPI-positive bands in their centromeric region, but their 45S rDNA chromosomal site showed DAPI-negative bands. Chromosomes 4 and 17 did not show any DAPI band. Taken in conjunction with the karyomorphology, the two 45S rDNA sites could be correlated to one site each in the secondary constriction region of chromosomes 5 and 10, and 5S rDNA site in the intercalary region proximal to secondary constriction region of chromosome 5. The occurrence of reduced DAPI fluorescence in the satellite region of chromosome 5 in the 45S rDNA-rich region seems to be a unique observation with respect to molecular composition of this region in *H. niger*.

The genus *Hyoscyamus* (family: Solanaceae) constitutes a group of 11 species distributed from Canary Islands over Europe and North Africa to Asia (Good 1953). Most of the species in the genus are medicinally important, and at least three species, namely *H. muticus, H. niger* and *H. albus*, are cultivated as a source of the tropane alkaloids hyoscyamine, hyoscine and atropine. Species *H. niger*, commonly known as black henbane or Indian henbane, is naturalized in India. It has diverse uses in traditional as well as modern system of medicine for its mydriatic, antispasmodic, anticholinergic, analgesic and sedative action owing to its biologically active compounds. Although extensive efforts have been made to facilitate agricultural enhancement, including realization of stable polyploids (Lavania 1986; Srivastava and Lavania 1990; Lavania and Srivastava 1991) in all the three commercially important species, and monosomics in *H. muticus* (Tyagi and Dhawan 1988), only limited information is available on karyomorphological details of these species (Srivastava and Lavania 1987; Tyagi and Dubey 1989). Efforts on linear chromosome identification based on C-banding analysis have been made in *H. muticus* (Tyagi and Gill 1990), and on localization of ribosomal DNA sites in *H. niger* (Lavania 1998a), but no precise effort has been made in the latter species on chromosome identification based on any kind of banding analysis coupled with location of rDNA sites. The present study was therefore aimed to localize the chromosomal positions of 5S and 45S rDNA sites and use the data together with DAPI banding patterns to establish fine ideogram of *H. niger* to facilitate linear chromosomal differentiation.

Materials and methods

Plant material and somatic chromosome analysis

Inbred seeds of variety 'Aela' of *H. niger* L. (family: Solanaceae) developed at the Central Institute of Medicinal and Aromatic Plants, Lucknow, India, were used in this study. Seeds presoaked overnight in water were placed in moist germination dish in dark at room temperature (≈25°C).

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Root tips from fast growing primary roots reaching about 1 cm were excised and pretreated in saturated aqueous solution of para-dichlorobenzene for 3 h at 12–14°C followed by fixation in ethanol:acetic acid (3:1) overnight. For metaphase chromosome analysis, the fixed roots were softened by warming in freshly prepared solution of 1N HCl:2% aceto–orcein (1:9) for 1 min at 60°C and then kept at room temperature for 2 h. The stained root-tips were squashed to obtain good metaphase spreads. At least five well spread metaphase plates with proper chromosome contraction and intact cell wall were analysed to prepare the standard idiogram.

Preparation of metaphase plates for FISH and DAPI banding

To prepare metaphase plates for *in situ* hybridization, the fixed root tips were washed in 1× enzyme buffer at pH 4.6 (prepared by mixing 40 mL of 10 mM citric acid and 60 mL of 10 mM trisodium citrate) to remove the fixative, and then transferred to an enzyme solution (mixture of 3% (v/v) pectinase from *Aspergillus niger* + 2% (w/v) cellulase (1.8% cellulase from *A. niger* and 0.2% 'Onozuka' RS cellulase)) for 45 min at 37°C to soften the cell wall in order to facilitate squashing. The softened material was again washed in 1× enzyme buffer, and meristematic cells from the root tip zone were squeezed out in 45% acetic acid on a clean slide and squashed under a cover glass. The cover glass was removed after freezing the slide on dry ice, and the preparations allowed to air dry. The air-dried preparations were stored in a moisture free card box at 37°C for 12 h. The slide preparations were then incubated in 100 μg/mL DNase free RNase in 2× SSC (saline sodium citrate) for 1 h at 37°C, followed by washing in 2 × SSC for 10 min. The slides were then post-fixed in 4% (w/v) para-formaldehyde at pH 7.5 in water for 10 min, washed in 2 × SSC for 10 min, dehydrated in a graded ethanol series, and air dried.

Probe labelling and fluorescence in situ hybridization

The two r-DNA probes, i.e., pTa71 for 45S rDNA obtained from wheat genomic DNA by cloning in pUC19 and 5S rDNA probe obtained from onion genomic DNA by cloning in pBR322 were used for physical localization of rDNA sites on somatic chromosomes. The 45S rDNA probe was labelled with biotin-11-dUTP (Roche Diagnostics, Germany) by nick translation, and 5S rDNA probe with digoxigenin-11-dUTP (Roche Diagnostics, Germany) during PCR amplification, according to manufacturer's instructions. The hybridization mix contained 0.05 μg each of probe DNA in 50% (v/v) deionized formamide, 2× SSC, 10% (w/v) sodium dextran sulphate, 2 μg of sheared and denatured salmon sperm DNA in a final volume of 20 μL. Probe mix, 10 μL, was dropped on each slide, covered with a cover-slip and incubated at 80°C for 2 min to denature the probe DNA and then incubated at 37°C overnight. After hybridization, the slides were washed in 2 × SSC at 30°C for 2 min to remove excess hybridization solution, followed by stringent washing in 50% formamide, 2 × SSC, pH 7.0 at 42°C for 3 × 5 min, and then briefly in 2 × SSC at 42°C.

The detection of FISH signals was done according to Lavania *et al*. (2005) by incubating the slides for 1 h at 37°C in the detection mix. The biotinylated 45S DNA probe was detected by avidin DN conjugated with Cy3 (0.025% v/v), and digoxigenin-labelled DNA was detected by anti-digoxigenin (*Fab* fragment) conjugated with FITC (fluorescein isothiocynate, 1%) in 5% (w/v) BSA (borine serum albumin). All further incubation steps were carried out at 37°C for 30 min, each followed by washing steps (4× SSC, 4× SSC with 0.1% Triton X-100, 4× SSC) of 10 min each at room temperature in the dark. Subsequently, the slides were rinsed with 2 × SSC and mounted in DABCO solution (1.25% DABCO (1,4-diazabicyclo (2,2,2) octane) in 90% glycerol) with 2.0 ng/μL DAPI as counter-stain. Hybridization signals were observed under fluorescence microscope. The observations on localization of rDNA sites at metaphase chromosomes were recorded from at least three preparations each, and maximum occurrence of *in situ* hybridization sites were taken into account for final computation. The microscopic images were captured under different band pass filters for DAPI (blue fluorescence), FITC (green fluorescence) and Cy3 (red fluorescence), and subsequently merged to obtain a composite image.

Results

Localization of 45S and 5S rDNA loci and DAPI banding patterns

Two color FISH with biotin-labelled 45S rDNA and digoxigenin-labelled 5S rDNA probes revealed one prominent locus homologous to 45S rDNA each in the secondary constriction region of chromosomes 5 and 10, and one small locus homologous to 5S rDNA in the intercalary region proximal to the secondary constriction on chromosome 5. A representative metaphase plate showing localization of rDNA sites is shown in figure 1A.

DAPI banding patterns were obtained from the same metaphases that were exposed to FISH. Of the 17 chromosomes, 15 were found to show DAPI positive bright fluorescing bands. Whereas two chromosomes, 5 and 10, that support secondary constriction had thin DAPI fluorescing bands in the centromeric region, the remaining 13 chromosomes had one DAPI positive band each in the terminal region of the short arm. A representative metaphase plate showing DAPI bands is shown in figure 1B. An idiographic reconstruction depicting karyomorphological features combined with DAPI banding patterns and rDNA loci is shown in figure 1C.

Discussion

Linear chromosome differentiation

Fluorescence *in situ* hybridization (FISH) is an effective tool for physical mapping of DNA sequences on chromosomes that hold the potential to delineate their direct physical

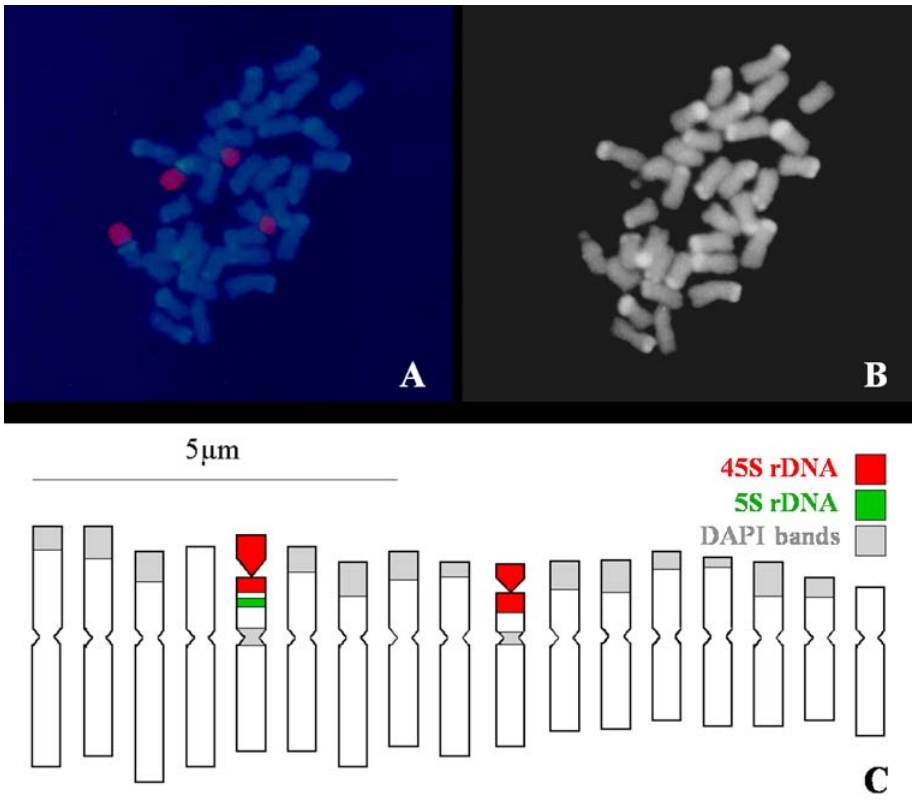


Figure 1. Simultaneous two colour *in situ* hybridization and DAPI banding in *Hyoscyamus niger.* (A) Metaphase plate showing two colour *in situ* hybridization with rDNA probes: 45S rDNA (labelled with biotin detected with Cy3 as red fluorescing signal); (B) DAPI banding on the same metaphase plate, and (C) idiographic representation on karyotype depicting linear differentiation for chromosome markers for primary and secondary constriction, rDNA sites and DAPI bands.

visualization under microscope. Availability of multicolor detection systems and reprobing of microscopic preparations have further facilitated simultaneous detection of several sequences and their linear ordering *in situ* (Lavania 1998b; Mukai 2004). Further the same metaphase preparation could be used for delineation of DAPI bands. After FISH, DAPI preferentially stains heterochromatic regions (Tagashira *et al*. 2009). Although it does not necessarily reflect corresponding AT-rich regions, but the DAPI positive bands have proved to be similar to C-banding or Q-banding in most cases (Heng and Tsui 1993; Ali *et al*. 2000). As such, the combination of DAPI banding pattern, localization of rDNA loci and conventional chromosome markers could be an effective tool to refine karyotypes facilitating chromosomal structural changes and identification of individual linkage groups (Ali *et al*. 2000; Abd El-Twab and Kondo 2009; Tagashira *et al*. 2009).

The present study on *H. niger* provides useful information on the distribution of the evolutionary conserved 45S and 5S rDNA sites vis-á-vis DAPI banding pattern and conventional chromosome markers that have value in chromosome identification. The study revealed several heterochromatic DAPI bands and two pairs of rDNA sites. In all 15 of the17 chromosomes showed DAPI bands; 13 showed prominent bands in the telomeric region and two showed thin centromeric bands on the secondary constriction carrying chromosomes. Two pairs of prominent 45S rDNA loci are located in the NOR region and one pair of 5S rDNA locus in the satellite chromosome. The occurrence of reduced DAPI fluorescence in the satellite region of chromosome 5 in the 45S rDNA-rich region seem to be unique observation with respect to molecular composition of this region in *H. niger*.

Evolutionary conserved ribosomal RNA (rRNA) multigene families consisting of the 18S-5.8S-26S (45S) and 5S rRNA genes are organized as families of tandemly repeated units located at specific chromosomal sites in higher eukaryotes. They may be unlinked on the same chromosome arm, or located on different chromosomes, but variation in locus size/copy number is known to occur across genotypes and geographical regions (Tagashira *et al*. 2009). Although the occurrence of 5S rDNA sites has been shown to be associated with nucleolus in soybean, common bean (Shi *et al*. 1996) and wheat (Mukai *et al*. 1991), the occurrence of two rDNA gene families on the same chromosome as observed in *Chlorophytum* (Lavania *et al*. 2005) as well as here is unusual and may serve as a marker to aid chromosome identification. In the last two decades, FISH studies have been conducted

in numerous plant species to elucidate the number and localization of rDNA sites. It is observed that most of the diploid plants have two sites (i.e. a single locus) of both 5S and 45S rDNA (Mishima *et al*. 2002), although some diploids may have multiple sites (deBustos *et al*. 1996; Lavania and Srivastava 1999), and the same is the case here in *Hyoscyamus* that has two prominent rDNA loci.

Karyoevolutionary differentiation

Of the 11 species in the genus *Hyoscyamus*, all except *H. muticus* has $2n = 28$, support the haploid chromosome number 17 or its multiple, e.g. *H. niger* has $2n = 34$, but *H. albus* supports $2n = 68$, whereas *H. georfei* has $2n = 102$, the latter being an interspecific hybrid (Federov 1969). *H. muticus* ($2n = 28$) has a relatively asymmetrical karyotype (Tyagi and Dubey 1989) compared to *Hyoscyamus niger* ($2n = 34$), and appears to have been derived from $x = 17$. This is also reflected in the presence of intercalary C-bands proximal to centromeres in three pairs of chromosomes. A closer look at the C-banding pattern in *H. muticus*, wherein 10 chromosomes are with telomeric bands, three with interstitial bands proximal to centromere and two chromosomes without any band (Tyagi and Gill 1990) compares well with 13 chromosomes with telomeric DAPI bands and two chromosomes with no DAPI band, indicating a possibility that Roberstsonian fusion involving three pairs in ancestral genome may have given rise to $2n = 28$ in *H. muticus*.

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