Chromosome identification and karyotype analysis of *Podophyllum hexandrum* Roxb. ex Kunth using FISH

Akshay Nag · Subramani Rajkumar

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Abstract *Podophyllum hexandrum* is an important high altitude medicinal plant from Himalaya. Somatic chromosomes of this species were studied to delineate and physical mapping of repetitive rDNA sites to provide landmarks in chromosome identification. The karyotype formula of this species was found to be $6m + 2sm + 2st + 2t$ with secondary constriction in the chromosome 1 and 7. The FISH analysis of rDNA sites showed 4 sites for 18S rDNA and 2 sites for 5S rDNA. The chromosome number 1, 2, 5 and 6 can be identified based on 18S rDNA sites in their short arm and chromosome 1 and 2 can be identified by 5S rDNA site in the centromere region. The estimated genome size of this plant is 16.07 pg (1C).

Keywords *Podophyllum hexandrum* · FISH · Flow cytometry · Genome size · rDNA localization

Introduction

Podophyllum hexandrum (Himalayan Mayapple) of family Berberidaceae is an important high altitude medicinal plant

of Western Himalaya (found at 2,800–4,000 M). The rhizomes of *P. hexandrum* yield several lignans which possess antitumor activity. Podophyllotoxin is the most active cytotoxic natural product. It is used as starting compound for the synthesis of anticancer drug etoposide and teniposide. Podophyllotoxin acts as an inhibitor of microtubule assembly. These drugs are used for lung cancer, testicular cancer, neuroblastoma, hepatoma and other tumors. Besides this, it also shows antiviral activities by interfering with some critical viral processes. (Giri and Narasu 2000). The utilization of in situ hybridization technology is of particular interest those engaged in chromosome walking and genome mapping projects. FISH has been used in many plants to identify chromosome accurately using species specific repetitive sequences, ribosomal genes and unique genes. Because of the universal occurrence and redundancy, the ribosomal genes are great value for karyotype analysis and comparative genome analysis. FISH techniques using fluorescence allows the visualization of multigene families such as 5S and 18S-5.8S-26S ribosomal RNA genes for their localization on the chromosomes. In some lower plants, such as *Marchantia polymorpha*, 5S rDNAs are encoded within the 45S rDNA repeat units (Sone et al. 1999) in contrast to the arrangement in the chromosomes of higher plants, where 45S and 5S rDNA are physically separate and map independently. These two classes of rRNA genes co-localize on the chromosomes bearing the nucleolar organizing regions. The high copy number and tandem organization of these genes together provide useful markers for chromosome identification and karyotyping. The copy number of rDNA repeats can vary from hundreds to thousands of copies, per genome (Weider et al. 2005). Hence rDNA clusters can be easily visualized by FISH, which makes them good targets for initial studies on physical genome mapping. Physical

A. Nag · S. Rajkumar
Institute of Himalayan Bioresource Technology,
Council of Scientific and Industrial Research,
P.O. Box. 6, Palampur 176 061, India

S. Rajkumar (✉)
National Research Centre on DNA Fingerprinting,
National Bureau of Plant Genetic Resources (ICAR),
Pusa Campus,
New Delhi 110 012, India
e-mail: rajnbpgr@gmail.com

S. Rajkumar
e-mail: srjihbt@yahoo.co.in

Fig. 1 FISH based localization of two rDNA sites in *P. hexandrum* **a** Metaphase chromosomes with 5S rDNA signals **b** Interphase with 5S rDNA signals **c** Metaphase chromosomes with 18S rDNA signals **d** Interphase with 18S rDNA signals (Bar=10 μ m)

localization and multiple copy of the above genes have been located in the chromosomes of *Pinus* (Liu et al. 2003; Cai et al. 2006), *Triticum* (Mukai et al. 1990) *Brassica* (Armstrong et al. 1998), *Plantago* (Dhar et al. 2006), *Artemisia* (Hoshi et al. 2006), *Pseudotsuga* (Amarsinghe and Carlson 1998), *Lupinus* (Naganowska and Zielinska 2002), *Trifolium* (Ansari et al. 1999). Since chromosome identification constitutes the first step in genetic manipulation of a species, the present investigation was therefore undertaken to provide meaningful landmarks to facilitate unequivocal chromosome identification of individual chromosomes employing DNA : DNA in situ hybridization.

Materials and methods

Preparation of chromosome spread

Growing root tips of *P. hexandrum* from the seeds were excised and pretreated with 0.1 % colchicine at 4 °C for 4 h for metaphase arrest and subsequently fixed in Carnoy's fixative for 24 h and stored in 70 % ethanol at 4 °C afterwards. The fixative from the root tips was removed by washing in 1X enzyme buffer (citric acid, trisodium citrate buffer); and then macerated in enzyme solution at 37 °C (Mixture of 2 % w/v cellulase and 3 % v/v pectinase from *Aspergillus niger*) until the root tips get softened. The softened root tips were again washed with 1X enzyme buffer, followed by transfer to 45 % acetic acid. The chromosome spreads were prepared by conventional squashing.

Preparation of probe

Genomic DNA of *P. hexandrum* was isolated from the leaves, using CTAB method and used as a template for PCR amplification of rDNA. 18S rDNA was amplified using primers 5'-AACGGCTACCACATCCAAGG-3' and 5'-GGTGGTGCCTTCCGTCAAT-3', whereas 5S rDNA was amplified using primers using 5'-CGATCATAACAGCAC TAACG-3' and 5'-TACTACTCTCGCCCAAGCAC-3'

Fig. 2 Karyo-Ideogram showing the karyotype of *P. hexandrum* somatic chromosome and the position of hybridization signals from 5S and 18 S regions

(Kitamura et al. 2000). The PCR products were separated in 1.5 % agarose gels and the rDNA band, recovered and purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The purified rDNA was labeled using FISH Tag DNA kit (Molecular Probes, Invitrogen), with Alexa Fluor 594 dye (Molecular Probes, Invitrogen) according to the manufacturer's instructions.

Pretreatment and hybridization

Pretreatment of fixed chromosome was carried out for probe hybridization using method developed by Hesslop & Harrison with minor modification. The slides were then dehydrated in a graded ethanol series, and air dried. Double sided adhesive in situ frames (Eppendorf, Germany); were applied to the marked area on each slide. The hybridization mixture was prepared containing 10 μ l (1 μ g) of labeled DNA probe, 65 μ l of formamide, 10 μ l of 20X SSC and 15 μ l of nuclease free water. This hybridization mixture was then denatured at 72 °C, for 5 min, and then kept in ice for 3 min. The denatured hybridization mixture was then applied to the marked area on the slide, and covered with a plastic coverslip adhering to the in situ frame. The chromosomes along with the hybridization mixture were denatured, by denaturing the slide at 72 °C on an in situ adapter (Eppendorf, Germany); for 5 min, followed by 8 h incubation in a humid chamber at 37 °C. The slides were then washed with 2X SSC, for 5 min twice, at 40 °C, followed by a dip in autoclaved distilled water. The slides were then kept for air drying in dark. It was counterstained with DAPI with Prolong Gold Antifade reagent (Invitrogen), and observed under Zeiss Fluorescent Microscope (Zeiss AxioImager. M1), equipped with CCD camera. Images were captured, using appropriate filter. The merging of fluorescent signals from the probe and the counter stain was done using ISIS software (Metasystems).

Genome size estimation

Genome size estimation was done by Flow Cytometry. Approximately 50 mg fresh green tissue from the leaf of *P. hexandrum* seedlings was excised and placed in a sterile plastic petriplate, on ice, along with 50 mg fresh green tissue from the leaf of internal standard i.e. *Vicia faba* L. 'Inovec' (Doležel et al. 2007). The leaf tissue was chopped into small fragments in 1 ml of ice cold nuclei isolation buffer (MgSO₄ buffer). The nuclei were obtained through filtration and centrifugation. Fluorochrome PI was added along with RNase-A at 50 mg ml⁻¹ concentration. The stained nuclear suspension was analyzed on FACS station, (Becton Dickinson FACSCalibur) and the data was analyzed using Becton Dickinson CellQuest Pro software. For karyotype analysis, chromosome images were captured by a

CCD camera, on Zeiss Axioimager, using Ikaros software (Metasystems). The average of chromosome length, chromosome arm ratio and position of hybridization signal was recorded, using Isis and Ikaros software. The chromosome arm ratio and arm length ($r = p/q$), were used to classify the chromosomes.

Results and discussion

Metaphase Chromosomes in *P. hexandrum* (2n=12) are large in size, indicating towards a larger genome size, which was found to be 32.25 pg (2C content), using *Vicia faba* L. 'Inovec' (2C = 26.90), (Doležel et al. 2007) as internal standard. Three metaphase plates were used for chromosome measurement and classification. The classification system proposed by Levan et al. (1964) was used to classify the karyotype, in which the arm ratio ($r = q/p$), is used to classify karyotype (Moscone et al. 1999), in which the chromosomes are classified as *m*, metacentric ($r=1.00$ – 1.69); *sm* submetacentric ($r=1.70$ – 2.99); *st*, subtelocentric ($r=3.00$ – 7.00); and *t*, telocentric ($r>7$). The karyotype formula of *P. hexandrum* was found to be 6m + 2 sm + 2 st + 2 t. Four 18S signals identified (Fig. 1.), two of which are present on the metacentric chromosomes no. 1 & 2, towards the subtelomeric region; and are very light, whereas two very strong signals are found just near the centromeres, on the short arms of submetacentric chromosomes no. 5 & 7; whereas two very bright signals are found on the centromeres metacentric chromosomes no. 1 & 2. The respective positions are apparent in the ideogram (Fig. 2).

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References

- Amarsinghe V, Carlson JE (1998) Physical mapping and characterization of 5S rRNA genes in Douglas fir. *J Hered* 89:495–500
- Ansari HA, Ellison NW, Reader SM, Badaeva ED, Friebe B, Miller TE, Williams WM (1999) Molecular cytogenetic organization of 5S and 18S-26S rDNA loci in white clover (*Trifolium repens* L.) and related species. *Ann Bot-London* 83:199–206
- Armstrong SJ, Frasz P, Marshall DF, Jones GH (1998) Physical mapping of DNA repetitive sequences to mitotic & meiotic chromosomes of *Brassica oleracea* var *alboglabra*, using fluorescent in situ hybridization. *Heredity* 81:666–673
- Cai Q, Zhang D, Liu ZL, Wang XR (2006) Chromosomal localization of 5S and 18S rDNA in five species of subgenus *strobilus* and their implications for genome evolution of *Pinus*. *Ann Bot-London* 97(5):715–722
- Dhar MK, Griebel B, Kaul S, Gill BS (2006) Characterization and physical mapping of ribosomal RNA gene families in *Plantago*. *Ann Bot-London* 97:541–548

- Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content of plants using flow cytometry. *Nat Protocol* 2(9):2233–2244
- Giri A, Narasu ML (2000) Production of podophyllotoxin from *Podophyllum hexandrum*: a potential natural product for clinically useful anticancer drugs. *Cytotechnol* 34:17–26
- Hoshi Y, Matoba H, Kondo K (2006) Physical mapping of ribosomal RNA gene in the genus *Artemisia* L. (Asteraceae) *Caryologia* 59 (4):312–318
- Kitamura S, Inoue M, Ohmido N, Fukui K (2000) Quantitative chromosome maps and rDNA localization in the T subgenome of *Nicotiana tabacum* L. and its putative progenitors. *Theor Appl Genet* 101:1180–1188
- Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. *Hereditas* 52:201–220
- Liu ZL, Zhang D, Hong DY, Wang XR (2003) Chromosomal localization of 5S and 18S-5.8S-25S ribosomal DNA sites in five Asian pines using fluorescence in situ hybridization. *Theor Appl Genet* 106:198–204
- Moscone EA, Klein F, Lambrou M, Fuchs J, Schweizer D (1999) Quantitative karyotyping and dual-color FISH mapping of 5S and 18S-25S rDNA probes in the cultivated *Phaseolus* species (Leguminosae). *Genome* 42:1224–1233
- Mukai Y, Endo TR, Gill BS (1990) Physical mapping of the 5S rRNA multigene family in common wheat. *J Heredity* 81:290–295
- Naganowska B, Zielinska A (2002) Physical mapping of 18s-25s rDNA and 5s rDNA In *Lupinus* via fluorescent in situ hybridization. *Cell Mol Biol* 7:665–670
- Sone T, Fujisawa M, Takanaka M, Nakagawa S, Yamaoka S, Sakaida M, Nishiyama R, Yamato KT, Ohmido N, Fukui K, Fukuzawa H, Ohyama K (1999) Bryophyte 5S was inserted into 45S rDNA repeat units after the divergence from higher land plants. *Plant Mol Biol* 41:679–685
- Weider LJ, Elser JJ, Crease TJ, Mateos M, Cotner JB, Markow TA (2005) The functional significance of ribosomal rDNA variation: impacts on the evolutionary ecology of organisms. *Annu Rev Ecol Evol Systemat* 36:219–242