Pak. J. Bot., 39(5): 1511-1515, 2007.

DETECTION OF RIBOSOMAL DNA SITES IN CHICKPEA (*CICER ARIETINUM* L.) AND MUNGBEAN (*VIGNA RADIATA* (L.) WILTZEK) BY FLUORESCENCE *IN SITU* HYBRIDIZATION

G.S.S. KHATTAK[1], E. WOLNY[2] AND I. SAEED[1]

[1]Nuclear Institute for Food and Agriculture (NIFA), P. O. Box 446, Tarnab, Peshawar, NWFP, Pakistan, Email: gssktt@yahoo.com
[2]Department of plant anatomy and cytology, University of Silesia, Jabiellonska 28, 40-032 Katowice, Poland

Abstract

Fluorescence *in situ* hybridization (FISH) technique was used to detect rDNA sites in chickpea and mungbean. In chickpea, the rDNA sites were detected on three pairs of chromosomes. Among these three pairs of chromosomes, one pair exhibited both 25S rDNA and 5S rDNA sites, while in case of other two pairs of chromosomes the 25S rDNA and 5S rDNA sites were located separately on separate pair of chromosomes. In case of mungbean, rDNA sites were found on four pairs of chromosomes. Each 25S rDNA and 5S rDNA had separate sites on two pairs of chromosomes. Active Nucleolus Organizer Regions (NORs) of both the crops were detected through silver staining technique. One and two pairs of chromosomes were detected active for NORs in chickpea and in mungbean, respectively.

Introduction

Chickpea (2n=16) and mungbean (2n=22) are important grain legumes widely grown in tropics and sub-tropics. Chickpea is cultivated in winter whereas mungbean in summer season. Both are excellent source of dietary protein.

Chickpea and munbean have small, morphologically similar chromosomes, which makes karyotyping difficult using conventional cytogenetic techniques (Khattak *et al*. 1997; Lavania and Lavania, 1983). Molecular cytogenetic methods, like fluorescence in situ hybridization (FISH) have the potential to improve karyotyping through the use of chromosome or genome specific markers. FISH is an extremely useful cytological technique to detect and localize the repeatative DNA sequences of ribosomal DNA (rDNA) sequences, which are also called as nucleolus organizing regions (NORs). The high copy number and tandem organization of the two rRNA genes (45S rDNA and 5S rDNA) together provide useful markers for chromosome identification and karyotyping in diverse plant genera such as Aegilops (Badaeva *et al*. 1996), Arabidopsis (Fransz *et al*. 1998), Horddeum (Taketa *et al*. 1999) and various plant species (Maluszynska, 2002). Analysis of the chromosomal localization of 45S rDNA and 5S rDNA sites (Snowdon *et a*l., 2002; Kulak *et al*., 2002; Hasterok *et al*., 2005 & 2006) was performed for different Brassica species. Chickpea rDNA detection and localization reports are scanty whereas about mungbean no report has been found in world literature. The aim of the present study was to detect the number of rDNA sites in chickpea and mungbean through FISH and active rDNA sites through silver staining.

Material and Methods

The research work was carried out at the department of plant anatomy and cytology, University of Silesia, Jabiellonska, Katowice, Poland and the seed of desi chickpea and

mungbean varieties were obtained from Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan.

Chickpea seeds were soaked for 24 hours under running tap water at room temperature. The seed coats were removed carefully and placed on a filter paper sprinkled by tap water in a petridish, and incubated at room temperature in dark for 48 hours. The root tips (5 mm) of the primary roots were cut off and the seedlings were incubated again in dark for 2 days to germinate lateral or secondary roots. The root tips of the secondary roots were cut off at length of 1 cm for metaphase arrest. The excised roots were treated with ice as cold treatment for 24 hours and then fixed in 95% methanol-glacial acetic acid (3:1) for 12 hours at 4°C. Similarly, mungbean seeds were placed on a filter paper sprinkled by tap water in a petridish, and incubated at room temperature in dark for 48 hours. Root tips (1 cm) of the primary roots were cut off for metaphase arrest. The excised roots were pre-treated with 0.05% aqueous solution of colchicine for 5 hours at room temperature in dark and then fixed in 95% methanol-glacial acetic acid (3:1) for 12 hours at 4°C.

Fixed roots were washed in 0.1 M citric acid-sodium citrate buffer (pH 4.6-4.8) for 20 minutes and digested enzymatically in a mixture comprising 4 % w/v cellulase "Onozuka"(Serva 16419) and 40 % (v/v) pectinase (Sigma P-0690) at 37˚C in humid chamber for 4 hours. Root tips were transferred into 45 % acetic acid and separated meristematic tissue from the rest of the roots (removed root cap and tissue above meristem) under stereomicroscope. Root tips were dispersed carefully with needles in a drop of 45% acetic acid on a slide and covered with coverslip and pressed lightly. Examined preparation under a phase contrast microscope and removed coverslip by freezing from the selected slides. The preparations were post-fixed in 3:1 ethanol:glacial acetic acid, followed by dehydration in absolute ethanol and air-dried.

To detect the number of rDNA sites, the 5S rDNA probe was amplified and labeled with rhodamine-5-dUTP (Roche) from the wheat (*Triticum aestivum*) clone pTa794 (Gerlach and Dyer, 1980), using PCR with universal M13 sequencing primers. The 25S rDNA probe was obtained by nick translation with digoxygenin-11-dUTP (Roche) of a 2.3 kb *Cla*I sub-clone of the 25S rDNA coding region of *Arabidopsis thaliana* (Unfried and Gruendler, 1990). The labeling procedure was followed as described by Hasterok *et al.,* (2000). The hybridization mixture consisted of 50% deionized formamide, 20% dextran sulphate, 2x SSC and salmon sperm blocking DNA in 50-100x excess of labeled probes. The ribosomal DNA probes were mixed to a final concentration of about 2.5 ng μl^{-1} and pre-denatured (80ºC for 10 min). The slides with chromosome material were then denatured along with the hybridization mixture (70ºC for 4.5 min) and allowed to hybridize for 12-18 h in a humid chamber at 37ºC. The post-hybridization washes were carried out in two changes of 20% deionized formamide in 0.1x SSC, 5 min each, at 42ºC, which is equivalent to 85% stringency. Detection of digoxigenated probes was done according to standard protocol using FITC-conjugated anti-digoxygenin antibodies (Roche). The preparations were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5 µg ml^{-1} of DAPI (4′, 6-diamidino-2-phenylindole; Serva).

To find the active sites of rDNA, silver staining was done according to the method of Hiume *et al*. (1980) with some modifications (Hasterok and Maluszynska 2000). Enzymatic preparations were rinsed in di-sodium tetra borate buffer (1.9 g $Na_2B_4O_7$ x 10 H_2O/500 ml distilled water or Merck, Art.1645 ready available solution) and dried in air at room temperature. Applied 50 µl aqueous solution of silver nitrate (0.25 g $AgNO_3$ (Merck, Art.

1.01512)/0.5 ml de-ionized water) to the marked area on slides and covered with polyamide cloth (silk & progress, 50-150 μm mesh size) coverslips. Slides were incubated in humid chamber at 42°C for 25-30 min and rinsed for 5 changes by distilled water and removed polyamide cloth cover slips. The slides were air dried at room temperature and made permanent by mounting in DPX and placed glass coverslip on the slides.

Images of both the FISH and silver staining were acquired using an Olympus Camedia C-4040Z digital camera attached to a Leica DMRB epifluorescence microscope or a Hamamatsu C5810 CCD camera attached to an Olympus Provis AX epifluorescence microscope and processed uniformly using Micrografx (Corel) Picture Publisher software.

Results

The green fluorescent signal on chromosomes indicate 25S rDNA sites, which consist of ribosomal genes responsible for the synthesis of large ribosome subunit's protein, and the sites with red fluorescent signals on chromosomes indicate 5S rDNA probe region, which consist of ribosomal genes responsible for the synthesis of small ribosome subunit's protein (Fig. 1). Three pairs of rDNA sites were observed in 40 somatic metaphase cells of 10 cultivated chickpea varieties (Fig. 1(a)). Among these three pairs of chromosomes, one pair exhibited both 25S rDNA and 5S rDNA sites, while in case of other two pairs of chromosomes, the 25S rDNA and 5S rDNA sites were located separately on separate pair of chromosome. The co-localized site of 5S rDNA appeared with low fluorescent signals compared to the independent 5S rDNA site. This could be due to the lower copies of ribosomal genes or more divergent sequence than the other 5S rDNA site.

Four pairs of rDNA sites were detected in 60 somatic metaphase cells of 12 cultivated mungbean varieties (Fig. 1(b)). Each 25S rDNA and 5S rDNA had separate sites on two pairs of chromosomes. One of the 5S rDNA pair of chromosomes exhibited very low fluorescent signals sites compared to the same type of sites on the other pair of chromosomes. It could be due to the lower copies of 5S rDNA ribosomal genes or more divergent sequence on this pair of chromosomes than the 5S rDNA site on the other pair of chromosomes.

The active NORs were detected through silver staining technique. One pair of chromosomes was detected active for NORs in chickpea (Fig. 2(a)) whereas two pairs of chromosomes were active in mungbean for NORs (Fig. 2 (b)).

Discussion

Hybridization sites of an rDNA probe coding for 18S, 5.8S and 26S genes were detected for the first time by Abbo *et al*. (1994) in chickpea. He has reported three pairs of rDNA sites in cultivated chickpea as detected in the current findings. No other report was found about the detection of rDNA sites in chickpea. The present investigation has an advantage by detecting rDNA sites of large ribosome subunit's protein and small ribosome subunit's protein of chickpea separately through the simultaneous use of 25S and 5S rDNA FISH probes. In addition, the active NORs were also detected through silver staining. The detection of 25S and 5S rDNA sites through FISH and active NORs through silver staining in mungbean described in this paper is the first report.

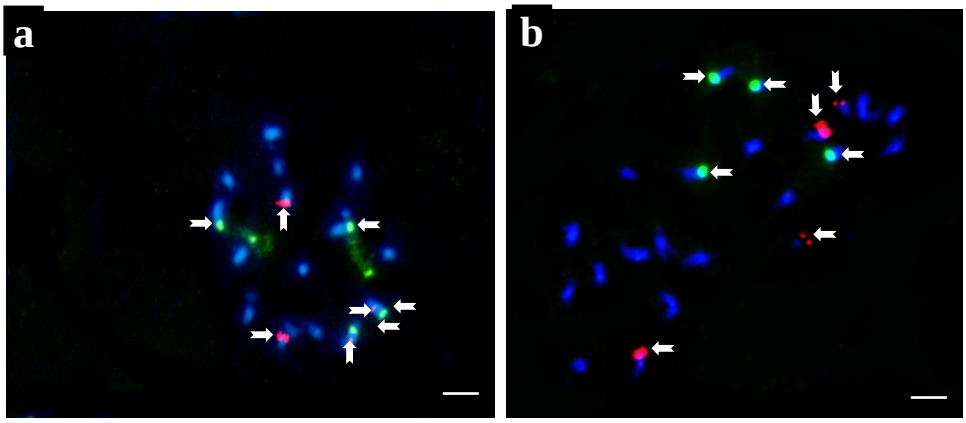

Fig. 1. Fluorescence *in situ* hybridization of 5S rDNA (red) and 25S rDNA (green) probes to somatic metaphase chromosomes of (a) Chickpea, (b) Mungbean.

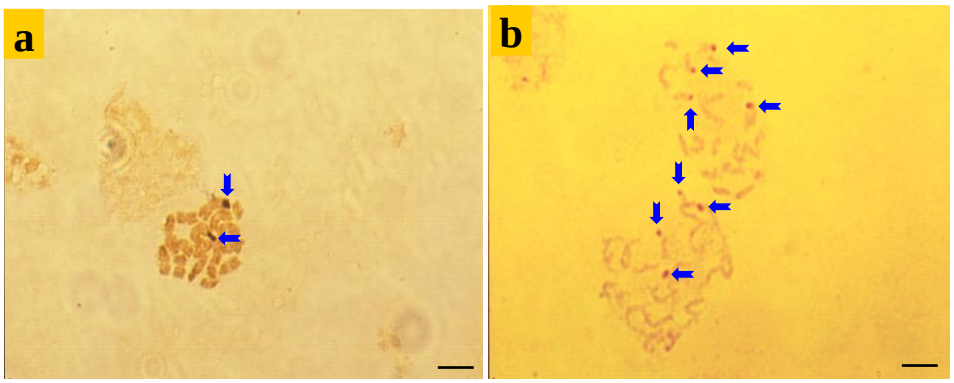

Fig. 2. Silver staining to somatic metaphase chromosomes of (a) Chickpea, (b) Mungbean
All scale bars =5 µm.

Further large scale FISH study of meiotic configuration using cultivated and wild species is required to understand the distribution of total rDNA sites and active NORs in chickpea and mungbean.

Acknowledgement

The first author is indebted to the International Atomic Energy Agency, Vienna for fellowship support at the department of plant anatomy and cytology, University of Silesia, Jabiellonska, Katowice, Poland.

References

Abbo, S., T.E. Miller, S.M. Reader, R.P. Dunford and I.P. King. 1994. Detection of ribosomal DNA sites in lenti and chickpea by fluorescent in situ hybridization. *Genome*, 37: 713-716.

Badaeva, E.D., B. Friebe, B. and B.S. Gill. 1996. Genome differentiation in Aegilops. 1. Distribution of highly repetitive DNA sequences on chromosomes of diploid species. *Genome*, 39: 293-306.

Fransz, P., S. Armstrong, C. Alonso-Blanco, T.C. Fisher, R. Torres-Ruiz and G. Jones. 1998. Cytogenetics for the model system Arabidopsis thaliana. *Plant Journal*, 13: 867-876.

Hasterok, R., E. Wolny, M. Hosiawa, M. Kowalczyk, S. Kulak, Ksiazczyk, T. Ksiazczyk, W.K. Heneenand, J. Maluszynska. 2006. Comparative analysis of rDNA distribution in chromosomes of various species of Brassicaceae. *Annals of Botany*, 97: 205-216.

Hasterok, R., E. Wolny, S. Kulak, A. Zdziechiewicz, J. Maluszynska and W. K. Heneen. 2005. Molecular cytogentic analysis of *Brassica rapa-Brassica oleracea* var. Alboglabra monosomic addition lines. *Theoretical and Applied Genetics*, 111: 196-205.

Hasterok, R. and J. Maluszynska. 2000. Nucleaolar dominance does not occur in root tip of allotetraploid *Brassica* species. *Genome*, 43: 574-579.

Khattak, G.S.S., Razi-ud-Din and R. Ahmed. 1997. Standardization of cytological techniques for chromosomal studies and pollen fertility of mungbean. *Sarhad J.Agric*., XIII(1): 67-70.

Kulak, S., R. Hasterok and J. Maluszynska. 2002. Karyotyping of Brassica amphidiploids using 5S and 25S rDNA as chromosome markers. *Hereditas*, 136: 144-150.

Lavania, U.C. and S. Lavania. 1983. Karotype studies in Indian Pulses. *Genetica Agraria*, 37: 299-308.

Maluszynska, J. 2002. *In situ* hybridization in plants-methods and applications. In: *Molecular techniques in crop improvement*. (Eds.) S.M. Jain, D.S. Brar, B.S. Ahloowalia. Dordrecht: Kluwer Academic Publishers. pp 299-326.

Snowdon, R.J., T. Friedrich, W. Friedt and W. Kohler. 2002. Identifying the chromosomes of the A- and C- genome diploid Brassica species *B. rapa* (syn. *Campestris*) and B. oleracea in their amphidiploid *B. napus. Theoretical and Applied Genetics*, 104: 533-538.

Taketa, S., G.E. Harrison and J.S. Heslop-Harrison. 1999. Comparative physical mapping of the 5S and 18S-25S rDNA in nine wild Hordium species and cytotypes. *Theoretical and Applied Genetics*, 98: 1-9.

Unfried, I. and P. Gruendler. 1990. Nucleotide sequence of the 5.8S and 25S rRNA genes and of the internal transcribed spacers from Arabidopsis thaliana. *Nucleic Acids Research*, 18: 4011.

(Received for publication 14 March 2007)