A Study of Nucleolar Organizers in Lentil Using FISH and Spore Quartet Analysis

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Summary The number of nucleolar organizing chromosomes in lentil (Lens culinaris Medik.) was ascertained using classical and molecular cytogenetic approaches, since in an earlier report, only one pair of nucleolar organizing chromosomes was resolved by FISH, although 1–3 nucleolar organizing chromosomes were reported in literature. During the present study, data on the number of nucleoli per cell in the normal lentil material (cv. PL639) was recorded at diakinesis and spore quartet stages of meiosis, and the number of bivalents associated with nucleoli were recorded at diakinesis. Use of a ribosomal DNA probe (pTa71) for FISH revealed only one pair of nucleolar organizing chromosomes on mitotic metaphase chromosomes (as also reported in an earlier study), but at spore quartet stage, 2 signals were observed in a spore (presumably due to decondensed state of chromosomes), suggesting the presence of 2 nucleolar organizing chromosomes. An interchange heterozygote, which had a nucleolar organizing chromosome involved in the interchange, was also used in this study; although at mitotic metaphase, only one pair of nucleolar organizing chromosomes was resolved by FISH, at spore quartet stage, frequencies of nucleoli per spore suggested the presence of 2 nucleolar organizing chromosomes. Taken together the results suggested that in the lentil material used in the present study, there are at least 2 pairs of nucleolar organizing chromosomes.

Nucleolar organizing regions (NORs) located on nucleolar organizing chromosomes (also called satellited chromosomes) are responsible for nucleologenesis in the cell. The physical location of ribosomal DNA (rDNA) loci on the chromosomes also corresponds with the NORs. In a chromosome complement, NORs can be marked by the presence of secondary constrictions in the mitotic metaphase chromosomes or by the association of nucleolar organizing chromosomes with the nucleolus at diakinesis stage of meiosis. In lentils, reports vary with respect to the number of satellited chromosomes, which range from one to three (Bhattacharjee 1951, Sharma and Mukhopadhayay 1962, Sinha and Acharia 1972, Gupta and Singh 1981, Lavana and Lavana 1983, Naithani and Sarbhoj 1973, Nandanwar and Narkhede 1991, Sinha and Keshwani 1973). However, in somatic preparations using fluorescence in situ hybridization (FISH) with rDNA probe, only one pair of chromosomes has been shown to carry distinct rDNA loci (Abbo et al. 1994, Balyan et al. unpublished); this may be, at least partly, due to poor resolution of weak NORs on somatic chromosomes, so that the number of nucleolar organizing chromosomes in diploid cultivated lentils still remains to be resolved.

Various approaches, both classical and molecular, can be used to resolve the question of the number of nucleolar organizing chromosomes in a crop, where one or more NOR(s) may be weak and therefore, not detectable in somatic chromosomes. For instance, the number of NORs may be determined using an interchange heterozygote, where a satellited chromosome is involved in the interchange. In such an interchange heterozygote, the number of nucleoli in different spores of a quartet would vary. The data on variation in the number of nucleoli in different spores of a quartet

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provide information on the occurrence of crossing over and different types of chromosome segregations (adjacent 1, adjacent 2, alternate) (Burnham 1950), which can be used to determine the number of NORs. Another approach to determine the number and positions of NORs in any organism is through fluorescence in situ hybridization (FISH) using decondensed prophase chromosomes (e.g. in spore quartets). During the present study in lentils, both these approaches have been used to resolve further the question of the number of nucleolar organizing chromosomes and the positions of corresponding NORs on these chromosomes.

Materials and methods

Plant material

Lentil material belonging to cv. PL639 and that of an interchange heterozygote (T₁₅) derived from it and maintained at the Department of Agricultural Botany, Ch. Charan Singh University, Meerut were used in this study.

Slide preparation

Meiotic preparations for classical cytogenetic studies were made using immature floral buds of both the normal plants and the interchange heterozygote. Somatic chromosome preparations were made using fixed root tips. For FISH, chromosome spreads at somatic metaphase and meiotic quartet stage were prepared in 45% acetic acid followed by treatment with 2% cellulase Onozuka R10 (E. Merck, Germany) and 20% pectinase (Sigma) for 40 min and 10 min, respectively. Slides were then frozen in liquid nitrogen and cover slips of frozen slides were flicked off. The slides were then air dried, dehydrated in ethanol series (70, 90, 100%) and stored in 100% glycerol at 4°C for subsequent use in FISH experiments.

Fluorescence in situ hybridization (FISH)

A probe consisting of ribosomal DNA of wheat (pTa71) was labelled with biotin-14-dCTP using random primer labelling system "Biotin High Prime (GIBCO-BRL)" according to the manufacturer’s directions. The hybridization procedure and signal amplification were followed as described by Leitch et al. (1994). The slides were counterstained by DAPI (4’,6-diamidino-2-phenylindole). The in situ signals were visualized under a fluorescence microscope (Leica) using high-performance universal objectives and by using suitable epifluorescence filter set (No. 513808). The photomicrographs were taken using Kodak 400 film.

Results

The number of nucleolar organizing chromosomes in lentil material used in the present study was ascertained using the following classical and molecular cytogenetic approaches.

Study of nucleoli during meiosis

In 25 of the 50 PMCs scored in control lentil (cv. PL-639) plants, a large nucleolus was found associated with 2 bivalents. In the remaining 25 PMCs, 2 nucleoli were observed (one normal, and the other smaller in size), one each associated with a bivalent (Fig. 1a). At spore quartet stage of meiosis, each spore in a quartet showed 2 nucleoli (Fig. 1c).

FISH at mitotic metaphase and at spore quartet stage

Mitotic metaphase chromosomes, when hybridized with rDNA probe, showed hybridization signals on 2 metacentric chromosomes, that were apparently homologous. On each chromosome, the signal is co-localized with the centromere (Fig. 2a). In contrast to this, during meiosis each
Fig. 1a–d. Number of nucleoli and associated chromosomes at diakinesis and spore quartet stages: (a) A PMC of normal lentil material, at diakinesis showing 2 nucleoli, one large and one small, each associated with a separate bivalent. (b) A PMC in an interchange heterozygote (T₁₁), at diakinesis showing one large nucleolus associated with one chain quadrivalent and a bivalent. (c) A spore quartet in an interchange heterozygote (T₁₃) showing 2 nucleoli in each spore (2:2:2:2 distribution). (d) A spore quartet in interchange heterozygote (T₁₅) showing 3:1:3:1 distribution of nucleoli in the spores.

Fig. 2a–d. Detection of nucleolar organizing chromosomes in lentil (L. culinaris Medik.) using rDNA probe (pTa71) by DNA:DNA fluorescence in situ hybridization (FISH). (a) A metaphase spread showing two FISH signals coincidental with the centromeres in a pair of metacentric chromosomes, (b) A metaphase spread of an interchange heterozygote (T₁₁) showing one FISH signal each on a metacentric and a sub-metacentric chromosome (inset shows the heteromorphic pair of nucleolar organizing chromosomes with signals). (c–d) Spore quartets showing two FISH signals in each spore with haploid genome.
spore (haploid genome) in a spore quartet, when hybridized with rDNA probe, invariably exhibited 2 hybridization signals (Fig. 2c, d).

**Nucleoli in an interchange heterozygote**

Since interchanges involving nucleolar organizing chromosomes can give useful information about the number of NORs, spore quartets from such an interchange heterozygote (T15) were also examined. The involvement of a nucleolar organizing chromosome in this interchange heterozygote was confirmed through the association of interchange quadrivalent with the nucleolus (Fig. 1b). This was confirmed by cross shaped figure at pachytene stage, where out of 4 chromosomes in the cross, 2 chromosomes contained secondary constriction near the break point (Fig. 3a). In mitotic chromosomes also, FISH gave signals in 2 chromosomes, both of which were metacentric in control, but in the interchange heterozygote, one was sub-metacentric, suggesting an unequal translocation involving a nucleolar organizing chromosome (Fig. 2b).

The theoretical expectations on the segregation of NORs in the above interchange were made assuming the presence of 2 pairs of chromosomes carrying NORs, only one of them being involved

![Figure 3a-b](image-url)

**Fig. 3a-b.** Chromosome associations and spore quartet types in an interchange heterozygote (T15), involving 2 pairs of nucleolar organizing chromosomes, one of which is involved in the interchange: (a) left to right, a photomicrograph of a cross shaped quadrivalent at pachytene, a line drawing of cross shaped quadrivalent, and a line drawing of a pair of nucleolar organizing chromosome, not involved in the interchange (the NOR of this pair is not resolved in the acetocarmine preparations). (b) spore quartet types resulting from the chromosome associations shown in (a), due to 3 types of disjunctions (adjacent 1, adjacent 2, alternate) of the quadrivalent; only one of the two nucleolar organizing chromosomes is involved in this quadrivalent, the other pair of nucleolar organizing chromosomes forms a bivalent as shown in (a); drawings in column 1 and 2 of (b) respectively represent a bivalent and a quadrivalent; in each case, on either side of a straight horizontal bar, a chromosome with 2 chromatids is shown; a bold dash (●) represents a chromatid with a satellite, and a light dash (-) represents a chromatid without a satellite.
in the interchange (Fig. 3). As can be seen, the following 2 types of quartets are expected due to adjacent 1, adjacent 2 and alternate types of disjunction of the quadrivalent: (i) all the 4 spores with 2 nucleoli each and (ii) 2 spores each with 3 nucleoli and the remaining 2 spores each with 1 nucleolus.

The data on number of nucleoli in spores at quartet stage in the above interchange heterozygote is summarized in Table 1. The normal type of quartets (in which all the 4 spores had 2 nucleoli each; 2 : 2 : 2 : 2; Fig. 1c) were observed in a very high frequency (58.06% to 82.22%) in all the 5 plants studied, but quartets with 3 : 1 : 3 : 1 distribution of nucleoli (Fig. 1d) were also frequent (13.33% to 41.93%). However, in 2 of the 5 plants, quartets with 3 : 1 : 2 : 2 distribution of nucleoli were observed, in a very low frequency (2.38% to 4.44%). This type of quartets could result due to nondisjunction of chromatids at anaphase II in the above interchange.

Discussion

The results of the present investigation provide evidence for the presence of 2 pairs of nucleolar organizing chromosomes in lentil (at least in the material used in the present study), although in mitotic chromosomes, NOR is resolved in only one of these 2 pairs in preparations based on acetocarmine, as well as FISH. The presence of 2 pairs of nucleolar organizing chromosomes was initially indicated in a study of PMCs, where, 2 different pairs of chromosomes were associated with 1 nucleolus or 1 chromosome pair each was separately associated with each of the 2 different nucleoli. The presence of 2 nucleoli in each spore of a quartet in a normal material of lentil, and the 2 FISH hybridization signals observed in each spore of a quartet also supported the presence of 2 NORs in the present material. The detection of only 1 pair of nucleolar organizing chromosomes during FISH conducted on mitotic metaphase chromosomes may be attributed to a failure of signal. Such a failure of signal for target sequences, <1–3 Mb long, at mitotic metaphase stage has been reported in several studies (Lawrence et al. 1990, Lichter et al. 1990, Zhong et al. 1996). The inference also receives support from the presence of 2 FISH signals in each spore of a quartet, possibly due to enhanced resolution of FISH signals in decondensed chromosomes at this stage. The above evidence using several approaches, when taken together suggests that the present material of lentil has 2 pairs of nucleolar organizing chromosomes, one with high copy number of rDNA, that is detectable at mitotic metaphase stage (Abbo et al. 1994, Balyan et al. unpublished) and the other with slightly low copy number of rDNA, that is not resolved at the mitotic metaphase stage. In the quartet spores of an interchange heterozygote (T₁₅) also, the presence of normal type of quartets with 2 : 2 : 2 : 2 distribution of nucleoli, and that of abnormal quartets with 3 : 1 : 3 : 1 distribution of nucleoli is in agreement with the theoretical expectations based on the assumption of 2 pairs of nucleolar organizing chromosomes, as shown in Fig. 3b.

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References


