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FISH MAPPING OF 18S-5.8S-26S rRNA GENES AND FLUOROCHROME BANDING IN THE TRIPLOID VIVIPAROUS ONION *ALLIUM × CORNUTUM* CLEMENTI EX VISIANI, 1842

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Triploid viviparous onions [*Allium × cornutum* Clementi ex Visiani 1842, syn *Allium cepa* L. var. *viviparum* Metzg. (ALEF.), auct.] (2n = 3x = 24), are known in some countries only as rare relict crops. In other parts of the world they are still traditionally or even commercially cultivated. In previous cytogenetic studies of the Croatian triploid viviparous onion Ljutika, Giemsa C-banding, chromosome pairing analysis during meiosis, and genomic hybridization in situ indicated a complex hybrid with highly heterozygous karyotype structure, with possible triparental genome organization. This study continues an analysis of the karyotype structure of Ljutika. Staining with fluorochromes CMA_3 (Chromomycin A_3) and DAPI (4,6-diamidino-2-phenylindole) confirmed previous results from Giemsa C-banding and revealed GC-rich heterochromatic regions associated mainly with chromosome ends and nucleolus organizing regions (NORs), and only a few interstitial bands. FISH mapping of the ribosomal 18S-5.8S-26S genes revealed two major rDNA signals on the short arms of two subtelocentric satellite chromosomes in almost all metaphase plates of Ljutika. The largest subtelocentric chromosome lacked rDNA signals. A significantly smaller rDNA signal was occasionally located on one small submetacentric chromosome. These results are in agreement with previously published results from identification of NORs by silver-staining technique, which confirmed a maximum three nucleoli in interphase nuclei. We discuss the molecular mechanisms underlying rearrangements and activity of ribosomal genes in the triploid karyotype.

Key words: *Allium*, triploid viviparous onions, hybrids, karyotype, fluorescence in situ hybridization, 18S-5.8S-26S genes, fluorochrome banding, silver staining.

INTRODUCTION

The genus *Allium*, originally confined almost entirely to areas of northern temperate climate, includes 750 species (Stearn, 1992). According to the FAO (2001) it represents one of the world's most economically and ecologically important crops. As in all angiosperms, hybridization among *Allium* species is very common. A large number of studies have examined the formation and characteristics of spontaneous or induced hybrids of the most cultivated *Allium* species, *A. cepa* (Jones and Mann, 1963; Koul and Gohil, 1971; McCollum, 1974; Puizina, 1992, 1997; Maass, 1997; Friesen and Klaas, 1998).

The first report on natural triploidy in onions comes from Singh et al. (1967), who described the morphological and cytogenetic characteristics of an Indian viviparous onion, Pran, 2n=3x=24, extensively grown in the northern Indian provinces of Kashmir and Jammu. Puizina and Papeš (1996) reported finding a spontaneous triploid viviparous onion, Ljutika, 2n=3x=24, traditionally cultivated and widespread throughout the coastal region of Croatia. Clones of triploid viviparous onions are cultivated locally in France as *Ciboule vivace*, in the Netherlands as *Sint Jan's ui*, in the region of Württemberg, Germany, as *Johannislauch*, and in Antilles as *Cive rouge*; there are also reports of their cultivation in Canada and other parts of the world (Maass, 1997; Friesen and Klaas, 1998). The results of restriction fragment-length polymorphism (RFLP) and isozyme analyses of several European and Asian triploid clones confirmed their hybrid constitution. Results indicating an identical random amplified

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polymorphic DNA (RAPD) banding pattern and identical DNA content (2C = 43.4 pg) indicate high genetic similarity and the unique origin of the Croatian clone Ljutika (Puizina and Papeš, 1996) and the Indian clone Pran (Singh et al., 1967) as well as other triploid clones (Friesen and Klaas, 1998). The Croatian viviparous onion Ljutika was first named *Allium cepa* L. var. *viviparum* Metzg. (ALEF.), auct., but Friesen and Klaas (1998) suggested the more appropriate botanical name *Allium* × *cornutum* Clementi ex Visiani 1842, according to the first record of this bulbiliferous onion form in Dalmatia (Croatia) by Visiani (1842). The new name also takes into account the hybrid origin of Ljutika.

However, the genomic origin and structure of Ljutika, like those of other triploid viviparous onions, is complex and not fully understood. Previous karyological analyses of Ljutika, using conventional and Giemsa C-banding, revealed hybrid and highly heterozygous karyotype structure (Puizina and Papeš, 1996). Analyses of chromosome pairing during meiosis showed frequent disturbances, and normal production of gametes was not possible. In the inflorescence of Ljutika, small bulbils for vegetative reproduction were developed among the sterile flowers. The frequent occurrence of complex multivalents (4–11 paired chromosomes) suggested that intergenomic pairing, translocations and chromosomal rearrangements were involved in the evolution of the triploid karyotype of Ljutika. The most frequent occurrence of heterotrivalents suggested partial homology of the three genomes. This observation is in accord with our previous results of GISH on triploid Ljutika, which identified at least two parental species, *A. cepa* L. and *A. roylei* Stearn.

In this study we extended the karyotype analyses of this interesting natural allopolyploid to include fluorescence in situ hybridization (FISH). We discuss the data from 18S-26S rRNA gene mapping in Ljutika in the light of our previous results on NOR activity (Puizina and Papeš, 1997). We also use fluorochrome (chromomycin A3 (CMA_3) and 4,6-diamidino-2-phenylindole (DAPI)) staining techniques to analyze the distribution and composition of the constitutive heterochromatin in the karyotype of Ljutika.

MATERIALS AND METHODS

PLANT MATERIAL

This work used *Allium* × *cornutum* clones from the Dalmatian coastal region. We collected bulbs from local gardens in four locations – Rogoznica (Kanica), Cista provo, Klis and Split – and germinated them in tap water.

CHROMOSOME PREPARATION

Pretreatment and fixation of roots followed Puizina et al. (1999). After washing in 0.01 mol citric buffer/L (pH 4.8), root tips were macerated in an enzyme solution containing 3% w/v cellulase (Sigma, Vienna, Austria) and 0.3 volume fraction of pectinase (Sigma) in the same buffer at 37°C (1–2 h), and squashed with cover glass in a drop of 0.45 volume fraction acetic acid. Chromosome spreads were cooled at –20°C for 30 min, the cover glass was removed and the slides were air-dried. Feulgen-orcein staining was performed for conventional karyotype analysis.

BANDING TECHNIQUES

Fluorochrome banding with chromomycin A_3 (CMA_3) and 4,6-diamidino-2-phenylindole (DAPI) was performed according to the protocol of Schweizer and Ambros (1984).

DNA PROBES AND FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Plasmid pRZ18 (Torres-Ruiz and Hemleben, 1994) containing the 18S rDNA fragment from pumpkin (*Cucurbita pepo*) was labeled with Cy3-dUTP (Amersham) using the BioNick labelling system (Life Technologies). Chromosome preparations were pretreated according to Leitch et al. (1994). Denaturation, in situ hybridization and detection followed the method of Heslop-Harrison et al. (1991) as modified by Pedrosa et al. (2001). 18S-5.8S-26S rDNA probe hybridization was detected using Cy3 fluorochrome. All preparations were counterstained with DAPI and mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, U.S.A.). Photographs were taken with a Zeiss Axioplan (Karl Zeiss, Vienna, Austria) microscope equipped with a mono cool-view CCD camera.

RESULTS

Staining of root tip metaphase chromosomes with Feulgen-orcein confirmed the highly heterozygous karyotype of Ljutika, Kanica clone (Fig. 1a), and clones from Cista provo, Klis and Split (Lepen, 2008), and its similarity to five previously analyzed clones (Puizina and Papeš, 1997). The homology between the 24 chromosomes was weak and occasional, and it was very difficult to identify homologous pairs of chromosomes. Therefore we lined up the chromosomes with respect to size. The exceptions were the three subtelocentric chromosomes, which we put at the end of the karyotype.

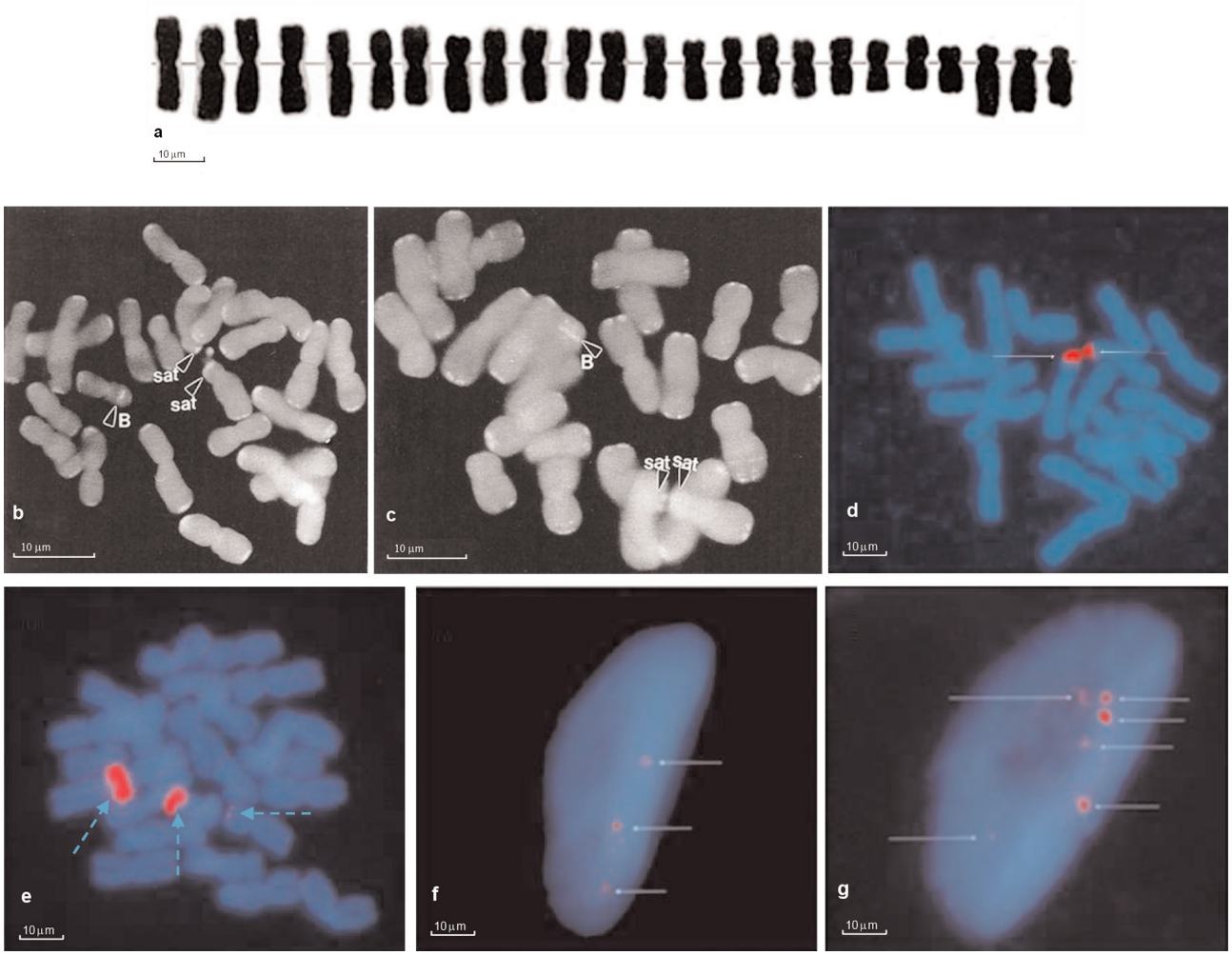

Fig. 1. Mitotic chromosomes of root tip cells in *Allium* × *cornutum*, 2n = 3x = 24. (**a**) Chromosomes after Feulgen-orcein staining, (**b–c**) Chromosomes after fluorochrome CMA_3 staining; GC-rich heterochromatic bands of different intensity are visible at chromosome termini of almost all chromosomes. Arrowheads marked "sat" indicate NORs of two smaller subtelocentric chromosomes. Association of NORs is visible in both pictures. Arrowheads marked "B" indicate the chromosome carrying the strongest intercalary CMA_3 band, (**d–g**) Fluorescence in situ hybridization (FISH) of CY3-labelled 18S-5.8S-26S rDNA probes (red signals) to *A.* × *cornutum* metaphase chromosomes counterstained with DAPI (blue). (**d**) Metaphase chromosomes of *A.* × *cornutum* with two strong rDNA signals were regularly observed on two subtelocentrics, (**e**) Third weak hybridization signal of terminal chromosome position, additional to two common strong hybridization signals, (**f**) An interphase nucleus with three or four hybridization signals was regularly observed, (**g**) An interphase nucleus with a maximum six hybridization signals was occasionally observed.

Staining with fluorochrome CMA_3 confirmed previous results of Giemsa C-banding and revealed GC-rich heterochromatic regions associated mainly with telomeres and NORs, and only a few interstitial CMA bands (Fig. 1b,c). Staining with DAPI revealed no AT-rich heterochromatic regions (Fig. 1d,e).

In order to identify the position and number of rRNA genes on chromosomes of Ljutika, we applied FISH technique with Cy3-labelled 18S-5.8S-26S rDNA probe. More than 20 well-spread metaphase plates were examined. Hybridization signals were observed in interphase nuclei and metaphase chromosomes (Fig. 1d–g). Two major rDNA signals on two shorter subtelocentric chromosomes (Fig. 1d,e) were observed regularly in all examined metaphase spreads in all four analyzed clones. In ~20% of the metaphase spreads we noticed a third 18S-5.8S-26S rDNA signal. It was much smaller and located on one of the smaller metacentric chromosomes (Fig. 1e). Similar results were obtained by localizing the Cy3-labelled 18S-5.8S-26S rDNA probe on interphase nuclei. Three or four 18S-5.8S-26S rDNA signals were visible in ~80% of the analyzed nuclei (Fig. 1f). In ~20% of the interphase nuclei examined,

probably as a result of decondensation of active loci, up to six rDNA signals were visible as separate and sometimes small spots, and usually two or three hybridization signals were much larger than other hybridization signals (Fig. 1g).

DISCUSSION

Triploid viviparous onions, a minor vegetatively reproduced crop which has been cultivated worldwide, probably represent an ancient natural hybrid, whose origin and parental species are still not fully understood (Maass, 1997; Friesen and Klaas, 1998; Puizina et al., 1999). In this study we continued its karyotype analysis. The results of fluorochrome CMA_3 staining are in agreement with previously published results of Giemsa C-banding (Puizina and Papeš, 1996). GC-rich heterochromatin was localized primarily in regions of chromosome ends and NORs. Only a few intercalary GC-rich heterochromatic bands were observed on a few chromosomes. These results are consistent with reports from Barnes et al. (1985), Pich et al. (1996a, b), and Pich and Schubert (1998) that the heterochromatin of chromosome termini in *A. cepa* and other species of sect. *Cepa* consists mainly of a tandemly organized and GC-rich 375-bp satellite sequence, except for the NOR-bearing short arm of chromosome 6. These satellite sequences, together with En/Spm-transposable element-like sequences and rDNA in NOR-bearing chromosomes, represent the most likely candidate sequences that may have acquired telomere functions in chromosomes of *Allium* species, which lack typical TTTAGGG telomeric repeats (Pich and Schubert, 1998). Do et al. (2001) identified a new class of satellite sequences (a 314 bp satellite sequence partially homologous with the satellite sequences ACSAT1, ACSAT2 and ACSAT3 of *A. cepa*) and located it by FISH technique to Giemsa-stained heterochromatic regions.

Against the commonly held view that the number and position of rDNA sites are species-specific and represent valuable chromosome landmarks useful in phylogenetic analyses (e.g., Maluszynska and Heslop-Harrison, 1991, 1993; Cerbah et al., 1998; Moscone et al., 1999; Adams et al., 2000; Šiljak-Yakovlev et al., 2002, 2003; Šestek et al., 2005), there is growing evidence for mobile NORs with a high potential to change their position within the same and/or closely related species (e.g., Schubert, 1984; Schubert and Wobus, 1985; Frello and Heslop-Harrison, 2000; Weiss and Maluszynska, 2000; Weiss et al., 2008).

Using FISH technique, Ricroch et al. (1992) and Pich et al. (1996b) showed that the haploid genome of *A. cepa* and other closely related species contains two 18S-5.8S-26S rDNA rDNA loci: a major one on the satellite chromosome and a smaller one on a small metacentric chromosome. Do et al. (2001), however, identified only a single 18S-5.8S-26S rDNA locus on the satellite chromosome of *A. cepa*. Considering that, in triploid hybrids of *A. cepa* with related species, in this case Ljutika, one would expect from three to six rDNA signals (three major ones containing the majority of ribosomal genes, and three smaller ones). Our results of FISH mapping of 18S-5.8S-26S rDNA probe revealed three rDNA signals on Ljutika's metaphase chromosomes. This result is in agreement with previously published results from silver staining technique, which marks nucleolus organizing regions (NORs) and transcriptionally active rDNA clusters. When applied to interphase nuclei of Ljutika it usually revealed two equal-sized nucleoli, whereas the third, small nucleolus could be identified only occasionally. The same technique applied to metaphase chromosomes of Ljutika revealed two active NORs on the two shorter subtelocentric chromosomes (Puizina and Papeš, 1997).

Our results confirmed the absence of one of the major rDNA signals on the largest subtelocentric chromosome, which is in accord with data from silver staining. In the Indian triploid Pran, sliver staining confirmed the presence of active rRNA genes on a single subtelocentric chromosome (Puizina and Papeš, 1997). Based on numerous similar findings among other allopolyploid species, several mechanisms can be suggested to account for the observed localization and active rDNA array homologous recombination, unequal crossover and illegitimate recombination, the epigenetic phenomenon of nucleolar dominance, and rDNA transposition.

Garrido-Ramos et al. (1992) attributed the significant variability of number of NORs in *Allium spherocephalon* to recombinant exchange of ribosomal genes between homologous and non-homologous chromosomes. In most eukaryotes the NORs of chromosomes frequently are terminally positioned, so that the nucleoli derived from them are located near the nuclear envelope as a result of transport of rRNA into the cytoplasm as ribosomal particles (Bourgeois et al., 1987). Such a position of NORs probably facilitates recombinant exchange and/or transposition, and deletion of ribosomal repeats. As mentioned above, homologous recombination between tandem rDNA repeats has been detected in rDNA during DNA replication of tandem repeats in several model organisms (reviewed in Richard et al., 2008).

The epigenetic phenomenon of nucleolar dominance is a very common cause of ribosomal gene silencing and/or loss in hybrids and polyploids (reviewed in Comai, 2000; Pikaard, 2000; Volkov et al., 2007). Dadejová et al. (2007) and Kovarik et al. (2008) suggested that active rDNA units in *Nicotiana*

allopolyploids are subjected to homogenization, which probably acts to reduce the mutational load across the active array. The rDNA units that are epigenetically silenced may be less prone to sequence homogenization and are likely to accumulate mutations and eventually be eliminated from the genome. Such may have been the scenario in the case of rDNA arrays that were lost from the largest subtelocentric chromosome of Ljutika, as well as the rDNA arrays on two subtelocentric chromosomes of Pran. Interestingly, there are some documented allopolyploids in which it seems that nucleolar dominance does not occur (i.e., in actively dividing root tip cells of allotetraploid *Brassica* species; Hasterok and Maluszynska, 2000; Hasterok et al., 2005).

Based on several morphological, cytogenetic and molecular studies, the triploid viviparous onion *A.* × *cornutum* is now recognized as a separate *Allium* crop and a new taxonomic unit. Since all its clones except for the Indian clone Poonch are practically identical, a monophyletic origin of this widely distributed crop is likely, and northern India (Kashmir) might be their place of origin. Although humans must have contributed to the maintenance and spread of this vegetatively reproduced crop, findings of Ljutika in abandoned vineyards and other less-favored agricultural areas suggest that this plant is very tolerant to drought and poor soil and is able to persist even in wild habitats.

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