Simultaneous Detection of 5S and 45S rRNA Genes in *Orychophragmus violaceus* by Double Fluorescence *in situ* Hybridization

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Summary Ribosomal RNA (5S and 45S) genes were determined simultaneously by fluorescence *in situ* hybridization (FISH) in the crucifer *Orychophragmus violaceus*. It carried twenty-two 5S and eight 45S gene sites. All rDNA sites were at the terminal parts of chromosomes and all eight 45S rDNA sites were colocalized together with 5S rDNA sites with the latter ones being more proximal. Double 5S rDNA sites—two sites of different fluorescent intensities on one chromosome arm separated by a short distance—were found on terminal parts of four chromosomes, with the distal one being stronger than the proximal one. No chromosomes with double 5S rDNA sites carried 45S rDNA sites. The implications of the relative order and distribution of both rDNA sites for the genome structure of the species and the identification of its individual chromosomes in wide hybrids with other species were discussed.

Key words Fluorescence *in situ* hybridization (FISH), *Orychophragmus violaceus*, 5S rDNA genes, 45S rDNA genes, Genome structure

Nucleolus organizer regions (NORs) are recognized as secondary constrictions in satellited (SAT) chromosomes. The 18S–5.8S–25S ribosomal RNA genes (45S rDNA) and intergenic spacer regions (18S–25S rDNA) exist as tandem repeats at the NORs and at other chromosomal sites where they may not be associated with an NOR. The 18S–5.8S–25S rRNA genes are present in several hundreds of tandemly repeated units of the three genes with intergenic spacers, organized in one or more clusters within the genome. Recent *in situ* hybridization experiments using the 18S–25S rDNA as probe have revealed the presence of minor 18S–25S rDNA sites in addition to the major 18S–25S rDNA sites with NOR-forming activity in various cereals (Mukai *et al.* 1991, Leitch and Heslop-Harrison 1992, Jiang and Gill 1994, Pedersen and Linde-Laursen 1994, Taketa *et al.* 1999) which are of importance in phylogenetic studies. The origin of minor 18S–25S rDNA sites is not well understood; silver-staining or an analysis of the meiotic association of nucleoli cannot detect their expression. The genes that code for 5S rRNA (5S rDNA) make up an independent multilocus, multigene family and are organized into clusters of tandem repeats with up to thousands of copies of repeated units (Appels and Honeycut 1986). Every repeat unit consists of a transcribed region of approximately 120 bp and a non-transcribed spacer region varying in size and sequence. The coding sequences are highly conserved across a broad taxonomic range. This variation within the non-transcribed spacer region was found to be useful for the phylogenetic reconstruction of species and even for cultivar identification (Benabdelmouna *et al.* 2001, Baum and Bailey 1997, Baum and Johnson 1994, Kitamura *et al.* 2001). In most higher eukaryotes, the 5S rRNA genes are

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arranged in tandem arrays at one or more chromosomal loci, mostly separated from the 45S rDNA (Fedoroff 1979).

During the last decade, the site number of rDNA of various plant species has been determined by fluorescence *in situ* hybridization (FISH) (Bauwens *et al.* 1991, Griffior *et al.* 1991, Mishima *et al.* 2002 and numerous other studies). These studies revealed that most of the diploid plants examined had two sites (*i.e.* a single locus) of both 5S and 18S–5.8S–26S rDNA, although some diploids had multiple sites (Fukui *et al.* 1994b, Ansari *et al.* 1999, Raina and Mukai 1999, Zhang and Sang 1999). The analysis of the molecular diversity of the rDNA genes (5S and 18S–5.8S–25S rDNA) and their chromosomal organization have already been shown to be suitable for the karyological characterization of species with small and similarly sized chromosomes as well as for establishing taxonomic and phylogenetic analysis (Appels and Baum 1991, Maluszynska and Heslop-Harrison 1991, Baum and Appels 1992, Baum and Johnson 1994, Schmidt *et al.* 1994, Kolipara *et al.* 1997, Zoldos *et al.* 1999, Benabdelmouna *et al.* 2001, Shibata and Hizume 2002).

Orychophragmus violaceus (L.) O. E. Schulz (syn. *Moricandia sonchifolia* (Bunge) Hook Fil.) ($2n=24$), belonging to *Cruciferae*, is cultivated as an ornamental plant in China. Its wild species occur in China and Korea. There are some controversies about its phylogenetic position and its placement in the tribe *Brassicaceae* has been questioned (Al-Shehbaz), but was supported by the study on isozyme variation (Anderson and Warwick 1995). The distant relationship between *O. violaceus* and the cultivated *Brassica* species was revealed by the chromosomal behaviors in their hybrids (Li *et al.* 1995, 1998, Li and Heneen 1999) and other studies (Inaba and Nishio 2002). The objective of the present study was to localize the 18S–5.8S–28S and 5S rDNA sites in *O. violaceus* by double-target fluorescence *in situ* hybridization, in order to provide molecular markers for certain chromosomes and give new information about its genome structure.

Materials and methods

Plant materials and chromosome preparation

The seeds of *O. violaceus* were placed on wet filter paper in a petri dish to germinate in an incubator at 22°C in the dark. Root tips from 1–2 cm roots were harvested and directly fixed in ethanol:acetic acid solution (3:1) without any chemical pretreatment. After fixation at room temperature (RT) overnight, they were stored in 70% ethanol at 4°C. Chromosome preparation was carried out by the enzymatic maceration/air drying (EMA) method (Fukui 1996) and by the procedure (Zhong *et al.* 1996). The slides were checked under phase-contrast microscope and kept at RT until utilization for *in situ* hybridization analysis.

Preparation of DNA probes

The 5S rDNA was amplified by PCR from the genomic DNA of *O. violaceus*, using a universal primer set specific for 5S rDNA. The sequences of primers 5SL1 and 5SL2 were 5'-CGGTG-CATTAATGCTGGTAT-3' and 5'-CCATCAGAACTCCGCAGTTA-3', respectively (Hizume 1993). PCR was performed using conventional thermal conditions. For the 45S rDNA probe we used the fragment from rice. The biotin-labeled 5S rDNA and digoxigenin-labeled 45S rDNA probes were prepared by nick translation.

Multi-color fluorescence in situ hybridization (McFISH)

The procedure of *in situ* hybridization was conducted according to Shishido *et al.* (1998) with some modifications. Chromosome spreads were treated with 100 ng/μl of RNase at 37°C for 1 h. The hybridization mixture consisted of 50% formamide, 2×SSC and labeled probes at a final concentration of 10 ng/μl for 45S and 5S rDNA, respectively. Salmon sperm DNA (1.5 μl per slide) was added as a blocking reagent to the hybridization mixture. The mixture was denatured at 70°C

for 10 min and kept on ice for 5 min. A 20- μ l aliquot of the hybridization mixture was applied onto each slide, covered with a coverslip, and sealed with paper bond. Both chromosomal DNA and probe DNA on the slides were denatured together in a thermal cycler at 80°C for 5 min, and hybridized with each other at 37°C overnight in a humid dark box.

After hybridization the chromosome spreads were washed twice in 2 $\times$ SSC, once in 50% formamide/2 $\times$ SSC, and once in 4 $\times$ SSC, each for 10 min at 42°C. Fluorescein-avidin (1%, Vector Laboratories, Calif.) in 5% NFM (non fat milk) 4 $\times$ SSC Tween was dropped onto the chromosome spreads, which were then incubated at 37°C for 60 min. After the fluorescein-avidin solution was rinsed with BT buffer (0.1 M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) three times at 37°C for 10 min each, the biotinylated anti-avidin (1%, Vector Laboratories) for secondary amplification and the anti-digoxigenin-rhodamine in 5% NFM 4 $\times$ SSC Tween were dropped onto the chromosome spreads, and they were then incubated at 37°C for 60 min. After brief washing with BT buffer three times at 37°C for 5 min each, 1% fluorescein-avidin and anti-sheep-Texas Red (1%, Vector) in 5% NFM (non fat milk) 4 $\times$ SSC Tween were dropped onto them, and they were incubated at 37°C for 60 min. After rinsing them twice with BT buffer, and once with 2 $\times$ SSC, at 37°C for 10 min each, the chromosome spreads were finally counterstained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) in an antifadant solution (Vector Shield, Vector). Before each immunocytochemical step they were blocked with 1% NFM in 4 $\times$ SSC Tween at 37°C for 5 min.

The fluorescent signals of FITC (green), rhodamine (red) and DAPI (blue) were detected by fluorescence microscopy (Zeiss). The different fluorescent images were acquired separately in the IP Lab-PVCAM system through a cooled CCD camera (Photometrics). Then they were merged into single composite images. The signal images were digitally stored in a personal computer (Quadra840AV, Macintosh) and analyzed by imaging software (IPLab Spectrum, Image and measurement, Signal Analytics).

Results and discussions

The double-target *in situ* hybridization with biotin-labelled 5S rDNA and digoxigenin-labelled 45S rDNA probes revealed up to twenty-two 5S rDNA signals and eight 45S rDNA signals. Up to twenty-two 5S rDNA signals had varying sizes and were located at the terminal parts of 9 chromosome pairs, and double 5S rDNA sites—two sites of two different sizes on one chromosome arm separated by a short distance—were found on four chromosomes, with the distal one being larger than the proximal one (Figs. 1, 2). Eight 45S rDNA sites of different sizes appeared at terminal parts of eight chromosomes and were colocalized together with 5S rDNA signals. The relative order of both rDNA genes could be distinguished and the 45S rDNA signals were distal in most cases. No chromosomes with double 5S rDNA sites carried 45S rDNA sites. The sites of rDNA hybridization corresponded generally to the more strongly DAPI stained terminal parts, which was more prominent on the less condensed chromosomes (Figs. 1, 2). The results were in contrast to those from the cultivated *Brassica* species, where the sites of rDNA hybridizations were less strongly DAPI stained than others (Maluszynska and Heslop-Harrison 1993).

In previous FISH experiment with 45S rDNA of wheat as probe, four pairs of signals with different sizes were detected in interphase nuclei of *O. violaceus* root-tips and up to four pairs of chromosomes were found to show signals of different sizes at terminal parts of short arms (Li *et al.* 2003). Among the four chromosome pairs with 45S rDNA sites, three pairs had obvious satellites and the remaining one showed no satellite. The studies on the karyotypes of some variants of *O. violaceus* in China indicated 1–3 pairs of satellited chromosomes in the same or different variants (Li *et al.* 1994). In the present study, four or six signals from 45S rDNA were shown in majority of cells at meta- or prometaphase and the results were consistent with those from karyotyping. The fourth locus of 45S rDNA genes detected previously and at present was apparently inactive and not

Figs. 1–2. Double FISH of 5S and 45S rDNA gene loci on the mitotic chromosomes of *O. violaceus* in two cells with different condensation degrees. The digoxigenin-labelled 45S rDNA probe was detected by anti-digoxigenin-rhodamine (red), the biotinylated 5S rDNA probe by Fluorescein-avidin (green), and the chromosomes were counterstained with DAPI (blue). A, Merged image. B, DAPI staining. C, Sites of 5S rDNA. D, Sites of 45S rDNA. Four 5S rDNA genes were separated by a short distance in each of four chromosomes. Two and three satellites were separated from the chromosomes in Figs. 1 and 2, respectively. The scale bars indicate 10 μ m.

associated with NOR, and the absence of one signal (Fig. 1) in the present study might result from the heterozygous karyotype from open pollination. The low frequency of the fourth locus also showed its much fewer copies than those of the other three.

In the two *Brassicaceae* diploids *Sinapis alba* and *Raphanus sativus*, their chromosomes carried four 5S rDNA sites (Schrader *et al.* 2000). The presence of both 5S and 25S rDNA genes in one chromosome pair was common to both *S. alba* and *R. sativus*. But the different position and arm-location of 5S compared to the 25S rDNA suggest distinct evolutionary pathways for this chromosome pair, in both species, from a common ancestor. However, in tetraploid *Brassica napus* originating from the natural hybridization between *B. rapa* and *B. oleracea*, 16 sites from 5S rDNA on 12 chromosomes were detected, and two pairs had closely linked double signals near to the centromeres, and double FISH of the 5S and 25S rDNA genes in this species revealed 12 signals for both 5S and 25S rDNA (Schrader *et al.* 2000). In six chromosomes both rDNA genes were localized together, of which two pairs were closely linked. One pair with double signals from 5S rDNA probe might be of *B. oleracea* origin, for Armstrong *et al.* (1998) demonstrated only one location of double signals on pachytene chromosome of *B. oleracea*. The high number of 5S rDNA sites can probably be related to another progenitor *B. rapa*. The A-genome had also been partly subject to nonreciprocal translocations (Sharpe *et al.* 1995, Parkin *et al.* 1995) that could potentially enhance the number of rDNA sites. Additionally, the sites of rDNA may have changed their position as a result of chromosome fission (Hall and Parker 1995) and a jumping of the nucleolus organizing regions (Schubert and Wobus 1985), as demonstrated by the centromeric, telomeric and interstitial localizations of rDNA sites in *Brassica* species (Maluszynska and Heslop-Harrison 1991, Fukui *et al.* 1998, Schrader *et al.* 2000).

The polyploidy nature of *O. violaceus* genome had been inferred by the frequent occurrence of multivalents during the meiosis of its pollen mother cells (Li *et al.* 1995). The basic chromosome numbers of 6 and even 3 were suggested from the cytogenetics of its hybrids with *B. napus* (Li *et al.* 1995, 1996) and progeny (Wu *et al.* 1997), respectively. The high number of both rDNA genes might give more evidence for the polyploid origin of its chromosome complement. All terminal localizations of both rDNA genes showed that their position underwent little changes, in contrast to different positions in *Brassica* species. According to the classification standards of karyotype symmetry (Stebbins 1971), the karyotype of *O. violaceus* belonged to 1A–2A, the most symmetric one. There were little length differences between two neighboring pairs and the relative lengths ranged from 6.81% to 10.06% averagely (Li *et al.* 1994) with gradual gradients. Then *O. violaceus* maintained the primitive karyotype. The results from the detection of active NORs (Cheng and Heneen 1995) and rDNA loci by FISH (Schrader *et al.* 2000) supported the conclusion that the number of three chromosome pairs carrying the 25S rDNA genes was basic for the *Brassicaceae*. Several authors had reported that the diploid species of *Brassicaceae* have evolved from a common ancestor but with different basic chromosome numbers of $x=6$ (Röbbelen 1960) and $x=3$ (Hussein and Abobakr 1976, Chen and Heneen 1991, Cheng and Heneen 1995). The detection of three chromosome pairs with 25S rDNA sites or active NORs in the *Brassica* diploids and *Sinapis* supports the hypothesis of a basic chromosome number of $x=3$ (with one active NOR-chromosome) in these genera (Schrader *et al.* 2000). The detection of four pairs of rDNA loci (three active) in *O. violaceus* also hinted that the species had $x=3$ ($2n=8x=24$). So this species probably was at a primitive evolutionary position among *Brassicaceae* species, for the lowest chromosome number in the family is $x=4$ for the species of the tribe *Lepidieae*.

The larger size of *O. violaceus* chromosomes compared to those of *Brassica* species has been shown in karyotype analysis and their hybrids. The characteristics of chromosome sizes and morphology were useful for distinguishing parental chromosomes in mitotic and meiotic cells of hybrids (Li *et al.* 1995, 1996, 1998, 1999). The length of *O. violaceus* chromosomes at mitotic prometaphase was generally more than 10 μm (Figs. 1, 2), while the length of the longest ones in

Brassica diploids was shorter than 10 μm (Fukui *et al.* 1998). All the chromosomes of *Brassica* diploids demonstrated clear heterochromatic blocks in the proximal regions of all the chromosomes at prometaphase stage and some chromosomes also had faint, unstable and small condensation at the end of the chromosome arms (Fukui *et al.* 1998). The condensation pattern was used to identify individual chromosomes. The phenomenon of uneven condensation on *Brassica* chromosomes was not observed on *O. violaceus* chromosomes which showed even condensation at prometaphase and had no heterochromatic blocks in the centromeric and other regions. The different condensation patterns of chromosomes from *Brassica* species and *O. violaceus* probably contributed to their asynchrony in the mitotic and meiotic divisions of their hybrids and consequently the separation of parental genomes and elimination of the *O. violaceus* chromosomes (Li *et al.* 1995, 1998, Li and Heneen 1999). Owing to the occurrence of partial genome separation in the mitotic divisions of these hybrids, the additional plants with whole *Brassica* complements and one to several *O. violaceus* chromosomes and the plants with substituted *Brassica* complements by some *O. violaceus* chromosomes were available among F1 plants. The homeologous relationship might exist between these *O. violaceus* chromosomes added to or included in *Brassica* complements and certain *Brassica* chromosomes.

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