Physical Mapping of 5S rRNA Genes in *Cycas revoluta*

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Among the cycads *Cycas revoluta* ($2n=22$) has been the most investigated species cytologically. The informations on the chromosomes of *C. revoluta* are deposited regarding to meiosis (Nakamura 1929), karyotype (Sax and Beal 1934, Segawa *et al.* 1971, Selvaraj 1980, Kokubugata and Kondo 1994, Kondo *et al.* 1995), C-banding (Tanaka and Hizume 1980), fluorescent banding using base specific fluorochromes, NOR (nucleolar organizing region) staining, Cd-staining and *in situ* hybridization (ISH) with 18S-5.8S-26S rRNA gene (Hizume *et al.* 1992). Genomic DNA content of *C. revoluta* nucleus was 10.3 or 12.7 pg per haploid genome (Kurdi-Haider *et al.* 1983, Ohri and Khoshoo 1986). There is a few molecular analysis on nuclear genome of this species. Base sequence was reported only in 5S rRNA (Erdmann and Wolters 1986) and DNA sequence organization in the genome was investigated by *Cot* analysis by Kurdi-Haidar *et al.* (1983).

While 18S-5.8S-26S rDNA had been mapped physically on the metaphase chromosomes using the heterogenous rDNA cloned from wheat genome (Hizume *et al.* 1992), no other sequence was mapped. As the 5S rDNA composed of highly conservative 5S rRNA sequence and the spacer variable in length and sequence among taxa is tandemly arrayed in the eukaryotic genome (Long and Dawid 1980), the spacer of 5S rDNA repeat unit was easily amplified in several plants by PCR using the universal primers possessing the base sequence correspond to a part of 5S rRNA consensus sequence (Cox *et al.* 1992). The 5S rDNA amplified by PCR has been successfully used as a probe for ISH in *Vicia faba*, *Crepis capillaris* (Hizume 1993), *Allium cepa*, *A. fistulosum* (Hizume 1994), *A. sativum* (Hizume *et al.* 1994), *Hordeum vulgare* (Fukui *et al.* 1994) and *Oryza sativa* (Kamisugi *et al.* 1994). I report here the physical mapping of 5S rRNA genes on the somatic chromosomes in *C. revoluta*.

Materials and methods

Young leaves collected from a male plant of *Cycas revoluta* Thunb. in mid June were cut into small pieces about 1 mm across and treated with 0.05% colchicine or 2 mM 8-hydroxy-quinoline at room temperature for 6 hr. The materials were fixed with acetic acid-ethanol (1:3), and then washed with 70% ethanol and distilled water each for 10 min and treated with 2% Cellulase Onozuka RS (Yakult Honsya) and 0.5% Pectolyase Y-23 (Seishin Pharmaceutical) in 0.05 M citrate buffer (pH 4.5) at 37°C for 1 hr. After washing with distilled water for 15 min, the materials were squashed in 45% acetic acid on glass-slides. The coverslips were removed from glass-slides by dry-ice technique and then the chromosome preparations were air-dried.

Total genomic DNA was extracted from young leaves by CTAB method. The 5S rDNA fragment was amplified by PCR (Saiki *et al.* 1988) using a template of the genomic DNA and the primer set of parts of highly conserved 5S RNA gene. Based on a comparison of sequences of 5S rRNA of *C. revoluta* (Erdmann and Wolters 1986) and several angiospermous plants (Ellis *et al.* 1988, Wolters and Erdmann 1988). The sets of primers which directed inside or outside of 5S rRNA sequence were synthesized. The sequences of the primers were as follows:

5SS1: 5'-AGGTGCGATATACCAGC-3' (nt 1-18 of the 5S rRNA sequence) and 5SS2: 5'-GGGTGCAATATTAGGACTT-3' (nt 118-100) for 5S rRNA region, and 5SL1: 5'-CGGTGCATTAATGCTGGTAT-3' (nt 10-30), 5SL2: 5'-CCATCAGAACTCCGCAGTTA-3' (nt 34-54) for repeat unit. Detail of PCR conditions have been described by Hizume (1993). Sizes of amplified fragments were analyzed by electrophoresis on 2% agarose gel with a size marker of the 100 bp ladder (BRL). The amplified DNA was labeled with biotin-dATP by nick translation or PCR, and then used as a probe for ISH. The probe mixture of 10 μ l containing 25 ng labeled DNA in 50% formamide and 2 $\times$ SSC was applied to the chromosome preparation. After sealing under a coverslip with rubber cement the chromosomal and probe DNAs in the preparation were simultaneously denatured on a hotplate set at 78°C for 10 min. The other ISH protocol was performed following the procedure of Hizume *et al.* (1992) and Hizume (1993). After the ISH, the chromosome arms were counterstained with 2% Giemsa in 2 $\times$ SSC for 2 min.

Results and discussion

The DNA fragment amplified from the template of total *C. revoluta* genomic DNA by

Fig. 1. Electrophoresis of 5S rDNA fragment amplified by PCR using the primer for 5S rDNA repeat unit from genomic DNA of *Cycas revoluta* on 2% agarose gel. A: PCR amplified fragment with about 1300 bp, B: 100 bp ladder (BRL).

PCR using the primer sets for 5S rDNA repeat unit or 5S rRNA sequence region were about 1300 bp (Fig. 1) or 120 bp, respectively. The 1300 bp 5S rDNA repeat unit of *C. revoluta* given was somewhat shorter than the previous data (1572 bp) analyzed by southern hybridization of *Bam*HI digested genomic DNA (Gottlob-Mchugh *et al.* 1990). The biotin-labeled 5S rDNAs were hybridized *in situ* to the *Vicia faba* chromosomes in which the 5S rDNA loci are well known. Two ISH signals appeared at the satellite of the longest metacentric chromosome (data not shown) same as previous reports (Knälmann and Burger 1977, Schubert *et al.* 1978, Hizume 1993). The fact indicated that 5S rDNAs were successfully amplified by PCR in this experiment.

Interphase nuclei and mitotic chromosomes had clear, heavy signals indicating hybridization with the probe of 5S rDNA repeat unit or 5S rRNA sequence region (Fig. 2). All interphase nuclei had two signals each (Fig. 2A). The 5S rDNA sites of homologous chromosomes separated always and did not fuse in any phases of mitosis (Figs. 2B, D). The karyotype of *C. revoluta* was composed of two pairs of large submetacentric chromosome, five pairs of medium-sized telocentric chromosomes and four pairs of small metacentric chromosomes, and confirmed that reported previously (Sax and

Fig. 2. Interphase nucleus (A), somatic prophase chromosomes (B) and metaphase chromosomes ($2n=22$) (C,D) of *Cycas revoluta* hybridized *in situ* with biotin-labeled 5S rDNA repeat unit (A,B,D) and 5S rRNA coding region (C). Bar = 10 μm .

Beal 1934, Segawa *et al.* 1971, Selvaraj 1980, Kokubugata and Kondo 1994, Kondo *et al.* 1995). The 5S rDNA locus was localized at approximate terminal region of the longest pair of telocentric chromosomes (the 3rd pair) (Fig. 2D). After counterstaining with Giemsa a pair of dots appeared at the centromere of each metaphase chromosomes was stained with Giemsa like Cd-bands (Hizume *et al.* 1993), and was not a signal of ISH. When the probe of 120 bp 5S rRNA gene sequence was used, the signals were somewhat weaker than those obtained using the whole repeat unit and the location of the signals was the same as that of the repeat unit (Figs. 2C, D). The fact indicates that any homologous sequences to the spacer of the 5S rDNA were not distributed on the chromosome of *C. revoluta* excepting for 5S rDNA locus.

The site of 5S rDNA can be compared with C-bands reported by Tanaka and Hizume (1980) and chromomycin A₃ (CMA)- and DAPI-bands, Ag-NORs and sites of 18S-5.8S-26S rDNA reported by Hizume *et al.* (1992). The 5S rDNA locus was exactly the same location as the C-band or the CMA-band at approximate terminal region of the long arm of the longest pair of telocentric chromosomes (the 3rd pair). As the karyotype has been very similar to each other in *Cycas* species (Kondo *et al.* 1995), the 5S rDNA locus may be useful marker for comparative karyotype analysis in the genus.

In other gymnosperms the size of 5S rDNA repeat unit was 525 and 850 bp in *Pinus radiata* (Cullis *et al.* 1988, Moran *et al.* 1992), 378 bp in *Junipers virginiana* (Gottlob-Mchugh *et al.* 1990). It is not clear that the long 5S rDNA repeat unit observed in *C. revoluta* is specific to

cycads in general, the genus *Cycas* or the species. The analysis of sequence of 5S rDNA may supply useful information for clarifying phylogeny of gymnosperm or cycad.

Summary

The 5S rRNA gene (5S rDNA) was physically mapped on the somatic chromosomes ($2n = 22$) in *Cycas revoluta* Thunb. by *in situ* hybridization using biotin-labeled probes amplified from total genomic DNA. The 5S rDNA was localized at approximate terminal region of the longest pair of telocentric chromosomes. The site of 5S rDNA were the same as C- or chromomycin A₃-band.

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