

Characterization of Spinach Chromosomes by Condensation Patterns and Physical Mapping of 5S and 45S rDNAs by FISH

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ABSTRACT. Molecular cytogenetic techniques and computer-aided karyotyping were applied to characterize the chromosomes of spinach (*Spinacia oleracea* L., $2n = 12$). Chromosome lengths, arm ratios, and degrees of condensation at prometaphase chromosomes were analyzed using a software Chromosome Image Analyzing System III (CHIAS III). DNA probes prepared from rice (*Oryza sativa* L.) rDNA were applied to the spinach chromosomes by the fluorescence in situ hybridization (FISH) method. Three 45S rDNA loci were detected at the nucleolar organizing region (NOR) of Chromosome 5, and at terminal positions of short arms of Chromosomes 2 and 6. The loci of 5S rDNA were also found at three locations. One was at the subtelomeric region of the long arm of Chromosome 2 and the other two were at the proximal region of the long arm of Chromosome 5. All spinach chromosomes were identified which will provide valuable information for mapping genes on these chromosomes.

Spinach (*Spinacia oleracea*) is a highly nutritious vegetable and has been cultivated in most regions of the world. The chromosome number of spinach was found to be $2n=12$ by Stomps (1911). Subsequently, the chromosomes were numbered based on their morphological characteristics, i.e., total length, arm ratio, and presence of satellite (Ellis and Janick, 1960; Sugiyama and Suto, 1964). Two of the six chromosomes are submetacentric and the others are subacrocentric with similar morphological characteristics. But the identification of spinach chromosomes is still difficult and it is necessary to develop a new method for identification of each chromosome.

The procedure for constructing an idiogram based on condensation pattern (CP) of chromosomes was developed by Fukui and Mukai (1988) for identification of small plant chromosomes but this procedure is difficult to characterize chromosomes based on their morphology or banding patterns. So far, chromosomes have been

characterized by CP in saitbush (*Atriplex rosea* L.) (Fukui and Mukai, 1988), rice (*Oryza sativa*) (Fukui and Iijima, 1991), soybean (*Glycine max* L. Merrill) (Yanagisawa et al., 1991), three diploid *Brassica* L. species (Fukui et al., 1998) and wild sugarcane (*Saccharum spontaneum* L.) (Ha et al., 1999). CP is now considered to be a useful procedure to characterize morphological traits and identify the chromosomes in many plant species.

In situ hybridization (ISH) is an effective method for physical mapping of specific RNA and/or DNA sequences on chromosomes. A fluorescence in situ hybridization (FISH) method has enabled us to obtain high sensitivity in chromosome analysis and to shorten the detection period for gene mapping. Loci of 18S-5.8S-25S and/or 5S ribosomal RNA (45S and 5S rDNA) have already been mapped using the FISH method in plant species with small chromosomes, such as rice (Kamisugi et al., 1994; Fukui et al., 1994) and *Arabidopsis thaliana* (L.) Heynh (Murata et al., 1997).

In the present report, chromosomes of spinach were characterized and karyotyped based on the CP of the chromosomes using the imaging system, CHIAS III, and numerical data of chromosome length and arm ratio. We also localized the sites for 45S and 5S rDNAs by the FISH method.

Materials and Methods

PLANT MATERIALS AND CHROMOSOME SAMPLE PREPARATION. Chromosome samples were prepared from *Spinacia oleracea* 'Minsterland'. 'Minsterland' is a major cultivar and has been used

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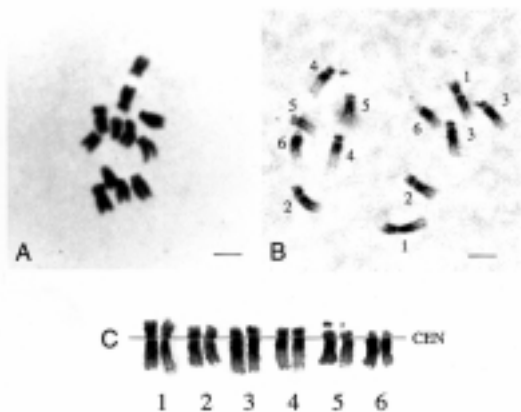


Fig. 1. Giemsa stained chromosomes of spinach. (A) Metaphase and (B) prometaphase; numbers indicate the chromosome number designated in the present paper. Bars = 5µm. (C) Alignment of the chromosomes in numbered order. CEN shows centromeric positions.

for breeding. We used 'Minsterland' as a representative of spinach cultivars since the preliminary survey for karyotype and FISH analysis indicated similar results among several cultivars. Root tips ≈1 cm long were collected from young plants after a few weeks growth in 500-mL pots. The root tips were pretreated with distilled water at 0°C for 20 h, and fixed in Farmer's fluid [3 ethanol : 1 acetic acid (v/v)] at least 1 d before preparation. Chromosome preparations were made by an enzymatic maceration and air-drying method (Fukui, 1996; Fukui and Iijima, 1991). The root tips were washed with water and apices 1 mm long were excised. The root apices were then macerated by incubation in 50 µL enzymatic solution (4% Cellulase Onozuka RS, Yakult Honsha Co., Ltd., Tokyo; 1% Pectolyase Y-23, Seishin Pharmaceutical Co., Ltd., Tokyo, pH 4.2) for 1 h at 37°C. After rinsing the root apices with distilled water, each apex was placed on a slide glass, tapped with the tip of fine forceps into small fragments and a few drops of the fixative [3 ethanol : 1 acetic acid (v/v)] was added and then air dried. For identification of the chromosomes, specimens with well spread chromosomes were used. Chromosomes were stained with a 1.5% Giemsa solution (Merck, Darmstadt, Germany) dissolved with 1/15M phosphate buffer, pH 6.8, for 3 min. All well spread metaphase and prometaphase chromosomes were photographed with black and white film (Neopan F, ISO 32, Fuji, Tokyo) through a microscope (Axiophoto, Zeiss, Oberkochen, Germany).

IMAGE ANALYSIS. Condensation pattern analysis of chromosomes stained with Giemsa solution was carried out using CHIAS III (Chromosome Image Analyzing System III). The density of staining was measured along the midrib of chromatids, expressed as gray values (for detail, see Kato and Fukui, 1998). Data for arm length and the degree of condensation in each chromosome were collected from 72 chromosomes in six chromosome spreads.

FLUORESCENCE IN SITU HYBRIDIZATION (FISH). Two types of ribosomal RNA genes (rDNAs), 45S rDNA consisting of a 18S-5.8S-25S rDNA gene cluster (Sano and Sano, 1990) and 5S rDNA (Kamisugi et al., 1994), were used. Both rDNAs were isolated from the genome of rice. The 45S rDNA was labelled with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) by a random priming method. 5S rDNA was amplified and labeled with biotin-16-dUTP simultaneously by polymerase chain reaction (PCR) using 5S rDNA-sequence-specific primers (5'-GATCCC-ATCAGAACTCCGAAG-3' and 5'-CGGTGCTTTAGT-

GCTGGTATG-3') (Kamisugi et al., 1994).

FISH was carried out according to the procedures of Ohmido and Fukui (1997). Detection of the biotin-labeled probe was performed with Fluorescein Avidin DCS (Vector Laboratory, Burlingame, Calif.) and Biotinylated anti-Avidin D (Vector Laboratory) was applied as the secondary amplification of the fluorescent signals. Chromosomes on the slides were counterstained with 10µg·mL⁻¹ 4',6-diamidino-2-phenylindole (DAPI). Fluorescent signals of FITC (fluorescein isothiocyanate) and fluorescent chromosomes with DAPI were observed independently using a fluorescence microscope (BX60, Olympus, Tokyo) using two different filters, B10 and UV01, respectively. Each image was recorded separately through a high-sensitive CCD camera (PXL1400, Photometrics, Tucson, Ariz.). Chromosomal identification was performed by microscopic observation. CPs of the chromosomes were measured by CHIAS III, and signals of loci for 45S and 5S rDNAs were identified on the chromosomes by FISH.

Results and Discussion

CONDENSATION PATTERN (CP) OF SPINACH CHROMOSOMES. A squashed preparation of metaphase chromosomes stained with a Giemsa solution is shown in Fig. 1A. All six pairs of chromosomes including Chromosome 5 which is known to have a satellite, were difficult to distinguish from each other. Prometaphase chromosomes stained with Giemsa solution are shown in Fig. 1B. Chromosome regions condensed at the prometaphase stage could be recognized and distinguished, as densely stained regions, which were more condensed at the early stage of metaphase than other regions. Also, variations in condensation patterns such as the degrees and regions were observed among the chromosomes. Fig. 1C shows the karyotype obtained from Fig. 1B, in which the numbers 1 to 6 were assigned for each chromosome according to the nomenclature by Sugiyama and Suto (1964).

The degree of condensation of each chromosome was quantified and analyzed on six prometaphase cells using CHIAS III for discriminating the chromosomes. Density profiles from Giemsa-stained images of the chromosomes are shown in Fig. 2. Each panel of this figure shows the average condensation pattern of six prometaphase chromosomes plates and indicates that each of the six chromosomes had its own characteristic pattern of condensation. In the profiles, lower threshold (Gray value = 96) corresponded to the visual boundary between darkly and lightly stained regions. The condensed regions indicating higher Gray values than the lower threshold are represented by dotted regions in the idiogram. The highly condensed regions, which were discriminated by the upper threshold (Gray value = 152), are represented by solid boxes in the idiogram. Both arms of Chromosome 1 had dark regions. Chromosomes 3 and 4 were quite similar in their morphologies, but were different in the characteristics of the gray values on the long arms, such as Chromosome 3 had a very dense region on the long arm. The long arm of Chromosome 6 had a highly dark region close to the centromere. On the other hand, the short arm was very short and had no highly condensed regions.

Morphological data of the six chromosomes of spinach, which were expressed as the average of six prometaphase plates, are presented in Table 1. From the arm ratios, Chromosomes 1 and 2, Chromosomes 3, 4, and 5, and Chromosome 6 were submetacentric, subacrocentric, and telocentric chromosomes, respectively. Chromosome 5 was a satellite chromosome.

PHYSICAL MAPPING OF 45S AND 5S RDNA. FITC hybridization signals on the chromosomes stained by DAPI are shown in Fig. 3.

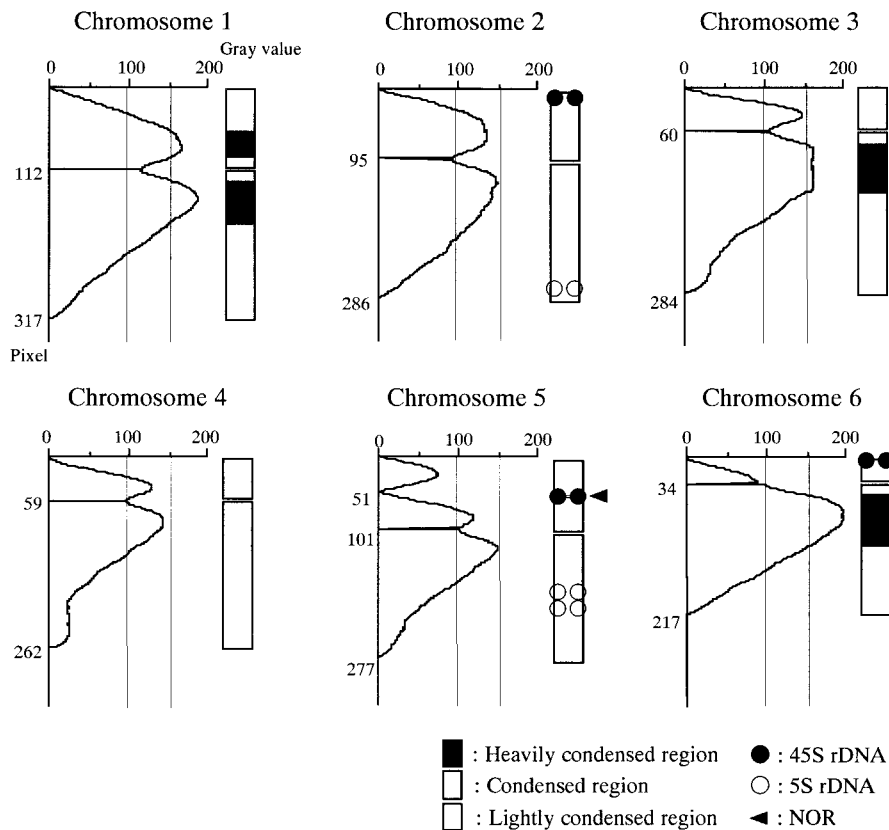


Fig. 2. Density profiles and idiogram of the spinach chromosomes. The vertical and horizontal axes represent the number of pixels along the chromosome and the gray value, respectively. The highly condensed regions are represented by the upper threshold in the panels, corresponding to solid regions in the idiogram. The moderately condensed regions discriminated by the lower threshold in the panels, are represented by dotted regions in the idiogram. The 45S and 5S rDNA loci are shown by solid and open circles on the idiogram, respectively.

Six 45S rDNA sites labelled with biotin were clearly observed as yellowish signals of FITC on the three pairs of chromosomes (Fig. 3A). Among the three pairs of the signal sites, a pair of the largest one was located on the short arms of Chromosome 5 which were satellite chromosomes. Another prominent signal site was also found on the short arms of Chromosome 6. The rest of 45S rDNA sites were recognized as small signals and located at the telomeric regions of the short arms on Chromosome 2.

The 5S rDNA signals were detected at three sites on the spinach chromosomes (Fig. 3B). Four signals of the two sites were located closely on the proximal regions of the long arms of Chromosome 5. In the same preparation, these two 5S rDNA loci were sometimes observed as one site due to the degree of condensation. Another pair of the signals were found on the subtelomeric regions of the long arms of Chromosome 2.

The 45S and 5S rRNA multicopy genes consist of highly repeated sequences arranged in tandem at several loci in eukaryote genomes. Although in situ hybridization is not a fully quantitative method, differences in signal strength reflect variation in copy number of the target loci (Leitch and Heslop-Harrison, 1993; Shishido et al., 1999). Since the strengths of the 45S signals were different among loci in the present study, it is suggested that each loci are varied in repeat numbers of the unit length. The major site of the 45S rDNA loci corresponded to the NOR of Chromosome 5. High sensitivity of the FISH technique made it possible to detect not

only the major loci, but also additional 45S rDNA loci, as shown at the distal parts of the short arms of Chromosomes 2 and 6. Although the secondary constrictions were not clearly observed at these locations, a small satellite was observed in some preparations on the short arm of Chromosome 6.

CHARACTERIZATION OF EACH SPINACH CHROMOSOME. In Fig. 2, the locations of the 45S and 5S rDNA loci are shown on the idiogram based on morphology of the chromosomes. Characteristics of each spinach chromosome on CPs and the locations of rDNAs are summarized as follows:

CHROMOSOME 1: Submedian and the longest chromosome among the six. Almost the whole region of the chromosome is condensed, except the ends of the chromosome.

CHROMOSOME 2: Submedian and shorter than Chromosome 1. The condensed region occupies the proximal two-thirds of both arms. The site of 45S rDNA is located on the telomeric region of the short arm and 5S rDNA site is located on the subtelomeric region of the long arm.

CHROMOSOMES 3 AND 4: Subterminal chromosomes. Chromosome 3 is a little longer than Chromosome 4, although the lengths of their short arms are almost the same. Chromosome 3 has a large condensed region on

the long arm, although Chromosome 4 has a small effect of condensation. Moreover in Chromosome 3, a much condensed region is observed at the middle of the long arm. It is remarkable that the condensed region on the long arm of Chromosome 3 is larger than that of Chromosome 4.

CHROMOSOME 5: Subterminal chromosome with a satellite. Condensed region is observed on the proximal half of the long arm. Satellite region is not condensed. 45S rDNA site is located on the terminal region of the short arm, corresponding to NOR. Two 5S rDNA sites are on the proximal region of the long arm.

CHROMOSOME 6: Subterminal and the shortest chromosome. The short arm is very small and not condensed. It has a highly condensed region near the centromere and a decondensed region at the terminal region of the long arm. 45S rDNA is located on the terminal region of the short arm.

As to the localization of genes on the chromosomes, it was attempted previously by Ellis and Janick (1960) and Sugiyama and

Table 1. Numerical data of the spinach chromosomes represented by relative length (total length in a cell = 100, equal to 52.8 μ m).

Chromosome no.	Short arm ^z	Long arm ^z	Total	Arm ratio ^y
1	7.0	13.0	19.9	0.35
2	5.9	12.1	18.0	0.33
3	3.7	14.2	17.8	0.21
4	3.6	12.8	16.4	0.22
5	3.2	11.1	14.3	0.22
6	2.1	11.4	13.5	0.15
Total	25.4	74.6	100.0	

^zThe average of six prometaphase plates. The length of the satellite was excluded from calculation.

^yShort arm/total length.

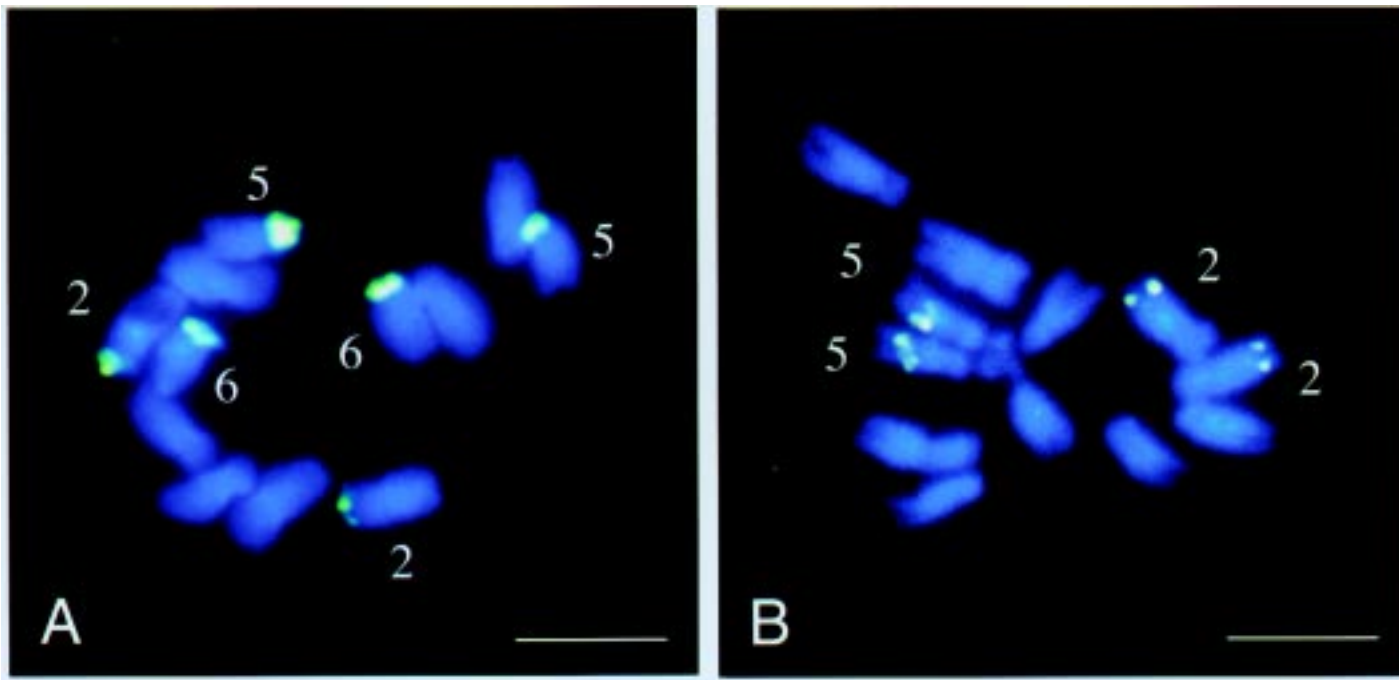


Fig. 3. Fluorescence in situ hybridization of spinach chromosomes using 45S rDNA and 5S rDNA probes. Yellowish signals show hybridization sites detected by FITC. (A) 45S rDNA signals on Chromosomes 2, 5, and 6, and (B) 5S rDNA signals on Chromosomes 2 and 5. The chromosomes are counterstained with DAPI (blue). Bars = 5 μ m.

Suto (1964) who localized the sex determining factors X and Y on Chromosome 1 by using trisomic series. Iizuka and Janick (1971) localized the factors on the short arm of Chromosome 1 by using a spontaneous translocation in Chromosome 1. However, no other reports on localization of genes have been found in spinach.

The present study is the first for physical mapping of genes using CP and FISH in spinach. In the near future, it is expected that FISH will be used efficiently for mapping single genes or low-copy sequences in spinach, as shown in *Brassica* sp. (Kamisugi et al., 1998) and rice (Ohmido et al., 1998). Therefore, it is concluded that development of a quantitative chromosome map as shown in the present report is quite useful not only for basic genome and chromosome researches but also for practical breeding and genetics of spinach. It is anticipated that the physical chromosome map as shown herein would be invaluable for localizing useful genes and systematic breeding.

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