Characterization; Genome Sizes and Morphology of Sex Chromosomes in Hemp (*Cannabis sativa* L.)

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Summary A dioecious plant, *Cannabis sativa* has two sex chromosomes (X and Y). The genome sizes of the diploid female and male plants were determined to be 1636 and 1683 Mbp, respectively, by flow cytometry. By the karyotype analysis, the X and Y chromosomes were found to be submetacentric and subtelocentric, respectively. The Y chromosome had the largest long arm with a satellite in the terminal of its short arm. Conspicuous condensation was specifically observed in the long arm and satellite of the Y chromosome during the prometaphase to metaphase stages. These results indicate that the Y chromosome, especially in its long arm, specifically differentiates in *Cannabis sativa* and might contribute to the sex determination.

The majority of flowering plants are hermaphrodites. In contrast, dioecious plants are reported to be approximately 4% hermaphrodites among angiosperm species (Yampolsky and Yampolsky 1922). The dioecious plants, *Cannabis*, *Rumex*, and *Silene* reportedly have heteromorphic sex chromosomes (X and Y chromosomes; Parker 1990, Chattopadhyay and Sharma 1991) and the sex of the plants is genetically determined.

Yamada (1943) revealed that the female plants of *Cannabis sativa* L. have two X chromosomes whereas the male plants have one X and one Y chromosome. The Y chromosome is much larger than the X chromosome and the autosomes. The roles of the sex chromosomes in sex determination have been studied using haploid, diploid, and triploid plants of *C. sativa* (Warmke and Davidson 1944, Nishiyama *et al.* 1947). It is reported that the X and Y chromosomes of *C. sativa* carry female and male-determining genes, respectively, and that the autosomes are not involved in sex determination. There is little information, however, characterizing the sex chromosomes of *C. sativa*. In the present study, we analyzed the genome size, karyotype, and specific condensation of the Y chromosome of *C. sativa* to elucidate the mechanisms of sex differentiation in plants.

Materials and methods

Plant materials

The female and male plants of *Cannabis sativa* (family: Cannabaceae) were grown in pots at 28°C under a photoperiodic regime of 16 hr of light and 8 hr of dark. Differentiation of the female or male was determined by the flowering under a photoperiodic regime of 8 hr of light and 16 hr of dark for 2 weeks. The plants were vegetatively propagated by cuttings. *Arabidopsis thaliana* (Colombia) was grown in pots at 21°C under continuous light.

Estimation of nuclear genome sizes

Nuclear genome sizes of *A. thaliana* and *C. sativa* were measured using flow cytometry. *A. thaliana* was used as the size standard. Fresh leaf samples (0.5 to 1 cm²) were collected from mature leaves and placed on plastic petri dishes with 300 μ l of solution A added for nuclei isolation (High resolution DNA kit, type P: Partec GmbH, Münster, Germany). Each leaf was chopped using a sharp scalpel and remained in the solution for 5 min. Suspensions were filtered through a 30- μ m nylon mesh and transferred to a sample tube. Solution B (2 ml) was then added for DAPI staining (High resolution DNA kit, type P: Partec GmbH) and they remained in the solution for 5 min. All samples were analyzed with a Partec PA flow cytometer (Partec PA, Partec GmbH), which enabled detection of the fluorescent signals from DAPI (4',6-Diamidino-2-phenylindole Dihydrochloride). In each sample, 15000 to 35000 nuclei were analyzed at a rate of 10 to 30 nuclei/s. Peak positions and coefficients of variation (CV) of G_{0/1} peaks were calculated using the program supplied by Partec.

Preparation of the chromosomes

Root tips (approximately 1 cm long) were excised and pretreated with distilled water at 11°C for 12 to 14 hr and fixed with ethanol:acetic acid (3:1). Chromosome samples were prepared by the enzymatic maceration/air-drying (EMA) method (Fukui *et al.* 1994, Ohmido and Fukui 1996). Root tips were macerated in the enzymatic mixture (2% Cellulase Onozuka RS, 1.5% Pectolyase Y-23, 0.3% Macerozyme R200, 1 mM EDTA, pH 4.2) at 37°C for 30 to 40 min. The root tip cells were spread on glass slides and air-dried. The chromosomes from prometaphase to metaphase stages were stained with a 0.004% Giemsa solution or 7.5 μ g/ml DAPI solution.

Karyotype analysis of the chromosomes

The chromosomal lengths were measured on a Macintosh computer using the public domain NIH image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>) and the CHIAS III system using the macro programs written by Kiichi Fukui, Seiji Kato, and Tamaki Hirose at Hokuriku National Agricultural Experiment Station, Japan (Kato *et al.* 1997).

Results

Distributions of the DNA content measured for the mixture of nuclei isolated from *A. thaliana* and female *C. sativa* or male *C. sativa* are shown in Figs. 1A and 1B, respectively. The peak number of 1, 2, 3, and 5 in Figs. 1A and 1B show diploid, tetraploid, octaploid and hexadecaploid *A. thaliana*, respectively as the standard. Peak number 4 shows diploid *C. sativa*. When the haploid genome size of *A. thaliana* is 130 Mbp (Kaneko *et al.* 1998), the diploid genome sizes in female and male plants of *C. sativa* are estimated to be 1636 ± 7.2 (S.D.) and 1683 ± 13.9 (S.D.) Mbp, respectively (Table 1).

Female and male *C. sativa* have XX and XY chromosomes with 18 autosomes, respectively. Condensation patterns were observed in the Giemsa-stained female and male *C. sativa* chromosomes at the pro-metaphase stage (Figs. 2A, 2B). The Y chromosome, the largest chromosome, is subtelocentric and has a large long arm with a satellite in the terminal of its short arm. The X chromosome was found to be submetacentric with a short satellite at the end of the short arm.

Conspicuous condensation was observed over the entire long arm and satellite of the Y chromosome from prometaphase to metaphase (Figs. 3A, 3B). Fig. 4A shows a model of the condensation pattern of the Y chromosome. Fig. 4B shows the dynamics and correlation of the length of the satellite, short arm and long arm against the total length of the Y chromosome from prometaphase to metaphase. The result indicates that the satellite and long arm of the Y chromosome condensed 5

Fig. 1. Nuclear DNA content isolated from leaf tissues of *C. sativa* and *A. thaliana*. A) The female plant of *C. sativa* (peak 4, CV=1.8–2.5%) and *A. thaliana*. B) The male plant of *C. sativa* (peak 4, CV=1.3–2.9%) and *A. thaliana*. Peaks 1, 2, 3 and 5 correspond to diploid, tetraploid, octaploid and hexadecaploid of *A. thaliana*, respectively.

Fig. 2. Chromosomes and karyograms with X and Y chromosomes of *C. sativa* at the pro-metaphase stage. A) female. B) male. Bars indicate 5 μm.

and 6.5 times more than the short arm, respectively.

Discussion

Flow cytometry of the nuclear DNA content has become an important technique for detection of genome sizes of various organisms. In many plant species, young leaves are commonly used for the flow cytometry. In the present study, we used only the mature leaves of *C. sativa* for flow cytometry, because young leaves are more contaminated by mitotic chromosomes. Cell division is probably still active in the

Table 1. Nuclear genome size of *Cannabis sativa*

Species	Genome size (diploid) (Mbp)
<i>Arabidopsis thaliana</i>	260
<i>Cannabis sativa</i> L.	
female (♀)	1636 (7.2)
male (♂)	1683 (13.9)

Each value ($\pm$ -S.D.) is the mean of results from six measurements.

Cell division is probably still active in the

Fig. 3. Chromosomes ($2n=20$) at the prometaphase to metaphase stages stained with DAPI. A a–d) Arrows indicate the Y chromosome in the male plant. Bars indicate $5\text{ }\mu\text{m}$. B a–d) are the enlarged Y chromosomes at each stage.

Fig. 4. Schematic illustration of specific condensation of the long arm and satellite of the Y chromosome. A) The correlation of the length of the Y chromosome represented graphically. B a–c) the satellite, short and long arms, respectively.

young leaves of *C. sativa*.

We detected differences in the DNA content between female and male *C. sativa*. The differences in the nuclear DNA content was approximately 47 Mbp (Table 1). This value occupies about 2.8% of the total genome size of the male plants and could correspond to the large long arm of the Y chromosome. In another dioecious plant, *Silene latifolia*, there is a 2 to 5% difference in the nuclear DNA content between females and males (Costich *et al.* 1991, Vagera *et al.* 1994, Doležel *et al.* 1995). Differences might be due to the large size of the Y chromosome.

The chromosomes of *C. sativa* condensed from the mitotic prometaphase to metaphase stages in a similar manner, except for the Y chromosome (Figs. 3A, 3B). The satellite and long arm of the Y chromosome were more rapidly condensed in comparison with the short arm, X chromosome and autosomes. These results indicate that the Y chromosome, especially in its long arm, specifically differentiates in *C. sativa* chromosome. This region might be associated with the accumulation of specific nucleotide sequences (Sakamoto *et al.* in press) and differentiation of the sex chromosomes.

In order to elucidate the structure of the Y chromosome of *C. sativa*, we compared female and male DNAs by the random-amplified polymorphic DNA (RAPD) technique. One DNA fragment, 729 bp in size, produced much more intense bands specific to male plants, in addition to less intense bands common to both the sexes in a gel-blot analysis of genomic DNA and was named *MADC1* (male-associated DNA sequence in *C. sativa*) (Sakamoto *et al.* 1995). A fluorescence *in situ* hybridization (FISH) experiment (Fukui *et al.* 1994, Ohmido and Fukui 1996, 1997) using metaphase chromosomes from root cells with *MADC1* as the probe detected a clear doublet signal only on the end of the long arm of the Y chromosome (Sakamoto *et al.* 1997). These results demonstrate that

some specific DNA sequences might be accumulated and associated with differentiation and functions of the sex chromosome, especially in the long arm of the Y chromosome. Further investigation of the molecular structure of the sex chromosomes will contribute to the elucidation of mechanisms of sex differentiation and heteromorphism of sex chromosomes in plants.

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