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The University of Osaka

Doctor Thesis

Development of quantitative chromosome maps and their utilization for visual analyses of genome information in higher plants

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2001

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Doctor Thesis

Development of quantitative chromosome maps and their utilization for visual analyses of genome information in higher plants

高等植物における定量的染色体地図の作成とそれに基づくゲノム情報の可視的解析

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2001

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Abbreviations

AFLP : amplified fragment length polymorphism

BAC : bacterial artificial chromosome

CAP : cleaved amplified polymorphic sequencence

CHIAS III : chromosome image analyzing system ver. 3

CP : condensation pattern

DAPI : 4',6 diamidino-2-phenylindole

EMA : enzymatic maceration and air-drying

FISH : fluorescence *in situ* hybridization

FITC : fluorescein isothiocyanate

FUSC : faint, unstable and small condensation

ISH : *in situ* hybridization

NOR : nucleolar organizing region

PCR : polymerase chain reaction

PI : propidium iodide

RAPD : random amplified polymorphic DNA

rDNA : ribosomal DNA

RFLP : restriction fragment length polymorphism

SSLP : simple sequence length polymorphism

YAC : yeast artificial chromosome

CHAPTER I

General introduction

In recent years, genome projects have rapidly proceeded in many organisms including worms, bacteria, fruit flies and human as targets for the nucleotide sequencing of the entire genome leading to an understanding of the genetic information of the organisms. In higher plants, *Arabidopsis thaliana* L. has been a model plant for research in plant biology, and was chosen as the target of a genome project (Kaiser, 1996), because of its small genome size and low contents of repetitive sequence contents (Luetwiler *et al.*, 1984). The sequencing of *A. thaliana* is close to being finalized, and all nucleotide sequences will be reported soon. Sequence analyses will be continued in other organisms as well. It is thus necessary to have some essential information of the genome to make projects effective in plants. First, the genome size is essential information, as it is important to estimate the total effort, funding and time for the project. Second, polyploidy is essential information for genome research, and can be obtained from cytogenetic studies including investigation of the number of chromosomes and chromosome karyotype. Third, genome analysis requires the positions of and/or the order of genes. One relevant map is a chromosome map, which shows the lengths of chromosomes in cytogenetic study. We can use it to analyze a whole genome at one time because the chromosomes are the visualized objects of genetic information constructed at the mitotic stage in a cell cycle. Others are a physical map in which sequence data is accumulated and correctly arranged, and a linkage map that represents recombination values.

Plant genome studies have progressed in many species, with progress as diverse as

the very advanced genome analysis of *Arabidopsis*, to plants whose chromosome numbers are unknown. Three species of plants, *Arabidopsis thaliana* L, *Lotus japonicus* L. and spinach (*Spinacia oleracea* L.) were chosen for the present study because all of them need to have chromosome maps to complete their genome research and also because their status in genome research is different..

Arabidopsis thaliana is a member of the Brassicaceae and diploid species with a short life cycle and being a small plant. The genome size of *A. thaliana* is approximately 130Mbp and is organized into five small chromosomes at the mitotic M phase, and is estimated to have 25,000 genes. Two maps, linkage and physical maps, of each chromosome have already developed. The linkage map is based on recombination frequencies that include the classical genetic map estimated location of mutant genes and a recombinant inbred map which locates cloned genes and molecular markers *i.e.*, restriction fragment length polymorphisms (RFLPs), simple sequence length polymorphisms (SSLPs) and cleaved amplified polymorphic sequencences (CAPSs). Many markers have been located on the linkage maps in *A. thaliana*. A physical map for *A. thaliana* has been developed and the nucleotide sequence analyses have been the most progressed among the higher plants. In the first phase of the *A. thaliana* genome project, yeast artificial chromosome (YAC) and bacteria artificial chromosome (BAC)-based physical maps of its chromosomes were constructed. In the second phase, the sequencing project has been proceeding based on the contig map that covers the entire chromosomes. It has been enabled by obtaining these data from the Web database (http://www.arabidopsis.org/). In contrast, a cytogenetic chromosome map has not been developed due to the small size of the mitotic chromosomes.

Lotus japonicus is a species in the Leguminosae plant family and the third target

plant for genome projects in Japan. Like the other model plant, *L. japonicus* possesses many biological and genetic advantages (Handberg and Stougaard, 1992). For example, it is a diploid, and a small genome size was estimated. It has also a short generation time and is self-fertilizing. *L. japonicus* has also been used for the study of nitrogen fixation and nodulation as a model legume plant. Also, a linkage map has been constructed. It was reported that molecular polymorphism was detected using a DNA amplification fingerprinting technique by a single arbitrary primer among the accessions, although a low-density marker map is available now (Jiang and Gresshoff, 1997). The sequence data and cytogenetic chromosome maps have not yet been presented, although sequencing of *L. japonicus* genome has been in planning.

Spinach is in the family Chenopodiaceae and is one of the most popular crops, a highly nutritious vegetable cultivated in most countries in the world. As in most vegetables, spinach genetics have not been analyzed in detail. The genome size was reported as 989 Mbp (Arumuganathan and Earle, 1991) and is relatively small in higher plants. However, genetical linkage, physical and cytogenetic chromosome maps have yet to be developed yet

In the presence study, the first priority was given to developing cytogenetic chromosome maps of these three plant species because no chromosome maps have been constructed for these three plants, regardless of the status of their genome analysis. We can visually trace chromosomal behavior and the localization of genes or DNA markers on chromosome maps. This is virtually the only method applicable for plants at the very infant stage of genome analysis. Moreover, once the chromosome map has been developed, we can analyze the whole genome at one time by physically mapping genes and markers, although centromeric and heterochromatic regions are rather difficult for physical

mapping the genes.

We have tried to develop the chromosome maps by using a computer image analysis method targeting the condensation patterns, *i.e.* uneven condensation of chromatin fibers appearing at the prometaphase chromosomes based on the method developed for the identification of small plant chromosomes (Fukui and Iijima 1991). Quantitative chromosome maps constructed by image analyses are being developed for various plants, and are accepted as objective maps in which researchers' fluctuation is excluded. So far, the chromosomes of *Atriplex rosea* (Fukui and Mukai 1988), rice (Fukui and Iijima 1991), oilseed rape (Kamisugi *et al.* 1998), three diploid *Brassica* species (Fukui *et al.* 1998) and wild sugarcane (Ha *et al.*, 1999) were identified based on the condensation patterns, and the quantitative chromosome maps were developed. The condensation pattern is now considered to be a key parameter to characterize small plant chromosomes and to facilitate the development of a chromosome map.

In situ hybridization (ISH) was first reported in 1969 as a molecular cytogenetical method to visually detect specific DNA or RNA sequences located on the chromosome, nucleus or a tissue sample using a microscope. In the first report, the radio-isotope (RI)-labeled ribosomal RNA was used to detect the localization of rRNA gene sequences on the human cell nucleoli (Gall and Pardue 1969). The ISH method has been greatly improved and is used for the physical mapping of DNA sequences on chromosomes in both animals and plants. Non-RI ISH method was developed using a biotin labeling method in 1981 (Langer *et al.* 1981). The safer, simpler and easier method of fluorescence *in situ* hybridization (FISH) method, which uses fluorochromes for the detection of the target nucleotide sequences, has since been widespread. Additionally, the great improvement of detection systems and the introduction of the image analysis method made the FISH method

sufficiently successful even for mapping of cosmid clones or RFLP markers size 1-2kb (Ohmido *et al.* 1998). The combination of several fluorochromes and epifluorescence filter sets, and the computing separation of spectra enabled the simultaneous discrimination of all the human chromosomes (Speicher et al. 1996, Schock *et al.* 1996). We used the FISH method to visually localize some DNA sequences on chromosomes and used mapping data for the construction of the quantitative chromosome maps.

In Chapter II, spinach chromosomes were visually characterized by condensation patterns and a quantitative chromosome map of spinach chromosomes was developed. Then, 45S and 5S rDNAs were visually mapped on the chromosomes by FISH.

In Chapter III, a quantitative chromosome map based on the condensation pattern was developed in *A. thaliana* with the locations of 45S and 5S rDNAs by combination of FISH and imaging methods.

In Chapter V, the genome size of *L. japonicus* was first determined and a quantitative chromosome map of *L. japonicus* was subsequently developed. 45S and 5S rDNAs and 500 cDNAs were also visualized on chromosomes due to research functional genes region.

Finally, in Chapter V, the significance of the results obtained in this study is summarized and discussed, and prospects for extending the study are discussed.

CHAPTER II

Development of a spinach chromosome map of imaging methods and physical mapping of 45S and 5S rDNAs by FISH

II-1. Introduction

Spinach (*Spinacia oleracea* L.) 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 sub-metacentric and the others are sub-acrocentric 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. 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. 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. 18S-5.8S-25S and/or 5S ribosomal RNA (45S and 5S rDNA) loci 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.

II-2. Materials and Methods

Plant materials and chromosome sample preparation

Chromosome samples were prepared from *Spinacia oleracea* cv. "Minsterland". Minsterland is a major cultivar and has been used 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 about 1 cm long were collected from young plants after a few weeks growth in 500ml volume 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 day before preparation. Chromosome preparations were made by an enzymatic maceration and air-drying (EMA) 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, Tokyo; 1% Pectolyase Y-23, Seishin Pharmaceutical, 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, Germany) dissolved with 1/15M phosphate buffer, pH 6.8, for 3 min. All well spread metaphase and pro-metaphase chromosomes were photographed with black and white film (Neopan F, ISO 32, Fuji, Tokyo) through the microscope (Axiophoto, Zeiss, Germany).

Image analysis

Condensation pattern analysis of the chromosomes stained with Giemsa solution was carried out using the computer program, chromosome image analyzing system ver. 3 (CHIASIII) that be obtained from the Web site (http://133.1.131.81/Eudejas/chias3/chias3.html) (Kato and Fukui 1998). The CHIASIII system is a program to quantify the uneven density on the chromosomes based on NIH Image (written by Wayne Rasband at the US National Institute of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd, Springfield, VA 22161, part number PB93-504868). A spread-sheet program, Microsoft Excel (ver. 5.0, Microsoft) was used for the calculation of averaged CPs or standard CPs to develop idiograms. To standardize data for arm length and the degree of condensation in each chromosome were collected from 72 chromosomes in six chromosome spreads.

DNA probes

Two types of ribosomal RNA genes (rDNAs), 45S rDNA consisting of 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 labeled with biotin-16-dUTP (Roche) 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'-GATCCCATCAGAACTCCGAAG-3' and 5'-CGGTG CTTTAGTGCTGGTATG-3') (Kamisugi *et al.*, 1994).

Fluorescence *in situ* hybridization (FISH)

FISH was carried out according to the procedures of Ohmido and Fukui (1997).

Prior to *in situ* hybridization, slides were incubated in 300μ 0.1mg/ml RNaseA (Sigma) solution dissolved with 2xSSC for 60 min at 37°C in a humid chamber. Subsequently, the slides were rinsed three times with 2xSSC for 5 min each at room temperature and dehydrated by submerging in 70, 95, and 100% (v/v) ethanol for 5min each and air-dried. A 20 μl probe solution (2.5μg/ml labeled DNA, 0.5mg/ml salmon sperm DNA in 50% formamide/2xSSC) was denatured for 10 min at 90°C and cooled rapidly on ice. Then, the probe solution was dropped on a chromosome preparation. The glass slide covered with a cover slip was denatured for 2 min at 80°C on a flat plate of thermal cycler (PHC-3, Techne, UK). Preparations were left to hybridize for 18 h in a humid chamber at 37°C.

Post-hybridization washes of the slides were done successively with 2xSSC, 50% formamide in 2xSSC, 2xSSC and 4xSSC for 10 min each at 40°C. To prevent excessive reaction, slides were blocked with 100μl blocking buffer containing 5%(w/v) non fat milk powder in 4xSSCT (0.05% Tween-20 in 4xSSC) for 5 min at 37°C. A 70 μl 0.1mg/ml Fluorescein Avidin DCS (Vector Laboratory) solution was dropped onto the glass slides, which were then incubated at 37°C for 60 min. After rinsing the FITC-avidin solution with BT buffer (0.1M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) three times at 40°C for 10 min each, immunological blocking was carried out with 100μl 5% goat serum in 4xSSCT. A 70 μl biotinylated anti-avidin solution (1mg/ml, Vector Laboratory) was dropped onto the glass slides, which were then incubated at 37°C for 60 min. After washing with BT buffer three times at 37°C for 5 min each, slides were blocked with 100 μl blocking buffer containing 5%(w/v) non fat milk powder in 4xSSCT for 5 min at 37°C. A 70 μl 0.1mg/ml Fluorescein Avidin DCS (Vector Laboratory) solution was dropped onto the glass slides, which were then incubated at 37°C for 60 min again. Then, these glass slides were washed with BT buffer two times and 2XSSC once at room temperature for 10 min

each.

Chromosomes on the slides were counterstained with 10μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 10 min. After brief washing with water, the chromosome preparations were then mounted with 10μl Vectashield (Vector Laboratories) and covered with 22 x 22mm slips. 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, 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.

II-3. Results and Discussion

Condensation pattern (CP) of spinach chromosomes.

A squashed preparation of metaphase chromosomes stained with a Giemsa solution is shown in Figure II-1A. All six pairs of chromosomes including Chromosome 5, which is known to have a satellite, were difficult to distinguish from each other. Pro-metaphase chromosomes stained with Giemsa solution are shown in Figure II-1B. Chromosome regions condensed at the pro-metaphase 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. Figure II-1C shows the karyotype obtained from Figure II-1B, in which the numbers 1 to 6 were assigned for each chromosome according to the nomenclature by Sugiyama and Suto (1964).

Image analysis

The degree of condensation of each chromosome was quantified and analyzed on six pro-metaphase cells using CHIAS III for discriminating the chromosomes. Figure II-2 shows a flow chart of the CP analysis and representative steps of the image manipulation for the CP analysis by using CHIAS III. Figure II-2A-K shows the same chromosomes. Figure II-2A shows the Giemza stained chromosomes image digitized by a film scanner at the size of 1200 x 1400 pixel. These chromosome images are digitally pretreated before the measurement of the numerical data of the CP from each 12 chromosomes. The background field image is structured using digitally filter, which represent

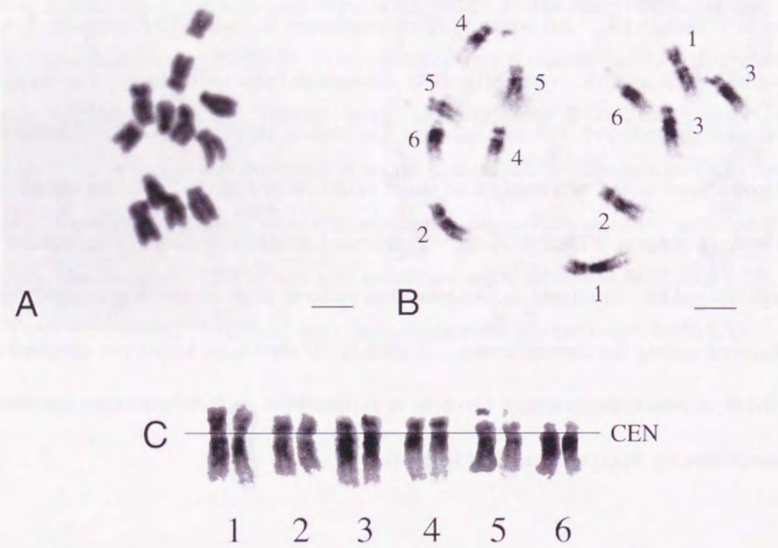

Figure II-1. Giemsa stained chromosomes of spinach. (A) metaphase and (B) prometaphase; numbers indicate the chromosome number designated in the present paper. Bars = 5μm. (C) alignment of the chromosomes in numbered order. CEN shows centromeric positions.

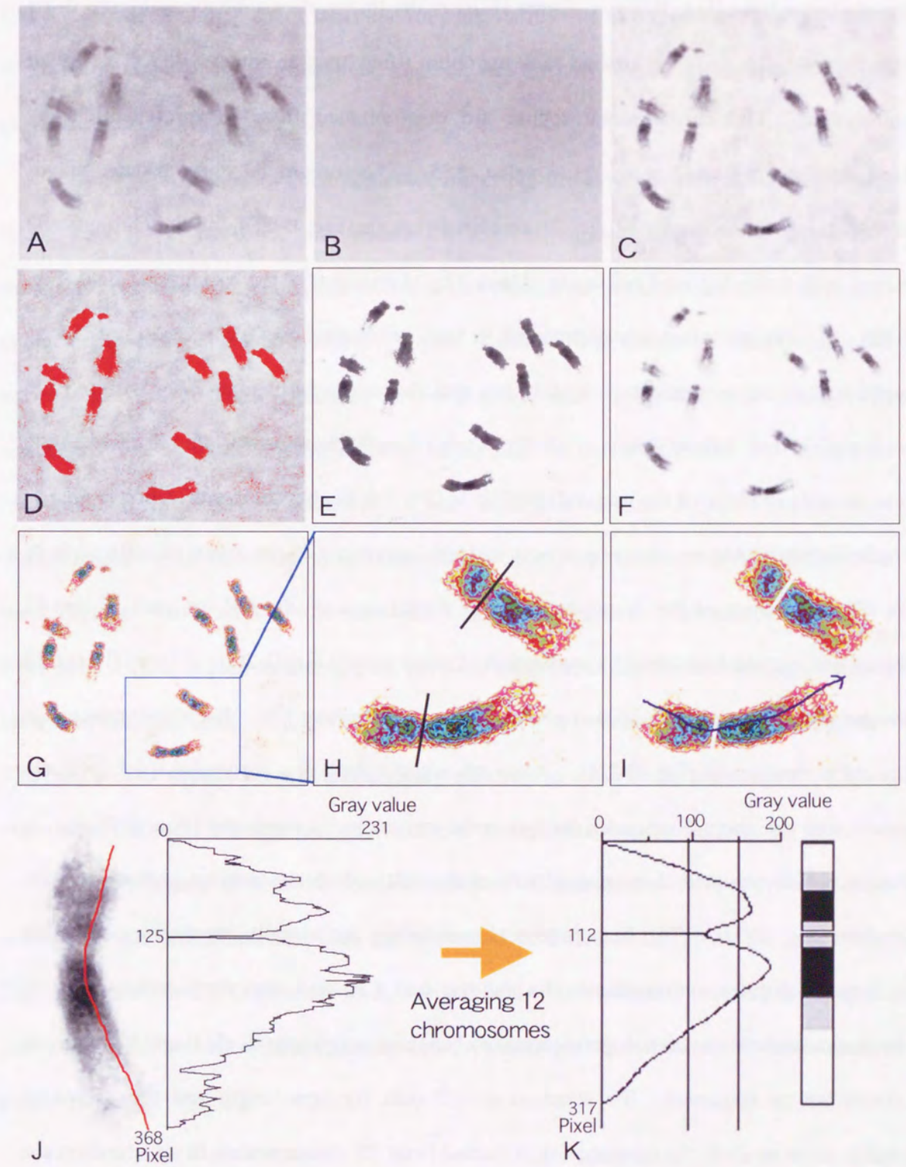

Figure II-2. Representative steps of the image manipulation for CP analysis. The details are described in the text.

irregularity of light (Fig. II-2B). Figure II-2C is the image after subtracting Figure II-2B from Figure II-2A and then applied rank operation filter (median filter) with a 3 x 3 pixel matrix size. The chromosomal region are discriminated from background field by threshold the gray level at the gray value, 155. The foreground chromosome images painted in red are recovered as the chromosomal regions (Fig. II-2D) and the chromosome images without background noises are obtained by elimination of the background field (Fig. II-2E). 12 chromosomes are normalized to their respective gray values of 1 to 254 by application of the normalization digital filter and then applied a median filter with a 5 x 5 pixel matrix size. Gray values of all the pixels of each chromosome are allocated to the new, broadened range of the gray values (Fig. II-2F). Alphabetical identification from a to f indicating homologous chromosomes is assigned according to the visual identification of the CP in advance of the acquisition of the digital data of the CP for each of the 12 chromosomes pseudo-colored by application of color tables (Fig. II-2G). Figure II-H and I are the zooming up image of two chromosomes for measure CP. Each chromosome is marked at centromere (Fig. II-2H). A line tracing a mid-rib of a chromatid is interactively drawn, and the gray value under the line were automatically measured (Fig. II-2I, J). A density profile obtained at the mid-rib line of the enlarged chromosome is plotted in a new window (Fig. II-2J). The homologous chromosomes are visually checked again within each panel for the chromosome morphology and CP, and necessary replacement of chromosomes between homologous group or change in assignment of short and long arms is carried out as requested. To standardize, CP data for arm length and the degree of condensation in each chromosome are collected from 72 chromosomes in six chromosome spreads.

Second, the plotted data of all the CPs are copied to the spread-sheet of Microsoft

Excel. The length in pixels of short and long each arms was averaged density profiles data of 24 chromatids per one chromosome number, and the corresponding gray value for each pixel is calculated (Fig. II-2K).

Density profiles from Giemsa-stained images of the chromosomes are shown in Fig. II-3. Each panel of this figure shows the average condensation pattern of six pro-metaphase 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 that were expressed as the average of six prometaphase plates are presented in Table II-1. From the arm ratios, Chromosomes 1 and 2, Chromosomes 3, 4, and 5, and Chromosome 6 were sub-metacentric, sub-acrocentric, and telocentric chromosomes, respectively. Chromosome 5 was a satellite chromosome.

Physical mapping of 45S and 5S rDNAs.

FITC hybridization signals on the chromosomes stained by DAPI are shown in

Figure II-4. Six 45S rDNA sites labeled with biotin were clearly observed as yellowish signals of FITC on the three pairs of chromosomes (Fig. II-4A). 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. II-4B). 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 sub-telomeric 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 rDNA signals were different among loci in the present study, it is suggested that each locus is 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 Figure II-3, 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-third of both arms. 45S rDNA site is located on the telomeric region of the short arm and 5S rDNA site is located on the sub-telomeric 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

Table II-1. Numeric and data of the spinach chromosomes represented by relative length. (Total length in a cell=100, 100 is equal to 52.8μm)

Chromosome No.	Short arm[a)]	Long arm[a)]	Total	Arm ratio[b)]
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	

[a)]The average of 6 prometaphase plates. The length of the satellite was excluded from caluculation.

[b)]Short arm/total length

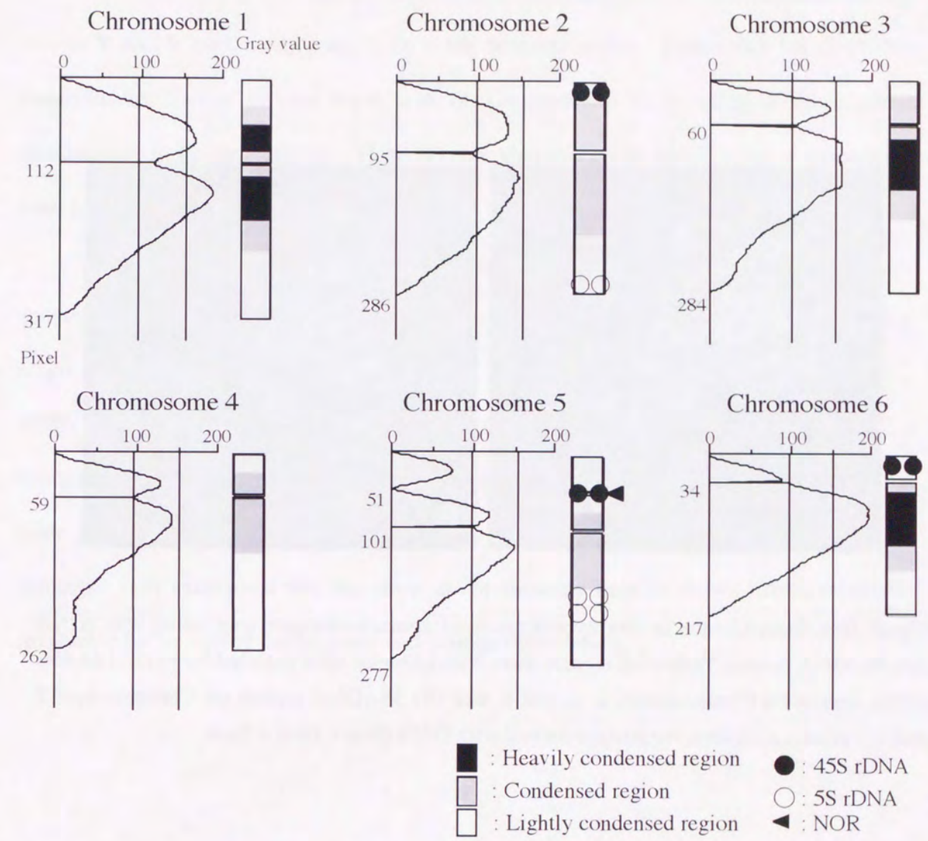

Figure II-3. Density profiles and idiogram of the spinach chromosomes. The vertical and horizontal axes represent the number of pixels along the chromosome and the gray value, respectively. The highly condensed regions are represented by the upper threshold in the panels, corresponding to solid regions 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.

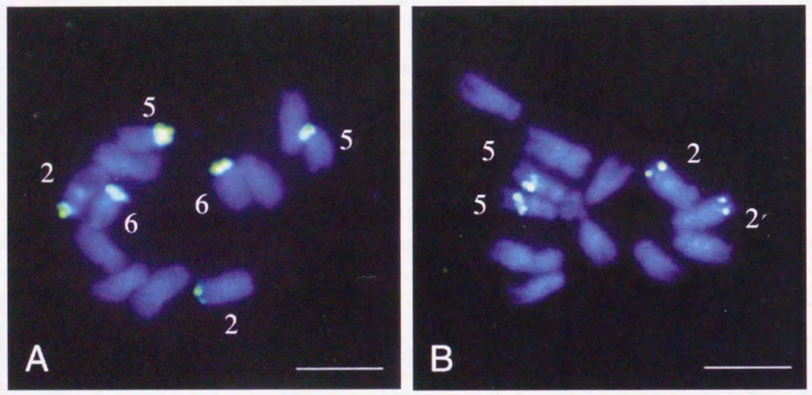

Figure II-4. Fluorescence *in situ* hybridization of spinach chromosomes using 45S rDNA and 5S rDNA probes. Yellowish signals show hybridization sites detected by FITC. (A) 45S rDNA signals on Chromosomes 2, 5, and 6, and (B) 5S rDNA signals on Chromosomes 2 and 5. The chromosomes are counterstained with DAPI (blue). Bars = 5μm.

Ellis and Janick (1960) and Sugiyama and 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* spp. (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.

II-4. Summary

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 3). 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 sub-telomeric 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.

CHAPTER III

Development of an *Arabidopsis thaliana* chromosome map by imaging methods and optical mapping of 45s and 5S rDNAs by Multicolor FISH

III-1. Introduction

Arabidopsis thaliana is a model plant that has been most extensively studied among higher plants. *A. thaliana* has the most detailed genetic maps based on molecular markers such as RFLPs, AFLPs, CAPS (http://genome-www.stanford.edu/Arabidopsis/). Recently, physical maps covering most of the *A. thaliana* chromosomes have been developed by YAC contigs (Zachgo *et al.*, 1996; Kotani *et al.* 1997; Campell *et al.*, 1992; Sato *et al.*, 1998). Sequence analysis of the *A. thaliana* genome has been accelerated (http://www.kazusa.or.jp/arabi/) (Terryn *et al.*, 1998). However, knowledge on the chromosomes is quite limited in contrast to the progress of sequence analyses. The main reason is that the *A. thaliana* chromosomes are small in size (2 μm long on average) at metaphase. As a result, detailed morphological characteristics are difficult to be detected by conventional methods. This fact has impeded the development of a reliable chromosome map of *A. thaliana*, which provides the basis for studies of genetics, genomics and molecular biology.

In the present research, we analyzed quantitatively the *A. thaliana* prometaphase chromosomes using the CHIAS 3. We also developed a quantitative chromosome map of *A. thaliana* based on the numerical data of the condensation patterns. In *A. thaliana*, two 45S (18S-5.8S-25S) and three 5S rDNA loci have visually been detected on the chromosomes by fluorescence *in situ* hybridization (FISH) up to now (Maluszynska and Heslop-Harrison, 1991; Murata *et al.*, 1997; Fransz *et al.*, 1998). We also detected the 45S and 5S rDNAs and accurately mapped the 45S and 5S rDNA loci on the quantitative chromosome map developed by image analysis methods in this study.

III-2. Materials and Methods

Plant materials and chromosome preparation

For the chromosome preparation, *Arabidopsis thaliana* L. ecotype Columbia ($2n$=10) was used. Root tips about 1 cm long were collected from young plants and fixed in Farmer's fluid (ethanol : acetic acid = 3:1) after pretreatment with distilled water at 11℃ for 3 h. Chromosomes were prepared by using the enzymatic maceration and air-drying (EMA) method (Fukui, 1996). The root tips cut 1 mm long were macerated in enzymatic solution (2% Cellulase Onozuka RS, Yakult; 0.5% Pectolyase Y-23, Seishin Co., pH 4.2) for 70 min at 37℃. Then, two to three root tips were placed on a glass slide and tapped with the tip of fine forceps with a few drops of fresh fixative (methanol : acetic acid, 3:1) and then air-dried.

Fluorescence *in situ* hybridization (FISH)

45S rRNA gene (45S rDNA) and 5S rRNA gene (5S rDNA) were used as the probes. FISH was carried out according to the method described by Chapter I. The 45S rDNA was labeled with digoxigenine-11-dUTP (Roche) by nick translation and was detected with Rhodamin and Texas red. 5S rDNA was amplified and labeled with biotin-16-dUTP (Roche) simultaneously by PCR using a 5S rDNA sequence-specific primer set, and was detected by FITC (fluorescein isothiocyanate) (Kamisugi *et al.*, 1994). Chromosomes were counterstained with 1 μg/ml 4',6 diamidino-2-phenylindole (DAPI). Each fluorescent signal was independently observed using a fluorescence microscope (BX60, Olympus) with three different filters (B10, G15 and UV01) and fluorescent images were captured

separately using a cooled CCD camera (PXL1400, Photometrics).

Image analysis of condensation pattern and FISH signals

Chromosomes stained with DAPI were identified by visual inspection under the microscope based on the condensation pattern (Fukui and Iijima 1991, Fukui *et al.* 1998). Numerical data of the density profile (CP) along the mid-rib of each chromatid, which represent the condensation pattern were measured by using CHIAS 3 described in Chapter II. The data for the arm lengths and the CPs were taken from 40 chromosomes in 4 chromosome spreads.

Image analyses of FISH signals by CHIAS 3/FISH version were as follows: digitally captured FISH signals and chromosome images were normalized to enhance the contrast of the original image. The CPs of the FISH signals and chromosomes themselves were independently measured. The lengths of the CPs were averaged among eight homologous chromosomes (16 chromatids). The profiles of the signal intensity after FISH were also averaged and adjusted the length to the chromosomal CPs. The averaged signal profile after FISH was superimposed on the CPs to localize the FISH signal precisely on the chromosomes.

III-3. Results and Discussion

Condensation pattern on prometaphase chromosomes in *A. thaliana*

Staining by fluorochromes enables to visualize the morphology of *A. thaliana* chromosomes at the prometaphase in detail. According to a common procedure using DAPI Figure III-1a shows 10 metaphase chromosomes evenly stained. Because the individual chromosome was small and similar in morphology, it was difficult to distinguish them from each other. In contrast to the metaphase chromosomes, prometaphase chromosomes showed an uneven condensation along the chromosome, which was depicted by intensely (condensed), and faintly stained (dispersed) regions (Fig. III-1b). Variations in the condensation patterns such as degrees and regions were recognized among the chromosomes. Based on the characteristic condensation pattern to each homologous chromosome, all the chromosomes were identifiable and the chromosome number was designated in accordance with the numbers of the linkage map (Murata *et al.*, 1997; Koornneef *et al.*, 1983).

Thus in this study, the uneven condensation of each chromosome was quantified by using CHIAS 3. The CPs of DAPI-stained chromosomes were shown in Figure III- 2. The CPs indicated that each of the five chromosomes displayed its own characteristics. The threshold (Gray value = 100) in the profiles corresponds to the visual boundary between intensely (condensed) and faintly stained (dispersed) regions. The condensed regions with the higher gray values are depicted as the gray regions in the idiogram.

Physical mapping of 45S and 5S rDNA loci on *A. thaliana* chromosomes

FISH signals on the chromosomes were shown in Figure III-1c and 1d. 45S

rDNA sites were detected as red signals on the short arms of Chromosomes 2 and 4. Signals from 45S rDNA on Chromosome 2 were stronger than those on Chromosome 4, which was consistent with the results obtained by Murata *et al.* (1997). 45S rDNA loci on Chromosomes 2 and 4 are estimated approximately as 3.5 and 4.0 Mb by the RFLP analysis, respectively (Copenhaver and Pikaard, 1996a). The visual difference in the signal intensity between the two 45S rDNA loci did not necessarily reflect the difference in the copy number of 45S rDNA. It may be caused by the differences in hybridization efficiency, due to the difference in the condensation degree of the chromatin fibers between these two NOR regions. Thus it was likely that the 45S rDNA on Chromosome 2 condenses more slowly than that on Chromosome 4 in *A. thaliana*. On the other hand, at the interphase, almost similar signal intensity between Chromosomes 2 and 4 was detected, as described by Maluszynska and Heslop-Harrison (1991), because the chromatin structure in interphase nuclei was more relaxed to the similar levels for both the 45S rDNA regions than in the prometaphase.

Six signals from the three 5S rDNA loci were detected on the short arms of Chromosomes 3, 4 and 5 (Fig. III-1d). The 5S rDNA signals on Chromosome 5 were slightly stronger than those at the other sites. The 5S rDNA located on the short arm close to the centromere of Chromosome 4. However, the molecular map localized the 5S rDNA, has been placed on the long arm close to the centromere (Schmidt *et al.*, 1995). The reason for this discrepancy between our FISH results and YAC contigs might be due to the difficulty in the determination of the centromere by the presence of repeat sequences around the centromeric region. Our FISH results were consistent with the previous FISH results using mitotic chromosomes (Murata *et al.*, 1997) and pachytene chromosomes (Fransz *et al.*, 2000). One 5S rDNA locus is closely localized to the 45S rDNA locus on the short arm of

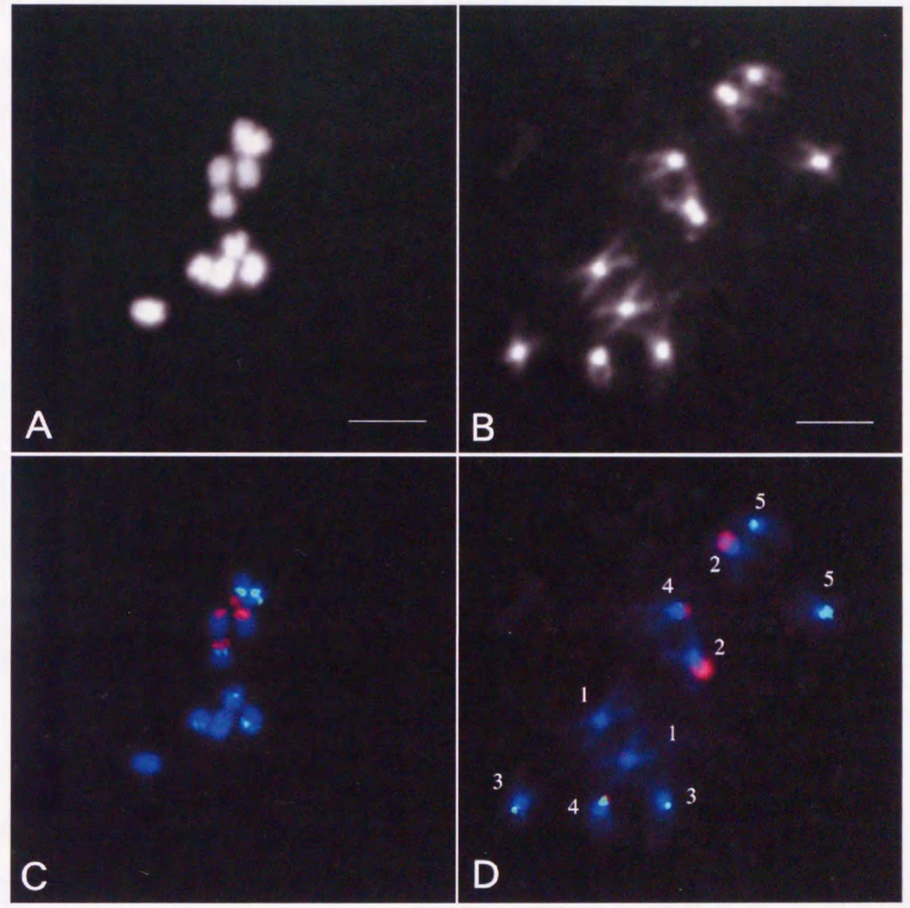

Figure III-1. *A. thaliana* chromosomes at metaphase (A,C) and prometaphase (B,D). DAPI stained chromosomes for CP analysis (A,B). Multiprobe FISH with the 45S (red) and 5S (green) rDNA probes (C,D). 45S rDNA signals locate on chromosomes 2 and 4, and 5S rDNA signals on chromosomes 3, 4 and 5. Chromosomes were stained with DAPI (blue). Scale bar shows 5μm.

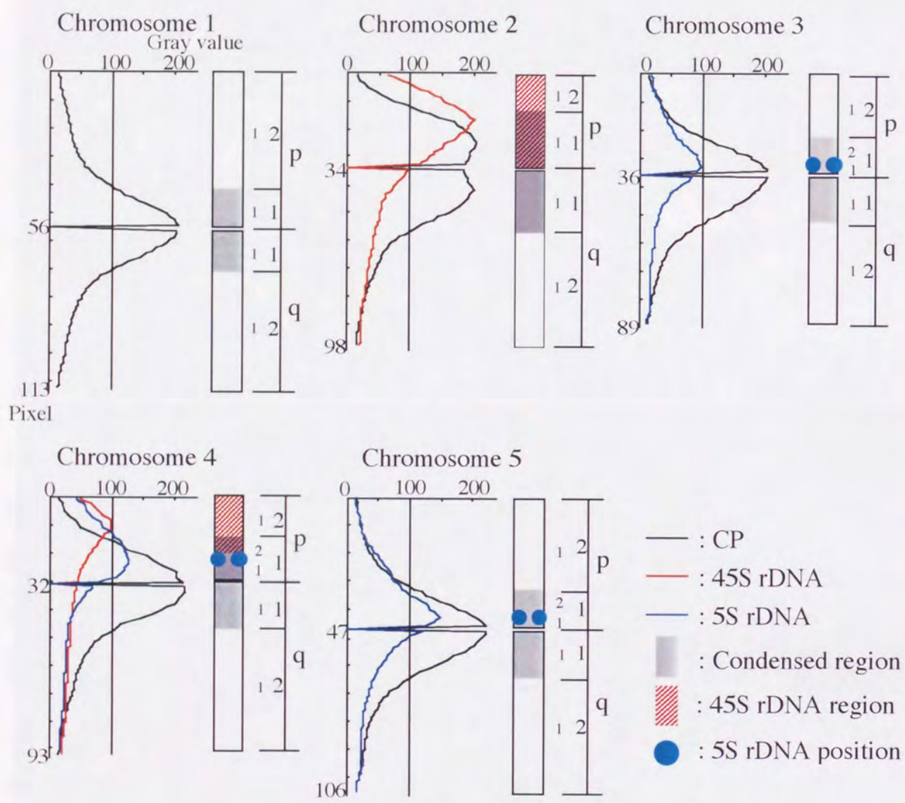

Figure III-2. CPs and density profiles for FISH signals, and idiograms with the addresses of the *A. thaliana* chromosomes. The vertical and horizontal axes represent the number of pixels along the chromosomes and the gray value, respectively. The condensed regions indicated by the threshold (Gray value = 100) are represented by gray regions in the idiogram. 45S and 5S rDNA loci are designated by the red regions and solid blue circles on the idiogram, respectively. p and q indicate short and long arms, respectively. The 5S rDNA loci are included to the address p1.1 of chromosome 3, 4 and 5.

Chromosome 4. Close proximity of 45S and 5S rDNAs is not common among plant species but have also been reported in *Brassica napus* (Kamisugi *et al.*, 1998), *B. rapa* (Fukui *et al.*, 1998), *Oryza officinalis* and *O. eichingeri* (Shishido *et al.*, 2000).

The precise mapping of rDNA loci on the defined chromosomal regions was performed using the CHIAS 3/FISH version. The CPs and signal profiles of the rDNAs after FISH were shown in Figure III-2. The averaged profile of rDNAs signals clearly indicated that the most probable locations on Chromosome 2, 3, 4 and 5. The locations of the two 45S and three 5S rDNA loci were depicted on the idiogram.

FISH signals of 45S rDNAs on the Chromosomes 2 and 4 covered extended regions reaching the end of the short arms. Each unit sequence of 45S rDNA was ca. 10 kb long and the repeat occurred in long tandem arrays occupying 8% of the genome (Pruitt and Meyerowitz, 1986; Copenhaver and Pikaard, 1996a). Molecular evidence revealed by RFLPs of 45S rDNA using pulsed field gels electrophoresis indicated that the rDNAs reach the ends of short arm for both Chromosomes 2 and 4, adjoining the telomere sequences (Copenhaver and Pikaard, 1996b). Thus, the 45S rDNA loci on the idiogram were not depicted by dots like 5S rDNA loci, but by the regions covering from the end of short arms to the peaks of the signals and the same extension towards the centromere. 5S rDNA is organized in tandem arrays of ca. 500 bp unit length and contained approximately 1000 copies per haploid genome in *A. thaliana* (Campell *et al.*, 1992). Thus 5S rDNA loci were indicated by the dots on the idiogram.

Characterization of each *A. thaliana* chromosome

Table III-1 shows the morphological data and the positions of rDNA loci, which are averaged for eight prometaphase chromosomes. The total length of the haploid

Table III-1. Numerical data of somatic chromosomes of *A. thaliana*.

Chr	Short arm		Long arm		Arm ratio[a]	Total length			45S rDNA	5S rDNA
No.	length (μm)	S.D.	length (μm)	S.D.		length (μm	S.D.	Relative length (%)	region (μm)[b]	position (μm)[b]
1	1.83	0.18	1.90	0.29	1.04	3.73	0.42	22.68		
2	1.10	0.11	2.15	0.28	1.96	3.24	0.36	19.69	0 - 1.10	
3	1.16	0.13	1.76	0.36	1.51	2.92	0.46	17.74		1.00
4	1.02	0.09	2.04	0.42	1.99	3.06	0.50	18.57	0 - 0.65	0.76
5	1.54	0.30	1.96	0.32	1.27	3.51	0.61	21.32		1.36

S.D.: standard deviation

[a]Long arm length / Short arm length.

[b]Signal positions from the short arm end.

prometaphase chromosomes is 16.5 μm, which was about 1.8 times the size of the metaphase chromosomes. The characters of each chromosome and locations of the rDNA loci are summarized as follows: Chromosome 1 is a metacentric and the longest chromosome. The chromosome shows condensation only in the proximal regions of the centromere. Chromosome 2 is a submetacentric chromosome with a 45S rDNA locus in the telomeric region of the short arm. It has longer condensed region than Chromosome 4, which is a key character to identify Chromosome 2 from Chromosome 4. Chromosome 3 is a metacentric and the shortest chromosomes. Chromosome 3 has 5S rDNA locus at the proximal region on the short arm. Chromosome 4 is a submetacentric chromosome with both 45S and 5S rDNA loci on the short arm. Chromosome 5 is a metacentric chromosome with a 5S rDNA locus close to the centromere on the short arm.

The present study enables to develop the chromosome map of *A. thaliana* by using imaging method based on the condensation patterns. It is possible to visualize not only rDNA loci but also single copy genes or low-copy sequences on the *A. thaliana* chromosomes by FISH as already shown in *Brassica* (Kamisugi *et al.*, 1998) and rice (Ohmido *et al.*, 1998). The quantitative chromosome map is useful for mapping the DNA sequences visually detected. It is concluded that the quantitative chromosome map developed in the present study is useful not only for cytological studies but also for genomics and molecular biology of *A. thaliana*.

III-4. Summary

A. thaliana L. has 10 chromosomes that are small and similar in morphology. Molecular cytogenetics and image analyses were applied to characterize the somatic chromosomes. Prometaphase chromosomes were quantitatively analyzed based on their patterns of condensation, because they show a prominent uneven condensation along the chromosomes. The total length of the haploid prometaphase chromosomes is 16.5 μm, which is about 1.8 times the size of the metaphase chromosomes. We also detected the 45S (18S-5.8S-25S) and 5S rDNA loci by fluorescence *in situ* hybridization (FISH) and determined their locations on the chromosomes by the imaging methods. As a result, a quantitative chromosome map based on the condensation pattern was developed for the first time in *A. thaliana* with the locations of rDNAs by using imaging methods. It is now possible to localize genes precisely on this map.

CHAPTER IV

Development of *Lotus* chromosome maps and determination of their genome sizes

IV-1. Introduction

Legumes are the main sources of plant protein both for man and domestic animals. Legumes are also important due to their ability to fix nitrogen by symbiosis with rhizobia. The small genome size, short life cycle, diploidy, self-fertility and availability of transformation make *Lotus japonicus* a suitable plant species for a genome project such as *Arabidopsis thaliana* and *Oryza sativa* L. *L. japonicus* has been used as a model plant species (Handgerg and Stougaard 1992).

The genus, *Lotus*, Fabaceae, consists of more than one hundred species of diploids ($2n$=10, 12, 14), tetraploids ($2n$=24, 28) and hexaploid ($2n$=36) species. The basic chromosome numbers were reported as x=6 and x=7 (Fernandes and Queiros, 1978; Ortega, 1986; O'Donoughue *et al.*, 1990; Sammour *et al.*, 1991; Small *et al.*, 1984). *L. japonicus* (Regal) K. Larsen (= *L. corniculatus* L. var. *japonicus* Regal) is a diploid species ($2n$=12) (Kawakami, 1930; Kodama, 1989; O'Donoughue *et al.*, 1990; Yeh *et al.*, 1986), while *L. corniculatus* L., a morphologically similar species, includes diploid ($2n$=12), tetraploid ($2n$=24) (Small *et al.*, 1984) and hexaploid ($2n$=36) (Baltisberger, 1990).

Lotus polymorphism has been studied using isozyme analyses (Raelson and Grant, 1989; Raelson *et al.*, 1989). The RAPD patterns were analyzed (Campos *et al.*, 1994), and restriction site variations of chloroplast DNA were reported (Gauthier *et al.* 1997). Jiang and Gresshoff (1997) identified molecular polymorphism between the two accessions Gifu B129-S9 and Funakura B581 of *L. japonicus* using a DNA amplification fingerprinting technique with a single arbitrary primer.

The identification of *L. japonicus* chromosomes at somatic metaphase under a

microscope has been difficult because of their small size and morphological similarity. Quantitative chromosome maps were developed for these species based on digital image analyses of their condensation patterns. The condensation pattern is now considered a key character to identify and characterize small plant chromosomes and facilitates the construction of a chromosome map (Ito *et al.*, 2000a; Ito *et al.* 2000b; Fukui and Ohmido, 2000; Fukui *et al.*, 2000). Physical mapping of 45S and 5S rDNAs was also performed by fluorescence *in situ* hybridization (FISH) method to localize these genes on the two accessions of *L. japonicus* to confirm the chromosome identification based on the condensation patterns. Moreover, a mixture of c.a. 500 cDNA clones were labeled and detected on *L. japonicus* chromosomes by the FISH method to determine the distribution of functional genes on the *Lotus* chromosomes.

In this thesis, we report identification of the chromosomes from two accessions of *L. japonicus*, Miyakojima and Gifu. Their genome sizes were also determined by flow cytometry.

IV -2. Materials and Methods

Plant materials and flow cytometry

Two accessions of *L. japonicus*, Miyakojima MG-20 and Gifu B-129, were used. These materials were kindly provided by Prof. Masayoshi Kawaguchi (University of Tokyo) and Prof. Makoto Hayashi (Osaka University). The nuclear DNA content was measured by flow cytometry according to the method described by Arumuganathan and Earle (1991) and Uozu *et al.* (1997) with minor modifications. *L. japonicus* nuclei were isolated from the young leaves from 2-month-old plants by chopping the leaf tissues and filtration (30 μm pore size). Nuclear samples were stained with 0.1mg/ml of propidium iodide (PI) for 60 min. Each sample was analyzed using a flow cytometer (FACS Calibur, Becton Dickinson) equipped with an air-cooled argon-ion laser tube with an emission wavelength of 488nm. The fluorescent signals of PI were measured after passing through a 585nm bandpass filter. Isolates of each material were analyzed using at least 500 nuclei in triplicate. The nuclei from leaves of *Arabidopsis* ecotype Columbia prepared by the same method were used as an internal standard. The value 260Mb/2C (The Arabidopsis Information Resource: http://www.arabidopsis.org/) was adopted as the nuclear DNA content of *A. thaliana*.

Chromosome preparation

Seeds of Miyakojima and Gifu were scarified by stirring in 95% sulfuric acid for 10 min and washed by tap water and then put on 0.7% agarose after sterilization. They were germinated at 26°C in the dark for 3 days. Root tips about 3 cm in length were collected from germinated seedlings and fixed in Farmer's fluid (ethanol : acetic acid = 3:1) without

any pretreatment. Chromosomes were prepared using an enzymatic maceration and air-drying (EMA) method (Fukui, 1996) with a few modifications. The root tips were cut into 1 mm length, and macerated in the enzymatic mixture (2% Cellulase Onozuka RS, Yakult; 0.5% Pectolyase Y-23, Seishin Pharmaceutical Co., pH 4.2) for 40 min at 37°C. Then, the root tips were placed on glass slides and exposed to steam over a water bath at 76°C for 2 sec, tapped with fine forceps with a few drops of a new fixative (methanol : acetic acid = 3:1), exposed again to steam, and air-dried on a hot plate at 60°C.

Cloning and nucleotide sequencing of 5S rRNA gene (5S rDNA)

To amplify the DNA fragments containing 5S rDNA, PCR was carried out using KOD polymerase (Toyobo, Japan) and a pair of primers, 5'-GATCGCATCCGTAAAGTTATGCGGG-3', 5'-CGATCATACCAGCACTAATGCACCG-3', which hybridized to the center of the 5S rDNA coding sequence in the opposite orientation. The amplified fragments were ligated into the *Hinc*II site of pUC18 and transformed into *E. coli* (JM109). The inserts of the cloned DNA were sequenced using an ABI Prism 310 Genetic Analyzer (Perkin Elmer Co., USA).

Image analysis of condensation pattern.

Lotus chromosomes were stained with a 2% Giemsa solution (Merck) with 1/15M sodium phosphate buffer (pH 7.0) for 5 min. Well spread metaphase and prometaphase chromosome plates were selected under a microscope (BX50, Olympus). Each of 6 pairs of *Lotus* chromosomes at prometaphase were identified based on the condensation pattern (Fukui and Iijima, 1991; Iijima *et al.*, 1991) and photographed on black and white film (Neopan F, ISO 32, Fuji).

Analysis of the condensation patterns of Giemsa-stained chromosomes was performed using CHIAS 3 described in Chapter II. Eighty-four chromosomes from seven prometaphase plates of Gifu and thirty-three chromosomes from three prometaphase plates of Miyakojima were analyzed.

Fluorescence *in situ* hybridization (FISH)

45S rDNA from *Oryza sativa*, 5S rDNA from *L. japonicus* and c. a. 500 mixed cDNA clones from *L. japonicus* were physically mapped by FISH. The 500 cDNA clones from 1 to 2 kbp in size contained non-redundant and non-retrotransposon clones based on the database of 5' end expressed sequence tags (ESTs) from cDNA libraries constructed from young (2-week-old) *L. japonicus* plants (Asamizu, *et al.* 2000). FISH was carried out according to the method described in Chapter II. Briefly, 45S rDNA and cDNAs were labeled with biotin-16-dUTP (Roche) by the nick translation method and were detected with fluorescein isothiocyanate (FITC) as green fluorescence signals. 5S rDNA was simultaneously amplified and labeled with digoxigenin-11-dUTP (Roche) by PCR using the 5S rDNA sequence-specific primer set, and was detected with Texas red as red fluorescence signals. Chromosomes were counterstained with 1μg/ml 4', 6 diamidino-2-phenylindole (DAPI). Each fluorescent signal was independently observed using a fluorescence microscope (Axio Plan 2, Zeiss) with three different filter sets (01, 17, 15). The images of green, red and blue fluorescent signals were captured separately using a cooled CCD camera (MicroMax, Photometrics), and were merged using Photoshop (ver.5, Adobe).

IV -3. Results and Discussion

Determination of the genome size.

Table 1 shows the nuclear DNA content of the two accessions of *L. japonicus* determined by flow cytometry. The genome sizes of Miyakojima and Gifu were calculated as 472.1±3.98 and 442.8±2.32 Mbp (mean ± standard error), respectively. The genome size (1C) of Miyakojima was larger than that of Gifu by 30 Mbp. This difference was statistically significant (p=0.01).

Condensation pattern of the prometaphase chromosomes.

Figures IV-1A and 1B show the metaphase chromosomes of Miyakojima and Gifu, respectively. Both Miyakojima and Gifu had 6 pairs of chromosomes in each somatic cell. Chromosomes at the metaphase stage were stained evenly with the Giemsa solution indicating almost uniform condensation. The small size and morphological similarity made it difficult to distinguish them from each other. Prometaphase chromosomes of Miyakojima (Fig. IV-1C) and Gifu (Fig. IV-1D) appearing in the same magnification showed a prominent uneven condensation along the chromosomes. Condensed regions of the chromosomes were intensely stained, and dispersed regions were faintly stained. Small condensations were observed at the telomeric regions of Miyakojima chromosomes (Arrows, Fig. IV-1C), while no such condensations was observed at the telomeric regions of Gifu chromosomes (Fig. IV-1D).

Six pairs of prometaphase chromosomes in *L. japonicus* can be identified based on the chromosome specific condensation patterns. Three Miyakojima and seven Gifu

Table IV-1. Nuclear DNA contents and genome size of *L. japonicus* measured by flow cytometry[a]

Accessions	Nuclear DNA content (pg ± S.E.[b])	Genome size (Mbp ± S.E.[b])
Miyakojima	0.4891 ± 0.00412	472.1 ± 3.98
Gifu	0.4588 ± 0.00240	442.8 ± 2.32

[a] DNA contents of *L. japonicus* were calculated based on *Arabidopsis* genome size (130Mbp).
[b] standard error

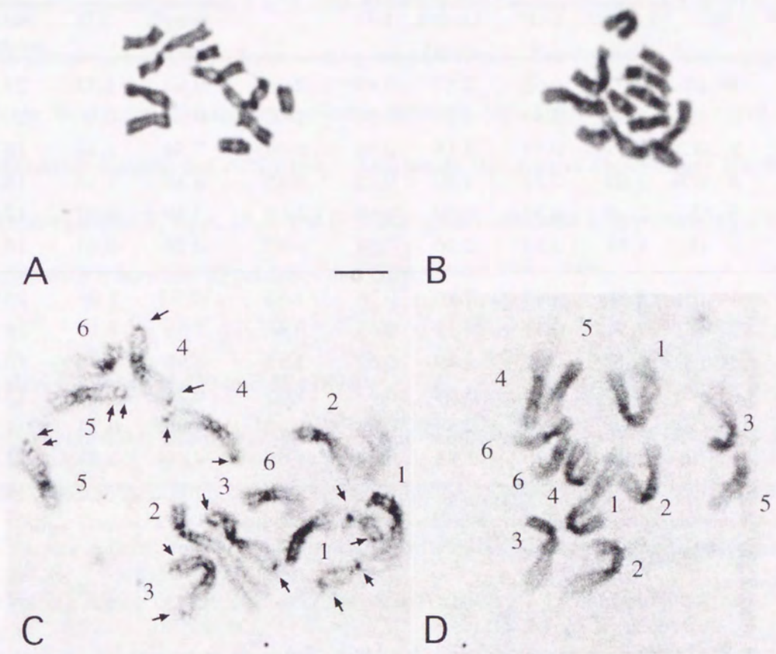

Figure IV-1. Giemsa-stained chromosomes of *L. japonicus*. (A) Metaphase plate of Miyakojima. (B) Metaphase plate of Gifu. (C) Prometaphase plate of Miyakojima. (D) Prometaphase plate of Gifu. Arrows show Miyakojima specific faint, unstable and small condensations (FUSCs). Numbers of 1 to 6 beside the chromosomes indicate the chromosome numbers designated in the present paper according to their length order. Bars = 5μm.

Table IV-2. Numerical data of somatic chromosomes of *L. Japonicus*.

Accessions	Chr. No.[a]	Short arm Length (μm)	Short arm S.D.[c]	Long arm Length (μm)	Long arm S.D.	Arm ratio[b]	Total length Length (μm)	Total length S.D.	Total length Relative length (%)
Miyakojima	1 (A)	2.07	0.63	7.57	0.89	2.75	9.64	1.32	23.29
	2 (B)	1.83	0.46	6.53	0.91	2.67	8.36	0.96	20.19
	3 (C)	3.35	0.52	4.18	0.80	0.94	7.54	1.16	18.21
	4 (D)	3.00	0.54	3.39	0.72	0.85	6.38	1.10	15.42
	5 (E)	2.19	0.38	3.00	0.68	1.03	5.19	0.97	12.54
	6 (F)	1.93	0.35	2.36	0.34	0.92	4.29	0.61	10.36
Gifu	1 (B)	3.51	0.47	7.26	0.76	1.55	10.77	1.06	25.95
	2 (C)	3.58	0.51	4.11	0.65	0.86	7.69	1.10	18.52
	3 (A)	2.49	0.28	4.49	0.62	1.35	6.98	0.80	16.80
	4 (D)	2.60	0.43	3.64	0.47	1.05	6.24	0.75	15.03
	5 (E)	2.12	0.34	2.84	0.56	1.00	4.96	0.74	11.93
	6 (F)	2.04	0.25	2.85	0.44	1.05	4.89	0.53	11.77

[a] The letters A-F indicate the corresponding chromosomes between Miyakojima and Gifu.
[b] Long arm / Short arm
[c] Standard deviation

chromosomal plates at mitotic similar stage with in the prometaphase stage were statistically selected and lengths of their chromosomal arms were measured using CHIAS 3. Table IV-2 shows the numerical data of the chromosomes of Miyakojima and Gifu. Chromosome numbers from 1 to 6 were designated in the decreasing order of relative length of Miyakojima and Gifu chromosomes, respectively. The letters A to F were used to indicate the corresponding chromosomes between Miyakojima and Gifu based on their condensation patterns and rDNA loci. As a result, the order of chromosome length in Gifu was chromosome B, C, A, D, E and F. Our results indicated a large difference in size of chromosome A between Miyakojima and Gifu.

Physical mapping of 45S and 5S rDNAs

Figure IV-2 shows 312 bp nucleotide sequences of 5S rDNA of Gifu. Boldface letters indicate the 120bp coding region of the 5S rRNA gene. The 5S rDNA sequence was the same between to Miyakojima and Gifu in both the coding and spacer sequences. The coding region of 5S rDNA of *L. japonicus* shows 97.4% homology to that of *Glycine max*, and 97.5% to that of *O. sativa*. The levels of sequence homology to *G. max* and *O. sativa* in the spacer regions are quite low.

Figures IV-3A and 3B show the positions of 45S and 5S rDNA loci on Miyakojima and Gifu chromosomes detected by FISH. The 45S rDNA loci were detected as green signals at the end of short arms of chromosome A (solid arrows) and the proximal region on the short arm of chromosome F (solid arrows). The fluorescent signal on chromosome A was usually larger than that on chromosome F. The 5S rDNA locus was detected as red signals on the proximal regions on the short arms of chromosome A (solid arrowheads). Thus, chromosome A has both the 45S and 5S rDNA loci on the short arm. The location

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  1 GGATGCGATC ATACCAGCAC TAATGCACCG GATCCCATCA GAACTCCGCA  50
 51 GTTAAGTGTG CTTGGGCGAG AGTAGTACTA GGATGGGTGA CCTCCTGGGA 100
101 AGTCCTCGTG TTGCATCCCC TTTTGCCGTT TTTTCGCAAT TCCTTATTTT 150
151 TTTTTTAATT TCTTAGAAGC CTCGGATGAA TACGAAGTGA ACTACAAAGT 200
201 TGAAGACAAA AATAAAGTAA TTGCACGCTT GTTTGCCGTT TTTTCGCAAT 250
251 TCCTTATTTT TTTTTAATTT CTTAGTTTAA CGCATATTAT GTTTTAACCC 300
301 GCATAACTTT AC 312
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Figure IV-2. Nucleotide sequences of a unit sequence of 5S rDNA. Boldface letters indicate the 5S rRNA coding sequences. The sequence data have been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB046116.

and number of 45S rDNA loci and 5S rDNA locus were the same in the two accessions.

Figure IV-3C shows the merged image of signals and chromosomes of *L. japonicus* chromosomes after FISH using 500 cDNA clones. Their signals were dispersed over all chromosome regions, with a tendency to accumulate on the terminal region rather than the centromeric and proximal regions of the chromosomes.

Quantitative chromosome maps of Miyakojima and Gifu

Figures IV-4A and 4B show quantitative chromosome maps based on the average density profiles (CPs), *i.e.*, standardized digital data of the condensation patterns (stCP) of Miyakojima and Gifu chromosomes, respectively. The visual boundaries between condensed and dispersed regions correspond to the border between gray and white bars on the chromosome maps. The condensed regions are divided into heavily condensed and condensed regions, which are presented by the black and gray bars on the chromosome maps, respectively. The gray value that delimits between heavily condensed and condensed regions was 83 and that between condensed and dispersed regions was 148 in the case of Miyakojima. The corresponding gray values of Gifu were 58 and 131, respectively. The 45S rDNA loci are indicated as double open circles and the 5S rDNA locus is shown by single open circles on the chromosome maps. Faint, unstable and small condensations (FUSCs) were only observed mainly at the terminal regions of Miyakojima. To differentiate FUSCs from the larger condensed regions constantly appearing in the proximal regions, small solid circles were placed on the chromosome map of Miyakojima.

Characteristics of each *L. japonicus* chromosome based on the chromosome maps (Fig. IV-4) are as follows. The condensation patterns and locations of rDNAs are shown diagrammatically in Figure IV-4, and the numerical data are presented in Table IV-2.

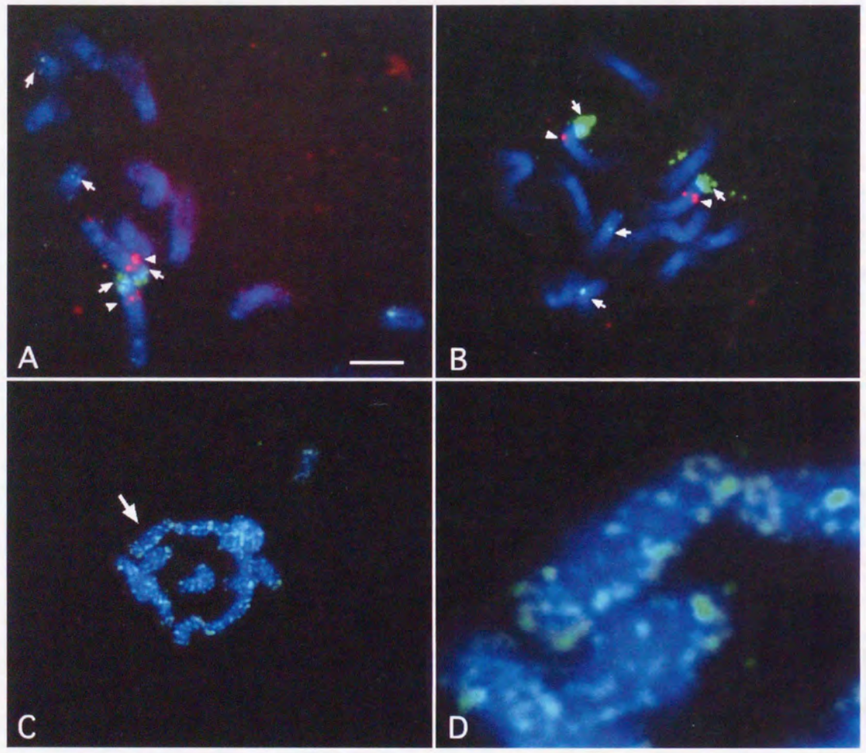

Figure IV-3. Detection of rDNAs and genes on the *Lotus* chromosomes by FISH. (A) 45S rDNA (arrow) and 5S rDNA (arrowhead) were detected in Miyakojima and (B) Gifu. Green fluorescent signals on chromosomes A and F show the 45S rDNA loci. Red fluorescent signals on chromosome A show the 5S rDNA locus. (C) Merged image of chromosomes and signals obtained with 500 cDNA clones as probes. (D) Enlarged image of part of the chromosome with signals indicated by the arrow in Fig. 3C. Bars = 5μm.

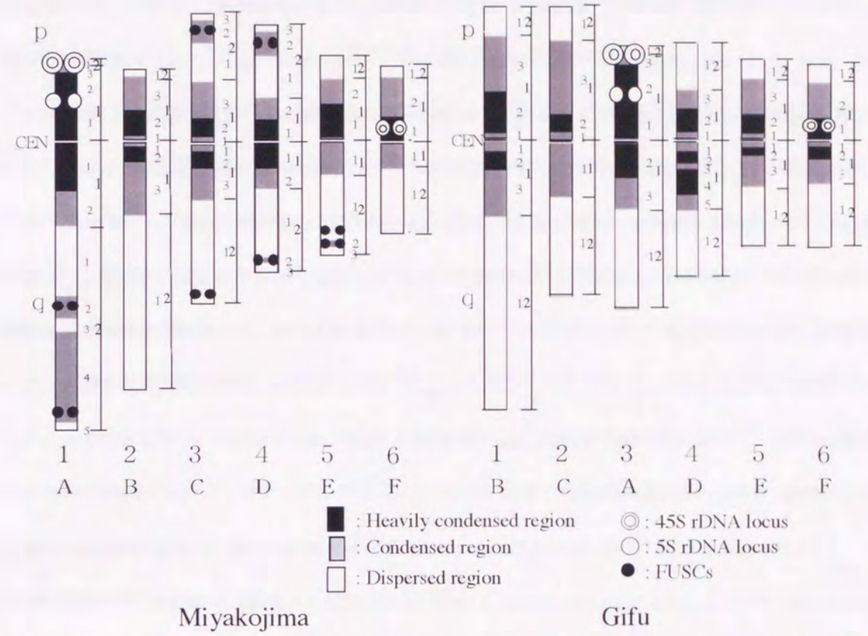

Figure IV-4. Quantitative chromosome maps with the addresses (Miyakojima and Gifu) based on the stCPs. CEN, p, q show centromeric position, short arm and long arm, respectively. Numbers 1 to 6 are the chromosome numbers based on their lengths, and A to F indicate corresponding chromosomes between the two accessions. Gray bars represent the condensed chromosomal regions, while solid bars represent the heavily condensed regions. The open bars correspond to dispersed regions of the chromosomes. The positions of 45S, 5S rDNA loci, and faint, unstable, small condensations (FUSCs) are shown by double, single open and solid circles on the maps, respectively.

Chromosome A: Miyakojima Chromosome 1 was the longest subtelocentric chromosome. It had heavily condensed regions throughout almost the whole region of the short arm and the proximal region of the long arm. Gifu Chromosome 3 was submetacentric, and had heavily condensed regions on both arms. Although the pattern of the condensed regions was similar to Miyakojima Chromosome 1, the distal regions of the long arm were quite different between Miyakojima and Gifu Chromosome A. Miyakojima Chromosome 1 had two more condensed regions at the distal and telomeric regions of the long arm, whereas Gifu Chromosome 3 had no such extension of the chromosomal long arm. The distal part of the long arm might have been lost during phylogenetic differentiation. Both the 45S rDNA and 5S rDNA loci were located on the short arm of Chromosome A in both accessions.

Chromosome B: Miyakojima Chromosome 2 was the second largest subtelocentric chromosome, while Gifu Chromosome 1 was the largest submetacentric chromosome among the 6 chromosomes. Condensed regions were distributed along most parts of the short arms of the two accessions. The proximal regions of the long arms were heavily condensed. The heavily condensed regions on the short arms are larger than those on the long arms.

Chromosome C: Miyakojima Chromosome 3 and Gifu Chromosome 2 were metacentric, and the sizes of these chromosomes were similar between the two accessions, although Miyakojima Chromosome 3 had a faint, unstable, and small condensation (FUSC) at the termini of both arms. Heavily condensed regions were also different from each other. Miyakojima Chromosome 3 had heavily condensed regions on both short and long arms, whereas Gifu Chromosome 2 had only one heavily condensed region on the short arm.

Chromosome D: Miyakojima and Gifu Chromosome 4 were metacentric for both the accessions. Condensed regions were located at the proximal regions and, in the case of Miyakojima FUSCs were located on the terminal parts of both arms.

Chromosomes E and F: Over all morphology, Chromosomes E and F were similar to each other. Both Chromosomes E and F were small-sized metacentric chromosomes. Proximal heavily condensed regions on the short arms of both Chromosomes E and F were larger than those on the long arms in both accessions. The heavily condensed regions were located at the proximal region of the short arms only in Miyakojima Chromosomes 5 and 6 and the proximal regions of both arms in Gifu. A small terminal condensed region and two FUSCs were observed on the long arm of Miyakojima Chromosome 5. A small 45S rDNA locus was detected on the short arm of Chromosome F in both accessions.

Differentiation between Miyakojima and Gifu

The DNA content per haploid genome of *L. japonicus* Gifu was reported to be 0.5 pg as determined by microdensitometry (482.5 Mbp) (Cheng and Grant 1973, Sz.-Borsos 1973, Bennett and Smith 1976). The estimated genome size is similar to that of the rice genome (Arumuganathan and Erale 1991). In this study, different genome sizes were obtained for the two accessions, Miyakojima (472.1 Mbp) and Gifu (442.8 Mbp). The 29.7 Mbp larger genome size of Miyakojima could be accounted for by an increase in heterochromatic regions only in Miyakojima. Heterochromatic regions are often clusters of tandem repeated sequences as shown in the case of Tandem repeat sequence A (TrsA) of *Oryza sativa* (Ohtsubo and Ohtsubo, 1994). The TrsA is similar to morphologically the terminal condensation of Miyakojima (Ohmido and Fukui 1997). Ohmido *et al.* (2000) compared two rice types, Indica and Japonica, and found that Indica rice has 6 TrsA sites at the end of

12 chromosomes, while Japonica rice has 2 TrsA sites. In addition, the genome of Indica rice is a larger by 37 Mbp due to the accumulation of other repeated sequences including TrsA (Ohmido *et al.* 2000). The observation that tropical or subtropical Indica rice has a larger number of TrsA than the temperate Japonica rice is consistent with the results obtained, *i.e.* geographical distribution *Lotus* species and the number of the FUSCs and genome sizes.

Cheng and Grant (1973) reported the karyotype of *L. japonicus* as five pairs of submetacentric and one pair of metacentric chromosomes. Although intra-specific variation of the karyotypes among *Lotus* species was not reported in metaphase chromosomes, different features of *Lotus* chromosomes were revealed, when the prometaphase plates of the two accessions were compared. For example, Chromosomes 1, 3, 4 and 5 of Miyakojima carry FUSCs close to their telomeric regions. The FUSCs could be observed when Miyakojima chromosomes were only at the prometaphase stage. No FUSCs were observed in Gifu chromosomes. Thus, we concluded that the prometaphase chromosomes are suitable for analysis of minute morphological characteristics among the *Lotus* chromosomes. FISH results using rDNAs as probes further supported the identification by condensation patterns. Especially, Chromosomes A and F could be identified as Miyakojima Chromosome 1 and Gifu Chromosome 3, although the simple chromosome lengths were quite different from each other. FISH was effective to discriminate between Chromosomes E and F, because Chromosome F has one small 45S rDNA locus at the proximal region on the short arm.

In the present study, two kinds of rDNA loci were located at similar positions on the same chromosomes of the two accessions, although the positions and number of rDNA loci have been reported to be quite variable even among phylogenetically closely related species

(Shishido *et al.* 2000). The similar rDNA locations represent further evidence that the *Lotus* genes are located at similar positions on chromosomes between the two accessions.

Localization of 500 cDNA on chromosomes

The FISH signals using 500 DNA clones as probes were dispersed on all chromosomal regions except for the centromeric regions (Fig. IV-C,D). They tended to be concentrated as small signal clusters in dispersed regions of the chromosome, which corresponded to the regions shown by white bars on the chromosome maps. Although further studies are necessary, these observations suggested that the expressed genes locate on late condensed regions on the *Lotus* chromosomes similarly to the barley chromosomes (Künzel *et al.* 2000).

The results obtained in this study provide useful information for genome analysis of *L. japonicus*.

IV -4. Summary

Genome and chromosome dimensions were determined using two accessions of *Lotus japonicus*, Miyakojima MG-20 and Gifu B-129. The genome sizes of Miyakojima and Gifu were determined as 472.1 and 442.8 Mbp, respectively. Both the accessions were diploid ($2n$=12) and six chromosomes were identified and characterized based on the condensation patterns and the locations of rDNA loci. The obvious polymorphism observed in the genome size and the chromosome morphology between the two accessions, revealed specific accumulation of heterochromatin in Miyakojima or elimination in Gifu. The chromosomes *L. japonicus* were numbered according to their length. Quantitative chromosome map of two accessions were also developed by the imaging methods using the digital data of the condensation pattern. 45S rDNA loci were localized on Chromosomes A and F, and 5S rDNA locus was localized on Chromosome A by fluorescence *in situ* hybridization (FISH). Identification of the chromosome and genome sizes and development of the quantitative chromosome map represent significant contribution to the *L. japonicus* genome project as the basic information.

CHAPTER V

Conclusion

This study has been carried out to research plant genomes using the advanced methods of image analysis and molecular cytology. Cytological chromosome maps of three plants, *Arabidopsis thaliana, Lotus japonicus* and spinach (*Spinacia oleracea*) have been developed, as there are no chromosome maps developed for these plants, although they are different in status in their genome analysis. Thus the development of the chromosome map is only a method enabling the localization of genes or marker DNAs through the entire genome in the case of *L. japonicus* and spinach. The quantitative chromosome maps of the three plants were successfully developed by image analysis, and mapped physically genes on the chromosome maps using FISH methods. It was a prerequisite to obtain as many good chromosome samples as possible for FISH experiments. For this purpose, several conditions of the growth of roots from young plants, and the best conditions for pretreatment to collect cells at mitotic stage were examined. The method for preparing chromosome samples was improved because prometaphase chromosomes are long and are difficult to be sufficiently spread. Secondly, imaging technology was adopted to confirm the accuracy of the chromosome maps. Signal positions in *A. thaliana* were measured using CHIAS 3 after modification of the program. Thirdly, FISH procedures were improved, especially the denaturing conditions of chromosomal DNA in order to achieve distinct signals on chromosomes by FISH. The improvement and development of all these basic cytological methods enabled the successful development of chromosome maps.

In Chapter II, it was found that the method based on the condensation pattern in prometaphase enabled all the 6 chromosomes of spinach to be distinguished. Chromosome lengths, arm ratios and the degree of condensation at prometaphase chromosomes were analyzed using a Chromosome Image Analyzing System III (CHIAS 3). In this study, the FISH method was first applied to spinach chromosomes. Three 45S rDNA loci were detected at terminal positions of short arms of Chromosomes 2, 5 and 6. Three loci of 5S rDNA were also found at three locations at the long arm of Chromosome 2 and 5.

Small chromosomes of *A. thaliana* had limited uses in the cytogenetic study. In Chapter III, five chromosomes are characterized, and 45S and 5S rDNAs different colors on the chromosomes are simultaneously detected by technical improvements. Therefore, a quantitative chromosome map was developed with accurate localization of 45S and 5S rDNAs using image analysis. A suit of three maps, linkage and chromosome maps and nucleotide sequences are now developed in *A. thaliana*. In the future, these maps will be adequate for their purposes. Mapping genes on chromosomes using FISH is a powerful tool for investigating the sites of transgenes, translocation and recombination of chromosome segments.

In Chapter IV, the determination of the genome size of *L. japonicus* was carried out using flow cytometry. Consequently, it was shown that the genome size of one accession, Miyakojima, was about 30Mbp larger than that of the other accession Gifu. Although clear differences in karyotypes between Miyakojima and Gifu based on the quantitative chromosome maps developed were revealed, the successful identification of both chromosomes of Miyakojima and Gifu was achieved using the CP of the chromosomes and rDNAs positions mapped by FISH. Moreover, it was estimated that the expressed genes

were localized at late condensed regions or terminal regions on the *L. japonicus* chromosomes by FISH using 500 cDNAs as the probe. This molecular cytogenetic information will be useful for advancing genomic analyses in *L. japonicus*.

The development of quantitative chromosome maps, as shown in this study, is a basis of and is useful for genomic and cytogenetic research. The development of a multicolor FISH method has been essential for progress in the analysis of the physical mapping of genes and the order of genes on the chromosome. It will enable us to understand the relationship between the actual chromatin structure and the chromosome map. A distribution pattern of individual genes and repeated sequences will contributed to the understanding of physical organization and genome dynamics of the chromosome and nucleus. It is anticipated that the quantitative chromosome maps, as shown in this thesis, will be invaluable for both basic genome research, such as localizing useful genes on chromosomes, and also on the applied side, such as systematic breeding in crop plants.

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LIST OF PUBLICATIONS

Concerning this thesis

Mikako Ito, Nobuko Ohmido, Yukio Akiyama, Kiichi Fukui and Takato Koba (2000) Characterization of spinach chromosomes by condensation patterns and physical mapping of 5S and 45S rDNAs by FISH. Journal of the American Society for Horticultural Science. 125: 59-62.

Mikako Ito, Nobuko Ohmido, Yukio Akiyama and Kiichi Fukui (2000) Quantitative chromosome map of *Arabidopsis thaliana* L. by imaging methods. Cytologia 65: 325-331.

Mikako Ito, Junko Miyamoto, Yumiko Mori, Satoru Fujimoto, Toshiki Uchiumi, Mikiko Abe, Akihiro Suzuki, Satoshi Tabata and Kiichi Fukui (2000) Genome and chromosome dimensions of *Lotus japonicus*. Journal of Plant Research (in press)（12 月号に掲載）

The other publication

Eva Sykorova, Jiri Fajkus, **Mikako Ito** and Kiichi Fukui (2001) Transition between two forms of heterochromatin at plant subtelomeres. Chromosome Research (in press)

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